

Serum Prostacyclin Binding Defects in Thrombotic Thrombocytopenic Purpura

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Abstract

To understand the pathophysiologic significance of abnormal serum prostacyclin (PGI_2) binding activities in thrombotic thrombocytopenic purpura (TTP), we evaluated the PGI_2 binding characteristics in three chronic TTP sera and 19 normal sera. PGI_2 binding by serum was rapid and reversible. The binding activity in TTP sera ($22.1 \pm \text{SD}, 4.4\%$) was significantly lower than that of normal sera ($42.2 \pm 6.2\%$). Moreover, the antiaggregating activity and 6-keto-prostaglandin $\text{F}_{1\alpha}$ ($6\text{KPGF}_{1\alpha}$) content in the gel filtrates representing the binding peak was proportionally lower in a TTP serum than normal serum. Although normal and TTP sera bound [^{14}C]arachidonate with similar activity, and neither bound [^3H]6KPGF $_{1\alpha}$, there was a difference in prostaglandin E_1 (PGE_1) binding. Binding of [^3H]PGE $_1$ was subnormal in two TTP sera (W.J. and T.G.) and normal in the third (H.S.). Normal serum corrected the binding defects of TTP serum. Interestingly, the mixture of two TTP sera (W.J. and H.S.) mutually corrected their PGI_2 binding defects. In addition, although *in vivo* plasma transfusions improved the PGI_2 binding activity of W.J. and H.S., there existed a striking difference in the nature of their response. These observations indicate that there are at least two types of PGI_2 binding defects in TTP.

Our data indicate that TTP is associated with diminished serum binding of PGI_2 . This defect may reduce the availability of PGI_2 to damaged vascular sites and decrease an important modulator of platelet thrombus formation at times of severe vascular insult.

Introduction

Thrombotic thrombocytopenic purpura (TTP)¹ is a clinical syndrome characterized by microangiopathic hemolytic anemia, thrombocytopenia, fever, and neurologic and renal abnormalities (1). The pathophysiologic basis for these clinical manifestations is thought to be due to the deposition of platelet and fibrin thrombi in the microcirculation. Although the pathogenetic mechanisms of this disorder are not entirely clear, recent studies have disclosed several possible mechanisms such as endothelial cell damage by immune complexes and platelet

aggregate formation by platelet aggregating factor (2) and the presence of abnormal Factor VIII: von Willebrand Factor multimers (3). We have recently detected accelerated serum prostacyclin (PGI_2) degradation (nonenzymatic hydrolysis) in a patient with chronic TTP (4). Detailed clinical and laboratory correlates suggest that reduced PGI_2 bioavailability may play an important role in the pathogenesis of TTP. Subsequently, we measured serum PGI_2 binding activity by gel filtration and found a significantly lower binding activity in this patient (5). To understand the pathophysiologic significance of abnormal serum PGI_2 binding in TTP, we have investigated the binding defects in three patients with chronic or relapsing TTP.

Methods

Serum. Serial serum samples were obtained from three patients (W.J., a 42-yr-old male; T.G., a 33-yr-old female; and H.S., a 32-yr-old female) who had the typical clinical pentad of fever, microangiopathic hemolytic anemia, thrombocytopenia, neurological symptoms, and renal dysfunctions. None of the patients had underlying diseases. Patient W.J. (4, 5) and H.S. (6) have been reported. All three patients initially recovered after plasma exchange therapy but relapsed subsequently. Patients W.J. and T.G. relapsed at regular intervals of 3–4 wk and have required plasma therapy for 27 and 30 mo, respectively. Patient H.S. relapsed twice but responded to plasma exchange and/or infusion on each occasion. Serum samples were prepared from the patients and 19 normal subjects by incubating venous blood at 37°C for 2 h, centrifuging samples at 1,500 *g* for 20 min and storing samples at –70°C until use. Fresh and frozen samples gave identical binding and degradation results.

Materials. [^3H] PGI_2 methyl ester (12 Ci/mmol), [^3H] 6-keto-PGF $_{1\alpha}$ (6KPGF $_{1\alpha}$) (150 Ci/mmol), [^{14}C] arachidonic acid (55.8 Ci/mmol) and [^3H] prostaglandin E_1 (PGE $_1$) (59.5 Ci/mmol) were obtained from New England Nuclear, Boston, MA. [^3H] PGI_2 methyl ester was reduced to [^3H] PGI_2 sodium and purified by thin-layer chromatography (7). PGI_2 sodium salt and 6KPGF $_{1\alpha}$ were generous gifts of the Upjohn Co., Kalamazoo, MI. Sephadex G-25 medium and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Trypsin (type III) and α -chymotrypsin (type II) were purchased from Sigma Chemical Co., St. Louis, MO. A series of human albumin preparations (i.e., Cohn fraction V, crystalline, and defatted albumin) were purchased from Research Plus, Bayonne, NJ. Crystalline and defatted human albumin preparations were also obtained from Sigma Chemical Co.

Serum PGI_2 binding was evaluated by gel filtration (8). Sephadex G-25 medium gel was swollen in 0.05 M Tris buffer, pH 7.4, and packed in a plastic column (21.5 \times 0.8 cm) that was washed with 3 bed vol of buffer. The flow rate was ~ 1 ml/min. [^3H] PGI_2 was incubated with serum before gel filtration. An incubation time of 3 min and temperature of 22°C were deemed optimal based on our pilot experiments. After discarding an initial 3-ml vol, 0.35-ml fractions were collected and the radioactivity of each fraction was determined in a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). As the radioactivity recovered in all the fractions was 99–100% of the radioactivity added, the recovered radioactive counts were used as the basis for determining the percentage of binding. The percentage of total radioactivity eluted with the non-included serum proteins (peak I, Fig. 1) gave a measure of PGI_2 binding. In the

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Received for publication 10 March 1982 and in revised form 10 September 1984.

1. Abbreviations used in this paper: 6KPGF $_{1\alpha}$, 6-keto-prostaglandin $\text{F}_{1\alpha}$; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PAGE, polyacrylamide gel electrophoresis; PGI_2 , prostacyclin; PGE $_1$, prostaglandin E_1 ; TTP, thrombotic thrombocytopenic purpura.

J. Clin. Invest.

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0021-9738/85/01/0168/07 \$1.00

Volume 75, January 1985, 168–174

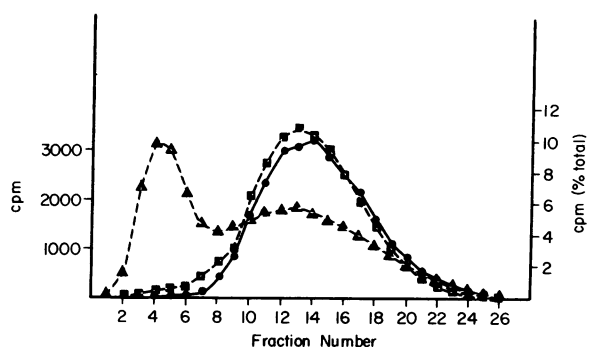


Figure 1. A representative gel filtration pattern of normal serum. [^3H]PGI $_2$ or [^3H]6KPGF $_{1\alpha}$ (10 nM) was incubated in serum or 0.05 M Tris buffer (pH 7.4) at 22°C for 3 min and 0.35 ml of the buffer, pH 7.4. Fractions were collected and the radioactivity and percentage of total added radioactivity were determined. Two peaks were noted with the PGI $_2$ -serum mixture (▲ — ▲). Only a single peak was noted with the 6KPGF $_{1\alpha}$ -serum mixture (■ — ■) and the PGI $_2$ -buffer mixture (● — ●), both of which resembled the pattern obtained with a 6KPGF $_{1\alpha}$ -buffer mixture.

dose-response experiments, increasing [^3H]PGI $_2$ concentrations were used in the gel filtration. In all other experiments, unless otherwise specified, 10 nM [^3H]PGI $_2$ was used. Serum binding of [^3H]PGE $_1$ (10 nM), [^3H]6KPGF $_{1\alpha}$ (10 nM) and [^{14}C]arachidonic acid (46 nM) was measured by the same gel filtration technique.

Bioassay for PGI $_2$ was based upon the inhibitory effect of PGI $_2$ on ADP-induced platelet aggregation. Venous blood was drawn from normal donors and collected in plastic tubes containing 1/10 vol of 3.8% sodium citrate. The blood sample was centrifuged at 200 g for 10 min to obtain platelet-rich plasma (PRP) and the remaining sample was centrifuged at 1,500 g for 15 min to prepare platelet-poor plasma (PPP). The platelet concentration in the PRP was adjusted to 3×10^8 /ml with autologous PPP. Platelet aggregation was measured by the turbidimetric method using a Chronolog aggregometer (9). ADP (1–10 μl) was added to 0.5 ml PRP in a cuvette containing a stirring bar and the extent of increment in light transmission was measured in 3 min. In each experiment, the minimal ("threshold") concentration of ADP that caused maximal aggregation was determined and this concentration was used for the bioassay. Increasing concentrations of standard PGI $_2$ sodium were incubated with PRP in the aggregometer for 15 s followed by the addition of ADP. A calibration curve was constructed in each experiment. The quantity of PGI $_2$ in an unknown sample was obtained by relating the inhibition of platelet aggregation to the calibration curve.

Radioimmunoassay for 6KPGF $_{1\alpha}$. Antiserum against 6KPGF $_{1\alpha}$ was obtained by repeated immunization of rabbits with 6KPGF $_{1\alpha}$ coupled to bovine serum albumin. The anti-6KPGF $_{1\alpha}$ was used at a final dilution of 1/8,000. [^3H]6KPGF $_{1\alpha}$ was used as the labeled antigen. The antiserum showed little cross-reactivity with PGF $_{2\alpha}$ (4.2%), PGE $_1$ (1.3%), thromboxane B $_2$ (<0.03%), arachidonic acid (<0.01%), PGF $_{1\alpha}$ (5.7%) and 6-keto-PGE $_1$ (6.4%). The sensitivity of the assay was 10 pg/tube (i.e., B/Bo = 90%).

PGI $_2$ content in gel filtrates. Unlabeled PGI $_2$ sodium at various concentrations was incubated with 0.35 ml serum at 22°C for 3 min and then applied to a Sephadex G-25 column. Fractions representing peaks I and II as identified by the [^3H]PGI $_2$ binding experiments were collected. PGI $_2$ activity was measured by bioassay and 6KPGF $_{1\alpha}$ was measured by radioimmunoassay (see above).

Serum PGI $_2$ degradation. PGI $_2$ degradation was determined by a modification of a procedure described (4). In each experiment, a PGI $_2$ concentration that inhibited 75–95% of ADP-induced platelet aggregation was incubated with normal or patient serum at 37°C. The PGI $_2$ concentrations ranged from 10 to 20 nM. Aliquots (50 μl) of the

mixture were removed at 1, 5, 15 min, etc., and the decline in PGI $_2$ activity was measured as the decline in antiaggregatory activity. The half-life of PGI $_2$ was determined as the time interval at which 50% of the added PGI $_2$ activity had disappeared.

Ammonium sulfate precipitation of serum was used for the preliminary separation of the active binding component(s). Saturated ($(\text{NH}_4)_2\text{SO}_4$) was added to serum until it had reached 30, 60, and 80% saturation, respectively. The supernatant was removed, concentrated, and dialyzed against phosphate-buffered saline. The binding percentage of the supernatant was determined by gel filtration as described above. The 60% ($(\text{NH}_4)_2\text{SO}_4$ supernatant (fraction D) contained the active component and was used for further biochemical studies. The protein concentration was determined by the method of Lowry (10).

Chromatographies. DEAE chromatography was performed by applying fraction D to a DE-52 column (Whatman, Inc., Clifton, NJ) that had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0. The column was washed with buffer until the ultraviolet 280 absorbance stabilized and then eluted with buffer containing 1 M NaCl. Hydroxylapatite chromatography was performed by diluting fraction D with an equal volume of 20 mM sodium phosphate buffer, pH 7.4 and mixing the sample with 1 ml of Bio-Gel HT (Bio-Rad Laboratories, Richmond, CA). The Bio-Gel HT material was removed by centrifugation and washed with 1 M sodium phosphate buffer, pH 8.5 to remove bound proteins. Dye ligand chromatography was performed by applying 0.5 ml serum to dye matrix columns (Amicon Corp., Danvers, MA). The columns were washed and then eluted with 20 mM potassium phosphate buffer, pH 7.4, containing 1.5 M KCl. All fractions were dialyzed and concentrated on an Amicon PM 30 membrane before testing.

Electrophoresis. Preparative isoelectric focusing was used to identify proteins in fraction D. Fraction D was electrofocused for 18 h with 8 W constant power at 8–10°C. A blot was made and stained. Then the gel bed was divided into fractions, the pH measured and the proteins removed by washing the gel in buffer. The fractions were titrated to pH 7.4 and concentrated by ultrafiltration on an Amicon PM 30 membrane.

SDS polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% polyacrylamide gels according to a modified method of Laemmli (11). All the reagents were obtained from Bio-Rad Laboratories, including the Tris/acetate/SDS buffer (Tris 24.84 g/liter, acetic acid 11.7 ml/liter, SDS 1 g/liter; pH of buffer 6.4, pH of gel 6.1). Fraction D, whose protein content was standardized at 33 μg for all samples, was pretreated with SDS and dithiothreitol (Sigma Chemical Co.) and then applied to the SDS-polyacrylamide gels. The protein bands were stained with Coomassie Brilliant Blue.

Results

PGI $_2$ binding characteristics of normal and TTP sera. Two peaks were noted when normal human serum preincubated with PGI $_2$ was subjected to gel filtration (Fig. 1). Peak I, which coincided with the void volume represents PGI $_2$ binding to a macromolecular serum component and peak II represents the standard 6KPGF $_{1\alpha}$ peak. The first peak was absent when 6KPGF $_{1\alpha}$ was added to serum, indicating that 6KPGF $_{1\alpha}$ does not bind to this serum component. The binding of PGI $_2$ to the serum component was rapid (i.e., it had reached a maximum in ≤ 30 s) and reversible. The reversibility of PGI $_2$ binding was demonstrated by the displacement of [^3H]PGI $_2$ by cold PGI $_2$. Normal serum was incubated with [^3H]PGI $_2$ (10 nM) for 3 min and divided into three aliquots. One aliquot was chromatographed immediately and showed a binding activity of 40.9%. Cold PGI $_2$ (10^{-2} M) and buffer were added to aliquots two and three, respectively, and after an additional 3-min incubation, they were chromatographed. The binding activity

in the aliquot containing cold PGI_2 was 7.5% while the activity in the buffered aliquot was 34.1%.

The binding of PGI_2 by serum was linear with increasing $[^3\text{H}]\text{PGI}_2$ at all concentrations tested (maximum, 200 nM of $[^3\text{H}]\text{PGI}_2$) (Fig. 2 A). Since saturation of serum binding with labeled PGI_2 was impractical, two alternative approaches were taken to estimate the PGI_2 concentration necessary to saturate serum binding. First, increasing concentrations (up to 10 mM) of unlabeled PGI_2 were incubated in the serum with a standard concentration of $[^3\text{H}]\text{PGI}_2$ and the reduction in the percentage of bound $[^3\text{H}]\text{PGI}_2$ as a result of PGI_2 displacement was determined (Fig. 2 B). Reduction in the binding began to be noted at 0.5 mM PGI_2 and a hyperbolic dose-response curve was obtained. The second approach was to add increasing concentrations of unlabeled PGI_2 to the serum and to measure the $6\text{KPGF}_{1\alpha}$ content to peak I (Fig. 2 B). A similar dose-response curve was observed.

The PGI_2 binding of three TTP sera was examined and a reduced first peak was found in each (Fig. 3 A). The percentage of total $[^3\text{H}]\text{PGI}_2$ in peak I was significantly lower in TTP sera ($22.1 \pm \text{SD}, 4.4\%$) when compared with that of normal sera obtained from 19 age- and sex-matched donors ($42.2 \pm 6.2\%$). The PGI_2 binding was measured multiple times on two different sera from each TTP patient (except T.G.). There was little variation in the binding values. The mean value of each TTP serum was W.J. $24.4\% \pm \text{SD}, 0.3$; T.G. 17.1% , and H.S. $24.9\% \pm 0.3$.

The effect of protein binding upon the PGI_2 degradation rate was studied by assessment of antiaggregatory activity and $6\text{KPGF}_{1\alpha}$ content in peak I and peak II serum fractions. The antiaggregatory activity of protein-bound PGI_2 decreased at

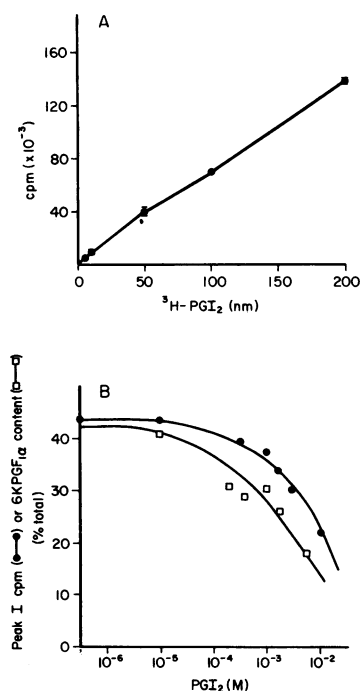
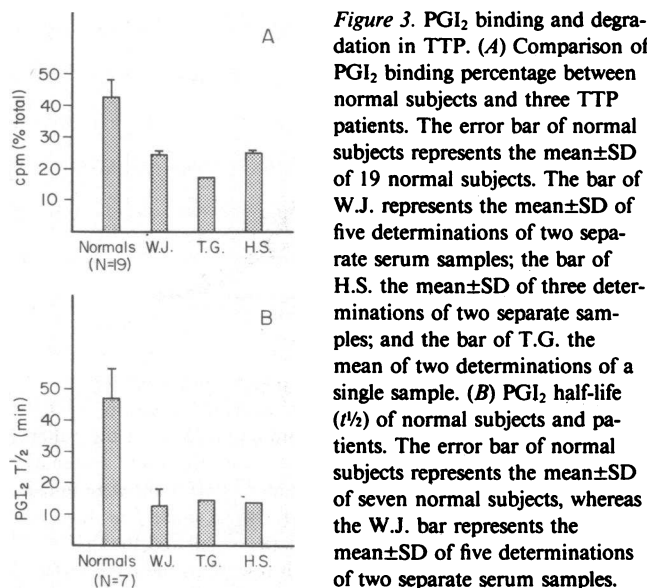


Figure 2. Dose-response curves of normal serum PGI_2 binding (A) Increasing concentrations of $[^3\text{H}]\text{PGI}_2$ were added to normal serum and applied to a Sephadex G-25 column. Percentages of total radioactivity present in peak I were calculated for each concentration. A linear curve was noted even at 200 nM $[^3\text{H}]\text{PGI}_2$. (B) Increasing concentrations of unlabeled authentic PGI_2 were incubated in serum with 16 nM of $[^3\text{H}]\text{PGI}_2$ and the percentages of total radioactivity present in peak I were determined and plotted against the unlabeled PGI_2 concentration (\bullet — \bullet). A hyperbolic reduction (displacement of peak I radioactivity) was observed. Each value represents the mean of two experiments. In parallel experiments, increasing concentrations of unlabeled PGI_2 were added to serum and the $6\text{KPGF}_{1\alpha}$ content (\square — \square) in peak I measured by radioimmunoassay and plotted against the PGI_2 concentration. A hyperbolic binding curve (similar to that obtained for the displacement) was obtained.

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The bars of H.S. and T.G. represent a mean value of two determinations.

slower rate than the activity of unbound PGI_2 (Fig. 4). In 10 normal sera, there were positive correlations between the PGI_2 binding, PGI_2 antiaggregatory activity and $6\text{KPGF}_{1\alpha}$ content in peak I (Table I). The antiaggregatory activity and $6\text{KPGF}_{1\alpha}$ content of peak I of TTP serum (W.J.) were proportionally reduced when compared with normals.

To confirm that defective binding leads to accelerated PGI_2 degradation, PGI_2 was added to normal and TTP sera, and the half-life of PGI_2 was determined. The PGI_2 half-life was

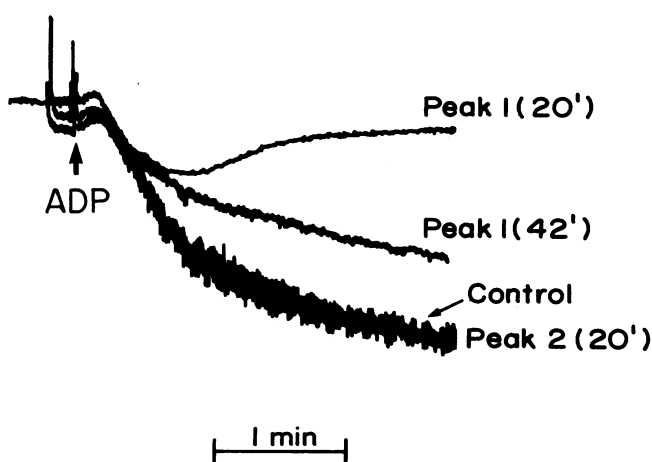


Figure 4. Inhibition of ADP -induced platelet aggregation by the two representative peaks collected from Sephadex G-25 gel filtration. The tracings show changes in light transmission in an aggregometer. $30 \mu\text{l}$ of fractions representing peak I or peak II were added to 0.5 ml human PRP (3×10^8 platelets/ml) at 37°C for 1 min before ADP ($3 \mu\text{M}$) was added. Peak I exhibited a potent anti-aggregatory activity when it was eluted from the column (~ 20 min after it was applied to the column). Furthermore, it retained antiaggregatory activity up to 42 min after its addition to the column. Peak II, similar to PGI_2 chromatographed in the absence of serum, had no anti-aggregatory activity.

Table I. Comparison of PGI₂ Binding with PGI₂ Anti-aggregatory Activity and 6KPGF_{1α} Content in Gel Filtration

Subjects	Peak I						Peak II					
	PGI ₂ radioactivity		PGI ₂ activity		6KPGF _{1α} content		PGI ₂ radioactivity		PGI ₂ activity		6KPGF _{1α} content	
	cpm (×10 ³)	%	ng	%	ng	%	cpm (×10 ³)	%	ng	%	ng	%
W.J.	8.1	23.4	260	21.9	300	25.3	26.5	76.6	0	0	883	74.7
A normal serum	13.9	41.5	518	43.9	542	40.7	19.6	58.5	0	0	790	59.3
Pooled normal serum (n = 10)	15.2	44.7	518	43.9	539	43.8	18.8	55.3	0	0	692	56.2

10 nM of [³H]PGI₂ and 10 μM of PGI₂ sodium were added to serum for 3 min and applied to a Sephadex G-25 column. Fractions representing peak I and peak II were collected. The radioactivity, PGI₂ antiaggregatory activity and 6KPGF_{1α} in both peaks were determined.

significantly shortened in all three TTP sera (Fig. 3 B). To determine the specificity of the binding abnormality, serum binding of [¹⁴C]arachidonic acid, [³H]PGE₁ and [³H]6KPGF_{1α} was compared in normal and TTP sera. Serum PGE₁ binding appeared to be reduced in W.J. and T.G. but was normal in H.S. (Fig. 5). On the other hand, there was no difference in arachidonic acid or 6KPGF_{1α} binding between the TTP and the normal sera.

Mixing experiments. Since plasma replacement was beneficial in all three patients, we examined the in vitro and in vivo effects of serum or plasma on PGI₂ binding and stability. The addition of normal serum to TTP sera significantly increased PGI₂ binding and half-life (Table II). T.G. and H.S.

seemed to have a greater response to a 50:50 mixture of their sera with normal sera than W.J., suggesting that the defects might differ between W.J. and the other two (T.G. and H.S.). The in vivo effect in two patients (H.S. and W.J.) following infusion of 3 U of fresh frozen plasma was investigated. Despite similar clinical benefit, the effect of plasma upon PGI₂ binding was quite dissimilar in these two patients. In H.S. there was an immediate rise of PGI₂ binding followed by a slow decline over 35 d (Fig. 6). By contrast, improvement in the PGI₂ binding of W.J.'s serum was delayed and the level of elevation was suboptimal when compared with that of H.S. These data provide further evidence to suggest that more than one defect in PGI₂ binding might exist in these two patients.

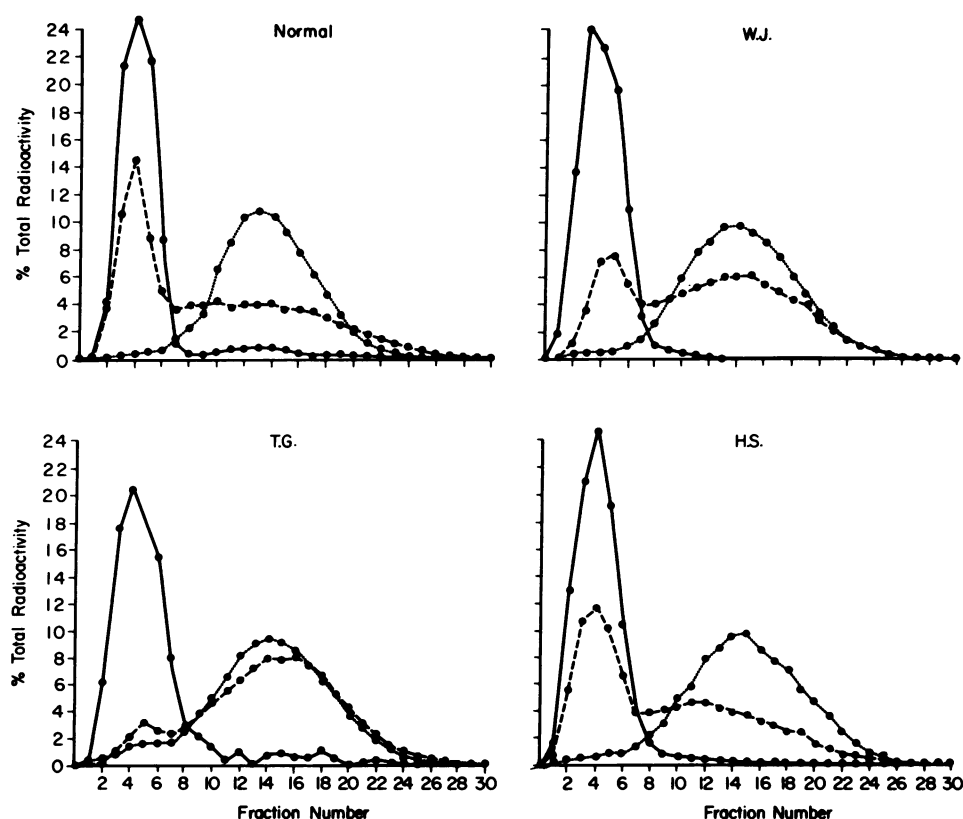


Figure 5. Comparison of the serum binding of [¹⁴C]arachidonic acid (●—●), [³H]PGE₁ (●---●) and [³H]6KPGF_{1α} (●····●) between a normal subject and three patients. None of the sera bound 6KPGF_{1α}. The percentage of PGE₁ binding was reduced in W.J. (28.8%) and T.G. (11.0%) but was normal in H.S. (50.3%) when compared with a normal subject (46.5%). There was no difference in [¹⁴C]arachidonic acid binding between the normal and any of the patients.

Table II. Correction of Abnormal PGI₂ Binding and Degradation by Normal Serum

Patient serum	Normal serum	PGI ₂ binding			PGI ₂ t _{1/2}		
		W.J.	T.G.	H.S.	W.J.	T.G.	H.S.
vol %	vol %	%	%	%	min	min	min
100	0	24.5	17.0	24.0	17	15	14
75	25	34.0					
50	50	34.5	33.1	37.9	30	38	30
25	75	38.6					
0	100	44.9	40.6	40.6	43	43	43

Various volumes of patient and normal sera were mixed and incubated at 37°C for 30 min. 0.35 ml of the mixture was removed and added to [³H]PGI₂ (10 nM) at 22°C for 3 min and the percentage of binding was measured. In parallel experiments, unlabeled PGI₂ sodium was added to the serum mixture and the half-life (t_{1/2}) of PGI₂ activity was determined by bioassay.

To evaluate this possibility, equal volumes of serum from W.J. and H.S. were mixed and the PGI₂ binding capacity of the mixture was compared that of the individual serum. The percentage of binding in the mixture was 33.3%, significantly higher than either of the individual values (W.J. 24.8%, H.S. 24%).

Since serum albumin has been implicated in prolonging PGI₂ activity (12–15), we investigated the effect of several albumin preparations on serum PGI₂ binding in W.J. and H.S. sera (Table III). When used alone, Cohn fraction V albumin preparations did not bind PGI₂, crystalline albumin bound only a small percentage of PGI₂, while defatted albumin bound a quantity of PGI₂ equivalent to that bound by serum. When these albumin preparations were added in physiologic concentrations (40 mg/ml) to W.J. and H.S. sera, the following results were obtained. Cohn fraction V did not influence the PGI₂ binding in H.S. but suppressed the binding in W.J. Crystalline albumin increased the PGI₂ binding in both patients. Defatted albumin elicited an additive response in PGI₂ binding in H.S. serum, but no effect in W.J. serum (Table III).

Characterization of binding defects in TTP sera. For the reason that TTP sera appear to be deficient in components that normally prevent rapid PGI₂ hydrolysis and hence increase PGI₂ bioavailability, normal serum was studied to identify these protective components. When normal serum was treated

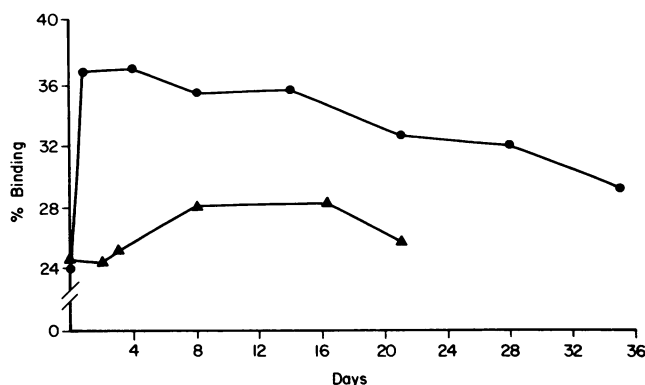


Figure 6. Responses of serum PGI₂ binding to transfusions of 3 U of fresh frozen plasma in H.S. (● — ●) and W.J. (▲ — ▲).

Table III. Binding of PGI₂ by Several Albumin Preparations and Their Effects on Defective PGI₂ Binding in TTP Patients

Albumin preparations	Albumin alone	PGI ₂ binding			
		W.J.	H.S.	W.J.	H.S.
		̄ alb.	̄ alb.	̄ alb.	̄ alb.
	%	%	%	%	%
Cohn fraction V	<1.0	11.7	24.5	31.4	24.0
Crystalline albumin	4.3	31.3	24.5	41.4	24.0
Defatted albumin	53.6	53.3	24.5	70.3	24.0

[³H]PGI₂ (10 nM) was added to the albumin preparation (40 mg/ml) at 22°C for 3 min and applied to a Sephadex G-25 column. Percentage of total radioactivity present in peak I was calculated. In the mixing experiments, albumin was added to serum and the percentage of radioactivity in peak I determined. ̄ alb. denotes that 40 mg albumin was added and ̄ alb. denotes that no albumin was added. Each value represents a mean of two experiments with a variability of <5%.

with 30, 60, 70, and 80% saturated (NH₄)₂SO₄, the binding activity was recovered only in the supernatant of the 60% (NH₄)₂SO₄ solution (fraction D). In the process, the specific binding activity was raised from 1.51±SD, 0.22%/mg protein (serum, n = 10) to 2.78±0.70%/mg protein (fraction D, n = 10). The binding activity of W.J. and H.S. sera was also located in the 60% (NH₄)₂SO₄ supernatant and this fractionation process produced an increase in specific PGI₂ binding activity that was proportional to the increase observed with normal serum. Normal fraction D corrected the PGI₂ binding of W.J. and H.S. serum (Table IV).

The binding components in fraction D were nondialyzable. The binding activities were completely eliminated by trypsin (1 mg/ml), chymotrypsin (1 mg/ml), and by boiling for 3 min. Neither heating the serum at 56°C for 30 min nor exposure to neuraminidase had an effect on binding activity. Further experiments were initiated to isolate the binding proteins. When fraction D was mixed with hydroxylapatite, 85% of the applied protein was unadsorbed and the remaining 15% could be eluted with 1.0 M sodium phosphate buffer at pH 8.5. However, despite a virtually complete recovery of the applied protein, binding activity was undetectable in either fraction (Table V). Similar loss of binding activity despite good protein recovery was observed with dye ligand chromatography. It is of particular interest to note that despite the fact that 97% of the applied protein was unadsorbed to Orange A and the remaining 3% was completely eluted, there was no significant binding activity in the unadsorbed or the eluted samples.

Table IV. Effect of Serum Fraction D on Patient's PGI₂ Binding and Degradation

Patient serum	Fraction D	PGI ₂ binding			PGI ₂ t _{1/2}		
		W.J.	T.G.	H.S.	W.J.	T.G.	H.S.
vol. %	vol. %	%	%	%	min	min	min
100	0	24.5	17.0	24.0	17	15	14
50	50	33.4	39.5	37.7	55	37	95
0	100	51.6	51.6	51.6	150	150	150

Table V. Summary of Chromatographic Purification of Fraction D*

Chromatography	Nonadsorbed fraction			Eluted fraction		
	Protein	Albumin	Binding activity	Protein	Albumin	Binding activity
	%	%	%	%	%	%
Hydroxylapatite	85	NT‡	<5	15	NT	<5
Green A	11	8	<5	89	86	<5
Red A	49	34	<5	51	47	<5
Orange A	97	83	<5	3	0	<5
Blue A	9	5	<5	58	55	<5
DEAE(DE-52)	69	NT	10.8	31	NT	<5

* See text for details. ‡ NT, Not tested.

When fraction D (specific binding activity 3.1%/mg protein) was applied to a DEAE-Sephacel column equilibrated in 50 mM sodium phosphate buffer, pH 7.0, 69% of the protein passed through the column unadsorbed. However, most of the activity was lost and the specific binding activity of the unadsorbed portion was reduced to 1.8%/mg of protein. The protein that bound to the column was eluted with 1 M NaCl. The eluted portion by itself had no binding activity; however, when it was mixed with the unadsorbed protein, the binding activity of the mixture was greater than either one of the individual fractions and approached that of fraction D, i.e., 2.9%/mg of protein.

Three major protein bands with pI's of 5.0, 5.2, and 5.6 were consistently noted when normal fraction D ($n = 10$) and TTP fraction D (W.J. and H.S.) were isoelectrofocussed. There was no apparent difference between the normal and TTP fraction D. Three distinct bands were also noted when normal and TTP fraction D were applied to SDS-PAGE; a heavily stained band (estimated molecular weight of 67,000) and two minor bands (80,000 and 45,000 mol wt, respectively). Again, there was no apparent difference between normal and TTP fraction D. Preparative electrofocusing over a pH range of 4.0–8.0 was used to subdivide fraction D into four pH zones (i.e., pH 4.0–5.1, 5.1–5.3, 5.3–5.7, and 5.7–8.0). Each of the first three zones contained a major protein band. No single pH zone exhibited active binding activity; however, when all four zones were recombined and tested, binding activity could be partially recovered (i.e., 42–81% of the initial binding activity) in 3 of 10 experiments. In the three experiments with partial recovery of the activity, the binding activity was primarily found in a mixture of the pH zones between 5.1 and 5.7.

As TTP sera contained erythrocyte and platelet fragments that might bind PGI₂, normal sera ($n = 3$) and two TTP sera (W.J. and H.S.), were ultracentrifuged at 103,000 g for 1 h to remove cell fragments and the supernatant was tested for PGI₂ binding. There was no difference between the uncentrifuged and the ultracentrifuged samples. To determine whether TTP samples contained platelet aggregating activity as described by Lian et al. (2), we added TTP PPP or heated serum (56°C for 30 min) to washed normal platelet suspensions and measured platelet aggregation in an aggregometer. None of our TTP samples exhibited platelet aggregating activity.

Discussion

This study indicates that human serum binds PGI₂. The binding occurs rapidly and is readily reversible. The components

responsible for the binding property are serum proteins that are soluble in 60% saturated (NH₄)₂SO₄. Further efforts to purify the protein(s) were hampered by an accompanying loss of binding activity. Reasons for this loss of activity could include protein instability or its presence in only trace amounts. An alternative explanation could be that PGI₂ binding may require the association of two components that are dissociated by purification procedures. The latter is supported by the observation that the binding activity lost after ion-exchange chromatography or preparatory isoelectrofocusing was recovered when their respective fractions were recombined. This hypothesis is further supported by the demonstration of two types of mutually correctable binding defects in chronic TTP.

The binding, half-life, and antiaggregatory activity of PGI₂ is markedly reduced in TTP serum as compared with normal serum. The association of diminished binding with diminished half-life and antiaggregatory effect suggests that PGI₂ binding may protect PGI₂ from degradation and modulate the delivery of PGI₂ to damaged vascular sites and thus provide a control over platelet aggregate formation. Diminished PGI₂ binding in TTP might therefore be an important determinant for the disseminated platelet aggregatory phenomena observed in TTP.

It is interesting to note that two TTP sera (W.J. and H.S.) with a clearly demonstrable impairment in PGI₂ binding were mutually corrective when mixed. The addition of normal serum, normal fraction D, or H.S. serum achieved a significant correction of the PGI₂ binding defect in W.J. serum. Similarly, the binding defect in H.S. was corrected by normal serum, normal fraction D, or W.J. serum. These findings indicate that the binding defects in W.J. and H.S. are different. Although plasma transfusions were efficacious in both patients, the response of PGI₂ binding to in vivo infusion of plasma was also quite different in these two patients. As compared to H.S., serum PGI₂ binding in W.J. responded suboptimally to in vivo plasma infusion and to in vitro addition of albumin.

Although a uniform theory could not be afforded to explain these intriguing defects, we speculate that the defects may be due to a deficiency of the binding protein, on the one hand, and its cofactor, on the other. This hypothesis provides a rational explanation for mutual correctability as well as correction of both defects by normal serum and its derivative, fraction D. However, the lack of an appreciable increase in W.J.'s serum PGI₂ binding after in vivo plasma infusion tends to mitigate against this hypothesis.

Serum albumin has been proposed as a PGI₂ stabilizing factor. Dye ligand experiments (Table V) and isoelectrofocusing experiments (albumin, pI 5.0) strongly suggest, however, that

an albumin "deficiency" was not responsible for the impaired PGI₂ binding observed in these two patients with TTP. This notion is further confirmed by the failure of several commercial human albumin preparations to increase PGI₂ binding. Moreover, since the albumin levels in all three TTP sera were normal, the binding defects are unlikely due to albumin abnormalities.

The pathophysiologic significance of abnormal PGI₂ binding and stability in TTP remains to be established. One may speculate that this abnormality may play an important role in predisposing patients to recurrent microangiopathy. Following vascular injury, PGI₂ production may be stimulated (16, 17) and protein binding may be needed to protect PGI₂ from rapid hydrolysis and catabolism (18). This may be critical to the bioavailability of PGI₂ for dispersion of platelet aggregates and prevention of new aggregate formation. Diminished binding activity may thus compromise PGI₂ bioavailability and lead to inadequate physiologic modulation of platelet thrombus formation at times of severe vascular insult. Further characterization of the binding proteins and their abnormalities in various vascular disorders must be pursued to advance our understanding of the pathophysiology and treatment of these important clinical problems.

Acknowledgments

The authors wish to thank Dr. D. Williams for providing T.G. serum; Dr. Udo Axen and Dr. John Pike of the Upjohn Co. for the generous supply of PGI₂ and 6KPGF_{1α}; and Ms. Molyneux for secretarial assistance.

This work was supported in part by a program project grant from the National Institutes of Health (P01-NS-18494) and a grant from the Chicago Heart Association (81-10).

References

1. Amorosi, E. L., and J. E. Ultmann. 1966. Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. *Medicine (Baltimore)*. 45:139-159.
2. Lian, E. C.-Y., D. R. Harkness, J. J. Byrnes, H. Wallach, and R. Nunez. 1979. Presence of a platelet aggregating factor in the plasma of patients with TTP and its inhibition by normal plasma. *Blood*. 53:333-337.
3. Moake, J. L., C. K. Rudy, J. H. Troll, M. J. Weinstein, N. M. Colannino, J. Azocar, R. H. Seder, S. L. Hong, and D. Deykin. 1982. Unusually large plasma Factor VIII: von Willebrand factor multimers in chronic relapsing TTP. *N. Engl. J. Med.* 307:1432-1435.
4. Chen, Y.-C, B. McLeod, E. R. Hall, and K. Wu. 1981. Accelerated prostacyclin degradation in thrombotic thrombocytopenic purpura. *Lancet*. II:267-269.
5. Wu, K. K., E. R. Hall, and A. Papp. 1982. Prostacyclin stabilizing factor deficiency in thrombotic thrombocytopenic purpura. *Lancet*. I: 460-461.
6. Rossi, E. C., F. del Greco, H. C. Kwann, and B. Lerman. 1980. Hemodialysis—exchange transfusion for treatment of thrombotic thrombocytopenic purpura. *JAMA. (J. Am. Med. Assoc.)*. 244:1466-1468.
7. Hsi, R. S. P., W. T. Stoelle, J. P. McGrath, and D. R. Morton. 1980. Tritium labeled prostacyclin sodium salt. *J. Labeled Compound and Radiopharm.* 18:1437-1447.
8. Acred, P., D. M. Brown, T. L. Hardy, and K. R. L. Mansford. 1963. A new approach to studying the protein-binding properties of penicillins. *Nature (Lond.)*. 199:758-759.
9. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond.)*. 194:927-929.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:261-275.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)*. 227:680-685.
12. Wynalda, M. A., and F. A. Fitzpatrick. 1980. Albumin stabilizes prostaglandin I₂. *Prostaglandins*. 20:853-861.
13. Orchard, M. A., and C. Robinson. 1981. Stability of prostacyclin in human plasma and whole blood. Studies on the protective effect of albumin. *Thromb. Haemostasis*. 46:645-647.
14. Pifer, D. D., G. L. M. Cagan, and C. M. Chesney. 1981. Stability of PGI₂ in human blood. *Prostaglandins*. 21:165-175.
15. Gimeno, M. F., L. Sterlin-Borda, E. S. Borda, M. A. Lazzari, and A. L. Gimeno. 1980. Human plasma transforms PGI₂ into a platelet antiaggregatory substance which contracts isolated bovine coronary arteries. *Prostaglandins*. 19:907-916.
16. Weksler, B. B., C. W. Ley, and E. A. Jaffe. 1978. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and ionophore A23187. *J. Clin. Invest.* 62:923-930.
17. MacIntyre, D. E., J. D. Pearson, and J. L. Gordon. 1978. Localization and stimulation of prostacyclin production in vascular cells. *Nature (Lond.)*. 271:549-551.
18. Rosenkranz, B., C. Frischer, K. E. Weimer, and J. C. Frolich. 1980. Metabolism of prostacyclin and 6-keto-PGF_{1α} in man. *J. Biol. Chem.* 255:10194-10198.