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PROJECT:

"PHYSIOPATHOLOGY OF BLOOD PLATELETS"
"A MODEL SYSTEM FOR STUDIES OF CELL-
to-CELL INTERACTION"

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SECTION 1.

ORIGINALLY STATED OBJECTIVES AND THEIR SIGNIFICANCE

INTRODUCTION

During the past few years it has become apparent that research on platelet preservation which began in 1960 with our demonstration that the then stored platelets had no hemostatic value (Baldini, M.G. et al., Blood 16:1669, 1960), has reached an impasse. Progress had been considerable in both preservation of platelet concentrates in the liquid and the frozen state, but limitations were still important and these could not be resolved without more basic knowledge about the platelets and their reactions. Also, new fields of study came to the forefront. Platelet aggregation and its relationship with thromboembolic phenomena as well as with the phenomenon of adhesion of all cells in general, became of essential importance. Platelet reactions with tumor cells and the relationship between tumor cells and platelet aggregability, and, also, the possible relationship between platelets and tumor cell proliferation took an important place in present-day research. The fact that platelets contain substances which can increase proliferation of other cells (like muscle cells of the vascular wall), also related platelets to a possible role in atherogenesis. The study of substances in the environment which can influence these platelet functions and the search for medications which can usefully modify platelet reactions has now begun to open new fields of investigation. In conclusion, there has been an explosion of platelet research which goes much beyond the borders of the platelet role in hemostasis. The platelet has, in reality, assumed almost a center role in cell research in general. Our present interests have followed these exciting developments in human and general biology and our objectives have, therefore, been modified accordingly.

OBJECTIVES

During the past three years, we followed three main objectives:

A. To complete our studies on platelet preservation and transfusion so that results previously obtained could assume a more practical meaning in patient care. In this area of research, we followed two specific objectives:

1. To improve a few technical details in the method of platelet preservation by freezing that we had originally described.

Frozen platelets can be preserved viable probably for an indefinite period of time. If the method can be attuned to the needs of a routine blood bank procedure, its value will enormously increase. This can

be done and frozen platelets are ready for large scale application, as soon as the FDA will approve the cryoprotective agent (DMSO) as a prescriptive drug.

2. To study a practical way by which acute bleeding in thrombocytopenic patients alloimmunized by previous platelet transfusions can be arrested even in the absence of HL-A matched platelets.

In spite of the fact that HL-A typing for platelets is available in most clinical centers, about 50% of patients polytransfused with platelets still become alloimmunized. Further platelet transfusions have little or no effect on them. In case of an acute hemorrhage, the physician must have a method by which platelet transfusions can become effective again, at least temporarily. This was obtained.

B. To continue basic studies of platelet function so that we could learn more about platelet properties and reactions and acquire notions which could be useful in areas of platelet research such as a) platelet kinetics, b) platelet aggregation, c) platelet metabolism and d) the platelet lesions caused by storage by various methods. Our detailed objectives in this area were as follows:

1. To study the stability of ^{51}Cr as a cell marker for platelets ^{51}Cr has been utilized in many studies of platelet kinetics in animals and humans. The results obtained in these studies would probably lose significance if we could demonstrate that the cell label was not firm.

This was, indeed, the case when antiplatelet antibodies were present in the circulation.

2. To investigate whether platelet adhesion to collagen, a basic phenomenon in hemostasis, is dependent on cell age.

Not all platelet functions have shown a clear age dependency and adhesion to glass has been shown by us (Scand. J. Haemat. 6:25, 1969) and others to be a random phenomenon with regard to platelet age.

3. To investigate the effect of serotonin on the cyclic nucleotide level in human platelets.

Serotonin is stored in electron dense subcellular organelles and released during platelet aggregation. There is good evidence that the reversible attachment of biologically active agents to specific sites in ma-

cromolecular elements may result in changes in their functional ability to synthesize substances which in turn may mediate cellular function. Serotonin and cyclic nucleotides indeed qualified in this context and positive results were obtained.

4. To measure adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) in platelets stored at 4°C and 22°C, and to correlate the intracellular levels of these cyclic nucleotides with the ability of the platelets to synthesize these compounds.

Cyclic nucleotides have been found to be mediators of a wide spectrum of cellular stimuli. No studies have been published on the intracellular levels of these compounds in stored platelets. Our investigations pointed to alterations in membrane receptor sites during storage, not to impairment of cyclic nucleotide production per se. This may explain also why platelets stored at 22°C rapidly lose the capacity to aggregate (see annual Progress Report).

5. To study the effect of α -tocopherol on platelet aggregation.

Our studies had previously demonstrated that platelet aggregation is associated with a sudden rise in malonaldehyde yielding lipid peroxidation. Other investigators showed, then, that malonaldehyde is a product accumulating during the production of prostaglandins and thromboxanes. Since we could also demonstrate that α -tocopherol inhibits lipid peroxide formation in stored platelets, it was logical that we investigated its potential to prevent lipid peroxidation which occurs during platelet aggregation. The hypothesis proved to be correct.

C. To initiate studies of cell-to-cell interaction using platelets as a model system.

During the past year we have begun an intense program of investigation of cellular interactions as a general biological phenomenon utilizing the platelets as a model for the study of interactions between cells of the same or different type and of factors which can influence these reactions. As this covers an extensive field we narrowed our objectives to certain well defined problems, as follows:

1. Investigation of platelet interaction with tumor cells.

There is good clinical and experimental evidence that platelets and tumor cells influence each other. Tumor

cells may cause platelet aggregation either by direct contact between platelets and neoplastic cells or by release of an aggregation inducing principle from the tumor cells. Furthermore, growth-promoting factors for several tissues and cells have been demonstrated in the platelets. Whether a growth-stimulating effect may also be seen with neoplastic cells is unknown and we are going to investigate it.

2. To establish a relation between the lipid composition of the platelet membranes and their fluidity, i.e. to investigate the fluidity of platelet membranes as a function of their lipid composition, and to relate changes in the physical behavior of the lipid composition of membranes to alterations in membrane-mediated functions, particularly aggregation and adhesion.

Microviscosity of the platelet membranes was measured using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). We are studying environmental factors which can influence platelet microviscosity, and we began with temperature. We also found that α -tocopherol which we had previously shown to be an antiaggregating agent by producing an inhibition of the platelet release reaction induced by any of the commonly used aggregating agents, also affected the fluorescence polarization of DPH-treated platelets, that is the platelet microviscosity.

3. To advance understanding of the interaction between platelets and vessel wall.

It has been demonstrated that platelets may release a growth factor for intimal smooth muscle cells. This may have importance in the development of atherosclerosis. In fact, several investigators have recently suggested a role of platelets in the proliferative response to endothelial injury and they have seen a positive correlation between platelet count and magnitude of proliferation of smooth muscle cells. Heparin can limit the proliferative response. It will be important to investigate, also, the possible role of antithrombin III in modifying the response to intimal injury. This area of research is of great current interest as it relates to the pathogenesis of atherosclerosis.

SECTION 2.

MAIN RESEARCH ACCOMPLISHMENTS AND THEIR SIGNIFICANCE

The major lines of investigation pursued during the past three years are listed here. A brief report and discussion on each subject of investigation is also given.

A. Preservation and Transfusion of Viable Human Platelets

1. Improved preservation of platelets by freezing.
2. Arrest and prevention of hemorrhage in allo-immunized thrombocytopenic patients.

B. Basic Studies of Platelet Function

3. In vivo elution of ^{51}Cr from labeled platelets induced by antibody.
4. Platelet age and adhesion to collagen.
5. Effect of serotonin on cyclic nucleotides of human platelets.
6. Response of cyclic nucleotides to stimulation in stored human platelets.
7. Effect of α -tocopherol on lipid peroxidation and platelet aggregation.

C. Studies of Cell-to-Cell Interaction Using Platelets as a Model System

8. Platelet interaction with tumor cells.
9. Studies of platelet membrane fluidity. The effect of α -tocopherol.

REFERENCES

A. PRESERVATION AND TRANSFUSION OF VIABLE HUMAN PLATELETS

1. IMPROVED PRESERVATION OF HUMAN PLATELETS BY FREEZING
(see list in Section 5, papers #2, 3, 7, 8)

The long search for an efficient method of platelet preservation by freezing has recently been attended with success. Prolonged, probably indefinite storage of viable and functional human platelets is now possible by freezing with dimethylsulfoxide (DMSO). It has only been six years ago that we (1,2) and others (3) demonstrated that human platelets stored after controlled freezing with 5 per cent DMSO in polyolefin plastic containers maintain a nearly normal viability as assessed by their in vivo survival after labeling with ^{51}Cr . In later studies (4), we demonstrated the hemostatic effectiveness of the frozen platelets in patients with severe thrombocytopenia. The method can now be considered nearly ready for the large-scale clinical application. As soon as the FDA will approve DMSO for prescriptive use, it can be expected that a very large percentage of platelet transfusions in medical practice will be with frozen platelets.

During the past three years studies were continued in our Division of Hematologic Research to confirm the clinical effectiveness of frozen platelets and to improve the technique of platelet freezing and to make it more adaptable for large-scale usage. We found that the medium by which to wash the platelets after thawing so that DMSO can be removed without damage to the platelets does not have to be pure plasma. A washing medium of 20 per cent plasma in Hanks' balanced salt solution is just as effective as pure plasma. Also the final platelet button could safely be resuspended in 50% plasma in Hanks' solution. A further reduction in plasma concentration was, however, deleterious to the platelets and caused reduction in their viability upon reinfusion. These two technical details allowed preservation of only 20 ml of autologous plasma for the resuspension of the platelet concentrate after washing with DMSO. This reduction in volume of autologous plasma for platelet resuspension has made it possible for us to design a new polyolefin bag for the simplified storage of plasma and platelet concentrate (see paper #8).

In further experiments we determined the shelf-life of human platelets after freezing and subsequent thawing. Using a number of in vitro tests and, also, in vivo survival studies to determine platelet integrity after freezing and thawing and subsequent preservation at 22°C, it was seen that the earliest significant changes in the platelets occurred already after six hours of storage and included a reduction in platelet count, a reduction of in vivo viability, an increased release of purine nucleoside phosphorylase and β -glucuronidase, a reduced degree of platelet

aggregability. After 24 hrs of shelf storage, ATP content was significantly reduced in comparison with the controls and platelet reversal reaction and serotonin uptake were also depressed. These tests demonstrated that previously frozen human platelets deteriorate rapidly during storage at 22°C and must, therefore, be infused soon after thawing (see paper #2). These studies also confirmed the significance of the in vitro test of platelet reversal reaction to osmotic stress, previously introduced by us as a test of platelet function, it is very valuable in predicting in vivo viability of preserved platelets (paper #9). Finally, our continued clinical studies with the use of frozen platelets showed that no side effects ever appeared in the patients from the infusion of trace amounts of DMSO, the potentially harmful cryoprotective agent that we must use in platelet freezing (see paper #3). This will contribute in helping the FDA to formulate a decision towards approval of DMSO for prescriptive use, therefore opening the field of platelet freezing to large-scale clinical application.

2. ARREST AND PREVENTION OF HEMORRHAGE IN ALLOIMMUNIZED THROMBOCYTOPENIC PATIENTS (see list in Section 5, paper #13, 14)

During the past two decades it has become clearly apparent that repeated transfusions of platelets to thrombocytopenic patients cause a state of refractoriness due to alloimmunization towards platelet antigens. This is clinically manifested by a progressively shortened survival of transfused platelets and failure of the platelets to produce hemostasis. We were the first to demonstrate that human platelets contain histocompatibility antigens (6,7), and that platelet alloimmunization in polytransfused patients is a natural and common biological event (5). Prevention of platelet alloimmunization has been difficult. The use of platelet donors matched on the basis of four major HL-A histocompatibility antigens has only partially resolved the problem. In fact, in a considerable number of patients, refractoriness to platelet transfusions has developed in spite of the matching of four HL-A antigens (8). Although studies are in progress and new findings may further elucidate this complex problem, it may still be expected that sufficiently matched donor platelets to avoid destruction after transfusion, may not always be available at the time of need and in sufficient quantities to arrest hemorrhage in severely allosensitized thrombocytopenic patients. Empirical trials have shown that the massive infusion of many platelet units together may, at times, produce a hemostatic response in the allosensitized patients. The scientific basis for this clinical observation was missing. We studied the mechanism of this phenomenon in rabbits, then in patients. Survival of ^{51}Cr -labeled allogenic platelets was repeatedly measured in rabbits previously sensitized by weekly intradermal injections of platelets from donor animals. When values of recovery and survival time were reduced to very low levels, a massive infusion of platelets from randomly selected donor rabbits was given and survival study was repeated soon after. A large increase in platelet survival and recovery was seen in every experiment. Similar results, although of lesser magnitude, were obtained with the infusion of platelets ^{made} ~~and~~ non-viable by prolonged storage and, also, of lyophilized platelets. A significant increase in life span and recovery of allogenic platelets was also observed in two patients with thrombocytopenia due to aplastic anemia, severely allosensitized to platelets. After one massive infusion of viable or of non-viable platelets, survival and hemostatic effectiveness of subsequently infused allogenic platelets was greatly improved in both patients. Experimental blockade of the RES and titration of the serum alloantibody by the ^{14}C -serotonin release method demonstrated that the favorable effect of the massive platelet infusions was due to the temporary adsorption of the alloantibody in the circulation of the sensitized animals. These results have demonstrated that even in severely alloimmunized patients, the massive infusion of platelets from

random donors causes an improvement in survival and hemostatic effectiveness of subsequently infused allogenic platelets because of adsorption of the alloantibody by the overwhelming excess of antigen infused. The fact that the antigen infused could be of fresh, stored or lyophilized platelets added clinical significance and practical value to these findings in the treatment of hemorrhage in selected, alloimmunized thrombocytopenic patients (see papers #13 and 14).

B. BASIC STUDIES OF PLATELET FUNCTION

3. IN VIVO ELUTION OF ^{51}Cr FROM LABELED PLATELETS INDUCED BY ANTIBODY. (see list in Section 5, papers #11, 12 and Abstracts #10, 11)

Chromium-51 sodium chromate has most widely been used as a radioisotopic label to study platelet survival and kinetics in experimental animals and in human subjects. A major prerequisite for a cell label in kinetic studies is that its binding to the cell be perfectly stable. In previous experiments (9) we demonstrated that antiplatelet heteroantibody in vitro can cause release of ^{51}Cr from labeled platelets. We have postulated that antiplatelet antibodies may cause elution of the ^{51}Cr -label from platelets also in the circulation. This would render inaccurate measurements of platelet survival and kinetics by the ^{51}Cr method in animals or patients with antiplatelet antibodies. To examine this hypothesis, we studied the effect of antiplatelet heteroantibody and alloantibody on ^{51}Cr labeled platelets infused in thrombocytopenic rabbits. Two thrombocytopenic patients alloimmunized by repeated platelet transfusions were also studied.

A potent guinea pig antirabbit-platelet serum was prepared. Rabbits with severe and stable thrombocytopenia produced by two injections of ^{32}P were infused with 4×10^{10} ^{51}Cr -labeled platelets from donor animals raising the platelet count to $180 - 250 \times 10^3$ per cumm. Diluted platelet antiserum was, then, injected I.V. and blood samples were drawn before and four times during 3 hr after injection. As soon as 15 min post-injection, platelet specific activity dropped to 30% of pre-injection value. Other experiments were done in rabbits first alloimmunized with platelets from random donor animals, then made thrombocytopenic by injection of ^{32}P . After infusion of 4×10^{10} labeled platelets from donor rabbits, platelet count and specific activity were measured during the subsequent 30 min to 24 hr. Platelet specific activity was 20% to 45% lower in alloimmunized than in non-alloimmunized animals. Two patients with severe amegakaryocytic thrombocytopenia, alloimmunized by prior transfusions of platelets, were also studied. At the time of study, their platelet counts were less than 6,000/cumm. They had both been severely alloimmunized to donor platelets with survival times of allogenic platelets of 3 hours and 3½ hours and recovery values of 30% and 31% respectively. They were infused with 9×10^{11} ^{51}Cr -labeled platelets and platelet counts rose to 70,000/cumm. The percent difference between recovery values measured by platelet enumeration and those measured by ^{51}Cr was significantly greater than the difference observed in normal subjects indicating that also in human subjects the presence of alloantibody in the circulation can cause a significant increase in ^{51}Cr elution from the circulating platelets. In conclusion, these experiments

clearly indicate that hetero- and alloantibodies can cause loss of ^{51}Cr label from the platelets in the circulation. These findings may cast doubt on the accuracy of platelet survival and kinetics measured by ^{51}Cr in the patients with antiplatelet antibodies and will raise great controversy on several studies published by other investigators during the past few years (see papers #11 and abstracts 10 & 11).

4. PLATELET AGE AND ADHESION TO COLLAGEN (see list in Section 5, paper # 5)

Since Wright's original proposal that newly formed platelets have greater "stickiness" than platelets which have been circulating for longer periods of time (10), there have been many studies demonstrating that circulating platelets are not homogenous with respect to size, density, metabolism and hemostatic function and that these differences may be related to platelet age. Not all the platelet functions, however, showed a clear age-dependency. While adhesion to glass has been reported by us and other investigators to be a random phenomenon with respect to platelet age (11), Johnson et al (1971) (12) describing a patient with thrombopoietin deficiency, found a difference in the adhesiveness to glass between young and old platelet populations. Controversy exists also about adhesion to collagen as a function of platelet age. Hirsh et al (1968) though demonstrating no difference in ADP-induced aggregation between young and old platelets, found selective adhesion of young platelets to collagen utilizing an ingenious method of in vitro and in vivo radioactive labeling of platelet population (13). Spaet and Lejnieks (1969) on the other hand found young and old platelets adhering alike in the presence of adequate amounts of collagen (14). The platelet-collagen interaction is characterized by definite structural and steric requirements on part of the collagen. Biochemical mechanisms of adhesion between collagen and platelet have been proposed. In the light of these hypotheses and the physiologic importance of platelet adhesion to collagen, a study of the platelet age dependency of this process was undertaken emphasizing the rate of adhesion as a possible parameter for detecting differences in adhesiveness between young and old platelets.

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 paper #5).

5. EFFECT OF SEROTONIN ON CYCLIC NUCLEOTIDES OF HUMAN PLATELETS (see list in Section 5, paper #6)

Serotonin which can be taken up by platelets against high concentration gradients is stored in electron dense subcellular organelles and released during platelet aggregation. Serotonin itself has been shown capable of inducing weak platelet aggregation. Specific receptors for this substance have been suggested in the central nervous system. In platelets, a ganglioside has been identified with serotonin-binding capacity (15). There is good evidence that the reversible attachment of biologically active agents to specific sites of macromolecular structural elements may result in changes of their functional ability to synthesize substances which in turn may mediate cellular functions. Serotonin and cyclic nucleotides qualify in this context which prompted us to investigate their relationship in platelets. In our experiments, serotonin produced a 6 to 10 fold increase of cyclic GMP over baseline levels of this nucleotide in platelets. Maximum stimulation was reached within 30 sec to 1 min after addition of serotonin and was dependent upon its concentration in the medium. Inhibition of serotonin uptake by methysergide, dihydroergotamine and chloroimipramine did not influence the serotonin-induced stimulation of cyclic GMP but glutaraldehyde and formaldehyde blocked it completely. Cyclic AMP levels in platelets were not affected by serotonin. The serotonin-induced stimulation of cyclic GMP is, therefore, independent of the uptake of this biogenic amine by platelets and is not due to platelet aggregation (see paper #6).

6. RESPONSE OF CYCLIC NUCLEOTIDES TO STIMULATION IN
STORED HUMAN PLATELETS (see list in Section 5,
Abstract #14)

There is growing experimental evidence that cyclic nucleotides have a regulatory role in platelet aggregation. The two major cyclic nucleotides of platelets, cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) seem to exert opposing effects on this platelet function. Cyclic AMP was shown to inhibit platelet aggregation when it was added to intact platelets (16). An increase of platelet cAMP by stimulation of adenylate cyclase activity and/or by inhibition of cyclic nucleotide phosphodiesterase activity was found to be associated with an inhibitory effect on platelet aggregation (17). On the other hand, a variety of agents that can induce platelet aggregation especially when mediated by the release reaction have been shown to increase platelet cGMP levels (18).

We have been interested for some time in the mechanism responsible for the development of the so-called "storage lesion" of platelets. Current methods of platelet storage both at 4°C and 22°C are severely limited by the length of shelf life which is able to provide viable, hemostatically active platelets. When platelets are stored at 22°C, their viability can be preserved satisfactorily for up to 3 days but their hemostatic function deteriorates rapidly. Conversely, 4°C stored platelets quickly lose their viability while their aggregability is well preserved or even enhanced (19). Recently we (20) and others (19) have demonstrated that platelets stored in frozen state are viable and are moderately active hemostatically.

The possibility that cyclic nucleotides may play an important role in the rapid loss of platelet aggregability during storage at 22°C was investigated. We have carried out a number of experiments measuring the levels of cyclic nucleotides in human platelets stored for up to 2 days, and their ability to synthesize these nucleotides in response to specific stimuli. For comparison we have also measured these parameters in platelets stored at 4°C as well as in platelets stored by freezing.

Levels of cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) and their response to stimulation by prostaglandin E₁ (PGE₁) and 5-hydroxytryptamine (5-HT) were studied in human platelets stored for up to 48 hours at 22°C and 4°C. Cyclic nucleotides were measured by radioimmunoassay. Platelet concentrates were continuously agitated during storage. The prestorage levels of cyclic nucleotides were 27.0 ± 4.1 ($\pm$ 1SD) pmoles per 10^9 cells for cyclic AMP and 2.3 ± 0.3 pmoles per 10^9 platelets for cyclic GMP. Storage of platelets at 4°C for up to 48 hours resulted in no significant

change in these cyclic nucleotides. However, slow and progressive decline of cAMP was observed in platelets stored at 22°C. The intracellular concentration of cAMP dropped to 22.7 ± 2.6 pmoles after 24 hrs and to 20.6 ± 2.8 pmoles per 10^9 platelets after 48 hrs of storage at 22°C. Cyclic GMP level in fresh platelets was 2.3 ± 0.3 pmoles per 10^9 cells. Storage of platelets at 4°C for up to 48 hrs resulted in no significant change in this cyclic nucleotide. At 22° however, a moderately rapid decline in cGMP level was observed during storage. Frozen platelets were studied twice. The mean levels of cAMP and cGMP were 19.0 pmoles and 1.9 pmoles per 10^9 cells, respectively. These concentrations were not changed by exposure of platelets to 5% DMSO, or by storage for 10 days in the frozen state.

The ability of platelets to synthesize cAMP and cGMP in response to stimulation was studied in the stored platelets after stimulation at 37°C for 2 min in the presence of prostaglandin E₁ (PGE₁) or 5-hydroxytryptamine (5-HT). PGE₁ caused a 2 fold increase in cAMP in platelets stored at 22°C for 48 hrs when compared to fresh controls. Storage at 4°C did not change this response. Platelets stored by freezing exhibited a moderate decrease in their cAMP response to PGE₁ stimulation. Storage at both 22°C and 4°C demonstrated an enhanced response of cGMP production by stimulation with 5-HT. Freezing with DMSO caused a depression of the cGMP response to 5-HT. A severe depression of the cGMP response to 5-HT stimulation was observed in platelets stored at 22°C. These results of the platelets' capacity to synthesize cyclic nucleotides in response to stimuli correlate well with their aggregability.

Kinetic studies of the cyclic nucleotide responses to stimulation by PGE₁ were also studied. It was concluded that storage at any temperature produces changes in the cyclic nucleotide response of platelets to stimuli. The evidence obtained so far points to alterations in membrane receptor-sites as the primary cause of different rates of cyclic nucleotide production in stored platelets. We have obtained no indication that storage at 4°C or at 22°C impairs cyclic nucleotide production per se. This is probably the first clear demonstration that storage causes a clear damage in the platelet membrane.

A paper on these results was presented at the 1978 Federation meeting in Atlantic City. A publication is now being prepared.

7. EFFECT OF ALPHA-TOCOPHEROL ON LIPID PEROXIDATION
AND PLATELET AGGREGATION (see list in Section 5,
Abstract #4, #2 and paper #4)

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 phytyl 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 paper #4).

C. STUDIES OF CELL-TO-CELL INTERACTION USING PLATELETS AS
A MODEL SYSTEM

8. PLATELET INTERACTION WITH TUMOR CELLS

An interaction of platelets with malignant cells has been reported by several authors. Recently several strains of malignant cells were shown to induce platelet aggregation in vitro (21). A considerable amount of time was devoted to the development of in vitro culture methods for malignant cell lines. In initial experiments we screened several malignant cell lines for platelet aggregating capability. The following cell lines were investigated: two mouse ascites tumors (Ehrlich and S-180), a mouse fibroblast cell line (3T3), virally transformed mouse fibroblasts (WI38-VA13 and 3T3-SV40) and human epidermoid carcinoma cells (HEp-2). Ascites tumors were maintained in syngeneic mice (A/J mice from the Jackson Laboratory, Maine) by weekly transplantation. Tumor cells were obtained as ascitic fluid at various time intervals after intraperitoneal inoculation. After washing with Ca^{++} -, Mg^{++} - free Hanks' balanced salt solution (BSS), cells were tested for platelet aggregation. All other cell lines were maintained in tissue culture. 3T3 and WI38 VA13 were obtained from American Type Culture Collection (Rockville, Maryland), while HEp-2 and 3T3 SV40 were obtained from Flow Laboratory (Rockville, Maryland). In general, cells were subcultured every 7-10 days in 75 cm² plastic flasks with Dulbecco's modified minimum essential medium (MEM) supplemented with 10% fetal calf serum at 37°C. Cells were collected at subconfluent density by scraping with rubber policemen. After washing with Ca^{++} -, Mg^{++} - free Hanks' BSS, cells were tested for platelet aggregation. Anticoagulated blood was obtained by mixing in a plastic syringe 1 volume of anticoagulant (3.8% sodium citrate or 200 units of heparin in 1 ml saline) with 9 volumes of blood. Human, rabbit and mouse blood was used in different experiments. Platelet-rich plasma (PRP) was prepared at room temperature by centrifugation at 160 x g for 15 minutes. Platelet poor plasma (PPP) was obtained by centrifuging the residual blood at 2000 x g for 10 minutes. A Chrono-Log aggregometer was used for platelet aggregation studies at 37°C. The aggregation potential of tumor cells for platelets was tested by varying the concentration of tumor cells suspended in 0.05 ml of Ca^{++} -, Mg^{++} - free Hanks' BBS which were added to 0.35 ml PRP. The aggregometer was calibrated with PPP to express 100% and PRP to express 0%.

Two mouse ascites tumors were tested with rabbit PRP. One of these, S-180 (sarcoma) did not induce platelet aggregation. Ehrlich tumor (anaplastic mammary carcinoma) also failed to induce platelet aggregation when ascitic fluid was aspirated 1 week post-transplantation, but did induce platelet aggregation when cells were harvested 2 weeks post-transplantation. Light microscopic and electronmicroscopic examinations suggested that platelet aggregates were attached to macrophages and not to

Ehrlich cells. This was confirmed by utilizing mouse macrophages induced by intraperitoneal injection of mineral oil. These cells aggregated rabbit platelets in the same manner as Ehrlich ascites cells collected 2-3 weeks post-transplantation. As contaminating macrophages were shown to be responsible for the rabbit platelet aggregation, this experimental system was abandoned.

HEP-2 cells induced immediate but completely reversible platelet aggregation with both human and rabbit heparinized PRP. This aggregation rapidly disappeared as function of time and was probably induced by ADP released from damaged cells (22).

WI38-VA13, 3T3 and 3T3-SV40 induced irreversible platelet aggregation with heparinized rabbit PRP. The aggregation curves were characterized by long lag periods (1-2 minutes) followed by complete and irreversible aggregation. None of these cells induced platelet aggregation with citrated rabbit or human PRP. Platelet aggregation was completely inhibited by aspirin (1 mM final concentration) or by addition of a system which rapidly destroyed ADP, viz. creatinine phosphate (CP, 100 mM)-creatinine phosphokinase (CPK, 40 μ g/ml) (23). These findings indicated that platelet aggregation induced by the above cell lines was associated with a secretory release. Citrate added as anticoagulant also completely inhibited platelet aggregation indicating that tumor cell-platelet interactions were strongly dependent on divalent cations. The possibility that thrombin generated in the heparinized PRP was responsible for the observed platelet clumping could be ruled out by an experiment in which hirudin, a specific inhibitor of thrombin, was added to the aggregation system in a final concentration of 1.5 U. Platelet aggregation was not inhibited under these conditions. When tumor cells were collected by brief exposure of the culture to trypsin (1:250), their capacity to aggregate platelets was lost. Trypsinized cells, however, were able to recover their platelet aggregating activity within 24 hrs by incubation in Dulbecco's MEM at 37°C.

To determine the role of divalent cations in the tumor cell-induced platelet aggregation, calcium and magnesium were added to citrated rabbit PRP containing 50 units heparin per ml to prevent fibrin formation. Addition of calcium up to a final concentration of 120 mM did not restore platelet aggregation but addition of magnesium overcame the inhibitory effects of citrate in concentrations as low as 0.8 mM. Thus it was concluded that the inhibitory effect of citrate on platelet aggregation induced by tumor cells was due to chelation of magnesium, and not due to depletion of calcium.

Our results can be summarized as follows:

- (1) Selected strains of tumor cell lines have platelet aggregating activity in heparinized plasma but not in citrated plasma.

- (2) Inhibition of platelet aggregation by citrate is due to its chelation of magnesium ions but not due to chelation of calcium ions.
- (3) Platelet aggregation induced by tumor cells is similar to aggregation induced by collagen in that it is preceded by a long lag period followed by irreversible aggregation.
- (4) Aggregation is accompanied by platelet release reaction, which is completely inhibited by aspirin or CP/CPK system.
- (5) Trypsinization of tumor cells abolishes platelet aggregation. Their aggregation potential can be restored by incubating the tumor cells in culture medium.

9. STUDIES ON PLATELET MEMBRANE FLUIDITY. THE EFFECT OF α -TOCOPHEROL (see list in Section 5, Abstract #12 and paper #10)

Studies in this subject area have concentrated until this time on the determination of the fluidity of normal platelet membranes. The method we are using allows us to obtain only an indirect measure of the microviscosity of platelet membranes but being based solely on actual, observed data it is not subject to the extrapolations and assumptions which have to be made in order to translate the results of fluorescence polarization measurements into microviscosity values. In our studies the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) whose vertical orientation with respect to the plane of the membrane has been well established, was used. Fluorescence polarization provides an estimate of the resistance to rotation of the microenvironment of the fluorescent probe. The hydrocarbon fluorophore DPH is localized in the nonpolar regions of membranes and is highly efficient in its emission of fluorescence. Briefly outlined, the fluorescence studies utilized a 2 mM stock solution of DPH which was prepared in tetrahydrofuran. Aqueous suspensions of DPH were prepared by diluting 1 vol of DPH stock solution into 1000 vol of phosphate buffered saline (PBS; 50 mM phosphate buffer, pH 7.3 containing 145 mM NaCl) at room temperature. The suspensions were stirred for a minimum of 2 hours, after which time little or no odor of tetrahydrofuran and only negligible fluorescence were noticeable. This DPH suspension was rapidly mixed with an equal volume of platelet or liposome suspension and incubated at 37°C for 1-2 hours. Initial experiments showed that DPH uptake leveled off within 30 min when the fluorescent probe was labeling intact platelets. Utilizing intact platelets, $2-5 \times 10^7$ cells/ml were incubated with the DPH suspension at a final concentration of 1 μ M. Fluorescence polarization and fluorescence intensity were measured with an MPF-44A Perkin-Elmer fluorospectrophotometer equipped with a temperature-controlled cuvette holder and a pair of polarizers. Measurements were made with an exciting wavelength of 360 nm and an emission wavelength of 430 nm. Exciting light was cut off by insertion of an appropriate filter. The instrument was used in ratio mode. Control samples of DPH suspensions alone and of unlabeled platelets or liposomes were examined with each experiment. These readings which amounted to approximately 5% and less than 3% of the total fluorescence for platelets and liposomes, respectively, were neglected. The rotational motion of the fluorophore was expressed as P_1 degree of polarization which was measured at right angle to the polarized excitation beam.

$$I_{11} - I_1 \text{ (G)}$$

$$P = \frac{I_{11} - I_1}{I_{11} + I_1} \text{ (G)}$$

I_{11} represents the fluorescence intensity parallel to and I_1 the intensity perpendicular to the plane of polarization of

the excitation beam. G is a correction factor for the inability of the instrument to transmit differently polarized light equally.

Fluorescence polarization was studied as a function of temperature in the range from 4°C to 44°C. There were no phase transitions recognized in platelet membranes. There were no appreciable differences in the temperature dependence of fluorescence polarization, P_1 of DPH-labeled intact platelets, isolated platelet membranes or liposomes prepared from the lipids of platelet membranes.

These investigations are now being extended to modified platelet membranes. Various free fatty acids (saturated and unsaturated) are being inserted into platelet membranes and their effect on membrane fluidity by the method described above evaluated. These studies are now in progress.

Alpha-tocopherol which produced an inhibition of the platelet release reaction induced by any of the commonly used aggregating agents also affected the fluorescence polarization of DPH-treated platelets. At temperatures <27°C the addition of α -tocopherol to intact platelets produced a moderate increase in fluorescence polarization but at temperatures >27°C α -tocopherol caused a slight decrease compared to normal, non-vitamin E-enriched platelets. Liposomes prepared from the lipids extracted from platelet membranes and then treated with α -tocopherol showed markedly increased polarization values over the entire temperature range tested. Preparation of liposomes from platelets of subjects on supplemental vitamin E showed no or a moderate decrease in fluorescence polarization. These studies were interpreted as possible evidence for a close association of α -tocopherol with boundary lipids and a moderate enhancement of platelet membrane fluidity at physiological body temperatures by vitamin E. The significance of reduced DPH fluorescence polarization of α -tocopherol-treated platelets is not clear at this time. However, it is noteworthy that recent studies suggest that high microviscosity values increase the availability or rotational mobility of surface receptors (24). The question whether the opposite behavior of surface receptors characterizes reduced microviscosity is still unresolved. Although it may seem reasonable to hypothesize that increased rotational mobility of surface receptors means increased aggregability and vice versa for decreased rotational mobility, the experimental evidence to support this theory is still incomplete. Another line of evidence, however, which is more firmly supported by experimental observations, seems to suggest a similar conclusion. High cholesterol content of membranes is relatively closely correlated with increased microviscosity (24,25). Platelets artificially loaded with cholesterol as also platelets from patients with a familial hyperlipoproteinemia associated with high cholesterol content have been reported to exhibit enhanced aggregability (26). One could speculate, therefore, that decreased fluorescence polarization may be associated with reduced aggregability of platelets. Although reduced polarization values cannot be directly equated with reduced microviscosities, the 2 parameters have similar trends.

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SECTION 3.

PLANS FOR CONTINUATION. NEW OBJECTIVES

Our research interests which were supported formerly by the Atomic Energy Commission, later by the Energy Research and Development Administration, and presently by the Department of Energy, have remained unchanged over the years focusing on the pathophysiology of blood platelets. Our specific aims in this framework of research have, however, undergone a considerable change. While in earlier studies our efforts concentrated mainly on various aspects of platelet preservation, in particular on maintaining functionally competent platelets which could survive on reinfusion, more recently our studies have turned towards exploring the basic mechanisms of some of the platelet functions.

The studies that we are presently pursuing represent an in depth investigation of certain aspects of the interaction between cells and the influence exerted by micro- and macro-environment. As a model system to examine this biological process in detail we have chosen the platelets whose primary physiological function depends upon contact phenomena, mainly cell to cell interactions.

In our current activities we are utilizing the platelet as a model for the study of interactions between cells of the same and different type. There are three specific areas in which we are concentrating our efforts: The interactions between platelet-to-platelet, between platelet-to-vessel wall, and between platelet-to-tumor cells. As this covers an extensive field we have narrowed our objectives to certain well defined problems:

I. One problem that we are presently investigating tries to establish a relation between the lipid composition of platelet membranes and their fluidity. Measurement of temperature-dependent changes in fluorescence polarization of fluorescent probes inserted into the plane of membranes in well characterized orientations allows conclusions to be drawn about the environmental resistance to motion of such probes. These measurements thus are related to the "microviscosity" of the area of insertion of the probe. Studies are now underway in our laboratory to clarify the functional significance of changes in microviscosity in platelet membranes. An increase in microviscosity has been shown by others to occur in cholesterol-rich platelets and was correlated to their enhanced aggregability and possibly thrombogenicity. In our own studies we have demonstrated that α -tocopherol reduces fluorescence polarization of diphenylhexatriene-treated platelets at temperatures above 25-27°C. Vitamin E treatment of platelets in vitro has also been shown to decrease platelet aggregation thus a relationship between membrane fluidity and aggregability may exist. We are

trying to extend these findings to other platelet functions which are initiated by an interaction of platelet plasma membranes with specific stimuli and conversely determine the effect of modifying the lipid composition of platelet membranes on various platelet functions and their microviscosity. Investigations of this type should clarify the physiological significance and role which fluidity plays in mediating membrane-related cellular functions. This area of research is still in its infancy and the implications for understanding the role of the plasma membrane in cellular functions are far reaching.

II. The second objective of our research is the investigation of platelet interaction with tumor cells. We have chosen this topic as it represents an example of interaction between different cells which is little understood yet of great practical and theoretical importance. There is good clinical and experimental evidence that platelets and tumor cells influence each other. Changes in number and size of metastases produced by certain tumor cell lines have shown a positive correlation to the number of platelets in the circulation. On the other hand, tumor cells may cause platelet aggregation either by direct contact between platelets and neoplastic cells or by release of an aggregation-inducing principle from the tumor cell. The existing literature in this field is quite unanimous in its conclusion that these phenomena are dependent upon the type of tumor cell used and upon maintenance of a relatively high concentration of divalent cations. The specific problems which we are working on are the demonstration of an aggregation-inducing factor elicited by tumor cells, its characterization and investigation of its mechanism of action. Virus-transformed 3T3 fibroblasts have produced aggregation of platelets which could be shown to depend on a high concentration of free Mg^{2+} in the medium. The interaction of these neoplastic cells with platelets initiates aggregation which is similar to that produced by physiological aggregation stimuli in being mediated by an ADP-dependent mechanism. We have also tested other cell lines and continue to do so. Non-virus transformed 3T3 cells are available as controls which makes this system very attractive for more extensive study.

Growth-promoting factors for several tissues and cells have been demonstrated in the platelet. Whether a growth-stimulating effect may also be seen with neoplastic cells is unknown. This is another area which we like to explore.

III. The third objective of our research is to advance the understanding of the interaction of platelets and vessel wall. As mentioned above platelets contain and are able to release growth-promoting substance(s) which stimulate the proliferation of smooth muscle cells. A role of platelets in the proliferative response to endothelial injury has been suggested by several

investigators who demonstrated a positive correlation between platelet count and magnitude of proliferation of smooth muscle cells. The nature of the growth factor(s) elicited by platelets is not known yet although studies in this area are progressing in other laboratories. The rationale for our studies of this problem is based on the observation that infusion of heparin was an effective means of limiting the proliferative response following an intimal injury. As thrombin's action is neutralized effectively by antithrombin III, the natural inhibitor of this clotting factor, through a mechanism which is markedly enhanced by heparin, we thought it important to investigate the possible role of antithrombin III in modifying the response to intimal injury. This plasma protein is able to bind fatty acids, aspirin and as we are showing now estrogens and other steroids. There is some evidence that such binding may change the inhibitory activity of antithrombin III and thus could alter the amount of thrombin available to exert a mitogenic action. We speculate that there may exist a variety of other substances which bind to antithrombin III and which may change its capacity to inhibit serine proteases. Of obvious interest would be the study of substances which have been linked to increased arteriosclerotic heart disease in human subjects. Lipids, prostaglandins, tobacco products and cadmium are but a few of the substances which are going to be examined for their effect on endothelial injury-induced smooth muscle cell proliferation. Studies of this problem have only recently begun and at this time are still concentrating on the development of optimal conditions for the experiments to be conducted in animals. The main problem is of a technical nature. It is required that animals (rats) move freely as they are perfused for days through a section of artery in which an endothelial injury had been induced. Concomitant experiments on the ligand activity of purified antithrombin III for a number of compounds described above are being carried out.

Although in these experiments the platelet is not taking center stage we are going to investigate its role in the response to endothelial injury especially with respect to characterization of the putative growth factor and conditions necessary for its release from platelets. We hope that the experiments we briefly sketched in these pages will provide the basis for further studies in the area of cellular interaction. The problems to which we address ourselves at present are quite specific and rather narrowly defined nevertheless each one concerns a field of research which may have far-reaching implications both on a basic and on a clinical level.

SECTION 4

INVESTIGATORS, POST-DOCTORAL FELLOWS AND STUDENTS WHO WORKED ON THIS PROJECT

The professional personnel of the Division of Hematologic Research who worked on this project included "permanent" and "non-permanent" investigators. The latter are engaged by the Division for a one-two year period of research and one year of clinical training in hematology, after which they return to their respective academic institutions.

The "permanent" investigators who worked actively on this project during the past three years were:

1. Mario G. Baldini, M.D., Director of Project (3 years)
2. Manfred Steiner, M.D., Ph.D., Associate Director (3 years)
3. Byung K. Kim, M.D., Senior Research Associate (3 years)

Post-doctoral Fellows in training were:

1. Yasuo Ikeda, M.D., Keio University, Tokyo, Japan
Research Associate (2 years)
2. Toshiro Nagasawa, M.D., Tsukuba University, Chiba, Japan
Research Associate (2 years)
3. Hiroshi Nagasawa, M.D., Tokyo Medical College, Tokyo, Japan
Research Associate (2 years)
4. Yoshikuni Hara, M.D., Keio University, Tokyo, Japan
Research Associate (1 year)
5. Kousaku Sugiura, M.D., Keio University, Tokyo, Japan
Research Associate (1 year)

Undergraduate and Postgraduate students were:

1. John Anastasi, Senior undergraduate student at Brown University (six months)
2. Robert M. Castellan, Senior Medical student at Brown University (two Summer periods)

The following Research Technologists were utilized:

1. Donna Field, Senior Research Technician (3 years)
2. Jane Raymond, Senior Research Technician (2 years)
3. Elaine Therien, Research Technician (2 years)
4. Joyce Merchant, Research Technician (2 years)

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SECTION 6.

NEEDED AND PLANNED FUTURE INVESTIGATIONS

We believe that an in depth investigation of certain aspects of the interaction between cells and the influence exerted by micro- and macro-environment is of great importance. As a model system to examine this biological process in detail, we have chosen the platelet whose primary physiological function depends upon contact phenomena. We are presently investigating three important aspects of cell to cell interaction and are examining with each those factors in the micro- and macro-environment (including also the diet) which may modify the anticipated response:

The first concerns the interaction between cells of the same type, exploring the phenomenon in relation to the lipid composition of platelet membranes. We postulate that their lipid component which is easily modified through exchange with the environment of the cell, influences the lateral diffusion of membrane proteins and can change the adhesive properties of platelets towards themselves or towards other cells and substances. We are approaching this problem by preparing liposomes of specific composition which will be allowed to exchange their lipids with those of platelet membranes. The change in lipid structure will then be correlated with possible alterations in the fluidity of these membranes which will be analyzed by measurement of the fluorescence polarization of certain lipophilic fluorescent probes inserted into the platelet membrane. Membrane fluidity or microviscosity will be related to the aggregative and adhesive properties of the platelet.

The second aspect of our present interests concerns the interaction between unlike cells. This phenomenon will be studied in terms of the contribution of both platelets and tumor cells to the interaction. The nature of the aggregation-inducing factor and of the adhesive characteristic of certain tumor cells for platelets is being investigated with tumors grown in vivo or propagated as ascitic malignancies. How platelets respond to contact with tumor cells and whether they contain and can release a growth factor for the latter represents another facet of this interaction that we believe deserves study.

A third problem of great interest is the interaction between platelets and intimal smooth muscle cells. This is an interaction which does not require intimate contact between the two reactants but relies on factors having long-range activity. Although the putative growth factor for intimal smooth muscle cells evolved by platelets is one subject of investigation, another line of study will be pursued in parallel which has at its center the role of antithrombin III in the intimal response to

injury. This area of research is one of great current interest as it relates to the pathogenesis of atherosclerosis. There are arguments in favor of a regulatory role of this inhibitor protein and we will do a series of experiments to test our hypothesis. As there is good evidence that this protein which occurs in center stage of the whole hemostatic process may be influenced in its activity by a variety of substances of small molecular size we are planning a detailed study of factors derived from the environment and largely toxic in nature which have been shown to be linked to an increased risk in the development of atherosclerosis. In our present studies our long-standing interest in platelets finds a fortuitous merger with an attempt to study a fundamental biological process. Although basic in nature, all of these studies have important clinical implication not only for the treatment but also the prevention of widespread disabling diseases.