MASTER

"PHYSIOPATHOLOGY OF BLOOD PLATELETS
AND DEVELOPMENT OF PLATELET SUBSTITUTES"

Progress Report
for Period August 1, 1975-July 31, 1976

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April 28, 1976

Prepared For

The U.S. Energy Research and Development Administration
Under Contract No. E(11-1)-2783

(previously E(11-1)-3234)

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Introduction

This progress report covers the period August, 1975 to July, 1976 and is submitted by Mario G. Baldini, M. D., Principal Investigator.

During the past year, work related to this project followed the guidelines of our project proposal, although not all the work proposed could be completed because of the excessive volume of it. The subjects which were investigated in depth were as follows:

1. Arrest and Prevention of Hemorrhage in Alloimmunized Thrombocytopenic Patients.
2. Cyclic Nucleotides and Platelet Function.
3. Effect of Alpha-Tocopherol on Lipid Peroxidation and Platelet Aggregation.
4. Effect of Platelet Age on Platelet Adhesion to Collagen.
5. Effect of Ionizing Radiation on Blood Platelets.
6. Further Improvements in Utilization of Frozen Platelets.

Other areas of investigation which had been proposed last year, were considered to a lesser extent with only spotty experiments. These areas were:

1. Prevention of Thromboembolic Phenomena by Inhibition of Platelet Function.
2. Nucleotide Pools and Platelet Aging.

Since our interest in these two areas of investigation has by no means faded, we are again including these two sections in our project proposal for next year.

Several publications were completed (see list) and recognition of our work was maintained at the national and international levels as shown by the following events: Dr. M. G. Baldini has been invited to chair a Platelet Symposium during the XIV Meeting of the International Society of Hematology in Kyoto, Japan (September, 1976) and to present a paper on "Pre-

vention and Arrest of Hemorrhage in Alloimmunized Thrombocytopenic Patients". He has also been invited to present a paper on "Preservation of Human Platelets with Special Reference to Platelet Freezing" at the next annual meeting of the American Society of Cryobiology in Washington, D. C. (August, 1976).

Drs. Y. Ikeda and M. Steiner presented a paper at the 1976 FASEB Meeting in Annaheim, California (April, 1976) on "Protein Kinase Activity of Platelet Tubulin". J. Anastasi (a volunteer senior-student at Brown University) who worked in our Division during the past year, will present a paper to the Am. Soc. for Clinical Nutrition in Atlantic City, N. J., next May 1, 1976, together with Dr. M. Steiner.

The professional investigators who worked actively on our projects during the past year were:

1. Mario G. Baldini, M. D. - Principal Investigator
2. Manfred Steiner, M. D., Ph.D. - Co-Principal Investigator
3. Byung K. Kim, M. D. - Co-Principal Investigator
4. Yasuo Ikeda, M. D. - Senior Research Associate
5. Toshiro Nagasawa, M. D. - Research Associate

PUBLICATIONS SINCE LAST PROGRESS REPORT*

1. Baldini, M. G. "Hemorrhage in Leukemia with Special Reference to Platelet Transfusions" in Therapy of Acute Leukemias, F. Mandelli, S. Amadori and G. Mariani, Ed., Minerva Medica, Turin, Italy, 1975, p. 209-222.
2. Kim, B. K., Tanoue, K. and Baldini, M. G. "Storage of Human Platelets by Freezing". Vox Sang. (in press), 1976.
3. Kim, B. K., Tanoue, K. and Baldini, M. G. "The Shelf-Life of Previously Frozen Human Platelets". Excerpta Medica, Int. Congr. Series, 357:457-461, 1975.
4. Baldini, M. G. "Discussion paper: Dimethyl Sulfoxide as a Cryoprotective Agent for Platelet Preservation by Freezing". Ann. New York Acad. Sciences 243:306, 1975.
5. Steiner, M. and Anastasi, J. "Vitamin E-An Inhibitor of the Platelet Release Reaction". J. Clin. Invest. 57:732-737, 1976.
6. Agarwal, K. C. and Steiner, M. "Effect of Serotonin on Cyclic Nucleotides of Human Platelets". Biochem. Biophys. Res. Communication (in press), 1976.
7. Castellan, R. M. and Steiner, M. "Effect of Platelet Age on Adhesiveness to Collagen and Platelet Surface Charge". Thrombosis, Diathesis Haemorrhagica (in press), 1976.

LIST OF PAPERS PUBLISHED IN ABSTRACT FORM

1. Kim, B. K., Tanoue, K. and Baldini, M. G. "Storage at 22°C of Previously Frozen Human Platelets." Cryobiology 12:565, 1975.
2. Anastasi, J. and Steiner, M. "Effect of Alpha-Tocopherol on Human Platelet Aggregation." Clin. Research 24:498A, 1976.
3. Baldini, M. G., Nagasawa, T. and Kim, B. K. "Arrest and Prevention of Hemorrhage in Severely Alloimmunized Thrombocytopenic Patients." The XVI Int. Congress of Hematology, Symposium on Platelet Immunology, Proceedings of Symposium.

*Note: Six reprints or copies of the manuscript are herewith submitted for each of the above papers.

Work performed during the past year will be summarized under the following headings:

1. Arrest and Prevention of Hemorrhage in Alloimmunized Thrombocytopenic Patients. Page 6
2. Cyclic Nucleotides and Platelet Function Page 16
3. Effect of Alpha-Tocopherol on Lipid Peroxidation and Platelet Aggregation Page 18
4. Effect of Platelet Age on Platelet Adhesion to Collagen. Page 20
5. Effect of Ionizing Radiation on Blood Platelets. Page 21
6. Further Improvements in the Utilization of Frozen Human Platelets. Page 22

Abstracts of papers presented at national or international meetings are included here Page 27

1. ARREST AND PREVENTION OF HEMORRHAGE IN ALLOIMMUNIZED THROMBOCYTOPENIC PATIENTS

It is well known that blood platelets are alloantigenic and that in addition to weak ABO red cells antigens and to specific platelet antigens they contain homotransplantation antigens with their inherent individual specificity. As could be expected on the basis of the above findings, it has now clearly been shown that repeated infusions of donor platelets invariably cause the rise of antiplatelet alloantibodies in the circulation of the recipient, after a variable number of infusions. The alloantibody causes reduction in survival and hemostatic effectiveness of the transfused donor platelets and defies the purpose of platelet transfusions.

Matching of major HLA-antigens between donor and recipient has prevented platelet alloimmunization, but only for limited periods of time in most cases (Yankee, R. A. et al in Baldini, M. G. and Ebbe S., Grune & Stratton, New York, 1974). This has also been our recent experience. The physician is frequently faced with an acutely bleeding thrombocytopenic patient in need of platelet transfusions which will, however, be ineffective because of alloimmunization. It has been our experience in the past that by infusion of a very large number of platelet units (6 to 20) an effect on hemorrhage could still be obtained. This procedure, however, was met with the difficulty of suddenly being able to procure very large numbers of fresh units of platelets for the acutely bleeding, alloimmunized, thrombocytopenic patient.

Experiments were planned to study the possibility of adsorbing the circulating platelet antibody, and infusing afterwards a small number of fresh platelets. If the antibody level could temporarily be reduced, fresh platelets infused after adsorption, would survive a nearly normal

or normal length of time and produce hemostasis in the bleeding thrombocytopenic recipient.

Rabbits and humans were used for these experiments. It was immediately realized that practical serological tests are presently too insensitive for the detection of platelet antibodies in the serum and that survival of allogenic platelets after labeling with Cr^{51} sodium chromate is the most accurate and sensitive method to measure allo-sensitization.

Rabbits were alloimmunized by repeated (weekly) intradermal injections of allogenic platelets (10^9 cells per injection). The circulating anti-platelet alloantibodies were detected by measuring the in vivo recovery and survival of Cr^{51} labeled allogenic platelets. As shown in Figure 1, the recovery value of Cr^{51} platelets was progressively diminished as the number of intradermal injections increased in the recipient animals (sensitization). After 12 infusions of allogenic platelets, sensitization was clearly evidenced by the very reduced recovery value of the infused allogenic Cr^{51} labeled platelets (Figure 1).

The level of circulating platelet alloantibodies was then reduced by adsorption with the infusion of a large number (10^{10}) of fresh allogenic platelets. After 30 minutes from the infusion a Cr^{51} platelet survival study with allogenic platelets was performed. The in vivo recovery values became significantly higher (59%) than the value observed immediately before adsorption (19%) as reported in Figure 2. In three experiments, a similarly higher recovery value was observed when the platelet survival study was performed soon after or even 12 hours from the infusion of unlabeled platelets to adsorb the circulating antibody. It appeared that efficiency of removal

of the circulating alloantibody was higher when fresh platelets were used than with stored (less viable) platelets (Figure 2) suggesting that stored platelets may be required in larger numbers than fresh platelets to produce the same effect because stored platelets are quickly removed from the circulation. This was also true when a powder of liophilized platelets was used for adsorption of the circulating antiplatelet alloantibodies (Figure 3).

In a subsequent experiment (Figure 4), a severely sensitized, 5 year-old boy with aplastic anemia was studied. The boy had severe thrombocytopenia ($<10,000$ platelets per cu mm) and was bleeding from various sites (nose bleeds, G.I. bleeding, severe purpura). Cr^{51} labeled donor platelets disappeared immediately from the circulation with a recovery value of 6% soon after infusion and less than 1% after two hours from infusion. After infusion of 6 units of donor platelets stored for one week at 4°C , survival of allogenic fresh platelets from the same donor was clearly improved with a recovery value of 30% soon after infusion. However, after 4 hours the recovery value dropped to about 10%. This indirectly indicated that alloimmunization was severe and the titer of the antibody very elevated. Subsequently, a more successful result was obtained by increasing the number of the platelets infused before survival of allogenic platelets was studied. These were, again, platelets which had been stored for one week at 4°C (and therefore non-viable). After infusion of 20 units of these non-viable platelets, survival of one unit of Cr^{51} labeled, fresh platelets was nearly normal, that is almost similar to survival of fresh platelets in a non-sensitized recipient. Also, the effect on hemostasis was clinically very evident with a more stable hemoglobin level and a partial disappearance of the purpura. The recovery value of the infused allogenic platelets was 75%, and it was 30% after 24 hours.

It is concluded that the treatment of thrombocytopenic hemorrhage in alloimmunized patients is feasible by the use of a two-step infusion of a large quantity of longly stored platelets collected as a by-product of blood banking, immediately followed by the infusion of one or a few units of freshly collected donor platelets. The rationale is based on the removal of the circulating platelet alloantibody by adsorption with the first infusion and the result is very encouraging. It will be possible in the future to arrest hemorrhage in a thrombocytopenic recipient even when he is alloimmunized to platelets. Also, the use of stored platelets for antibody adsorption will greatly reduce the use of freshly collected platelets which are more expensive and difficult to obtain.

Platelet Alloimmunization in the Rabbit.

Fig. 1-

Survival of Cr^{51} labeled allogeneic Platelets
after infusion of stored (dead) platelets.

Infusion of
non-labeled platelets

Fig. 2

Fig. 3

LEGEND

Figure 1: The in vivo recovery values of Cr^{51} labeled allogenic platelets in the rabbit with platelet alloimmunization. The shaded area represents Cr^{51} platelet recovery value in the non-sensitized rabbits (6 experiments). Sensitization was done with repeated intradermal injection of 10^9 allogenic platelets in one ml saline every week. The in vivo recovery values at one hour and 24 hours post-invasion (an average of 2 - 3 experiments) are reported after 3 to 5 sensitizing injections (-----), after 6 to 8 injections (-·-·-·-), 9 to 11 injections (-·-·-·-·-), and after more than 12 injections (—), indicating the progressive character of platelet isosensitization.

Figure 2: In vivo recovery and survival values of Cr^{51} labeled allogenic platelets in a alloimmunized rabbit before and after antibody adsorption by the infusion of stored (dead) platelets. Each value represents a mean of 2 to 3 experiments observed before adsorption of the circulating platelet alloantibody (—) and after adsorption with infusion of 10^{10} fresh platelets (-----), 1 day stored platelets at 4°C (-·-·-·-), and of one week stored platelets (-·-·-·-).

Figure 3: The in vivo recovery values of Cr^{51} labeled allogenic platelets in alloimmunized rabbits before (—) and after adsorption of circulating antibody with infusion of a liophilized platelet powder suspension equivalent to 10^{10} platelets (-----), 2×10^{10} platelets (-·-·-·-), and to 4×10^{10} (-·-·-·-).

Figure 4: Case I and II. Separate experiments in a thrombocytopenic child with aplastic anemia. Infusion of 6 units of stored (dead) platelets produced a

significant improvement in survival of allogenic Cr⁵¹ labeled platelets infused soon after (Case I). In the second graph (Case II) a larger infusion produced a near normalization of survival of labeled allogenic platelets.

2. CYCLIC NUCLEOTIDES AND PLATELET FUNCTION

The ability of various aggregating agents to influence and alter cyclic nucleotide levels in human platelets was examined. Most of our studies focused on the effect of serotonin. Addition of this biogenic amine to platelets caused a 6-10 fold increase in cyclic GMP levels within 30 sec which over a period of 10 min decreased to baseline levels of this cyclic nucleotide. A second addition of serotonin at that point resulted in a renewed increase of cyclic GMP which was followed by a much slower decline towards baseline levels than after the first addition of 5-hydroxytryptamine. The serotonin-induced elevation of cyclic GMP was related to the concentration of 5-hydroxytryptamine in the medium although not in a strictly linear fashion. Cyclic AMP levels of platelets did not change on addition of serotonin. The elevation of cyclic GMP was not related to platelet aggregation, in fact no aggregation was observed as platelets were not agitated and were suspended in artificial media. It was of great interest that even complete inhibition of serotonin uptake was unable to stop the increase in cyclic GMP when serotonin was added. Glutaraldehyde and formaldehyde, crosslinking agents of membrane proteins completely inhibited the serotonin-induced rise in cyclic GMP and depressed cyclic GMP production in normal non-serotonin incubated platelets below baseline levels.

The effect of thrombin and epinephrine on cyclic GMP levels were studied in platelets suspended in artificial media and autologous plasma. Although most experiments demonstrated an elevation of platelet cyclic GMP when these aggregating agents were added the stimulation of cyclic GMP was variable in degree and duration and no clear cut relation to the release reaction could be established. Aspirin, a potent inhibitor of the secretory release of platelets, was unable to prevent the rise in cyclic GMP levels

obtained with epinephrine and thrombin. Further studies on the rate of synthesis of cyclic nucleotides in aggregating platelets and correlation with the temporal sequence of the aggregation-induced platelet release will be necessary to settle the role of cyclic nucleotides in platelet function (see our paper in Biochem. Biophys. Res. Comm., 1976, in press. Manuscript included under separate cover).

3. EFFECT OF ALPHA-TOCOPHEROL ON LIPID PEROXIDATION AND PLATELET AGGREGATION

In previous studies we have shown that platelet aggregation is associated with a sudden rise in malonaldehyde-yielding lipid peroxidation. Recent studies from other laboratories have shown that malonaldehyde is a product accumulating during the production of prostaglandins and Thromboxanes. As alpha-tocopherol has been shown to inhibit lipid peroxide formation in stored platelets we have investigated its potential to prevent the lipid peroxidation which occurs during platelet aggregation. Our studies showed that alpha-tocopherol was able to inhibit the platelet release reaction when the vitamin was added in vitro in concentrations from 0.9 to 1.8 mM. The release from both the electron dense and the alpha granules was inhibited by vitamin E in a dose-dependent manner. The inhibition could be correlated with a reduction of the aggregation-induced rise in lipid peroxides and which paralleled the effect of acetylsalicylic acid. These in vitro studies were recently extended to in vivo investigation of the effect of oral vitamin E supplementation of the diet on platelet aggregation. At a dosage of 1200 I.U. daily alpha-tocopherol inhibited the secondary wave of aggregation induced by ADP and epinephrine and markedly reduced collagen-induced platelet clumping. No ill effects were noted at this or higher dosage of vitamin E (up to 2400 I.U. daily). In an effort to determine the mechanism of alpha-tocopherol action we examined the effect of alpha-tocopheryl quinone, a metabolite of vitamin E without antioxidant action. Added in vitro to platelet suspensions this compound suppressed platelet aggregation to a similar degree as alpha-tocopherol. This finding is considered strong evidence for the molecular theory of tocopherol action advanced by Lucy (Ann. N.Y. Acad. Sci. 203:4-11,

1972) which postulates a physicochemical interaction between the phythyl chain of alpha-tocopherol and unsaturated fatty acyl residues particularly those of arachidonic acid in phospholipids. Our studies showed that the platelet release reaction was completely inhibited when the ratio of arachidonic acid to alpha-tocopherol in platelets approached 20:1. As arachidonic acid is the principal substrate for cyclo-oxygenase in the production of prostaglandin endoperoxides and Thromboxanes, the observed effect of alpha-tocopherol inhibition of platelet aggregation also strengthens the hypothesis of a causal relation between thromboxane formation and platelet release reaction. Furthermore, our studies revealed an alternate means of preventing platelet aggregation other than by drugs with variable degrees of toxicity, by a physiological substance with no known toxicity and minimal side effects (see our papers in J. Clin. Invest. 57:732, 1976, reprint included here; and our abstract Clin. Research 24:498A, 1976, copy included under separate cover).

4. EFFECT OF PLATELET AGE ON PLATELET ADHESION TO COLLAGEN

The adhesivity of rat platelets to collagen was studied as a function of platelet age. Platelet populations predominating in young or old platelets were compared to randomly aged platelets in their ability to adhere to collagen. Platelet adherence to collagen was studied by measuring the rate of increase of optical transmission after adding an EDTA-containing platelet suspension to a weighed amount of bovine Achilles tendon collagen, homogenized and macerated and immobilized between 2 plastic-coated magnetic stirring bars. Absence of platelet aggregation was verified by Phase microscopy. Young platelets were harvested in rats during the recovery phase from immune thrombocytopenia induced by heterologous antiserum. Old platelets were prepared by blocking thrombopoiesis with a cytostatic agent. A moderate but significant increase in the rate of adherence to collagen was noted with young platelets. Old platelets did not differ significantly from randomly aged populations in this function. The electrophoretic mobility of platelets was also studied as a function of their age but no difference between the various platelet populations was observed. Collagen adhesivity decreased rapidly when platelets were stored and was abolished when collagen was denatured by heat treatment and markedly reduced when collagen was pretreated with collagenase. These studies suggested that in vivo or in vitro aging of platelets changes their surface membrane resulting in an age-dependent loss of specific receptor sites for collagen (see our paper in *Thrombosis and Diath. Haemorrh.*, 1976, in press. Manuscript included under separate cover).

5. EFFECT OF IONIZING RADIATION ON PLATELETS

Further studies on the effect of low doses of x-irradiation (up to 1000 r) on platelet function were carried out. In particular, the platelet release reaction and platelet aggregation were investigated. The results may be summarized as follows:

1. X-irradiation produced a dose-dependent release of ^{14}C -5-hydroxytryptamine from prelabeled platelets.
2. ADP and epinephrine-induced release was increased after platelets had been irradiated. The augmentation of the serotonin release could be completely accounted for by the magnitude of the preaggregation release.
3. Collagen-induced release in irradiated platelets was increased during the first 2 min after addition of collagen, but then fell below the level observed in corresponding controls.
4. Aggregation induced by ADP and epinephrine and measured by percentage of total clearing of the platelet suspensions was moderately enhanced but not aggregation induced by collagen.

These results together with our previous ones in this area, indicate that platelets exposed to ionizing radiation become "activated", i.e. have increased functional activity. X-irradiation with moderate doses (up to 1000 r) did not result in obvious changes in the distribution pattern of sodium dodecyl sulfate solubilized membranes or whole platelets, did not change the surface charge as revealed by cell microelectrophoresis and had no effect on adenylate cyclase activity.

Effect of X-Irradiation on Collagen-Induced Platelet Serotonin Release

* Significant differences ($p > .005$)

6. FURTHER IMPROVEMENTS IN THE UTILIZATION OF FROZEN PLATELETS

During the past year we have established an experimental "Frozen-Platelet Bank" at The Memorial Hospital. A special laboratory was established, equipped with a -85°C mechanical refrigerator (Harris LoTemp Model 12L-2-10) with a storage capacity for 350 platelet units and a liquid nitrogen vapor refrigerator (Union Carbide Corp., Model LR 310) with a storage capacity for 340 units. By pooling 3 to 5 units in single plastic bags, as we are presently doing, our maximum storage capacity will be increased to 1200 units which we presume will be sufficient for the needs of our Hospital (350 beds) since any predictable use of frozen platelets is regularly being replaced. To date, 186 platelet concentrates (average platelet content $50 \times 10^9/\text{unit}$) obtained as a byproduct of our routine blood banking and stored in individual plastic containers, and 14 autologous multiplatelet units (3 to 5 concentrates in one storage unit) obtained from individual living patients with leukemia or lymphomas under chemotherapy have been preserved (total 240 platelet concentrates) at -85°C for a period of a few weeks to 13 months.

Simultaneously, we have organized HLA-typing (lymphocyte cytotoxicity method) and trained a research technician for this purpose so that all platelet units being preserved in the frozen state are typed for HLA-antigens, in addition to the ABO-Rh antigens.

The research projects being presently studied by this organized frozen-platelet bank are mainly three:

1. The study of technical details involved in the large-scale application of preserved frozen platelets for human use. These include: the practicality of our newly designed Hemoflex platelet bag (UCAR#2031-4) provided

with two compartments, one for the platelet concentrate and one for plasma; the sterile procedure of platelet separation, transfer and freezing; the cost of platelet preservation by freezing; the practicality of utilizing platelets obtained from single blood units as a by-product of blood banking versus the utilization of the cell separator (Hemonetics) in selected platelet donors; the study of the platelet damage caused by the cell separator; the platelet damage caused by a delay of one to six hours from the time of blood collection to the time of platelet separation; the damage caused by a delay of one to six hours from the time of thawing to the time of infusion in the patient; the effect of temperature on the previously frozen platelets preserved for short intervals in the liquid state; the study of platelet function in vitro and platelet hemostatic function in vivo in thrombocytopenic patients, for all the above experimental situations; the possible toxicity of the trace amounts of DMSO infused in the patients with the frozen platelets, to reach a complete evaluation of the feasibility and practicality of the use of platelet preservation by freezing.

2. The effectiveness of HLA typing and matching for platelets with regard to the occurrence and severity of platelet isosensitization in multi-transfused recipients. It is our impression based on preliminary findings, that while isosensitization to donor platelets can be delayed by this technique, it cannot, however, be prevented.

3. The practical value of the long-term preservation of autologous platelets collected from leukemic (or other malignancy) patients during remission and re-infused at the time of need at the height of bone marrow suppression caused by chemotherapy.

The method of platelet separation and freezing that we have utilized is as follows:

Platelet-rich plasma obtained from the blood bank of our hospital is centrifuged and a platelet concentrate is prepared suspended in 15 ml plasma, at 22°C. The concentrate is transferred to a Hemoflex bag (experimental type UCAR #2031-4). A volume of plasma containing 10% DMSO is gradually added (0.5% gradient every minute) as previously described by us. The final volume of the platelet concentrate containing 5% DMSO in the large compartment of the UCAR hemoflex bag is 30 ml. A 20 ml autologous plasma is then preserved in the small compartment of the bag. Blood type, HLA-type, serology and Australian antigen are determined for each unit. Each platelet unit is labeled for identification, then placed in an aluminum press (freezing frame) and is kept in the middle portion of the lower level of our -85°C mechanical refrigerator overnight. In this position, cooling occurs at a rate of -1° to -2°C per minute. After freezing the unit is finally placed in the storage compartment of the freezer. When a freezing bag was used for a multiplatelet concentrate unit, both volume of platelet concentrate and plasma stored were doubled.

Platelet pheresis procedures for preservation of autologous platelets and transfusion of frozen platelets in the patients, their benefits and possible hazards, were discussed with each patient and relatives, and a signed informed consent was obtained. When the patients were in remission of their malignant disease with platelet counts higher than 200,000/ μ l, 3 to 5 cycles (equivalent to 3 to 5 pints of blood) for each pheresis were processed within 2 to 3 hours using either Haemonetics Model 30 cell separator or the conventional method by repeated phlebotomies. The procedure was at times repeated at weekly intervals.

At the time of use, platelets stored in the frozen state are thawed rapidly in a 37°C water bath and the volume of the platelet concentrate is

gradually diluted at the rate of a 0.5% gradient every minute using four volumes of Hank's balanced salt solution. The platelet button is obtained by centrifugation and is finally resuspended in 20 to 30 ml of 50% autologous plasma in Hank's balanced salt solution. The platelet suspension is, then, infused in the patient. A nearly normal life-span and a normal hemostatic effectiveness of autologous frozen platelets has so far been observed in all cases. The usefulness of this procedure applied on a larger scale is presently being evaluated.