PROGRESS REPORT

DOE Contract EY-76-S-02-2783

November 1, 1978 to October 31, 1979

"Physiopathology of Blood Platelets: A Model System for Studies of Cell-to-Cell Interaction"

INTRODUCTION

The enclosed Progress Report covers the work done during the past year of this project. Progress has been made in all the different areas which our proposal encompasses. However, in the section on "Platelet to platelet interaction," we had difficulties which somewhat delayed our work. In fact, in a study of platelet membrane fluidity, a defective instrument (a gaschromatograph) slowed our progress and only now are we again able to proceed with our studies. In the meantime, we have performed studies which fall into the category of platelet-macrophage interaction. Substantial progress and results have been obtained which are summarized in one of the sections of this report. Our specific aims have remained as previously stated, that is directed towards basic mechanisms of cellular interactions, utilizing platelets as a model system and, when possible, concentrating on the influence that environmental factors (nutritional, metabolic, cellular, and others) have on them.

In this report, we will limit ourselves to the detailed description of four major sections of our research done during the past year:

A list of the publications completed during the past year is given here in the following section. Reprints, manuscripts and abstract photocopies are included with the progress report:

Abstracts removed

1. Sugiura, K., M. Steiner and M. Baldini. "Characterization and Measurement of Normal Platelet Associated Immunoglobulin G by Fluorospectrophotometry." J. Biological Chem. Removed

2. Sugiura, K., M. Steiner and M. Baldini. "Physiological Significance of Nonimmune Platelet Associated Immunoglobulin G." In publication, J. Clin. Invest. Removed

3. Sugiura, K., M. Steiner and M. G. Baldini. "Platelet Antibody in Idiopathic Thrombocytopenic Purpura and other Thrombocytopenias." In publication, New Engl. J. Med. Removed

DISCLAIMER

4. Hara, Y., M. Steiner and M. Baldini. "Characterization of Platelet Aggregating Activity of Tumor Cells." In publication, Cancer Research. Removed

5. Hara, Y., M. Steiner and M. G. Baldini. "Platelets as a Source of Growth Promoting Factor(s) for Tumor Cells." In publication, Cancer Research. Removed

6. Kim, B. K. and M. Steiner. "Evaluation of Cyclic Nucleotide Responses of In Vitro-Aged Platelets." In publication, Proc. Int. Workshop, Oklahoma, Elsevier, N. Holland Publishing Co. Removed

7. Kim, B. K., M. Steiner and M. G. Baldini. "Response of Cyclic Nucleotides to Stimulation by Prostaglandin E_1 and 5-Hydroxytryptamine in Stored Human Platelets." In publication, Thrombosis Research. Removed

Publications which appeared in abstract form are listed as follows:

All the investigators listed in our project proposal worked hard on the individual projects during the past year. These included:

1. Mario G. Baldini, M.D. - Principal Investigator (20% of time)
2. Manfred Steiner, M.D., Ph.D. - Co-Principal Investigator (15% of time)
3. Byung K. Kim, M.D. - Senior Investigator (85% of time)
4. Toshiro Nagasawa, M.D. - Co-Investigator (100% of time)
5. Yoshikuni Hara, M.D. - Co-Investigator (100% of time)

Collaborating non-professional personnel were:

1. Susan Berry, Senior Research Technician (100% of time)
2. Jane Raymond, Senior Research Technician (50% of time)
3. Dennis Erminelli, animal and laboratory caretaker (50% of time)

A. Platelet Interaction with Tumor Cells

We have studied 2 aspects of this interaction, the platelet aggregating principle of certain tumor cells and the platelet-derived growth promoting factor(s) for tumor cells. The following presents a summary of the most important findings. A more detailed account of our studies can be found in the 2 manuscripts entitled "Characterization of Platelet Aggregating Activity of Tumor Cells" and "Platelets as a Source of Growth Promoting Factor(s) for Tumor Cells' which have been submitted for publication.

We have examined a series of animal tumors of which two, viz. mouse renal adenocarcinoma (RAG) and mouse neuroblastoma cell line (Neuro-2a) were found to possess aggregating activity for heparinized mouse or rabbit platelets. A closer examination of tumor cell-induced platelet aggregation revealed that addition of sodium citrate in concentrations of $\geq$ 0.19% completely inhibited aggregation. Mg^{2+} in concentrations > 0.8 mM overcame the citrate-induced inhibition. On the other hand, Ca^{2+} even up to 120 mM failed to restore platelet aggregation. Tumor cell membrane fragments isolated by incubation of tumor cell cultures with low ionic strength media were able to induce platelet aggregation in a dose-dependent manner. The aggregating principle of tumor cells extracted by this method was precipitable by centrifugation at 2,600 x g. Platelet aggregation induced by RAG cell membrane fragments was completely inhibited by preincubation of platelets with acetylsalicylic acid and by an ADP clearing system consisting of creatine phosphate:creatine phosphokinase. We found no indication that procoagulant activity derived from tumor cells was responsible for the observed effect. Hirudin, a potent inhibitor of thrombin did not change tumor cell-induced platelet aggregation. The aggregating principle was shown to be susceptible to destruction by heat at 85° but not at 56°, to trypsin digestion, to the action of neuraminidase, and phospholipase A_2 and ionic and non-ionic detergents. Also sonication completely abolished the aggregation-inducing potential of tumor cell membrane fragments. It was interesting to note that growth promoting activity for RAG cells was found in the supernatant of platelets aggregated by RAG cell membrane fragments. Control experiments ruled out the possibility that the growth promoting factor(s) were derived from sources other than platelets. Attempts to extract the platelet aggregating material from tumor cells as a soluble fraction were unsuccessful. Possible experimental approaches to circumvent this difficulty are suggested in the renewal proposal of this contract.

These results underline the importance of platelets in establishing and maintaining tumor metastases. They also indicate that the aggregation-inducing principle of tumor cells needs a highly organized structure to exhibit this function. Quite likely, it may be a polymer of a membrane polypeptide which needs the lipid matrix of the tumor cell membrane to form.

In other experiments we were able to show that human platelet lysate had growth promoting factor(s) for various tumor cell lines. Our studies demonstrated that platelet lysate promoted the growth of mouse neuroblastoma (Neura-2a), mouse renal adenocarcinoma (RAG), rat Leydig cell testicular tumor (R2C) and mouse mammary tumor (MMT 060562). The lysate of human platelets contained "survival factor," being able to support tumor cell proliferation in the absence

of plasma or serum, as well as mitogenic factor. The platelet-derived stimulation of tumor cell growth was dose-dependent and could be readily released into the supernatant from aggregated platelets. Inducers of α-granule release such as thrombin and collagen were found to release the growth promoting factor(s) but ADP which does not induce significant release from α-granules was ineffective. The growth promoting activity was non-dialyzable, was relatively heat stable at 56°C but was degraded at temperatures above 70°C, was sensitive to trypsin and lost its growth stimulant effect by periodate oxidation. Non-specific protease inhibitors were unable to inhibit its activity.

These results clearly indicate that platelets can support the growth of tumor cells. The factor(s) responsible for this effect appear to differ from those that promote growth of normal smooth muscle and fibrous tissue cells. Efforts are now underway to obtain the platelet-derived growth promoting activity for tumor cells in a soluble form. These studies are outlined in the renewal proposal of this contract.

B. Studies of the Interaction of Platelets with Macrophages

Experiments were performed to determine the amount of and the physiological significance of platelet-associated immunoglobulin G (PAIgG). Even normal platelets have a substantial quantity of IgG attached to their surface. One of the objectives of this study was the development of a sensitive and rapid method for the quantitive assay of this protein. We were able to accomplish this by the use of a fluorescent probe, fluorescein isothiocyanate (FITC) conjugated with purified human IgG. A major effort was directed at estimating the nonspecifically trapped IgG and determining the optimal conditions for preparing platelets for the assay. Our studies revealed that PAIgG could be readily mobilized from platelets by allowing them to remain for 30 - 45 min at room temperature. From the accurately measured molar ratio of FITC/IgG protein we were able to quantify the normal amount of PAIgG and enumerate its binding sites. High and low affinity binding sites for IgG were identified on platelets. Scatchard plot analysis of the binding data revealed 410 ± 200 high and 1800 ± 500 low affinity binding sites. The effect of pH and temperature was evaluated. Binding was greatest at 4°C, it was less at 22°C and least at 37°C. The kinetics of association and dissociation of PAIgG were evaluated. K_d, the dissociation constants for the high and low affinity binding sites were 3.3×10^{-7} M and 8.9×10^{-6} M, respectively. Our studies gave evidence that prebound PAIgG could be dissociated quite readily from the high affinity binding site with an apparent T/2 of 10 min.

The specificity of binding of FITC-conjugated IgG is essential to prove the accuracy of our assertion that IgG receptor sites were studied. Competition experiments with non-FITC-conjugated, normal human IgG were performed at 2 different concentrations of nonfluorescent IgG. The observed inhibition was found to be competitive in nature. The K_i's for low and high affinity sites were calculated from double reciprocal and Dixon plots. The K_i for the high affinity site which varied between $2.9 - 3.7 \times 10^{-7}$ M was almost equal to the K_d of 3.3×10^{-7} M. The K_i for the low affinity site was about 1.5 - 2 times larger than its respective K_d. All of these studies were performed with IgG which was 99% pure as demonstrated by gel electrophoresis and by immunoelectrophoresis. In addition no aggregated IgG was present in our final preparation used for these experiments as demonstrated by analytical ultracentrifugation. We found the method of PAIgG assay rapid, quantitative, sensitive and inexpensive.

In other experiments, we focused on the physiological significance of platelet-associated immunoglobulin G. In a series of experiments we investigated this problem, relating amount of PAIgG to platelet volume, serotonin release, adherence of platelets to monocytes (macrophages) and platelet senescence. Most of these studies were performed with human platelets. Platelets were freed of preexisting PAIgG by incubation at 22°C for 30 - 45 min. Such platelets were subsequently incubated with IgG in a series of concentrations ranging from 0.4 - 27.0×10^{-6} M. The amount of PAIgG bound to platelets was determined by the use of FITC-conjugated anti-IgG antibody. The latter was measured by fluorospectrophotometer after solubilizing the washed platelets by addition of sodium dodecyl sulfate. Nonspecifically trapped IgG in the platelet pellet was estimated and subtracted from the apparent quantity of IgG calculated from the fluorescence and the molar FITC/IgG protein ratio.

Platelet volume increased with the concentration of IgG in the medium. The maximal volume increase which was observed at an IgG concentration of 13.5 x 10^{-6} M was 4.1%. At that concentration of IgG 97.6% of the high affinity sites and 60.2% of the low affinity sites were saturated. The volume increase was almost completely reversible over a 30 - 45 min time interval. The volume increase associated with the binding of nonimmune IgG to platelets was fairly evenly distributed over the whole range of platelet sizes. The platelets treated with IgG in these experiments did not show any sign of shape change.

Platelets which were incubated with IgG released serotonin. The release increased sharply up to a concentration of 1.6 x 10^{-6} M IgG. From then on it rose slowly but steadily over the whole IgG concentration range that was tested. There was no apparent aggregation of platelets and the serotonin release when expressed as a fraction of the total [^{14}C] 5-hydroxytryptamine incorporated into platelets amounted only to 3 - 5%. As it was conceivable that platelets exposed to IgG could have an increased membrane permeability leading to such small release of serotonin from the cytoplasmic pool of this amine, we determined the volume change associated with the preparation of the platelets subsequent to their incubation with IgG. A minimal decrease in platelet volume was observed but its magnitude stood in no relation to the amount of serotonin released. Therefore, this possible explanation for the observed release of serotonin can be discarded.

As our working hypothesis surmised an opsonizing effect for the absorbed nonimmune platelet IgG we developed a quantitative assay utilizing an electronic cell sizer to assess this effect. Monocytes isolated by standard procedures from buffy coat layers of human blood were incubated with platelets exposed to varying concentrations of IgG. Adherence of the IgG-coated platelets to autologous monocytes (cells derived from the same donor as the platelets) was measured after fixation with formaldehyde. Our results showed a very clear increase of platelet adherence to monocytes with rising concentration of IgG. A plateau was reached when monocyte volume was increased by 14.8% above the control. This volume increase corresponds to 45 μm^3 and equals the volume of 7 - 8 platelets. A similar phenomenon was seen when platelets of patients with idiopathic thrombocytopenic purpura (ITP) were incubated with donor monocytes. A distinct and significant increase in adherence to monocytes was noted when ITP patients' platelets were compared to platelets of normal, healthy males. There was no or very little difference between the adherence of autologous and allogenic platelets of normal, healthy males to monocytes. The amount of PAIgG measured on the surface of platelets from ITP patients was significantly higher than that of control platelets.

Finally, we measured PAIgG and IgG binding sites in platelets of varying age. These experiments were performed with rabbit platelets. After producing platelets cohorts of different age during the recovery phase of immunologically induced thrombocytopenia in rabbits, available binding sites for IgG were measured by the use of fluorescent-labeled rabbit IgG. The number of binding sites increased with the age of the cohorts. The cohort consisting of 1, 2, 3 and 4 day old platelets was able to bind 1.8 times as much IgG as the cohort of 1 day old platelets. The PAIgG already present on the different aged platelets was measured with FITC-conjugated anti-rabbit IgG antibody. It also increased in an age-dependent manner. The cohort consisting of a mixture of 1 - 4 day old platelets had 1.9 times as much IgG bound as 1 day old platelets.

Serum had a significant inhibitory effect on the binding of IgG and also of anti-IgG antibody. Both normal and IgG-deficient sera inhibited the binding of immune and nonimmune IgG in a noncompetitive manner.

These results suggest that PAIgG plays a role in the clearance of senescent platelets. We assume that the age-dependent increase in PAIgG enhances the likelihood of such platelets to adhere and finally to be phagocytosed by macrophages. The increase in the number of potential binding sites for IgG in aged platelets is in line with this hypothesis.

Finally, the measurement of PAIgG preexisting on platelets was developed into a quantitative, clinically useful test whose applicability was explored in a considerable number of normal control individuals and a series of patients with immune and non-immune thrombocytopenias. We evaluated the variables of the method which uses FITC-conjugated anti-human IgG antibody to measure PAIgG. The test as set up in our laboratory is performed with an antibody concentration low enough to assure that under the usual circumstances the antibody/antigen ratio would not exceed 1.0. However, as this ratio also varies with the level of antigen, simple subtraction of the control value will somewhat overestimate the PAIgG in the sample because the low correction values of the control correspond to ratios < 1.0. Since most of our IgG measurements fell within a rather narrow range of values we have taken a constant binding ratio of 0.6 which provides close enough approximation in all but a very few experimental results. We have found an antibody concentration of 1×10^{-7} M most satisfactory for our experiments.

In developing an assay for the detection of PAIgG which is to be applicable also in pathological conditions we have attempted to set up a system by which platelet-reactive IgG in the serum can be absorbed by allogenic platelets freed of their indigenous PAIgG. The amount absorbed from the test serum could be quantified as described above. A range of normal values has been determined both for the direct assay, i.e. the measurement of platelet-bound IgG and for the indirect test which evaluates the amount of platelet specific IgG in the serum which can be absorbed by normal, control platelets (from donors of type 0, Rh+ blood). All patients with immune thrombocytopenias (acute and chronic idiopathic thrombocytopenic purpura and systemic lupus erythematosus) had elevated PAIgG values by the direct assay provided their platelet count was $\leq$ 100,000/µl. The indirect assay with sera from these patients gave positive results in 86% of the cases. All non-immunological thrombocytopenias showed normal results in these assays. In cases studied repeatedly during the course of their illness, an inverse relation was found to exist between platelet count and level of platelet-bound IgG. Patients with systemic lupus erythematosus presented exceptions to this rule in exhibiting unusually high levels of PAIgG which remained present even when the platelet count was in the normal range. Therefore, the IgG coating platelets in this group of patients does not appear to have an opsonizing effect. The latter, however, was clearly found in patients with auto- and alloantibodies.

Piloting experiments to develop this test into an assay of predictive value in patients who were alloimmunized have been carried out. We were able to demonstrate preferential absorption of alloantibodies by platelets of some donors but not by those of others. In one case we were able to show that one parent's

platelets were able to absorb the alloantibodies present in a patient's serum while the other parent's platelets did not absorb it. These results could be shown to correspond with the in vivo survival of ^{51}Cr-labeled platelets from the 2 parents in the patient. The first parent's platelets which avidly absorbed antibody survived very briefly while the 2nd parent's platelets which did not absorb the patient's antibody survived normally. These provocative results have encouraged us to seek further support for more extensive research in this field (see renewal proposal of contract).

C. Interaction of Platelets with Vessel Walls

So far we have concentrated on the isolation and characterization of antithrombin III. Antithrombin III is the natural inhibitor of thrombin through a mechanism which is markedly enhanced by heparin. It is known that infusion of heparin is an effective means of limiting the proliferative response of smooth muscle cells following intimal injury. It is our intention to investigate the possible role of antithrombin III in modifying the response to intimal injury. Furthermore, it is becoming increasingly clear that antithrombin III, a natural inhibitor of a variety of serine proteases, occupies a center stage position in hemostasis. Irrespective of whether the factors of the clotting cascade, platelet aggregation or fibrinolysis are considered, antithrombin III has a significant role in each area of the hemostatic process.

Specifically, our proposal is rooted on one hand in our finding that antithrombin III is a protein which can bind certain steroid hormones and as a result of this ligand activity its inhibition of thrombin and presumably of other serine proteases is altered. The second reason for this study is the recognition that a variety of different small molecular weight substances can interact with antithrombin III (1). It still remains to be established what effect such interaction has on the biological function of antithrombin III, but deviations from its normal activity can be expected at least for some of the substances which interact and bind to the inhibitor.

During the past year, as a first step in investigating possible modifications of the biological function of antithrombin III, we purified this inhibitor from human fresh plasma and verified its homogeneity.

The purification procedure was essentially based on the method of Abildgaard (2) as modified by Rosenberg et al. (3). The results are summarized in Table 1. The initial steps of heat defibrination, elimination of several coagulation factors by $BaSO_4$ adsorption and $Al(OH)_3$ gel adsorption-elution were performed as originally described by Abildgaard (2). The eluate of the $Al(OH)_3$ gel was concentrated by the use of an ultrafiltration method and it was then applied on a Sephacryl S-200 column (2.5 x 90 cm). Tris-HCl buffer solution (0.05 M, pH 8.3) containing 1 M NaCl was used as the eluent and the flow rate was controlled at 20 ml per hr by a peristalic pump. Fractions of 10 ml were collected and assayed for antithrombin III activity and protein content. Activity of the inhibitor in each fraction was measured according to a two-stage assay method similar to that of Gerendas (4) described by Rosenberg et al. (3). The original defibrinated plasma kept at 4°C was used as the standard for assay of the inhibitor activity and was arbitrarily set at one unit per ml. Protein content of the column effluent was recorded by measurement of absorbance at 280 nm.

Pooled fractions of antithrombin were exhaustively dialyzed against 0.1 M Tris-HCl buffer, pH 8.3, then, a linear gradient ion-exchange chromatography was performed on a DEAE-cellulose column (2.5 x 40 cm). A linear salt gradient was performed with the mixing chamber containing 600 ml of 0.1 M Tris-HCl buffer, pH 8.3 and the reservoir containing 600 ml of 0.3 M NaCl in 0.1 M Tris-HCl buffer, pH 8.3. Flow rates were maintained at 40 ml per hr with a peristalic pump and fractions of 6 ml were collected. The peak of inhibitor activity centered at an added ionic strength of 0.105 M NaCl.

The fractions of the DEAE column eluate containing antithrombin activity were pooled, concentrated and dialyzed against 1% glycine, then, applied to an isoelectric focusing column with an Ampholine gradient of pH 4-6. After 48 to 52 hrs of focusing the content of the column was removed at a flow rate of 2 ml per min. Fractions of 2 ml were collected. Activity of inhibitor, protein content and pH of each fraction were measured. In our study, antithrombin had an isoelectric point of 4.92 and was distributed predominantly over 0.17 pH unit. At this step the purity of the preparation tested by sodium dodecyl sulfate disc gel electrophoresis showed a more rapidly moving component which amounted to 12% of the major band.

Homogeneous antithrombin could be isolated from above preparation by an affinity chromatography (5). Heparin was conjugated to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the procedure described by the company. The preparation obtained from isoelectric focusing was dialyzed against 0.1 M Tris-HCl buffer (pH 7.4) containing 0.01 M citrate and 0.15 M NaCl and then applied to heparin-Sepharose gel column (1 x 30 cm). Antithrombin was eluted by a linear salt gradient from 0.15 M NaCl to 1.5 M NaCl in 0.1 M Tris-HCL-0.01 M citrate buffer, pH 7.4. Fractions of 2.5 ml were collected at flow rate of 20 ml per hr. The peak of inhibitor activity emerged at an added ionic strength of 1.08 M. This preparation exhibited a single band on disc gel electrophoresis obtained with 20 µg of protein and a single precipitation on an immunoelectrophoresis with antisera against whole human sera (Behring Diagnostics). It was identified by immunoelectrophoresis with specific antisera against antithrombin III (Behring Diagnostics).

Recent experiments have provided preliminary evidence that antithrombin III is absorbed by platelets. In the process the inhibitor becomes "activated," reacting rapidly with thrombin to neutralize the latter within seconds similar to the activation affected by heparin. This field is being actively investigated at present in our laboratory and more definite results are expected within the next few months.

References

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D. Studies on Cyclic Nucleotides in Stored Platelets

As previously demonstrated (1) platelets preserved at 4°C for up to 48 hr maintained their levels of cAMP and cGMP but storage at 22°C for the same length of time led to a decline of both cyclic nucleotides, especially of cGMP. The cyclic nucleotide response of stored platelets to increasing dosages of PGE_1 or 5-HT, specific stimulators for cAMP and cGMP respectively, showed a characteristic pattern. While platelets preserved at 22°C demonstrated enhanced responsiveness to PGE_1 (60 - 100% above the three-fold increase in cAMP production observable in fresh platelets), storage at 4°C did not significantly change their response rate compared to fresh platelets.

The response pattern to 5-HT, the cGMP stimulant, was a virtual mirror image of that to PGE_1. A greater than two-fold enhancement of 5-HT-induced stimulation of cGMP levels in 4°C stored platelets contrasted with a marked depression of this response in 22°C preserved platelets. There was no experimental evidence that the above changes in platelet cyclic nucleotide levels were due to an altered rate of phosphodiesterase activity.

To further investigate and delineate the response behavior of the 2 major cyclic nucleotides to their respective stimulants in stored platelets, the kinetic characteristics of 5-HT- and PGE_1-induced cGMP and cAMP production were determined and analyzed. The dose-response of cAMP synthesis to different PGE_1 concentrations revealed a progressive increase of the maximal cAMP production above baseline values with time of storage. The Δ cAMP of 140 pmol/l x 10^9 platelets increased to 260 pmol/l x 10^9 platelets stored at 22°C for 48 hr. The half maximal cAMP response dose for PGE_1 was 2.5×10^{-7} M, unchanged by storage.

Similar experiments performed with 5-HT as the stimulant of cGMP production revealed a maximal response rate which was unaffected by platelet storage. The half maximal response dose for this amine, however, was subject to storage-induced changes. An increased sensitivity to 5-HT in 4°C preserved platelets (8×10^{-6} M in 24 hr, and 2×10^{-6} M in 48 hr stored platelets) contrasted with a decrease in this parameter in 22°C stored platelets (2.2×10^{-5} M before storage and 5×10^{-5} M after 24 - 48 hr storage).

To provide further elucidation of these findings binding studies of 5-HT and PGE_1 were performed. The results are summarized in Table 2 and 3 which lists the characteristic binding data calculated by the method described above. There was clear evidence for the presence of at least 2 types of receptor sites for both of these substances, one of high and one of low affinity. While high and low affinity sites for 5-HT declined during platelet storage at either temperature, only the high affinity sites of PGE_1 decreased during storage at 22°C. Dissociation constants of both types of receptor sites for PGE_1 and 5-HT showed a tendency to decline but only those of the low affinity sites registered a significant degree of reduction.

The experimental results obtained are quite definite in their demonstration of an enhanced PGE_1-stimulated cAMP production and a depressed 5-HT-induced cGMP response in 22°C preserved platelets. Their interpretation is quite difficult and for the present remains somewhat speculative. Hormone-response cyclic nuc-

leotide production in cell membranes can be viewed as a three component system consisting of receptor, intermediate coupler and catalytic enzyme (2). While a change in the latter resulting in increased affinity for its substrate and enhanced production of cyclic nucleotides seems unlikely in stored platelets, an alteration of the coupling mechanism leading to increased or decreased efficiency of mediation appears quite plausible considering that membrane structure may undergo storage-related changes (3-5). A growing body of experimental evidence underscores the importance of the lateral mobility of cell surface receptors as a modifying influence on adenylate cyclase activity (6,7).

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TABLE 1

SUMMARY OF PURIFICATION OF HUMAN ANTITHROMBIN III:

A TYPICAL PREPARATION

	Total Protein	Specific Activity	Yield
Defibrinated Plasma (500 ml)	25,500*	0.02**	100%
Aluminum Hydroxide Adsorption-Elution	1,700	0.19	64.6
Sephacryl S-200	384	0.61	46.8
DEAE-cellulose	46	2.14	19.7
Isoelectric Focusing	11	7.35	16.2
Heparin-affinity	8.4	8.05	13.5

* Protein content was expressed in optical density unit.

** Specific activity was expressed in units of antithrombin activity per one O.D. unit of protein. The activity of antithrombin III contained in 1 ml of defibrinated plasma was defined as one unit.

TABLE 2

SEROTONIN BINDING DATA IN STORED PLATELETS

Storage Condition		K^a nmol	n^b site/platelet
Fresh	H:	2.1 ± 2.04	263 ± 92
	L:	1524 ± 1532	16814 ± 13136
24 hr, 22°C	H:	0.313 ± 2.03	101 ± 60
	L:	304 ± 69	9162 ± 1144
24 hr, 4°C	H:	0.17 ± 0.08	10 ± 25
	L:	470 ± 131	8735 ± 1908

The values presented in this table are the means ± SE of 6 experiments.

[a] Dissociation constant for binding of ligand (5-HT).

[b] Number of binding sites.

H, high affinity binding sites.

L, low affinity binding sites.

TABLE 3

PROSTAGLANDIN E_1 BINDING DATA IN PLATELETS STORED AT 22°C

Storage Time hr		K nmol	n sites/platelet
0	H:	2.04 ± 0.92	80 ± 17
	L:	735 ± 139	10710 ± 1607
24	H:	0.47 ± 0.67	17 ± 12
	L:	380 ± 41.4	9793 ± 747
48	H:	0.29 ± 0.86	9 ± 4
	L:	326 ± 26	9927 ± 596

The values presented in this table are the means ±SE of 5 experiments.

Abbreviations in this table are identical to those of table 2.