PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147**, 188–194 (1974)

Platelet Aggregation and Release Triggered by Antibody to Platelet Factor 4 (PF_4)[1] (38309)

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It is well established that immune injury of platelets will trigger their aggregation and the platelet release reaction. There are at least three possible mechanisms responsible for these phenomena: (a) Antibody to structural antigens or determinants adsorbed on the platelet membranes may sensitize the cell for the cytolytic action of complement. However, in the presence of higher concentrations of antibody, even in the absence of complement, platelet aggregation and subsequent platelet damage may occur (1). (b) Complexes of antigens (unrelated to platelets) with antibodies may bind to platelet membranes or may be phagocytosed by platelets resulting in release of platelet constituents (2). (c) Platelets may be aggregated by modified (aggregated) immunoglobulins (3).

Platelet factor 4 (PF_4) is a platelet constituent neutralizing the anticlotting activity of heparin. It is released from platelets during the platelet release reaction (4, 5). The purpose of this communication is to report that antibody to PF_4 can specifically trigger platelet aggregation and release.

Materials and Methods. Experiments were performed using platelet rich plasma (PRP) or suspensions of washed platelets of human or pig. Blood was collected either in acid citrate–dextrose (ACD) or in citrate (0.11 *M*); pH of PRP was adjusted to 7.4 before experiments. Human platelets were isolated according to the method of Mustard *et al.* (6). Platelets were washed at 37° with Tyrode–albumin solution containing potato apyrase prepared according to Molnar and Lorand (7). Final suspensions (count 10^9/ml of human platelets), kept at 37° without addition of apyrase, was used within 2–3 hr.

Suspensions of washed pig platelets were prepared according to the method of Ardlie *et al.* (8). Platelet count in the final washed suspension, if not otherwise stated, was 10^9/ml. Platelet count in PRP was approximately 4×10^9/ml. Bovine serum albumin (BSA) was omitted from the washing fluid when platelets were isolated for the purpose of purifying PF_4.

Pig PF_4 was prepared as previously described (9). Washed pig platelets were treated with collagen. Released PF_4 was precipitated by $ZnSO_4$ and further purified by diethyloaminoethyl–cellulose (DEAE-C) column chromatography. PF_4 was released from washed human platelets by either collagen or thrombin. Human PF_4 was recovered and purified following a method similar to the one used for pig PF_4. Purified human PF_4 was homogenous as tested by SDS acrylamide gel electrophoresis (10). Antiheparin activity of PF_4 is expressed in protamine sulfate (PS) units (11); one unit of PF_4 corresponds to 1 μg of PS.

Antisera to PF_4 were prepared in rabbits by injecting them with an emulsion of DEAE-C purified-PF_4 and Freund's-complete-adjuvant in the foot pads or subcutaneously at weekly intervals. Total pig PF_4 protein injected per animal distributed over eight injections was about 5 mg. Human PF_4 protein for the same period was 2 mg/animal. Animals were bled at periodic intervals from the ear vein or by cardiac puncture and blood was collected in ACD (1 part of ACD for 6 parts of blood). Platelet poor plasma (PPP) was

[1] Supported by NIH grants HL 15226 and HL 14217 and by grant in aid from Pharma-Research Canada Ltd. (Montreal, Quebec, Canada).

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obtained by differential centrifugation. PPP was heated to 56° for 30 min and precipitated fibrinogen was removed by spinning. Serum was separated and stored frozen without any preservative. Immunoglobulin was prepared by ammonium sulfate fractionation of this serum (12). Antigenic assay of PF_4 was done using double gel diffusion test of Ouchterlony (13) in the modification previously described (9). The titer of both anti-human PF_4 ($AHPF_4$) and anti-pig PF_4 ($APPF_4$) antisera was 1:8 when tested in gel diffusion. The concentration of protein of gamma globulin fraction of rabbit serum was approximately 20 mg/ml.

Aggregation of platelets was studied by the method of Mustard *et al.* (14) using a Payton aggregometer (Payton Associates, Inc., Scarborough, Ontario, Canada). Results were expressed in LTU (light transmission units) which correspond to the changes in light transmission through the platelet suspension.

Platelet release reaction and lysis were evaluated by measuring the amount of platelet constituents lost from the cells to ambient fluid. The released quantities or activities were expressed as percentages of the total present in the platelet suspension, homogenized either by Triton X-100 or by sonification. Labeling platelets with ^{3}H-serotonin (^{3}H-5-hydroxytryptamine, ^{3}H-5-HT) and measuring the release of radioactivity from labeled platelets were performed as described previously (15). ^{3}H-5-H-T (Amersham/Searle) had specific activity of 5.4 mCi/mg. Platelets were also labeled with ^{51}Cr to assess their lysis. 200 μCi of ^{51}Cr sodium chromate were incubated with the suspension of platelets, obtained during first washing, for 30 min at 37°. Then platelets were centrifuged, washed for the second time and resuspended in Tyrode–albumin solution. Specific activity for ^{51}Cr-sodium chromate (Amersham/Searle) was 500 μCi/μg Cr. The radioactivity released from platelets was counted in an automatic gamma spectrometer (Nuclear Chicago). Adenine nucleotides (AN) released from platelet suspension were measured as described previously (15). Lactic dehydrogenase (LDH) was assayed by the method of Bergmeyer *et al.* (16). β-*N*-acetylglucosaminidase was assayed as described by Day *et al.* (17). Antiheparin activity of platelet factor 4 was tested by the method of Poplawski and Niewiarowski (11).

Results and Discussion. Figure 1 shows that when anti PF_4 γ-globulin was added to a washed pig platelet suspension, aggregation of platelets occurred within a short time. Various dilutions of γ-globulin (1:10, 100, 1000, 3000, etc.) continued to aggregate platelets to the same extent, however, there was a progressive increase in the lag period or interval between the addition of

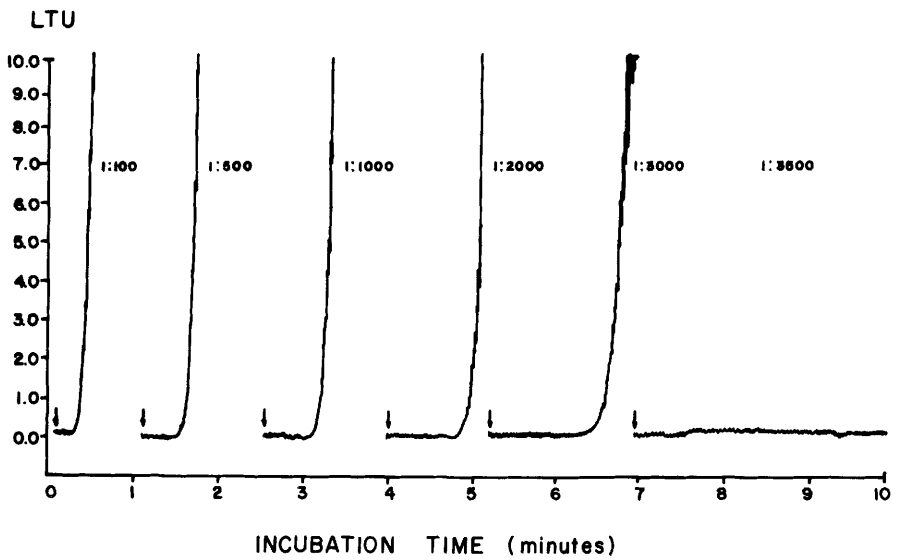

FIG. 1. Effect of various dilutions of anti-pig PF_4 γ-globulin on the aggregation of washed suspension of pig platelets. Aliquots of 0.1 ml of $APPF_4$ antibody diluted in 0.15 *M* NaCl were incubated with 0.9 ml platelets (10^9/ml) in Payton aggregometer cuvettes.

γ-globulin and the beginning of aggregation, with increasing dilution of the γ-globulin.

Table I shows the results of three different experiments in which aggregation of pig or human platelets by $APPF_4$ or $AHPF_4$ gamma globulin was studied. Preimmune rabbit γ-globulin did not show significant aggregating activity. Pig platelets were 10–30 times more sensitive to $APPF_4$ than to $AHPF_4$ antibody. Similarly human platelets were more sensitive to anti-human PF_4 antibody, thus indicating a species specificity of PF_4 antigen. It is evident that pig platelets were more sensitive than human platelets to antibody induced aggregation. Pig plasma appeared to contain a substance which blocked aggregation induced by $APPF_4$.

Table II compares the percentage release of adenine nucleotides, β-*N*-acetyl-glucosaminidase, ^{3}H-5H-T, lactic dehydrogenase and antiheparin activity from washed pig platelets when treated with $APPF_4$ γ-globulin. Release of platelet constituents was monitored at 1, 2, 3, 5, and 15 min intervals. Low concentration of lactic dehydrogenase (up to 6.7% after 15 min), a cytoplasmic marker, suggests that platelet lysis was minimal during the course of experiments. Adenine nucleotide and ^{3}H-5H-T radioactivity reached a 35–40% level within 1 min as compared to antiheparin and glucosaminidase activity which attained a 17–22% level in the same time. Release of PF_4 antigen, tested by gel diffusion, approached its peak in 2 min. ^{3}H-5H-T radioactivity attained a peak value in 2 min and then fell back slightly to reach a plateau at 3 min which remained more or less stable for 15 min. The release process was essentially completed within two minutes and an equilibrium was achieved in 3 min. The overall release of granular constituents amounted to approximately 50% of total, a value in agreement with the release induced by thrombin and collagen (18). PF_4 antigen estimated semiquantitatively by immunodiffusion, was released in parallel with antiheparin activity.

Human platelets labeled with ^{51}Cr and aggregated with $AHPF_4$ antibody released about 18% of AN and 3.7% ^{51}Cr radioactivity (Table III). Thrombin caused approximately 50% release of AN and 4.8% release of ^{51}Cr radioactivity. Complement caused the $AHPF_4$ antibody induced aggregation to commence about 30 sec earlier (not shown in Table III). However, the extent of aggregation was not influenced by complement. Release of AN appeared to be partially inhibited by this component of guinea pig plasma. C_1 of complement (100 units/ml) or C_1 inactivator (100 units/ml) (Cordis) added to the suspension of human platelets did not show any significant effect on $AHPF_4$ induced platelet aggregation.

$APPF_4$ induced aggregation of pig platelets was inhibited by the presence of prostaglandin E_1 (3×10^{-5} *M*, a gift of Upjohn, Kalamazoo, MI), potato apyrase (200 units), EDTA (5×10^{-5} *M*), dipyridamole (6×10^{-6} *M*) and RA-233 compound (3×10^{-6} *M*, gift of Pharma Research Canada, Ltd.) in the medium. Addition of purified pig PF_4 (4–16 units/ml) to the suspension of pig platelets prevented aggregation induced by $APPF_4$ antibody. Addition of lower concentration of pig PF_4 (1–2 units) resulted in delay of aggregation (Fig. 2). It is noteworthy that pig platelets contained a total of 20–68 units PF_4 per 10^9 platelets. The level of extracellular PF_4 in the suspension of pig platelets, 10^9/ml, as determined in six experiments, was 1.4 ± 1.5 units/ml. However, this value is on the borderline of the sensitivity of the method of PF_4 determination.

Low concentrations of heparin inhibited ag-

TABLE I. Titers of Antibodies Aggregating Pig or Human Platelets.

Platelets	Anti-pig PF_4 rabbit γ globulin	Anti-human PF_4 rabbit γ globulin	Preimmune rabbit γ globulin
Washed pig	350	35	0
Washed pig	500	50	0
Washed pig	3000	100	1
Washed human	1	5	0
Pig PRP	4	—	0
Human PRP	1	10	0
Washed human[a]	0	10	0

[a] In this experiment anti-PF_4 sera were used instead of γ globulin.

TABLE II. Release of Platelet Constituents Following Interaction of APPF4 Antibody with Washed Pig Platelets.[a]

Incubation time, min.	Platelet constituents in extracellular fluid, % of total					
	β-*N*-acetyl-glucosaminidase	^{3}H-5HT radioactivity	AN	antiheparin activity	PF4 antigen	LDH[b]
0	6.21 ± 2.1	2.2 ± 1.06	8.1 ± 2.3	2.3 ± 5.3	—	1.0 ± 0.0
1	17.0 ± 6.9	39.0 ± 24.7	36.1 ± 19.3	21.4 ± 9.0	+	1.1 ± 0.9
2	31.0 ± 15.1	55.5 ± 33.7	42.7 ± 26.0	55.5 ± 33.7	+++	1.8 ± 0.3
3	31.0 ± 15.8	46.8 ± 32.8	44.9 ± 29.0	41.4 ± 18.5	+++	2.2 ± 1.9
5	33.4 ± 17.0	47.4 ± 37.3	48.6 ± 33.8	44.3 ± 17.8	+++	4.8 ± 4.6
15	36.7 ± 18.5	50.8 ± 38.6	55.4 ± 30.0	47.8 ± 22.9	+++	6.7 ± 4.7

[a] Mean values and standard deviations from five experiments. 0.1 ml of APPF4 gamma globulin (dilution 1:100) was incubated with 0.9 ml of pig platelet suspension (9×10^8 platelets) in Payton aggregometer for various time intervals. Samples were immediately centrifuged in Eppendorff centrifuge and the supernatant tested for the released platelet constituents.

[b] Three experiments only.

gregation of pig platelets triggered by APPF4 antibody (Fig. 3). Heparin (Upjohn or Sigma) as low as 10^{-3} units/ml (approximately 0.01 μg heparin/ml) still significantly delayed this type of aggregation. Similar results were obtained with human platelets and AHPF4 antibody system although higher concentrations of heparin (1–10 μg) were needed to inhibit aggregation.

It has been found that heparitin-sulfate, chrondoitin-sulfate and dermatan-sulfate (kindly supplied by Dr. L. B. Jaques, Saskatoon, Canada) in concentration of 10–100 μg/ml also delayed immune aggregation induced by APPF4 antibody.

Immune complexes of BSA-anti-BSA caused aggregation of suspensions of washed pig platelets. High concentrations of heparin (40 μg/ml or more) inhibited this type of immune aggregation. The mechanism of interference by heparin is not understood.

It is unlikely that traces of thrombin contaminating anti PF4 sera were responsible for the reactions observed. Care was taken to minimize thrombin generation while preparing serum from plasma, moreover, any thrombin produced is likely to be inactivated by heating at 56° for 30 min. In addition preimmune sera from the same rabbits were processed in an identical fashion. Preimmune sera did not cause any significant aggregation or release reaction. Moreover, if nonspecific factor(s) in immune sera were responsible for aggregation, one would not expect

TABLE III. Loss of Platelet Constituents during Aggregation of Human Platelets by AHPF4 Antibody and Thrombin. Effects of Complement.[a]

Reagents added to platelets	Incubation time (min)	LTU	Platelet constituent in extracellular fluid, % of total	
			AN	^{51}Cr radioactivity
0.1 ml 0.15 *M* NaCl	3	10	14.4 ± 13.0	0.3 ± 0.1
0.1 ml AHPF4	15	10	18.0 ± 15.6	3.7 ± 3.8
0.1 ml complement	3	10	4.7 ± 8.1	0.7 ± 0.8
0.1 ml AHPF4	15	10	6.3 ± 10.9	0.9 ± 0.3
0.1 ml 0.15 *M* NaCl	3	0.0	0.0	0.8 ± 0.8
0.1 ml complement	15	0.83 ± 0.14	0.0	0.5 ± 0.3
0.1 ml 0.15 *M* NaCl	3	10	51.0 ± 7.6	4.3 ± 2.2
0.1 ml thrombin (2 U)	15	10	54.8 ± 3.7	4.8 ± 2.0

[a] Mean values and standard deviations from three experiments. The following reagents were added to 0.8 ml of platelet suspension (8×10^8): AHPF4 serum (absorbed with bovine serum albumin and human serum albumin) undiluted; complement (guinea-pig whole complement, Cordis Laboratories, Miami, FL.) reconstituted to original volume and diluted 1:10 with 0.15 *M* NaCl; thrombin (Parke Davis & Co.).

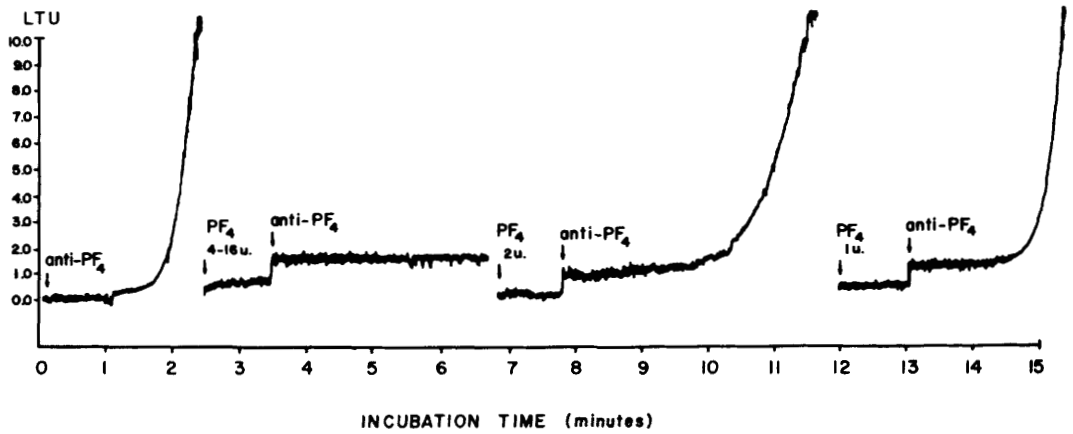

FIG. 2. Effect of purified pig PF4 on pig platelet aggregation induced by anti-PF4 antibody. Aliquots of 0.8 ml of washed platelets of pig were incubated for one minute with 0.1 ml of DEAE-C purified pig PF4. Then 0.1 ml of APPF4 antibody (diluted 1:500 in saline) was added. All reactions were carried out in Payton aggregometer cuvettes at 37°. The specific activity of pig PF4 was approximately 200 units/mg.

to see the observed species specificity (Table I). Therefore, it would be reasonable to believe that the observed aggregation is due to anti PF4 antibody. AHPF4 antisera were always absorbed with BSA. In addition its absorption with human albumin did not modify the ability of AHPF4 to cause platelet aggregation and release.

The aggregation of platelets, initiated by anti PF4 antibody appears to be independent of complement. The reaction occurred without platelet lysis as evidenced by low values of LDH and ^{51}Cr radioactivity in ambient fluid. Release and aggregation were more rapid in the washed platelet system than in PRP, and it was not significantly modified by the addition of guinea-pig complement.

There are three possible mechanisms of aggregation induced by anti PF4-antibody. PF4 may be released into the ambient fluid from some platelets during preparative procedure. PF4 antigen thus released may form complexes with the PF4 antibody and these complexes may then bring about platelet aggregation in a rather nonspecific fashion. However, contrary to expectation addition of purified PF4 to the platelet suspension inhibited immune aggregation. Enhancement of platelet aggregation by the added PF4 was not observed at any of the concentration of PF4 and antibody tested.

PF4-like antigen may be a constituent of platelet membranes and the reaction between PF4 antigen and antibody may occur on platelet

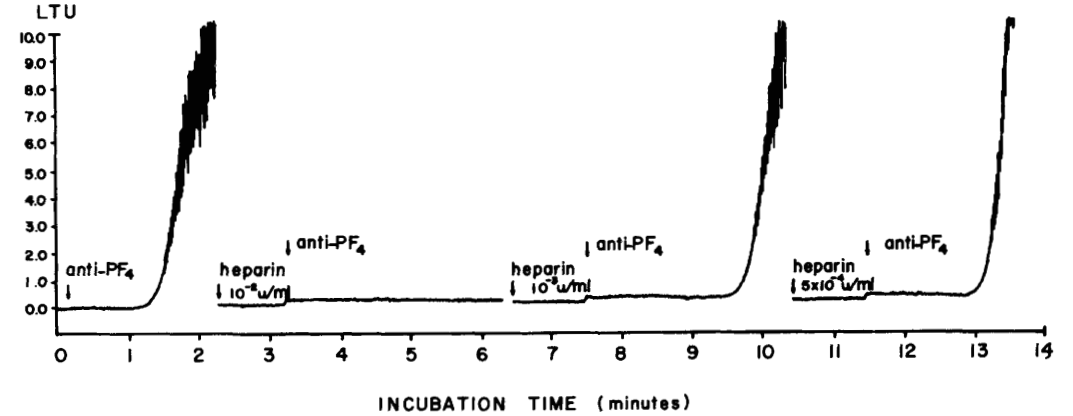

FIG. 3. Effect of heparin on pig platelet aggregation induced by anti-PF4 antibody. Aliquots of 0.8 ml of pig washed platelets were incubated for one minute with 0.1 ml heparin (Upjohn Co.). For other explanations see Fig. 2.

membranes thus leading to the release reaction or lysis. However, most investigators agree that PF_4 is contained in granular fractions of platelets (19, 20). In cooperation with Salganicoff, platelet membranes and platelet granules were prepared from pig platelets using a method which has been recently described (21). We found that pig platelet granules contained approximately 10 times more PF_4 antigen and PF_4-antiheparin activity per mg protein than pig platelet membranes. Small amounts of PF_4 antigen detected in the platelet membranes was either contamination from the lysed granules during handling of platelets or a small amount of PF_4 antigen was indeed an integral part of the membranes. PF_4 antigen in the membranes may form the "critical sites". It has been found that antiheparin activity on the platelet surface appears during treatment of unstirred platelets with ADP (22). The release reaction may be triggered following binding of specific antibody with platelet membranes.

It is conceivable that PF_4 antibody travels through the open cannaliculi of the platelets to reach platelet granules. Interaction of PF_4 antigen with antibody may thus take place within the cell. Time taken by antibody to travel through these channels might be responsible for the very pronounced lag period (see Fig. 1). This interaction, occurring during the lag period of 1–3 min may culminate in rapid release of platelet granules and platelet aggregation. At present there is no evidence that such a reaction does occur. Antibodies are usually considered incapable of penetrating into the biological interior of living mammalian cells (23) but several recent studies (24, 25) have indicated that malignant cells have a pronounced ability to take up proteins from their environment. For instance Ng and Gregory (26) have demonstrated that antibodies to lactic acid dehydrogenase and other enzymes can be taken up and accumulated by some malignant cells.

The specific inhibition of PF_4 antibody induced aggregation by heparin is compatible with our previous observations that heparin interferes with PF_4–anti PF_4 interaction and that PF_4 antibody inhibits the antiheparin property of PF_4 (9). It is well known that PF_4 is released from human platelets by thrombin in a form of high molecular weight complex with chondroitin sulfate (19). Heparitin sulfate, chondroitin sulfate and dermatan sulfate also combine stoichiometrically with PF_4 (19). Inhibition of $APPF_4$ aggregation by chondroitin sulfate or related compounds suggests that antigenic sites of PF_4 molecule may be situated in the same part of the PF_4 molecule as the site which binds heparin or other mucopolysachiarides. Our results also show that PF_4 antigens from human and pig platelets have low degree of cross reactivity.

Summary. Antibodies to PF_4 were prepared in rabbits injected with highly purified human or pig PF_4 preparations. Anti-human PF_4 ($AHPF_4$) caused aggregation of human platelets in plasma or in washed suspension. Anti-pig PF_4 ($APPF_4$) antibody caused aggregation of washed pig platelets. Slight cross reaction between the two species was observed. The aggregation was accompanied by the platelet release reaction, however, platelet lysis was not evident even on addition of complement. Aggregation could be blocked by prostaglandin E_1, EDTA, potato apyrase, purified or crude PF_4 and heparin. It is believed that platelet aggregation and the subsequent release reaction are triggered by the interaction of antibody with PF_4 antigen on or in platelets.

The authors wish to thank Dr. L. Salganicoff for his cooperation and helpful discussion. Technical assistance of A. Tai Sha and Carolyn Lowery is gratefully acknowledged.

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Received April 9, 1974. P.S.E.B.M., 1974, Vol. 147.