Biochemical Pharmacology, Vol. 40, No. 2, pp. 357–363, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

AMPLIFICATION OF PLATELET RESPONSE DURING ACUTE INFLAMMATION IN RATS*

RANJANA SRIVASTAVA and RIKHAB C. SRIMAL†
Division of Pharmacology, Central Drug Research Institute, Lucknow-226001, India

(*Received* 23 *February* 1989; *accepted* 8 *December* 1989)

Abstract—Enhanced aggregation of platelets was observed in platelet-rich plasma, but not in washed platelet suspension (WPS), during acute inflammation in rats. Incubation of WPS with inflammatory plasma increased the aggregatory response to ADP, but the plasma itself did not cause aggregation of platelets. It potentiated the aggregatory response of normal platelets, when platelets were stimulated with arachidonic acid, thrombin, calcium ionophore A23187 or phorbol-12-myristate-13-acetate. Pretreatment of rats with indomethacin (10 mg/kg) did not prevent the increased aggregation response of platelets due to inflammation. This response was not due to any of the biogenic amines nor was it due to platelet factor 4. The activity of the inflammatory plasma was reduced when it was incubated with phospholipase A_2, indicating the involvement of platelet-activating factor (PAF). The activity was present both in the lipid and the protein fraction of the inflammatory plasma. The results indicate that a substance(s) released in the circulation during inflammation renders the platelets hyperactive. This substance appears to be a protein which is present in the inflammatory plasma and acts together with PAF to cause increased aggregation of platelets.

Platelets play an important role in acute inflammation [1–3]. They accumulate at the site of injury and respond to injury by releasing important mediators like 5-hydroxytryptamine, prostanoids, platelet specific proteins, hydrolases [4] and platelet-activating factor (PAF‡) [5] which contribute to the inflammatory process. On the other hand, these substances also act as platelet agonists and could be responsible for the activation of platelets observed during the acute inflammatory response. We reported earlier [6] that increased aggregation of platelets is observed in carrageenin-induced inflammation for up to 72 hr. It was observed that, during acute inflammation, platelets become activated, as seen by scanning electron microscopic studies, and release their granular and lysosomal contents. In the present investigation, we have made an attempt to determine the factor(s) responsible for the increased aggregatory response of the platelets.

MATERIALS AND METHODS

Acute inflammation

Carrageenin-induced rat paw edema. Inflammation was induced in male rats of the Charles–Foster strain, by injecting 100 μL of 1% carrageenin in saline in one of the hind paws of the rat [7]. The volume of the paw was measured plethysmographically 4 hr after carrageenin injection.

Isolation of platelets

Blood was drawn from rats, by cardiac puncture, into 0.13 M sodium citrate (pH 6.5) at 0 and 4 hr of inflammation. Platelet-rich plasma (PRP) was obtained from the whole blood by centrifugation at 200 *g* for 20 min at room temperature.

Washed platelet suspension (WPS) was prepared by centrifuging PRP at 1800 *g* for 10 min. The sedimented platelet pellet was washed twice and suspended in modified Hepes-Tyrode's buffer, pH 7.35 (136 mmol/L of NaCl, 5.5 mmol/L of dextrose, 1 mmol/L of $MgCl_2$, 0.47 mmol/L of NaH_2PO_4, 11.62 mmol/L of $NaHCO_3$, 2.7 mmol/L of KCl, 10 mmol/L of Hepes and 0.3 g/dL of bovine serum albumin). The solution was made 1 mmol/L with respect to Ca^{2+}.

Aggregation studies

Aggregation studies were performed turbidimetrically [8] on a Payton's Aggregometer (Payton Associates, Buffalo, NY). Cuvettes containing 900 μL of PRP or WPS were stirred at 900 rpm at 37° for 1 min prior to addition of 100 μL of ADP, arachidonic acid phorbol-12-myristate-13-acetate (PMA). In experiments where plasma and/or drugs were used, the volume of the PRP was adjusted as described in the legends. The concentration of ADP used to study the aggregation response of PRP and WPS was 5.6×10^{-6} M and 5×10^{-6} M, respectively. This concentration was adjusted in later experiments, so as to clearly observe the responses which were higher or lower than the control response (see legends).

Dialysis and ammonium sulfate precipitation

Plasma obtained from the blood of rats with

* Communication No. 4028 from C.D.R.I., India.

† To whom all correspondence should be addressed.

‡ Abbreviations: PAF, platelet-activating factor; PRP, platelet-rich plasma; WPS, washed platelet suspension; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMA, phorbol-12-myristate-13-acetate; TMB-8, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate HCl; 5-HT, 5-hydroxytryptamine; PF4, platelet factor 4; PLA_2, phospholipase A_2; EGTA, ethyleneglycolbis(aminoethyl-ether)tetra-acetate; βTG, β-thromboglobulin; NSAID, non-steroidal anti-inflammatory drugs.

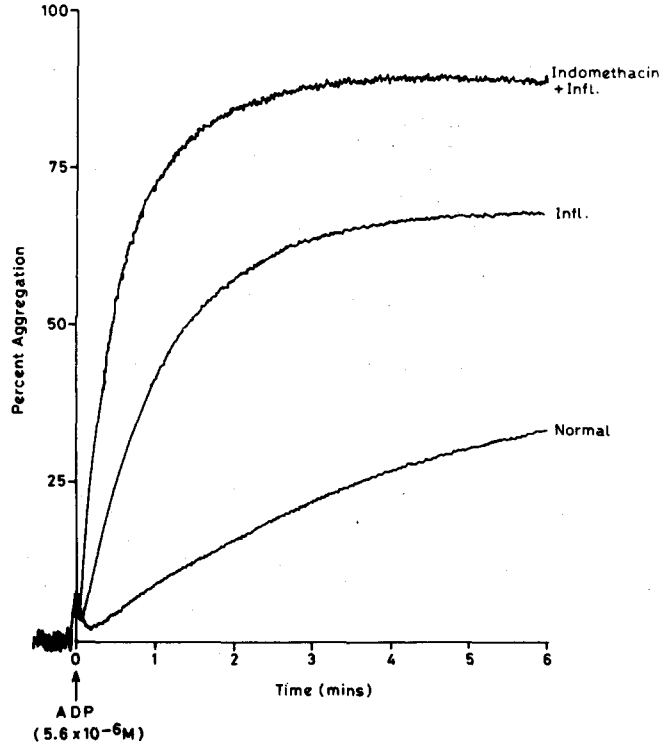

Fig. 1. ADP-induced aggregation of PRP obtained from normal, inflamed (Infl.) and indomethacin (10 mg/kg, p.o.) pretreated and inflamed (indomethacin + Infl.) rats. Aggregation was carried out as described in Materials and Methods.

inflammation was dialysed against 0.15 M NaCl for 18 hr at 4°. The dialysate was further purified by graded ammonium sulfate precipitation.

Extraction of lipids

The lipids from the plasma were separated by extracting twice with chloroform:methanol (2:1), and the organic layer was dried under N_2. The dried residue was reconstituted into Hepes-Tyrode's buffer.

Statistical analysis

All values are given as means ± SE. P values were calculated by Student's *t*-test.

Materials

Stock solution of arachidonic acid (Sigma) was prepared in 1 M Na_2CO_3. ADP, bovine thrombin, PMA, cimetidine, pancreatic phospholipase A_2 and 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate HCl (TMB-8) were purchased from the Sigma Chemical Co. (U.S.A.). Phentolamine and ketanserin were procured from Ciba (Sweden) and Janssen Pharmaceutica (Belgium) respectively.

RESULTS

Inflammatory response

The mean increase in the paw volume at 4 hr during carrageenin-induced inflammation was 0.665 ± 0.034 mL compared to normal paw.

Analysis of platelet responses

Aggregation response due to inflammation. Figure 1 shows ADP (5.6×10^{-6} M) induced aggregation of PRP obtained from normal rats, PRP obtained after 4 hr of carrageenin edema of untreated rats, and PRP from indomethacin-pretreated rats with inflammation. The increase in aggregation of platelets during inflammation, as well as after pretreatment with indomethacin, was observed. An increase in aggregation (61, 89, 38 and 34%) of platelets was also observed when other agonists like thrombin (1 unit/mL), arachidonic acid (5 μM), calcium ionophore A23187 (2 μM) and PMA (33 ng/mL), respectively, were used.

Inflammatory plasma induced enhancement of aggregation response of WPS. When WPS, obtained from plasma of normal rats, or that obtained from rats with inflammation, was stimulated with ADP, no significant difference in aggregation response was observed between the two. But when plasma (0.2 mL) obtained from the blood of rats with inflammation (henceforth referred to as inflammatory plasma) was incubated with WPS, an increase in ADP (5×10^{-6} M) induced aggregation of platelets was observed (Fig. 2). Plasma from indomethacin-pretreated rats also potentiated the aggregation response to ADP.

Effect of inflammatory serum on aggregation of WPS. An effect similar to that by plasma was observed when WPS was incubated with inflammatory serum. The aggregation of WPS was increased 3.75 times compared to normal serum.

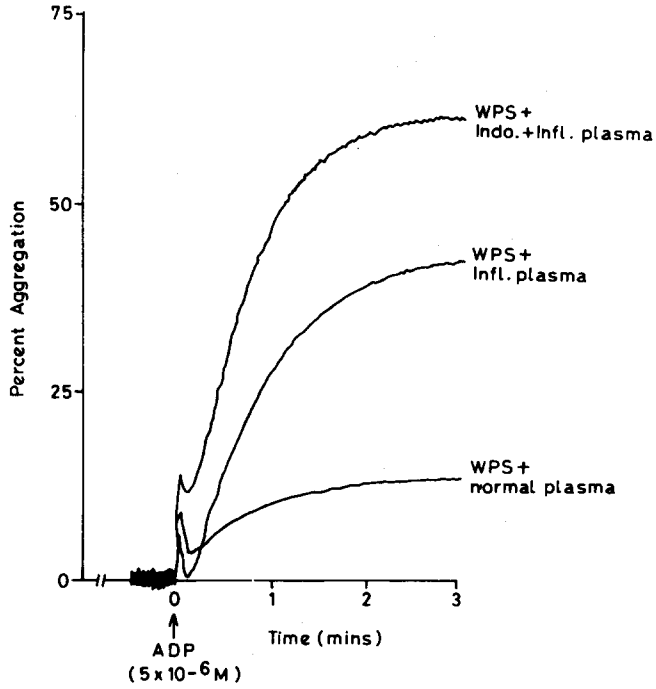

Fig. 2. Effect of ADP-induced aggregation of normal WPS, preincubated for 3 min with normal plasma (200 μL), inflammatory plasma (200 μL), or plasma (200 μL) obtained from indomethacin-pretreated rats. The aggregometer tracing is representative of a single experiment. The mean increase of aggregation (±SE, N = seven experiments) by the inflammatory plasma was 128.21 ± 21.0%, $P < 0.001$.

Table 1. Effects of phentolamine, ketanserin and cimetidine on inflammatory plasma induced increase of aggregation of WPS

	Compound	(%) Aggregation	Increase in aggregation vs respective controls (%)
1.	WPS + normal plasma (control)	37.50	
2.	WPS + inflammatory plasma	81.25	116.67
3.	WPS + phentolamine + normal plasma (control)	35.00	
4.	WPS + phentolamine + inflammatory plasma	75.00	114.29
5.	WPS + ketanserin + normal plasma (control)	27.50	
6.	WPS + ketanserin + inflammatory plasma	50.00	81.82
7.	WPS + cimetidine + normal plasma (control)	30.00	
8.	WPS + cimetidine + inflammatory plasma	56.25	87.50

Washed platelet suspension (WPS) was incubated with phentolamine (3.5×10^{-5} M), or ketanserin (1.8×10^{-4} M), or cimetidine (4×10^{-4} M) for 3 min prior to addition of normal or inflammatory plasma and subsequent stimulation with ADP.

Effects of phentolamine, ketanserin and cimetidine on inflammatory plasma induced increase of aggregation response. The increase in aggregation of platelets due to inflammatory plasma was not blocked when WPS was incubated with phentolamine (3.5×10^{-5} M), an α-adrenergic antagonist, or ketanserin (1.8×10^{-4} M), a 5-HT_2 blocker, or cimetidine (4×10^{-4} M), an H_2 antagonist, for 3 min prior to addition of inflammatory plasma and subsequent stimulation with ADP (Table 1).

Effect of inflammatory plasma, preincubated with PLA_2, on aggregation of WPS. Normal plasma, or inflammatory plasma, was incubated with pancreatic phospholipase A_2 (30 units/mL) and 1 mM $CaCl_2$ for 60 min at 37°, prior to addition to normal WPS and subsequent aggregation with ADP. The inflammatory plasma caused a partial increase in aggregation of normal WPS (Fig. 3a), which was 48.84% less than that caused by inflammatory plasma alone (Table 2). The lipids extracted from the inflammatory plasma partially retained the proaggregatory activity (Fig. 3b), which was, however, less than that of inflammatory plasma (Table 2).

Heparin-induced neutralization of platelet factor 4 (PF4) and its effect on platelet aggregation. When normal plasma or inflammatory plasma was preincubated with heparin (50 units/mL) at 37° for 10 min, before its addition to normal WPS and sub-

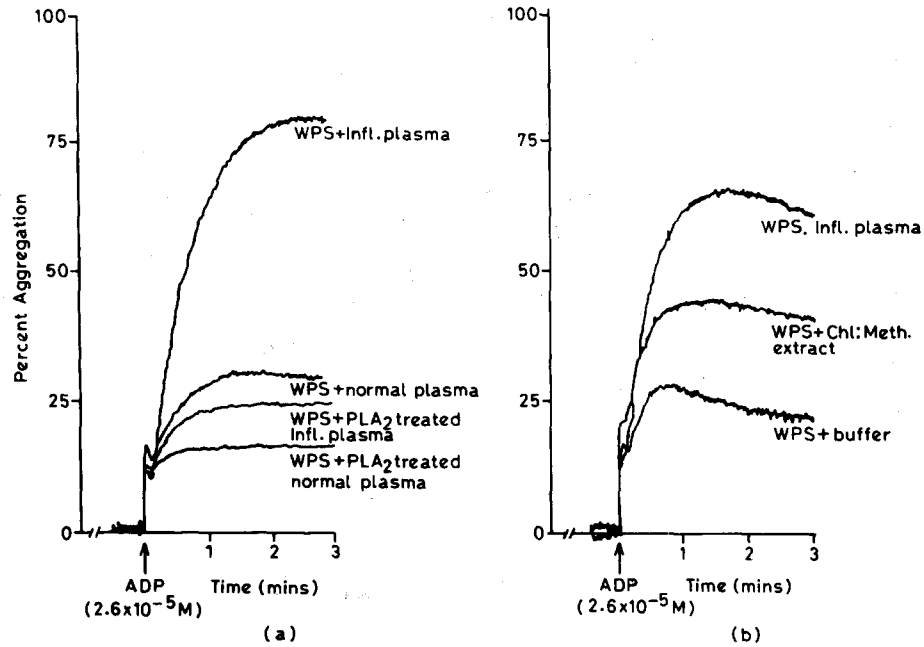

Fig. 3. Effect of inflammatory plasma, preincubated with PLA_2 (a) or chloroform : methanol extract (b), on aggregation of WPS. Pancreatic PLA_2 (30 units/mL) was incubated with normal or inflammatory plasma for 60 min at 37° in the presence of 1 mM $CaCl_2$. Panel a: ADP-induced aggregation of WPS preincubated with PLA_2-treated normal or inflammatory plasma. Panel b: Effect of preincubation of WPS with chloroform : methanol extract of inflammatory plasma. The concentration of ADP was increased to 2.6×10^{-5} M compared to that used in Fig. 2 so as to observe clearly the inhibitory response of PLA_2-treated plasma.

Table 2. Comparison of relative effects of protein and lipid fractions of inflammatory plasma on aggregation of WPS

	Fractions	Increase in the aggregation of WPS* (fold increase)	Inhibition of aggregation† (%)	Increase in aggregation† (%)
1.	Inflammatory plasma	2.4		
2.	PLA_2-incubated inflammatory plasma	1.33	48.84	
3.	Chloroform:methanol extract	1.48	38.33	
4.	Ammonium sulfate precipitable fraction I	1.30	45.83	
5.	Dialysate	4.00		66.66

WPS = washed platelet suspension.
* Compared to aggregation of WPS + normal plasma.
† Compared to aggregation of WPS + inflammatory plasma.

sequent aggregation with ADP, the inflammatory plasma caused an increase in the aggregation response compared to normal plasma. This increase was the same as that by unincubated inflammatory plasma.

Effects of dialysate and ammonium sulfate fractions on aggregation response. The inflammatory plasma was dialysed to eliminate substances of M_r less than 12,000. When 200 μL of dialysate (material inside the dialysis bag) was incubated with normal WPS, it caused a 66.6% increase in the aggregation response compared to the increase by inflammatory plasma (Fig. 4 and Table 2).

The dialysate of normal plasma added together with ADP caused 45–50% aggregation which was less than the aggregation response of the inflammatory plasma and much less than that produced by the dialysate of the inflammatory plasma. When the dialysate of inflammatory plasma was incubated with PLA_2 (30 units), the increase in aggregatory response was not diminished. Normal WPS was preincubated with 20, 50 or 75% ammonium sulfate precipitable fractions for 3 min at 37° before addition of ADP (1×10^{-6} M). A partial increase in aggregation was observed with the 20% ammonium sulfate fraction (Fig. 5), which was 45.83% less than that caused by inflammatory plasma alone (Table 2).

Effects of TMB-8 and EGTA on 20% ammonium sulfate fraction-induced increase in aggregation response. When normal WPS was preincubated with

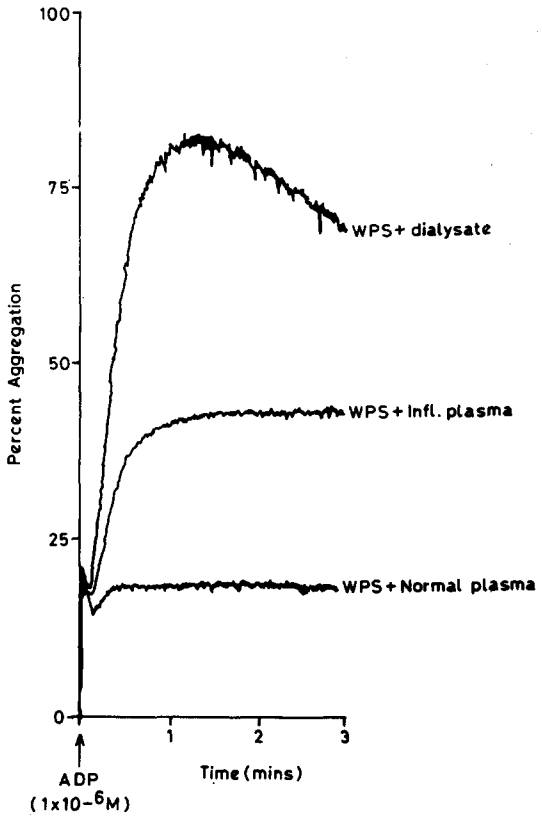

Fig. 4. Effects of normal plasma, inflammatory plasma and dialysate (obtained after dialysis of inflammatory plasma for 18 hr against normal saline at 4°) on ADP-induced aggregation of WPS. Two hundred microliters of each was preincubated with WPS for 3 min at 37° before addition of ADP. The concentration of ADP used was 1×10^{-6} M so as to clearly observe the increased response with the dialysate.

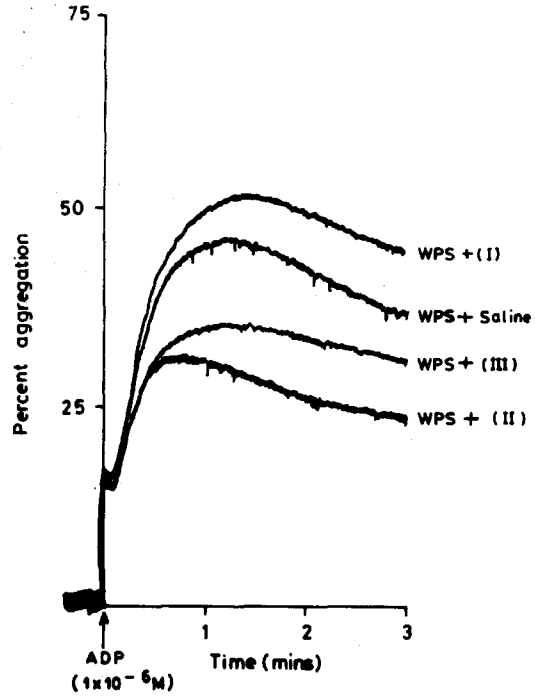

Fig. 5. Effect of 20% (I), 50% (II) and 75% (III) ammonium sulfate fractions (200 μL) on ADP-induced aggregation of WPS. The dialysate was subjected to graded ammonium sulfate precipitation. The precipitate was dialysed overnight and dissolved in Hepes-Tyrode's buffer, pH 7.4. The aggregometer tracing is representative of a single experiment. The increase in aggregation was 31.56 ± 2.12% (mean ± SE), $P < 0.05$.

1×10^{-4} M TMB-8, an intracellular calcium blocker, prior to addition of 20% ammonium sulfate precipitable fraction of inflammatory plasma, the increase in the aggregation response due to this fraction was not blocked. On the other hand, when normal WPS was preincubated with 1 mM EGTA, a calcium chelator, the increased response was partially (38%) blocked.

DISCUSSION

Platelets are known to participate in the production of tissue injury during the acute inflammatory response [1, 2, 9]. They secrete a number of mediators which contribute to inflammation, while many inflammatory mediators formed at the site of tissue injury or released from other inflammatory cells are potent platelet agonists. Of these, the most strongly implicated are the prostaglandins, leukotrienes, histamine, 5-hydroxytryptamine, bradykinin and platelet-activating factor.

We reported earlier [6] that platelets become activated during acute inflammation. However, Vincent *et al.* [2] report a diminished aggregation response 2 hr after carrageenin rat paw edema. In our studies, enhanced aggregation of platelets was observed when PRP, obtained from rats with inflammation, was stimulated by ADP, thrombin, arachidonic acid, A23187 or PMA, *ex vivo*. Since activation of platelets results in the hydrolysis of arachidonyl phospholipids [10], it was reasonable to assume, that the increase in aggregation of platelets could be due to an increase in the cyclooxygenase activity of the cell. But, it was observed that pretreatment of rats with indomethacin (10 mg/kg) did not prevent the increase in aggregation response (Fig. 1). Indomethacin at this dose also blocks the lipoxygenase activity [11]. Thus, the results clearly indicate that blockade of cyclooxygenase or lipoxygenase pathways does not prevent the increased aggregation of platelets during inflammation.

Incubation of normal WPS with inflammatory plasma/serum increased aggregation induced by ADP. This observation suggests that there is some agent(s) released into the circulation during inflammation, either from platelets or from other inflammatory cells, which is responsible for the activation of platelets during inflammation.

Biogenic amines are involved in the early phase of carrageenin edema [12], but preincubation of inflammatory plasma with phentolamine, ketanserin or cimetidine, at concentrations at which they effectively inhibit normal platelet aggregation, failed to

block the increased aggregation response of inflammatory plasma (Table 1). Thus, released adrenaline, 5-HT or histamine is not involved in the increased aggregation of platelets.

Activated platelets release specific (α) granule proteins like PF4, β-thromboglobulin (βTG) platelet-derived growth factor and thrombospondin, which are important contributors to the inflammatory process [13]. High-affinity PF4 binding sites are present on the platelets and PF4 binding results in increased sensitivity to aggregating agents [14]. Moreover, PF4 has a role in normal platelet aggregation in contrast to βTG-like proteins, which do not interact with platelets [14]. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis of platelets of normal rats and of rats with carrageenin edema shows a marked release of PF4 and βTG during inflammation (unpublished observations). These proteins have a high affinity for heparin [14]. Therefore, we incubated the inflammatory plasma with heparin to neutralize the effect of PF4. But, it was observed that the proaggregatory activity of the plasma was not inhibited, indicating that secreted platelet proteins like PF4 are not responsible for the hyperaggregation of platelets during inflammation.

PAF is a potent lipid mediator produced by stimulated neutrophils, basophils, alveolar macrophages, platelets and endothelial cells [15] and is involved in the first phase of carrageenin edema [16]. Pancreatic PLA_2 destroys the natural and synthetic PAF. It hydrolyses the acetate moiety at the 2-position of glycerol, which is required for the activity of PAF acether [17]. The loss of proaggregatory activity of inflammatory plasma due to incubation with PLA_2 suggests that PAF could be one of the substances responsible for the aggregatory response of platelets. Moreover, PAF-induced foot paw edema is not blocked by NSAID like indomethacin, even at a daily high dose of 10 mg/kg, nor do NSAID block PAF-induced platelet aggregation [16]. In our studies also, indomethacin did not inhibit inflammatory plasma induced increase in aggregation. Additionally, upon extraction of lipid fraction from plasma into chloroform:methanol, the proaggregatory effect was partially retained in the extract (Fig. 3b). Inflammatory plasma incubated with phospholipase A_2 caused a 1.33-fold increase in the aggregation of WPS compared to 2.4-fold increase by inflammatory plasma alone (Table 2). Similarly, the lipids extracted from the inflammatory plasma showed a 1.48-fold increase in aggregation, indicating that the lipid fraction accounts for only part of the enhanced aggregation response by the inflammatory plasma.

Part of the proaggregatory activity of the inflammatory plasma appears to be present in the protein fraction. When inflammatory plasma was dialysed to remove the proteins of lower molecular weight ($M_r < 12{,}000$), the dialysate caused a considerable increase in the aggregation response of normal WPS compared to that by inflammatory plasma (Table 2). Dialysis removed peptide-like kinins, which mediate the second phase of acute inflammatory response and have a molecular weight below 12,000 daltons. The increased activity (more than that by inflammatory plasma) is due to the concentration of proteins and the possible retention of the lipids in the dialysate. The observation that a protein(s) present in the inflammatory plasma is also involved in the proaggregatory response found further support from our observation that the proaggregatory activity was present in the 20% ammonium sulfate precipitable fraction of the inflammatory plasma which caused only a partial (1.3-fold) increase in the aggregation response (Table 2). Additionally, this activity was seen to be partly dependent on extracellular Ca^{2+} as there was a 38% reduction in the proaggregatory effect when EGTA was also included in the incubation medium.

The identity of this protein has not been established so far, and its purification is in progress. The possibility of it being either a protein released from inflammatory cells, like cathepsin G from neutrophils [18] which can initiate platelet aggregation, or it being one of the acute phase plasma proteins, like α_2-macroglobulin or C-reactive protein (CRP) which are formed during acute inflammation, cannot be ruled out. C-reactive protein, although not a major acute phase reactant protein in the rats, is also present in large amounts in normal rat plasma and its concentration doubles during acute phase response to injury [19] and activation of human platelets by PAF is augmented by CRP [20]. At the same time, it could be an altogether new protein, formed during inflammation, which renders platelets susceptible to aggregation.

The results reported in this paper show that during inflammation a protein of yet unidentified nature, together with PAF, causes increased aggregation of platelets, when the latter are stimulated with platelet agonists.

REFERENCES

1. Nachman RL and Weksler B, The platelet as an inflammatory cell. *Ann NY Acad Sci* **201**: 131–137, 1972.
2. Vincent JE, Bonta IL and Zijlstra FJ, Platelets and mediators in the carrageenin rat paw edema. *Agents Actions* (Suppl 2): 41–46, 1977.
3. Vincent JE, Bonta IL and Zijlstra FJ, Accumulation of blood platelets in carrageenin rat paw edema. Possible role in inflammatory process. *Agents Actions* **8**: 291–295, 1978.
4. Holmsen H, Sal Janicoff L and Fukami MH, Platelet behaviour and biochemistry. In: *Haemostasis: Biochemistry, Physiology and Pharmacology* (Eds. Ogston D and Beunett B), pp. 239–319. John Wiley, London, 1977.
5. Page CP, Archer CB, Paul W and Morley J, PAF-acether: A mediator of inflammation and asthma. *Trends Pharmacol Sci* **5**: 239–241, 1984.
6. Srivastava R and Srimal RC, Inflammation induced changes in platelets. *Indian J Pharmacol* (Suppl 1): O-2, 1986.
7. Winter CA, Risley EA and Nuss GW, Carrageenin induced edema in the hind paw of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol Med* **111**: 544–547, 1962.
8. Born GVR, Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* **194**: 927–929, 1962.
9. Ferreira SH, Ubatoba FB and Vane JR, Platelets, acute inflammation and inflammatory mediators. *Agents Actions* **6**: 313–319, 1976.

10. Gerrard JM, Phospholipid metabolism and cell stimulus activation coupling in platelets and other blood cells. *Surv Synth Pathol Res* **3**: 457–476, 1984.
11. Braqt PC and Bonta IL, Indomethacin inhibits the formation of the lipoxygenase product HETE (12-hydroxy5,8,10,14-eicosatetraenoic acid) during granulomatous inflammation in the rat. *J Pharm Pharmacol* **32**: 143–144, 1980.
12. DiRosa M, Giroud JP and Willoughby A, Studies of the mediators of acute inflammatory response induced in rats in different sites by carrageenin and turpentine. *J Pathol* **104**: 15–29, 1971.
13. Holt JC and Niewiarowski S, Biochemistry of alpha granule proteins. *Semin Hematol* **22**: 151–163, 1985.
14. Capitanio AM, Niewiarowski S, Rucinski B, Tuszynski GP, Cierniewski CS, Hershock D and Kornecki E, Interaction of platelet factor 4 with human platelets. *Biochim Biophys Acta* **839**: 161–173, 1985.
15. Vargaftig BB and Benveniste J, Platelet activating factor today. *Trends Pharmacol Sci* **4**: 341–343, 1983.
16. Hwang SB, Lam MH, Li CL and Shen TY, Release of platelet-activating factor and its involvement in the first phase of carrageenin induced rat foot edema. *Eur J Pharmacol* **120**: 33–41, 1986.
17. Pearson JD, Plasma factors regulating prostaglandin biosynthesis and catabolism. In: *Cardiovascular Pharmacology of Prostaglandins* (Eds. Herman AG, Vanhoutte PM, Denolin H and Goosens A), pp. 22–33. Raven Press, New York, 1982.
18. Rindler-Ludwig R and Braunsteiner H, Cationic proteins from human neutrophil granulocytes; Evidence for their chymotrypsin-like properties. *Biochim Biophys Acta* **379**: 606–617, 1975.
19. Kushner I, The acute phase response: An overview. In: *Methods in Enzymology* (Ed. DiSabato G), Vol. 163, pp. 373–383. Academic Press, New York, 1988.
20. Kohayakawa M and Inoue K, Augmentation of 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF)-induced human platelet activation by C-reactive protein. *Thromb Res* **41**: 649–657, 1986.