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Immunoregulatory activity of peptides related to platelet factor 4

(immunosuppression/suppressor T cells/antibody formation)

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ABSTRACT **Platelet factor 4 (PF4), a secreted platelet protein, alleviates concanavalin A-induced immunosuppression in mice. We now find that activity also resides in (*i*) the C-terminal tridecapeptide of PF4 (P13S), (*ii*) an analog of this in which arginine replaces the lysine residues and in which the last two amino acids are absent, (*iii*) the C-terminal 18 amino acids of low-affinity platelet factor 4, which is very similar to P13S, and (*iv*) peptide fragments of P13S that contain only 5–9 amino acids. P13S treated with fluorescamine to derivatize the free amino groups retained immunoregulatory activity but did not bind to heparin-agarose. The N-terminal and middle portions of PF4, polylysine, protamine, and three unrelated peptides were inactive in this assay.**

Platelet factor 4 (PF4), a 7800-kDa secretable platelet protein with anti-heparin and chemotactic activity (1–3), alleviates immunosuppression induced in mice by injection of concanavalin A (Con A) or γ-irradiated reticulum sarcoma cells (4, 5). Earlier results (5) showed that it was active at 0.2 μg per mouse and secretable from as few as 2×10^7 human platelets. Recent results have shown activity at 0.05 μg. Recombinant PF4 with additional amino acids at each end of the molecule and with aspartic acid instead of asparagine at position 47 is also active (6). We now report studies on the immunoregulatory activity of fragments of PF4.

MATERIALS AND METHODS

Source of Peptides. The amino acid sequences of the PF4 molecule and other peptides studied are shown in Fig. 1. T. Maione (Repligen, Cambridge, MA) donated recombinant preparations of (*i*) PF4 (rPF4) identical to the native molecule, (*ii*) the C-terminal 41 amino acids (designated C41), (*iii*) the N-terminal 29 amino acids (designated N29) of PF4, and (*iv*) synthetic P13S, the C-terminal 13 amino acids of PF4. Low-affinity PF4 (LA-PF4) was prepared from extracts of fresh platelets and was separated from PF4, β-thromboglobulin, and platelet basic protein by chromatography on CM-Sephadex (7). Peptides H18, H22, and H24 were derived from reduced *S*-pyridylethyl-modified native PF4 (900 μg) by digestion with 60 μg of protease isolated from *Staphylococcus aureus* V8 (Pierce) at 37°C for 3 hr in 0.05 M sodium phosphate (pH 7.8). The digest was fractionated on a Vydac wide-pore C_{18} reverse-phase column (The Separations Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid and was eluted with a gradient of 0–55% acetonitrile in the same solvent. Identity of the collected peaks was established by automated N-terminal sequencing.

The C-terminal octadecapeptide of LA-PF4, designated P18D, and a second preparation of P13S were synthesized by Peninsula Laboratories and were provided by Stefan Niewiarowski (Temple University). Peptides unrelated to PF4 were kindly given to us by T. Maione (Fig. 1*C*). Peptides H1, H2, and H3 (Fig. 1*D*) are portions of P13S prepared by incubating the tridecapeptide for 53 hr at 37°C in 0.1 mM $CaCl_2$/10 mM Hepes, pH 7.8, with a fibrinogenolytic enzyme from the salivary glands of the leech *Hementaria ghiliani* (hementin, a gift of N. Kirschbaum and A. Budzynski, Temple University) (8). The fragments of P13S were isolated by reverse-phase HPLC and identified by automated N-terminal sequencing. The C-terminal tridecapeptide of PF4 without the two terminal amino acids and with arginine residues replacing lysine was a gift from Daniel A. Walz (Wayne State University Medical School, Detroit, MI) (9) (Fig. 1*E*). Polylysine (average M_r, 14,000) was obtained from Sigma, and injectable protamine (10 mg/ml) was from Du-Pont Critical Care (Waukegan, IL).

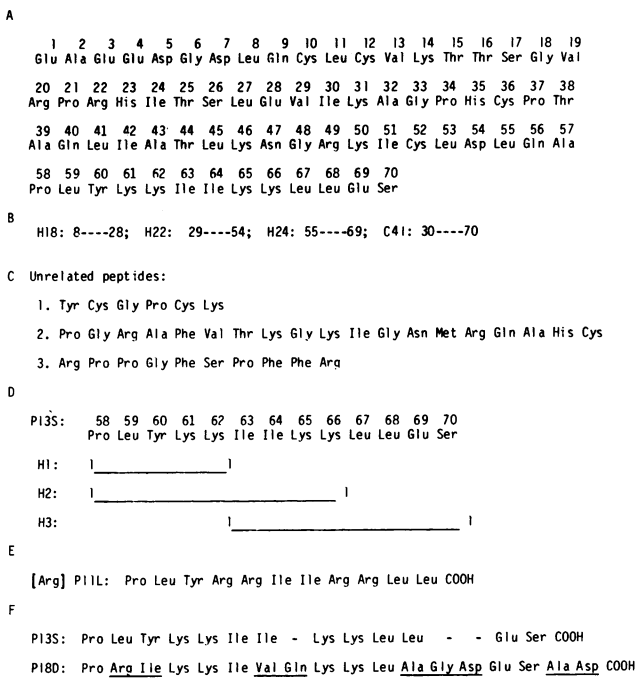

FIG. 1. Amino acid sequences of PF4 and peptides tested for immunoregulatory activity. (*A*) Amino acid sequence of PF4. (*B*) Fragments of PF4 tested. (*C*) Peptides unrelated to PF4. Peptide 3 is bradykinin. (*D*) C-terminal tridecapeptide of PF4 (P13S) and smaller peptides H1, H2, and H3. (*E*) Amino acids 58–68 of PF4 with arginine substituted for lysine. (*F*) C-terminal tridecapeptides of PF4 (P13S) and of LA-PF4 and β-thromboglobulin (P18D), aligned to indicate their homology (see ref. 3). Dissimilar amino acids are underlined.

Automated N-Terminal Sequencing. Sequencing was performed on a gas-phase sequencer (Applied Biosystems,

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Abbreviations: PF4, platelet factor 4; rPF4, recombinant PF4; P13S, terminal tridecapeptide of platelet factor 4; LA-PF4, low-affinity PF4; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

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Table 1. Immunoregulatory activity of fragments of PF4 and unrelated peptides

Material injected on day −1*	% of control, geometric mean of PFC per spleen (×/÷ SE)†				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
None	100 (1.2)	100 (1.1)	100 (1.2)	100 (1.1)	100 (1.1)
Con A	39 (1.2)	57 (1.3)	62 (1.2)	25 (1.1)	25 (1.1)
+ rPF4		285‡ (1.1)	80 (1.3)	70‡ (1.1)	70‡ (1.1)
+ H18					39 (1.2)
+ H22	35 (1.2)	79 (1.1)	28 (1.2)	29 (1.1)	38 (1.3)
+ H24	86§ (1.2)	219‡ (1.1)	162¶ (1.1)		
+ peptide 1				30 (1.1)	
+ peptide 2				21 (1.1)	
+ peptide 3				26 (1.1)	

*All mice were injected with SRBC on day 0. Peptides and rPF4 were used at 1 μg per mouse; their compositions are shown in Fig. 1.
†100% of PFC per spleen: 26,300, 8300, 29,700, 19,000, and 31,700 in experiments 1–5, respectively.
‡$P < 0.0001$ compared with Con A alone.
§$P < 0.05 > 0.01$ compared with Con A alone.
¶$P < 0.01 > 0.001$ compared with Con A alone.

model 470A) coupled to an on-line phenylthiohydantoin analyzer (Applied Biosystems, model 120A) (10, 11), both operated by the Macromolecular Analysis and Synthesis Laboratory of the Temple University Health Science Center. Manufacturers' standard protocols were followed for both Edman degradation and separation of the phenylthiohydantoin-conjugated amino acids by HPLC. Cysteine was detected as *S*-pyridylethylcysteine.

Fluorescamine Treatment. To derivatize the amino groups, rPF4 and P13S were treated with fluorescamine (Sigma) dissolved in dry acetone (12). The acetone was evaporated under nitrogen, and fluorescence was determined with a Farrand ratio fluorimeter 2 before and after passing fluorescent P13S over a column of heparin-conjugated agarose. As a control, rPF4 or P13S was similarly treated with acetone alone.

Assay. The method was described earlier (4, 5). Briefly, on day −1, BALB/c mice (Harlan–Sprague–Dawley) were injected i.v. with PF4 or peptides in 0.2 ml and 1 hr later with 10 μg of Con A (Sigma) in 0.2 ml. Each peptide was tested in four or five mice. On day 0, the mice were injected i.v. with 3–5 × 10^6 sheep erythrocytes (SRBC; Colorado Serum, Denver). On day 5, the numbers of plaque-forming cells (PFC) in the spleens were determined (13, 14), and the geometric mean ×/÷ SE (antilog) was calculated. When the SE was between 1.0 and 1.1, it was designated 1.1. In one experiment, suppression was induced by injecting γ-irradiated reticulum sarcoma cells (4) into SJL mice (The Jackson Laboratory). Low variability in response and consistent Con A-induced suppression required hepatitis-free mice, as this disease alters T-cell function (15).

PF4 and peptides were diluted either in mouse albumin at 0.5 mg/ml (Sigma) or crude human albumin at 1 mg/ml, which was the 50%-saturated ammonium sulfate supernatant of platelet-poor citrated human plasma dialyzed against 0.15 M NaCl/0.01 M Hepes, pH 7.4. Both solutions were treated with 1 mM diisopropyl fluorophosphate and dialyzed against saline/Hepes. These diluents did not affect the response.

RESULTS

Immunoregulatory activity of the peptides of Fig. 1 *B* and *C* is shown in Table 1. Peptide H24, comprising amino acids 55–69 of PF4, was active. The C-terminal 41 amino acids of PF4, prepared by recombinant techniques, also had activity (data not shown). In contrast, peptides comprising amino acids in the middle and N-terminal portions of PF4 were inactive (H22 and H18, Table 1; and N29, data not shown), as were three peptides unrelated to PF4 (Table 1).

P13S, the C-terminal tridecapeptide of PF4 (amino acids 58–70) had immunoregulatory activity at 0.7 μg per mouse but not at 0.07 μg (Table 2) or 0.2 μg (data not shown). P13S, like PF4 (4), reversed suppression in mice in which the antibody response was suppressed by injection of γ-irradiated cells from a reticulum cell sarcoma (RCS) rather than by Con A (γ-irradiated RCS alone, 33%; γ-irradiated

Table 2. Immunoregulatory activity of P13S, fluorescamine-treated rPF4, and P13S

Material injected on day −1*	% of control, geometric mean of PFC per spleen (×/÷ SE)†			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	100 (1.2)	100 (1.2)	100 (1.1)	100 (1.1)
Con A	32 (1.2)	36 (1.2)	13 (1.3)	17 (1.1)
+ P13S				
2.5–7 μg	98‡ (1.2)			
0.7–1 μg	94§ (1.1)	240¶ (1.1)	80§ (1.2)	
0.07 μg	36 (1.4)			
0.007 μg	51 (1.4)			
+ fluorinated P13S, 1 μg			62§ (1.1)	
+ rPF4, 1 μg			150‡ (1.2)	83§ (1.2)
+ fluorinated rPF4, 1 μg			58‖ (1.1)	153‡ (1.1)

*All mice were injected with SRBC on day 0. In experiments 1 and 2, $n = 10$; in experiments 3 and 4, $n = 5$.
†100% of PFC per spleen: 11,100, 17,500, 16,800, and 22,500 in experiments 1–4, respectively.
‡$P < 0.005$ compared with Con A alone.
§$P < 0.01 > 0.005$ compared with Con A alone.
¶$P < 0.0001$ compared with Con A alone.
‖$P < 0.05 > 0.01$ compared with Con A alone.

Table 3. Absorption of immunoregulatory activity of C-terminal tridecapeptide of PF4 by cells from mouse spleen or thymus

Material injected on day −1*	% of control, geometric mean of PFC per spleen (×/÷ SE)† Exp. 1	Exp. 2
None	100 (1.2)	100 (1.3)
Con A	35 (1.1)	38 (1.2)
+ unabsd. P13S‡	288 (1.1)	260 (1.1)
+ peptide absd. with normal spleen cells	103§ (1.1)	69 (1.6)
+ peptide absd. with normal thymus cells	236¶ (1.3)	
+ peptide absd. with spleen cells from athymic mice		150 (1.2)

*All mice were injected with SRBC on day 0.
†100% of PFC per spleen: 8200 in experiment 1 and 14,900 in experiment 2.
‡Unabsorbed (unabsd.) P13S at 0.5 μg per mouse in experiment 1 and 2.5 μg per mouse in experiment 2.
§$P = 0.001$ vs. unabsorbed peptide.
¶$P = 0.6$ vs. unabsorbed peptide; $P = 0.058$ vs. peptide absorbed (absd.) with normal spleen cells.

RCS + 1 μg of P13S, 71%; $P < 0.05 > 0.01$). P13S lost much more activity after incubation in the cold with mouse spleen cells than after incubation with normal thymus cells (Table 3, experiment 1) or with spleen cells from athymic mice (Table 3, experiment 2).

Table 4 shows the activity of other peptides related to PF4. The analog of the C-terminal tridecapeptide in which lysine was replaced with arginine and the last two amino acids were omitted ([Arg]P11L) was active (Fig. 1*E*), as were three fragments of the C-terminal tridecapeptide (H1, H2, and H3) (Fig. 1*D*) and P18D, the C-terminal octadecapeptide of LA-PF4 (Fig. 1*F*). LA-PF4 was tested in three experiments. In the first experiment, 1, 3, and 10 μg of LA-PF4 plus Con A did not significantly affect the number of PFC per spleen compared with Con A alone, whereas rPF4 was active at 0.05, 0.5, and 5 μg as expected ($P \approx 0.01$). In two subsequent experiments, the number of PFC per spleen with 1 μg of LA-PF4 plus Con A was significantly greater than that with Con A alone ($P < 0.01$) (data not shown).

Fluorescamine-treated rPF4 and recombinant P13S were active (Table 2), although measurement of fluorescence showed that they did not bind to heparin-agarose. Polylysine and protamine had no significant immunoregulatory activity (Table 5).

Table 5. Immunoregulatory activity of polylysine and protamine

Material injected on day −1*	% of control, geometric mean of PFC per spleen (×/÷ SE)† Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	100 (1.2)	100 (1.1)	100 (1.1)	100 (1.1)
Con A	45 (1.1)	58 (1.1)	30 (1.1)	38 (1.1)
+ rPF4				
0.05 μg	107‡ (1.1)			
0.2 μg		94§ (1.1)		
1.0 μg	129§ (1.2)	114¶ (1.1)	95¶ (1.1)	76‡ (1.1)
+ polylysine				
0.05 μg	51 (1.2)			
0.2 μg		81 (1.2)		
1.0 μg	58 (1.2)	81 (1.2)	27 (1.1)	25 (1.1)
+ protamine				
1.0 μg	66 (1.5)		33 (1.1)	
5.0 μg	69 (1.3)			

*All mice were injected with SRBC on day 0. In experiment 1, $n = 9$; in experiments 2 and 3, $n = 4$, in experiment 4, $n = 8$.
†100% of PFC per spleen: 7500, 14,900, 14,400, and 34,000 in experiments 1–4, respectively.
‡$P < 0.001$ compared with Con A alone.
§$P < 0.05 > 0.01$ compared with Con A alone.
¶$P < 0.01 > 0.001$ compared with Con A alone.

DISCUSSION

In view of the immunoregulatory activity of platelet-derived and rPF4 we have described (4–6) and the chemotactic activity of PF4's C-terminal tridecapeptide, P13S, reported by others (16, 17), we investigated the immunoregulatory activity of fragments of PF4 and related molecules. The C-terminal 41, 15, and 13 amino acids of PF4 (C41, H24, and P13S) were all active, whereas immunoregulatory activity was not detected in the 21–30 amino acid peptides from the middle and N-terminal portions of PF4 or in three unrelated small peptides with 6 to 19 amino acids.

PF4 and LA-PF4 belong to a family of structurally similar proteins containing four cysteine residues in homologous positions (reviewed in ref. 3) Some members of this group have mitogenic or chemotactic activity, and it will be of interest to determine whether any of them have immunoregulatory activity even though their C-terminal regions show little homology with the C-terminal regions of LA-PF4 and PF4.

Recombinant PF4 is consistently active at 0.05 μg per mouse, whereas P13S is active at 0.2 and 0.7 μg. In contrast, P13S is reported to have about 30-fold greater chemotactic activity toward monocytes than the parent compound PF4

Table 4. Immunoregulatory activity of peptide fragments of PF4 and β-thromboglobulin

Material injected on day −1*	% of control, geometric mean of PFC per spleen (×/÷ SE)† Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
None	100 (1.1)	100 (1.2)	100 (1.2)	100 (1.1)	100 (1.1)	100 (1.1)
Con A	39 (1.2)	16 (1.1)	31 (1.1)	29 (1.2)	31 (1.1)	27 (1.1)
+ P13S			92‡ (1.2)	60 (1.2)	59 (1.1)	
+ [Arg]P11L	111‡ (1.2)	67§ (1.4)				
+ H1			162‡ (1.1)	76‡ (1.1)	89§ (1.2)	
+ H2			97§ (1.3)	74§ (1.3)	102‡ (1.2)	
+ H3			96 (1.4)	79‡ (1.2)		
+ P18D			93§ (1.3)	105‡ (1.2)		107‡ (1.1)

*All mice were injected with SRBC on day 0. Peptides are described in Fig. 1 and were used at 1 μg per mouse. SRBC controls and Con A-suppressed mice are the same in experiment 1 as in experiment 1 of Table 1.
†100% of PFC per spleen: 26,300, 41,900, 16,600, 39,500, 12,000, and 35,100 in experiments 1–6, respectively.
‡$P < 0.01$ compared with Con A alone.
§$P < 0.05 > 0.01$ compared with Con A alone.

(17). Although these results suggest a dissociation between the *in vivo* immunoregulatory activity of PF4 and its *in vitro* chemotactic activity, considerations such as possible differences in the *in vivo* fates of the peptide and intact PF4 preclude a firm conclusion.

As with native or recombinant PF4 (4, 6, 18), the immunoregulatory activity of P13S is markedly decreased after incubation in the cold with spleen cells from normal mice but is relatively unaltered after incubation with those from athymic mice or thymus cells from normal mice.

LA-PF4, with about 50% homology to PF4 (2, 3), was inactive when first tested, but the same preparation was active in two subsequent experiments. The C-terminal peptide of LA-PF4, P18D, was consistently active. Physicochemical analysis (19) indicates that the active helical C-terminal region may be hidden in the native LA-PF4 molecule but not in PF4. Possibly, repeated freezing and thawing exposes the C-terminal region of LA-PF4, conferring immunoregulatory activity on the whole molecule.

Three fragments of P13S, H1, H2, and H3, were active. These small peptides have one or two pairs of adjacent lysines. An analog of a portion of P13S, [Arg]P11L, in which lysine residues are replaced with arginine residues, is also active. Although these results suggest that adjacent basic amino acids are necessary for activity, the basic proteins polylysine and protamine are inactive. Furthermore, activity is undiminished in rPF4 and C13 which had been treated with fluorescamine to derivatize the amino groups. These fluorescent compounds do not bind to heparin-agarose. Peptides from the C terminus of PF4 bind to heparin but are eluted by much lower concentrations of NaCl than are necessary to elute the whole PF4 molecule (1, 2, 6, 20). Thus, affinity for heparin does not seem to be responsible for the immunoregulatory activity of PF4 and related peptides. Possibly the hydrophobic character of the active peptides is important.

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