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Protease-induced immunoregulatory activity of platelet factor 4

(immunosuppression/suppressor T cells/antibody formation)

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ABSTRACT **Intravenous injection of human or mouse serum or platelet material secreted from appropriately stimulated platelets ("releasate") together with antigen alleviates the immunosuppression in SJL/J mice induced by injection of irradiated lymphoma cells or in (CB6)F_1 mice induced by injection of concanavalin A. We now report that injection of releasate from 10^6 human platelets restores plaque-forming cells to the unsuppressed number; greater amounts increase responses further. Immunoregulatory activity is released from platelets exposed to thrombin in parallel with other α-granule components. Heparin-agarose absorbs activity. Purified platelet factor 4 (PF4) has activity; β-thromboglobulin and platelet-derived growth factor have little or none. Activity in serum is neutralized by goat anti-human PF4. An enzymatic step is necessary for production of immunoregulatory activity. Releasates boiled immediately after platelet aggregation with 250 nM A23187 or those produced by adding A23187 in the presence of 100 μM serine protease inhibitor (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF) are ineffective, whereas releasates boiled or mixed with APMSF after incubation for 60 min are active. Activity is generated by incubating a mixture of heparin-absorbed releasate (as enzyme source) and heparin-agarose eluate of releasate made in the presence of APMSF (as substrate source). The enzymatic step does not alter the heparin-neutralizing activity of PF4. Apparently a secreted platelet protease converts PF4 to a form with immunoregulatory activity.**

We reported recently that injection of human or mouse serum reverses immunosuppression in mice (1). This immunoregulatory activity is not present in plasma but is found in material released from washed platelets by thrombin. It is absent from the serum of a patient with congenital deficiency of α-granules (1). Activity is absorbed from solution by thymus-derived cells (T cells) of the suppressor phenotype (2).

Platelet α-granules contain at least three secretable proteins not present in plasma (3): platelet factor 4 (PF4), a protein with heparin-neutralizing activity; different forms of β-thromboglobulin (β-TG); and platelet-derived growth factor (PDGF). We now report that purified PF4 has immunoregulatory activity and that generation of this activity in platelet material secreted from appropriately stimulated platelets ("releasates") requires the action of a secreted enzyme.

MATERIALS AND METHODS

Materials. Sources were as follows: [^{14}C]serotonin [5-hydroxytryptamine creatinine sulfate, 56 mCi/mmol (1 Ci = 37 GBq)], Amersham; heparin immobilized on agarose, Pierce; concanavalin A (Con A), Bio-Yeda Research Products, Israel, or Sigma; A23187, Calbiochem–Behring; (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF), California Medicinal Chemistry, San Francisco; ε-aminocaproic acid (εAhx, 6-aminohexanoic acid), Lederle Laboratories, Pearl River, NY; tranexamic acid and soybean trypsin inhibitor (SBTI), Millipore; bovine thrombin, Upjohn; heparin (Liquaemin sodium), 5000 units/ml, Organon; sheep erythrocytes (SRBC), Colorado Serum, Denver, CO.

PF4 was kindly given us by Stefan Niewiarowski (Temple University Health Science Center, Philadelphia) (4); it was >95% pure by $NaDodSO_4$/polyacrylamide gel electrophoresis and failed to react with antibodies to the platelet proteins β-TG, fibronectin, thrombospondin, albumin, and fibrinogen. β-TG was also given us by S. Niewiarowski (4); isoelectric focusing indicated that it was about 10% β-TG$^{pI\ 10}$ (platelet basic protein), 80% β-TG$^{pI\ 8}$ [low-affinity PF4 (LA-PF4)], and 10% β-TG$^{pI\ 7}$ (β-TG) (see ref. 5 for nomenclature; ref. 6). PDGF was kindly provided by Thomas Deuel (Washington University, St. Louis, MO) (7) and human α-thrombin was provided by John Fenton II (New York State Department of Health, Albany, NY) (8). Goat antiserum to pure PF4 was a gift from Karen L. Kaplan (Columbia College of Physicians and Surgeons, New York) (9).

***In Vitro* Assays.** Serotonin release was measured in platelets that had been incubated with 0.5 μM [^{14}C]serotonin (10). β-TG and PF4 were assayed by radioimmunoassays (9) with reagents from Amersham and Abbott, respectively, lactate dehydrogenase and β-glucuronidase were assayed with reagents from Sigma, with lactate and phenolphthalein glucuronide as substrates, respectively, and heparin-neutralizing activity was assayed by a modification of the method of Harada and Zucker (11).

Preparation of Serum and Platelet Releasate and Measurement of Secretion. Serum was prepared from human or mouse blood clotted alone or in the presence of inhibitors. Platelet releasate was prepared by washing platelets from citrated human blood (1) and suspending them in Tyrode's solution buffered to pH 7.4 with Hepes or *N*-[tris(hydroxymethyl)-methyl]aminoethanesulfonic acid (Tes) with no added $CaCl_2$ or protein. Albumin was not added as we and others (12) found that it may contain platelet secretory products. The suspension contained 0.5–2 × 10^9 platelets per ml and <1/1000th this number of leukocytes and erythrocytes. Very marked aggregation and secretion were induced by shaking samples with 1 unit of pure thrombin per ml or 250 nM A23187 (from 100 μM stored at 4°C in 95% ethanol). Samples were incubated up to 60 min at 37°C and centrifuged 2 min at 12,000

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Abbreviations: APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; εAhx, 6-aminohexanoic acid (ε-aminocaproic acid); PF4, platelet factor 4; LA-PF4, low-affinity PF4; PDGF, platelet-derived growth factor; PFC, plaque-forming cells; SRBC, sheep erythrocyte(s); T cells, thymus-derived cells; β-TG, β-thromboglobulin; RCS, reticulum cell sarcoma (lymphoma); SBTI, soybean trypsin inhibitor.

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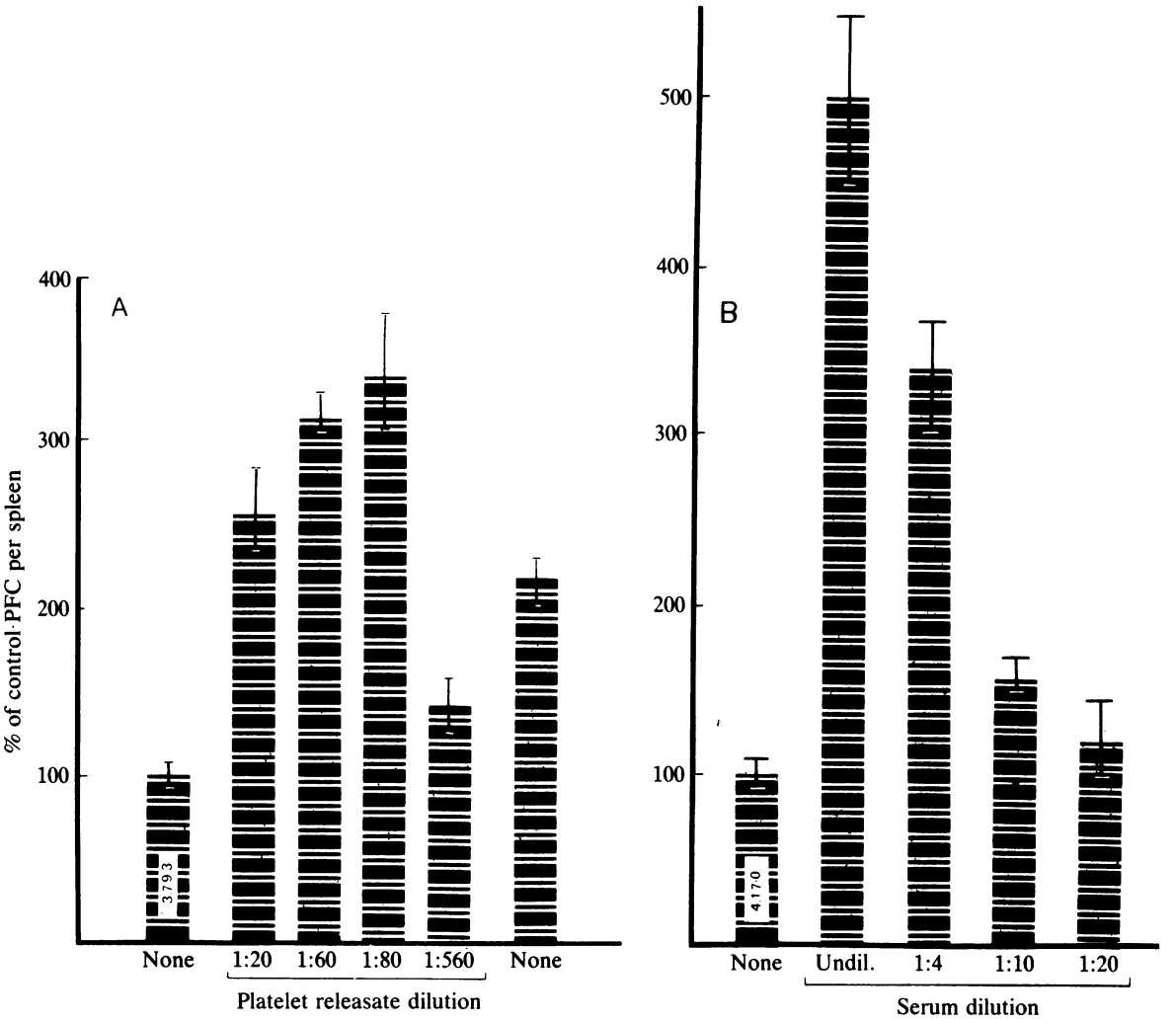

FIG. 1. Splenic PFC produced in response to injection of suppressive agent (γ-irradiated RCS cells), SRBC, and different concentrations of (*A*) releasate from 10^9 platelets per ml or (*B*) human serum. The geometric mean for PFC per spleen in the control suppressed mice is indicated in the lefthand bars. No γ-irradiated RCS cells were injected into the mice represented by the righthand bar in *A*. Undil., undiluted.

× *g*. When the protease inhibitor APMSF was used, samples were dialyzed before testing.

Absorption of Releasates. Platelet releasate was mixed for 2–12 hr with heparin-agarose. The beads were poured into a column, washed with 0.15 M NaCl/0.05 M Tris, pH 8.0, and eluted with Tris-buffered 1.5 M NaCl, sometimes preceded by Tris-buffered 0.5 M NaCl (13). The eluate was dialyzed against TES/Tyrode's solution or diluted 1:10 with distilled water.

Immunization and Assay for Plaque-Forming Cells (PFC). Female SJL/J (The Jackson Laboratory) and $(CB6)F_1$ mice (Charles River Breeding Laboratories) were used. Suppression was induced by 2×10^7 γ-irradiated SJL lymphoma

Table 1. Effect of εAhx on the generation of immunoregulatory activity from mouse or human platelets

Mice injected with SRBC			Geometric mean of PFC per spleen			
+ suppressive agent*	+ releasate or serum	+ εAhx	Exp. 1†	Exp. 2‡	Exp. 3‡	Exp. 4§
−	−	None	14,790	18,840	5,310	10,730
+	−	None	4,270	7,850	2,320	4,170
+	+	None	26,600	19,050	22,880	36,570
+	+	25 mM, pre	5,890¶	18,220	ND	24,560
+	+	250 mM, pre	ND	ND	6,950	14,070
+	+	25 mM, post	24,550	19,050	ND	31,820
+	+	250 mM, post	ND	ND	ND	ND

ND, not determined.
*γ-Irradiated RCS cells in SJL/J mice in exps. 1, 2, and 4; Con A in $(CB6)F_1$ mice in exp. 3.
†Mouse blood; εAhx added before or 45 min after clotting.
‡Human platelets; εAhx added before or 45 min after thrombin in exp. 2 and A23187 in exp. 3.
§Human blood; εAhx added before or 45 min after clotting.
¶Antilogarithm of SEM = 1.2.

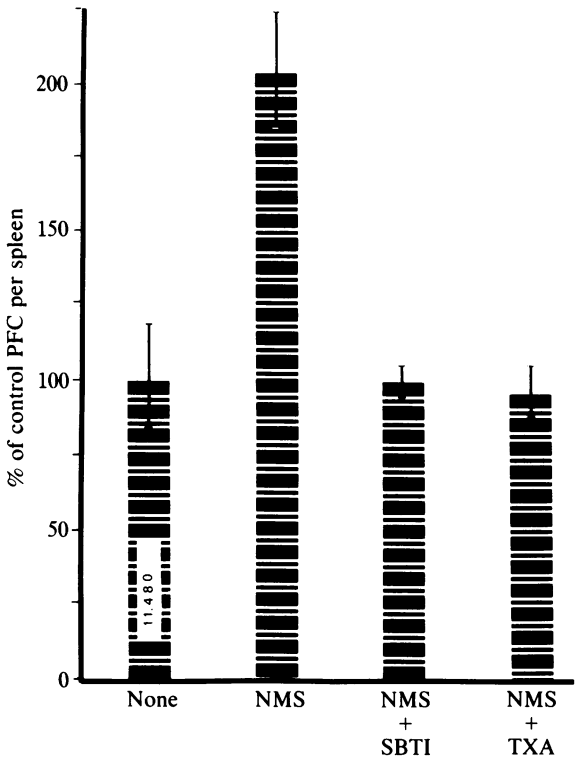

FIG. 2. Splenic PFC produced in response to SRBC in mice given no suppressive agent and injected 1 day before SRBC injection with serum from normal mouse blood (NMS) or serum from blood clotted in the presence of 10 μg of SBTI per ml or of 50 μM tranexamic acid (TXA). The geometric mean of PFC per spleen was 11,480 in control unsuppressed mice.

(RCS) cells (10,000 rads; 1 rad = 0.01 gray) injected i.v. into SJL mice simultaneously with 2×10^6 or 10^7 SRBC (1) or by 5 μg of Con A injected i.v. into $(CB6)F_1$ mice 1 day before i.v. injection of 10^7 SRBC (2). The two *in vivo* assay systems give essentially identical results (2). Platelet releasate was usually diluted to represent about 10^8 platelets per ml, and 0.2 ml was injected i.v. either 1 day before or on the same day as antigen injection.

Cell suspensions in Hanks' balanced salt solution were prepared from individual spleens of mice taken 5 days after antigen injection. None of the procedures caused any obvious increase in spleen size. Direct PFC were counted by means of a slide modification (14) of Jerne and Nordin's technique (15). At least four mice were used for each datum point. Results are expressed as geometric means of PFC per spleen; the SEM (antilogarithm) was ≤1.1 unless otherwise noted.

RESULTS

Dose–Response Curve of Immunoregulatory Activity. Undiluted serum had the largest effect, but even a 1:10 dilution increased the number of PFC above the control value (Fig. 1*A*). Releasate from a human platelet suspension had the greatest effect after injection of material from about 10^7 platelets (Fig. 1*B*); releasate from 10^6 platelets was still active. High concentrations of releasate increased the number of PFC above the number obtained in unsuppressed mice injected with antigen alone (Fig. 1*B*; Table 1, exps. 1, 3, and 4).

Comparison of Release of Immunoregulatory Activity with Release of Other Platelet Granule Components. In releasate from platelets incubated for 60 min with 0.025–1 unit of thrombin per ml, immunoregulatory activity, PF4, and β-TG were released most readily, followed by [^{14}C]serotonin and β-glucuronidase. Less than 8% of total lactate dehydrogenase was released at all thrombin concentrations (data not shown).

Effects of Enzyme Inhibitors or Boiling on Generation of Immunoregulatory Activity. Although fibrin formation was not noticeably impaired when mouse blood clotted in the presence of 10 μg of SBTI per ml or 50 μM tranexamic acid, this serum did not enhance the immune response (Fig. 2). Addition of 25 mM εAhx prior to clotting also prevented development of activity (Table 1, exp. 1). In contrast, 25 mM εAhx had little or no effect with human platelets exposed to A23187 or clotting whole blood, and 250 mM εAhx only partially inhibited development of activity (Table 1, exps. 2–4).

Releasates had little immunoregulatory activity when human platelets were stimulated with 250 nM A23187 in the presence of 100 μM serine protease inhibitor APMSF (16), although the size of the aggregates and amount of serotonin secreted were not decreased. Full activity was noted if APMSF was added after incubating samples for 60 min (Table 2). Little activity was also produced when platelets were aggregated with 1 unit of thrombin per ml and APMSF was added 2 min later, whereas activity was marked when the inhibitor was added 60 min after thrombin (data not shown). Development of immunoregulatory activity did not require the continuing presence of platelets after secretion had taken place: activity was the same in samples centrifuged 2 min after addition of A23187 and then incubated as in samples incubated prior to centrifugation. Little activity was found in samples centrifuged and boiled 2 min after aggregation with A23187, whereas activity was only slightly decreased by

Table 2. Inhibition by APMSF or by boiling of the generation of immunoregulatory activity from human platelets stimulated with 250 nM A23187

Mice injected with SRBC		Geometric mean of PFC per spleen				
+ suppressive agent*	+ platelet releasate	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
−	−	17,450	5,585	16,090	12,445	22,760
+	−	8,270	1,540	8,000	4,875	7,540
+	+	20,990	44,870	14,185	29,920	24,570
+	+ APMSF, pre†	8,900	8,709	6,240	3,940	6,630
+	+ APMSF, post‡	ND	ND	13,850	ND	25,120
+	Boiled before incubation§	9,550	ND	7,690	ND	8,785
+	Boiled after incubation¶	ND	28,820	12,940	ND	24,220

ND, not determined.
*γ-Irradiated RCS cells in exp. 1; Con A in exps. 2–5.
†100 μM APMSF added to platelet suspension before A23187, supernatant dialyzed after 60 min.
‡Suspension incubated 60 min with A23187, then 100 μM APMSF added and supernatant dialyzed.
§Suspension centrifuged 2 min after A23187, then boiled 5 min and incubated 60 min.
¶Suspension centrifuged 2 min after A23187, then incubated 60 min and boiled 5 min.

Table 3. Absorption of activity by heparin-agarose and interaction between heparin binding and nonbinding factors in the generation of immunoregulatory activity from human platelets

Mice injected with SRBC		Geometric mean of PFC per spleen			
+ suppressive agent*	+ additional material†	Exp. 1	Exp. 2	Exp. 3	Exp. 4
−	None	9,330	3,800	15,850	7,700
+	None	4,020‡	1,640	8,510	1,760
+	Releasate	11,450‡	6,990	22,910	ND
+	Releasate absorbed with heparin-agarose (I)	3,000‡	ND	7,410	4,210§
+	0.5 M NaCl eluate from releasate absorbed with heparin-agarose	ND	990	ND	ND
+	1.5 M NaCl eluate from releasate absorbed with heparin-agarose	10,540‡	5,210	26,060	11,940
+	1.5 M NaCl eluate from APMSF releasate absorbed with heparin-agarose (II)	ND	ND	8,320	1,920
+	Combination of I and II	ND	ND	27,540	9,620

ND, not determined.
*Con A in exps. 1 and 2; γ-irradiated RCS cells in exps. 3 and 4.
†Releasates and derivatives incubated for 45 min at 37°C.
‡Antilogarithms of SEM = 1.2; for all other determinations, it is ≤1.1.
§Releasate absorbed with plain agarose, 10,580. In another experiment, immunoregulatory activity was 20,740 in releasate, 14,470 in releasate absorbed with plain agarose, and 6590 in a 1.5 M NaCl eluate of plain agarose. Activity in releasate absorbed with heparin-agarose was 9330 and in the 1.5 M NaCl eluate activity was 17,080 PFC per spleen.

boiling samples after they had been incubated with A23187 for 60 min (Table 2).

Studies of Secreted Platelet Substances. Table 3 shows that heparin-agarose but not plain agarose removed immunoregulatory activity from solution. Activity was eluted with 1.5 M NaCl but not with 0.5 M NaCl. It was generated by incubating together the inactive 1.5 M NaCl eluate prepared from APMSF-inhibited releasate and the inactive flow-through obtained by passing active releasate through heparin-agarose.

PDGF (0.003 μg per mouse) had little or no activity, β-TG (0.6 μg per mouse) had a small amount of activity, and PF4 (0.2–0.6 μg per mouse) had very marked immunoregulatory activity, about the same as that released from 2×10^7 platelets (Table 4). Goat antiserum to PF4 nearly abolished the activity of normal human serum, whereas a similar amount of normal goat serum had no effect (Table 5).

Heparin-Neutralizing Activity. Ability of platelet releasate to neutralize the anticoagulant activity of heparin was the same whether or not APMSF was present during stimulation of the platelets with A23187. It was slightly decreased by boiling, but to the same extent whether the releasate was boiled immediately after platelet stimulation or 60 min later (data not shown).

DISCUSSION

The immunoregulatory material shown earlier (1) to be secreted by platelets is remarkably active. Injection of 0.2 ml of a 1:10 dilution of serum is effective and equivalent to $<10^7$ platelets per mouse. Similarly, 0.2 ml of platelet releasate diluted to represent material from $<10^6$ platelets retains slight activity. Injection of higher concentrations of serum or releasate increases the number of PFC far above the number produced by injection of antigen into mice in which antibody formation was not suppressed by Con A or lymphoma cells. Indeed, the material can enhance antibody formation even in mice not treated with an immunosuppressive agent (ref. 1; Fig. 2). Human serum enhances the response to trinitrophenylated Ficoll (1) without raising the number of anti-SRBC PFC in the spleen (I.R.K. and G.J.T., unpublished observations).

Assays of releasates prepared by stimulating platelets with thrombin confirmed the findings of others (17, 18) that the α-granule constituents PF4 and β-TG are released more readily than the contents of the dense granules (e.g., serotonin) and lysosomes. Immunoregulatory activity was released by low thrombin concentrations, consistent with its origin from the α-granules as suggested earlier (1).

An enzyme secreted from platelets plays a role in production of active material. Serum from mouse blood in the presence of εAhx, SBTI, or tranexamic acid lacked activity. εAhx was less effective in inhibiting the development of activity from human platelets; the other two agents were not tested. The irreversible serine protease inhibitor APMSF (16), added before platelet secretion was induced by the calcium ionophore A23187 or 2 min after it was induced by thrombin, prevented development of immunoregulatory activity without impairing secretion of [^{14}C]serotonin or aggre-

Table 4. Immunoregulatory activity of platelet α-granule constituents

Mice injected with SRBC		Geometric mean of PFC per spleen		
+ Con A	+ additional material	Exp. 1	Exp. 2	Exp. 3
−	None	16,090	12,450	32,590
+	None	8,000	4,880	12,920
+	Releasate*	14,190	29,920	30,740
+	PF4†	ND	22,910	34,420
+	β-TG‡	ND	9,440	ND
+	PDGF§	8,370	ND	ND

ND, not determined.
*From 2×10^7 platelets per mouse.
†0.6 μg (exp. 2) or 0.2 μg (exp. 3) per mouse.
‡0.6 μg (exp. 2) per mouse.
§0.003 μg per mouse.

Table 5. Reversal of immunoregulatory activity in human serum by goat antiserum to PF4

Mice injected with SRBC		Geometric mean of PFC per spleen
+ Con A	+ additional material*	
−	None	8,720
+	None	4,380
+	Human serum	51,590
+	Human serum + goat anti-PF4	13,190
+	Human serum + normal goat serum	49,470
+	Goat anti-PF4	10,000

*Human serum (0.05 ml) and/or goat antiserum to PF4 (0.008 ml) or normal goat serum (0.008 ml) were incubated for 20 min at 4°C prior to injection.

gation. Samples to which APMSF was added after incubation of the A23187-stimulated platelets were fully active. Development of activity was also prevented by boiling releasates separated 2 min after stimulating the platelets but not by boiling them 60 min afterwards, providing further evidence for the role of a secreted platelet enzyme.

Niewiarowski and his collaborators (4, 13, 19) showed that a secreted platelet serine protease, inhibited by phenylmethylsulfonyl fluoride, converts β-TG$^{pI\ 8}$ to the immunologically indistinguishable β-TG$^{pI\ 7}$ by catalyzing removal of four N-terminal amino acids and similarly converts β-TG$^{pI\ 10}$ to β-TG$^{pI\ 8}$ (5). Factor D, a serine protease required in the alternate complement pathway, is also secreted from platelet α-granules (18). Legrand *et al.* (20) purified trypsin-, chymotrypsin-, and elastase-like enzymes from platelet lysates.

The purified PF4 used in the present study has immunoregulatory activity; 0.2 or 0.6 μg was about as active as releasate from 2×10^7 platelets. Since it was made from lysed out-dated platelets, any changes produced by the putative platelet enzyme had presumably taken place before it was purified. The amount of PF4 in human platelets (18 μg per 10^9; ref. 21) and serum (5.3 μg/ml; ref. 22) is sufficient to account for their immunoregulatory activity. PDGF and β-TG, tested at about one-fourth the concentration in human serum (22, 23), had little or no activity. The active agent is absorbed from releasates by heparin-agarose, eluted by 1.5 M NaCl but not by 0.5 M NaCl, and neutralized by specific antibody to PF4, providing further evidence that it is PF4. The enzymatic change in PF4 that occurs after its secretion does not affect its ability to neutralize heparin. This observation is consistent with localization of heparin-neutralizing activity to the C-terminal end of the molecule and localization of the proteolytic cleavage site (if it is like that of β-TG) to the N-terminal end (3).

Activities have been described for PF4 in addition to its ability to bind heparin (24–27). Most relevant is its chemotactic activity for human leukocytes (26) and fibroblasts (27). Furthermore, a protein with considerable amino acid homology to PF4 but no known function is induced by treatment of human spleen and mononuclear blood cells with γ-interferon (28). Thus, the ability of PF4 to alleviate immunosuppression that we have described opens a new perspective on the functions of this family of proteins.

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