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Platelet inhibitory effects of CRP preparations are due to a co-isolating low molecular weight factor

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Summary. We previously reported that C-reactive protein (CRP), an acute phase reactant, inhibits platelet activation through an effect upon a factor(s) critical to ADP-mediated secondary wave platelet aggregation but independent of a direct effect upon platelet contractile elements. However, a role for an accessory factor in this inhibitory effect became of concern because of an inconsistency in the effects of CRP preparations upon the platelet: inhibition was lost upon storage and CRP preparations differed, on a weight basis, in inhibitory capacity and sensitivity to the presence of the CRP ligand C-polysaccharide (CPS). The studies presented herein were thus intended to assess whether an accessory factor was involved in the inhibition of platelet activation observed with CRP. We report that the activity of the inhibitory CRP preparations resulted from association with a low molecular weight factor (LMF) with an apparent nominal molecular weight of 8300–12,500 and an A_{280}:A_{260} ratio of ~0·4. Purified CRP did not inhibit platelet responsiveness but CRP with associated LMF (CRP-LMF) did. Moreover, the inhibitory capacity of CRP-LMF but not LMF was substantially reversed in the presence of CPS. These studies indicate that the platelet inhibitory properties of CRP preparations result from and are contingent upon the presence of a co-isolating low molecular weight factor.

INTRODUCTION

The classical acute phase reactant, C-reactive protein (CRP), described by Tillett & Francis in 1930, appears in markedly elevated concentration in the sera of individuals undergoing reactions of acute inflammation and tissue degradation and is found deposited at sites of tissue injury (Anderson & McCarty, 1950; Hedlund, 1961; Kushner & Kaplan, 1961; Claus, Osmand & Gewurz, 1976; Parish, 1976). CRP has a mol. wt of 115,000 and consists of five identical, non-covalently bound subunits (Osmand, Friedenson, Gewurz, Painter, Hofmann & Shelton, 1977; Oliveira, Gotschlich & Liu, 1979); it is one member of a recently described superfamily of proteins termed pentraxins (Osmand *et al.*, 1977).

We previously demonstrated that preparations of CRP inhibit platelet activation via an effect upon a factor(s) critical to ADP-mediated secondary wave platelet aggregation but independent of any direct

Abbreviations: CRP, C-reactive protein; LMF, low molecular weight factor; CRP-LMF, a complex of CRP and LMF; RID, radial immunodiffusion; PAGE, polyacrylamide gel electrophoresis; PRP, platelet-rich-plasma; ASC, acid soluble collagen; CPS, pneumococcal C-polysaccharide; TBS, tris-buffered saline; TBS^+, tris-buffered saline containing 10 mM $CaCl_2$; RSC, relative salt concentration; PC, phosphocholine.

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effects upon platelet contractile elements (Fiedel & Gewurz, 1976a, b; Fiedel, Simpson & Gewurz, 1977). We present herein evidence that the association of CRP with a low molecular weight factor (LMF) confers a platelet inhibitory property upon CRP not evident in the isolated molecule.

MATERIALS AND METHODS

Isolation of CRP

CRP was isolated from pleural or ascitic fluids obtained from patients undergoing diagnostic/therapeutic procedures. The fluids were centrifuged at 5000 *g* (15 min; 4°), further clarified by filtration through gauze and applied by gravity drip to a phosphocholine (PC)-Sepharose 4B affinity column (2·5 × 22 cm, Pharmacia Fine Chemicals, Piscataway, N.J.) prepared essentially as described (Volanakis, Clements & Schrohenloher, 1978). The column was washed with 20 mM Tris-buffered saline containing 10 mM $CaCl_2$ (TBS^+; pH 7·5) at 20 ml/hr for 36–48 hr after the A_{280} reached $<0·05$; elution of CRP was achieved using isotonic Tris-citrate buffer (Osmand, Mortensen, Siegel & Gewurz, 1975), pH 7·2, at a flow of 20 ml/hr. Fractions containing CRP, as judged by immunoassay, were pooled and exchanged on a DE-52 (Whatman Ltd, Kent) column (1·5 × 20 cm) with elution achieved using a 0·1–0·4 M linear salt gradient (20 ml/hr); CRP-containing fractions were pooled and concentrated in an Amicon UF cell (PM-10 membrane) (Amicon Corp., Lexington, Mass.) and sieved through a 3 × 40 cm Sephacryl S-200 superfine column (Pharmacia; S-200) in Tris-buffered saline in the absence of $CaCl_2$ (TBS; pH 7·2). In a number of experiments, CRP preparations obtained from the PC affinity column were directly passaged over S-200. All final CRP preparations were dialysed 1–3 days against 1000 vol of TBS before use. Final CRP concentration was determined by radial immunodiffusion (RID), Lowry and Bio-Rad (Richmond, Calif.) protein analyses and absorbance at 280 nm using an average extinction coefficient of 19·0 (g/dl; Wood & McCarty, 1951 and Gotschlich & Edelman, 1965). The purity of CRP was assessed by polyacrylamide gel electrophoresis (PAGE) in SDS and urea as described (Osmand *et al.*, 1977) and by a RID and double diffusion screen using a battery of monospecific antisera selected to detect components which we and others have observed to co-purify with CRP (Wood, 1963; Hokama & Riley, 1963; Osmand *et al.*, 1975; Pepys, Dash & Ashley, 1977) or in which we have particular interest. At a minimum, this involved testing with antisera to IgG, IgM and IgA, serum amyloid P component, C3 and human serum; many preparations also were tested using antisera directed against albumin, ceruloplasmin, Clq, Clr and Cls, fibrinogen, fibrinopeptides D and E, α and β-lipoproteins and orosomucoid. Reactions in RID were considered negative only after eight-fold application of highly concentrated CRP material such that, in most instances, 1–3 μg/ml non-CRP antigen could be detected in preparations containing 1·5–2·0 mg/ml CRP. Similarly, 50–100 μl (1–2 mg/ml) of CRP was subjected to SDS-PAGE to allow detection of trace amounts of non-CRP material. Final preparations of CRP were filter sterilized (Millipore Corp., Boston, Mass.) and stored at 4° in dosette vials. Marker molecules of known molecular weight which were used as standards in column chromatography and SDS-PAGE were obtained from Pharmacia Fine Chemicals.

Platelet aggregation

Platelet aggregation was monitored in a Model 300 BD aggregometer (Payton Associates, Buffalo, N.Y.) by adding CRP diluted in TBS (300 μl) to 450 μl of PRP (Fiedel & Gewurz, 1976b), permitting the total reaction mixture to equilibrate for 1 min at 37° (1000 r.p.m.), adding 10–20 μl of the test agent, and monitoring aggregation responses for a minimum of 4 min (Fiedel & Gewurz, 1976b; Fiedel *et al.*, 1977). Platelet agonists used in these studies included ADP, acid soluble collagen (ASC) and arachidonate obtained and prepared as previously described (Fiedel & Gewurz, 1976b; Fiedel *et al.*, 1977).

Pneumococcal C-polysaccharide (CPS)

CPS prepared as described by Liu & Gotschlich (1963) was the generous gift of Dr Carolyn Mold, Rush University, Chicago, Illinois; CPS was prepared at 0·5 mg/ml in isotonic saline and stored frozen at −70°.

RESULTS

Presence of a low molecular weight platelet inhibitory activity co-isolating with CRP

We had previously demonstrated that preparations of CRP inhibit platelet aggregation and suggested that CRP plays a role in the limitation of platelet-initiated reactivities (Fiedel & Gewurz, 1976a; Fiedel & Gewurz, 1976b; Fiedel *et al.*, 1977). A role for an accessory

molecule in these reactions now has been obtained with the initial experiments presented in Fig. 1. CRP isolated from ascites or pleural fluids by PC-affinity column chromatography was passaged over Sephacryl S-200 and selected fractions were pooled and tested for the ability to inhibit platelet aggregation stimulated with ADP or ASC (Figs 1a and 1b, respectively) in

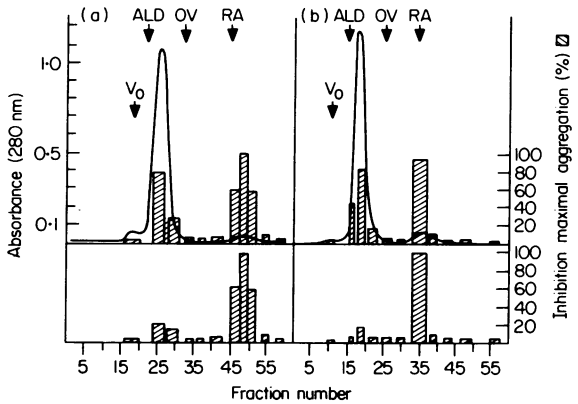

Figure 1. Passage of CRP prepared by PC-affinity chromatography through Sephacryl S-200 and the inhibitory action of the various pooled fractions on platelet aggregation stimulated by ADP (a) or acid soluble collagen (b). Upper panels reflect the inhibition in the absence and lower panels in the presence of C-polysaccharide. CPS was added to CRP-containing fractions to obtain a constant ratio (10:1 by weight) of CRP:CPS; in fractions devoid of CRP antigen, CPS was added at a variety of concentrations, all without effect. The column was calibrated with blue dextran 2000, aldolase (ALD), ovalbumin (OV) and ribonuclease A (RA). Absorbance 280 nm (—); percentage inhibition maximal aggregation (■).

both the absence and presence of CPS, a ligand for CRP. Platelet inhibitory activity generally was observed in two eluant zones, the first involving a factor migrating as a molecular species of ~115,000 mol. wt coincident with CRP antigenicity (main peak) and the second involving a low molecular weight factor migrating as a molecular species of 8300–12,500 mol. wt. Only the inhibition observed with the former was abrogated in the presence of CPS suggesting that the platelet inhibitory activities of CRP and LMF were distinct. However, rechromatography of the CRP main peak over Sephacryl S-200 yielded CRP devoid of platelet inhibitory activity as well as a separated low molecular weight factor.

An ultraviolet absorption spectrum of LMF is presented in Fig. 2 and reveals an absorption maxi-

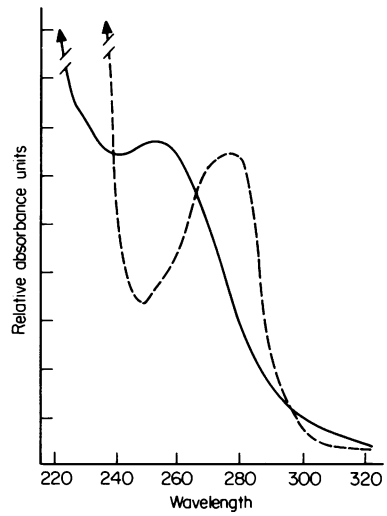

Figure 2. Ultraviolet absorption spectra of the low molecular weight factor (——) and a reference protein, bovine serum albumin (– – –).

mum at ~254 nm and an A_{280}:A_{260} ratio of <1·0 (~0·4); most proteins (e.g. bovine serum albumin) have absorption maxima at ~278 nm with A_{280}:A_{260} values of >1·7. When the absorption spectra of multiple preparations of CRP were analysed and correlated with platelet inhibitory activity, it became clear that only preparations with values <1·7 were inhibitory, and that the lower the A_{280}:A_{260} ratio of given CRP preparations the greater their inhibitory capacity on a weight basis. Collectively, these observations suggested that LMF co-isolated with certain CRP preparations and participated in the inhibition of platelet activation, and that monitoring the A_{280}:A_{260} ratio might serve as a means to detect the presence of LMF. This was assessed in the next series of experiments.

Relationship between A_{280}:A_{260} ratio, CRP antigenicity and inhibition of platelet aggregation

In order to more critically assess the presence of LMF, a portion of the PC-affinity eluates were passaged over a mini-column of Sephacryl S-200 (1·0 × 15 cm) and the A_{280}:A_{260} ratio, CRP antigenicity and platelet inhibitory capacity were evaluated for each fraction; data are presented in Fig. 3 and are representative of four separate PC-affinity eluates tested. A single large broad protein peak and a second smaller area of absorbance corresponding to the molecular weight of

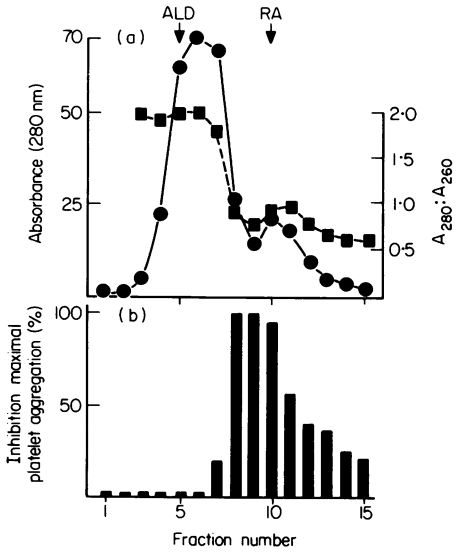

Figure 3. Chromatographic separation of the PC-affinity eluate over Sephacryl S-200 resin (a) and inhibition of platelet aggregation by the various fractions (b). The A_{280}: A_{260} ratios are shown (■—■). ADP was used as the platelet stimulator at a concentration yielding 50% maximal irreversible aggregation; similar data was obtained with collagen as the platelet activator. The column was calibrated with ALD and RA. Absorbance 280 nm (●—●).

the ribonuclease A marker were observed; the A_{280}: A_{260} ratio was relatively constant (1·9–2·0) across the first peak (fractions 5–7) but dropped to 0·9 at fraction 8 and remained low through fraction 15. Platelet inhibitory activity commenced in fraction 8, and remained markedly elevated through fraction 13. CRP antigenicity was present throughout the first peak but was associated with platelet inhibitory activity only in fraction 8; in addition, CRP antigenicity was anomalously present in the second peak, corresponding to a molecular weight slightly less than 13,700. When this low molecular weight region containing LMF activity was analysed by SDS-PAGE, both a 23,000 CRP subunit component and a larger > 240,000 mol. wt component were detected.

When LMF prepared in this manner was concentrated by Amicon ultrafiltration and then rechromatographed over Sephacryl S-200, a peak eluting at 115,000 mol. wt (antigenic identity to CRP; 23,000 in SDS-PAGE) as well as a large molecular weight peak free of CRP antigenicity eluting at the column Vo (> 240,000 in SDS-PAGE; A_{280}: A_{260} ratio ~ 1·0) were observed; little low molecular weight material was visualized. This suggested that under appropriate conditions the low molecular weight component might assemble into aggregates. Indeed, LMF as well as the high molecular weight component subsequently obtained during chromatography rapidly insolubilized upon storage.

Separation of CRP from LMF by an alternative isolation procedure

Since the PC-affinity procedure followed by a single gel-sieve chromatography step generally did not adequately isolate CRP free of LMF, we attempted to ascertain whether an ion exchange chromatography procedure intermediate between the PC-affinity isolation and molecular sieve chromatography steps better served the purification of CRP. CRP which eluted from the PC-affinity column was exchanged on DE-52 before passage over Sephacryl S-200; data are presented in Figs 4 and 5. Chromatography of the PC-affinity eluate (Fig. 4a) over DE-52 (Fig. 4b) yielded material eluting at 0·24–0·27 RSC which

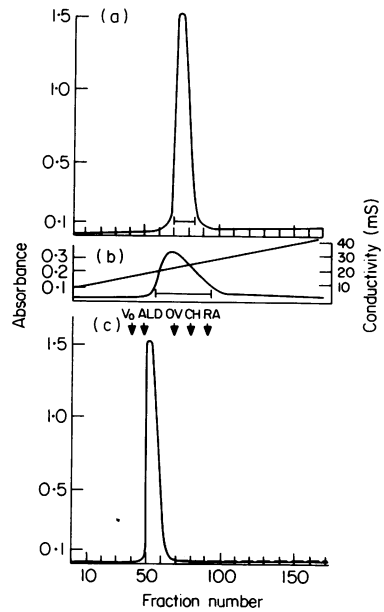

Figure 4. Isolation of CRP by PC-affinity column chromatography (a), with further purification using DE-52 (b) and Sephacryl S-200 (c). Pooled fractions are designated (⊢) and DE-52 material was concentrated by Amicon ultrafiltration. The gel sieve column was calibrated with blue dextran 2000, ALD, OV, chymotrypsinogen (CH) and RA. Absorbance: (——) 280 nm; (– – –) 260 nm.

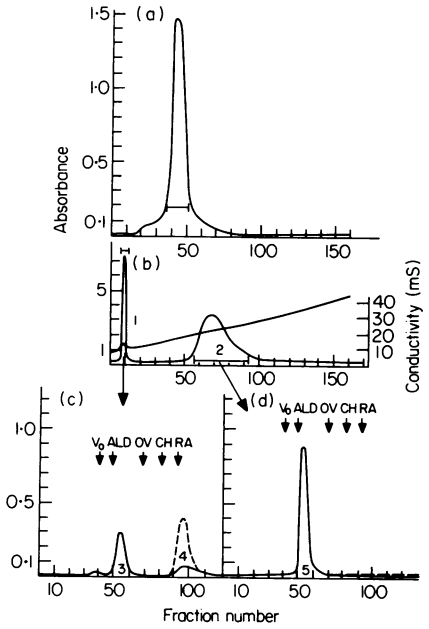

Figure 5. Isolation of CRP by PC-affinity column chromatography (a), with further purifications using DE-52 (b) and Sephacryl S-200 (c,d). Pooled fractions are designated (—) and all DE-52 material was concentrated by Amicon ultrafiltration before gel sieve chromatography; the gel sieve column was calibrated as in Fig. 4. Absorption: 280 nm (——); 260 nm (– – –).

migrated as a single 115,000 mol. wt peak over Sephacryl S-200 (Fig. 4c) with an A_{280}:A_{260} ratio ~ 1·9–2·1 and gave a 23,000 component in SDS-PAGE; these data are representative of 18 of 20 preparations. In 2 of 20 preparations, the PC-affinity eluate (Fig. 5a) resulted in an eluate from the DE-52 anion exchange resin at 0·17 RSC (Fig. 5b, peak 1) as well as at 0·27 RSC. The peak 1 material contained CRP antigenicity, had an A_{280}:A_{260} ratio of ~ 1·1 and gave both 23,000 and > 240,000 mol. wt components in SDS-PAGE. Peak 1 was resolved into two major peaks by passage over Sephacryl S-200 (Fig. 5c), one containing (peak 3) and the other lacking (peak 4) CRP antigenicity; the latter peak had an extremely low A_{280}:A_{260} ratio, chromatographed on Sephacryl S-200 with an elution volume identical to that of the low molecular weight factor, gave a molecular weight of > 240,000 in SDS-PAGE and precipitated from solution within 3–6 h of isolation. A high molecular weight peak (> 200,000) was also visualized (Fig. 5c, fractions 35–45) and this material rapidly insolubilized. The material which eluted from the ion exchange column at 0·24–0·27 RSC (Fig. 5b; peak 2), when resolved over Sephacryl S-200, chromatographed identical to the 0·24–0·27 RSC material described previously in Fig. 4, and gave a single 23,000 mol. wt component in SDS-PAGE. These data pointed to the appropriateness of an intermediate ion exchange chromatography step for the purification of CRP free of LMF.

Platelet inhibitory activity of CRP, CRP-LMF and LMF

The materials eluted from these columns next were tested for their ability to modulate platelet activation stimulated by ADP, ASC, and arachidonate in both the absence and presence of C-polysaccharide. Data for the platelet stimulator ADP are presented in Fig. 6, and are summarized for all the activators tested in Table 1. CRP with A_{280}:A_{260} ratios of 1·9–2·1 did not inhibit platelet activation in amounts to 200 μg/ml. However, in the presence of LMF (whether isolated as CRP-LMF or obtained by recombination of the separated components), inhibition of platelet activation stimulated by ADP (Fig. 6), ASC and arachidonate (Table 1) was observed and in a manner consistent with previous reports (Fiedel & Gewurz, 1976a; Fiedel *et al.*, 1977). Moreover, this inhibition was not evident in the presence of the CRP ligand, CPS. By comparison, isolated LMF (A_{280}:A_{260} ratio of < 0·4) in the absence of CRP profoundly inhibited responsiveness to each of the platelet activators, and

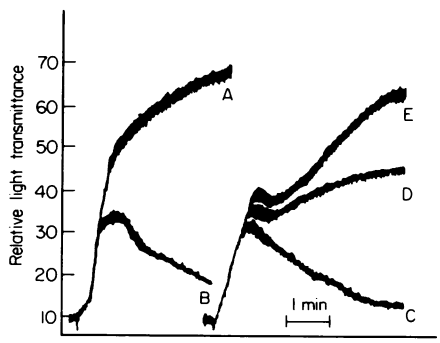

Figure 6. The comparative ability of CRP, CRP-LMF and LMF to inhibit platelet aggregation induced by 10^{-5}M ADP in the presence or absence of CPS. A represents buffer ± CRP; B represents CRP-LMF; C represents LMF ± CPS; D represents CRP-LMF + 10 μg/ml CPS; E represents CRP-LMF + 20 μg/ml CPS. CRP was used at 200 μg/ml; other conditions are as described in Table 1.

Table 1. Effects of CRP, CRP-LMF and LMF upon platelet aggregation*

Activating agent	CRP (1·9–2·1)†	CRP-LMF‡ (1·1–1·6)	LMF (≤0·4)
ADP	No effect	Inhibition§	Inhibition¶
ASC	No effect	Inhibition§	Inhibition¶
Arachidonate	No effect	Inhibition§	Inhibition¶
None	No effect	No effect	No effect

* Platelet aggregation was monitored as described in Materials and Methods. Platelet activators were used at that concentration which resulted in a 50% maximal aggregation response. CRP was at 200 μg/ml and LMF at various concentrations. CPS was added to CRP to obtain 20:1 and 10:1 ratios (by weight) CRP:CPS; CPS was added to LMF at a variety of concentrations.

† A_{280}:A_{260} ratio indicated in parentheses.

‡ CRP-LMF represents CRP preparations which contain LMF.

§ Inhibition by CRP-LMF was abrogated by CPS.

¶ Inhibition by LMF was unaffected by CPS.

this inhibition was not reversed by the presence of CPS. These data imply that association with LMF was responsible for the platelet inhibitory activity previously attributed to native CRP, and that this association accounts for the sensitivity of this inhibition to the presence of the CRP ligand, CPS.

DISCUSSION

Associated with the acute inflammatory response is an elevation in the circulating level of C-reactive protein, the classical acute phase reactant. We previously reported that CRP preparations inhibited platelet stimulation by a wide variety of platelet activators. A role for an accessory factor in this inhibitory effect first became of concern because of an inconsistency in the effects of CRP preparations upon the platelet: inhibition was lost upon storage and CRP preparations differed, on a weight basis, in inhibitory capacity and sensitivity to the presence of the CRP ligand, CPS. The studies presented herein were thus intended to assess whether an accessory factor was involved in the inhibition of platelet activation observed with CRP.

The data generated from these experiments clearly suggest that the previously described platelet inhibitory properties of CRP result from and are contingent upon its apparent association with a low molecular weight factor (LMF). This material, which often co-elutes with CRP isolated from pleural or ascites fluids during CPS or PC affinity column chromatography, can be separated from CRP by ion exchange on DE-52 followed by molecular sieve chromatography over Sephacryl S-200; passage of these post-affinity eluates directly over S-200 bypassing the ion exchange step generally does not provide for adequate separation. Though not yet identified as to its molecular species, this factor has a nominal mol. wt of 8500–12,000 as judged by column chromatography on Sephacryl S-200 and an A_{280}:A_{260} ratio of ~0·4. However, SDS-PAGE analysis yields an apparent larger mol. wt of >240,000 for this material, perhaps reflecting the formation of aggregates, and similarly, minimal concentration of LMF by Amicon ultrafiltration before rechromatography over S-200 results in the appearance of high molecular weight material (>200,000). This high molecular weight material does not possess the platelet inhibitory activity; thus, its relationship to LMF awaits further clarification. Attempts to characterize LMF have been hindered by its propensity to insolubilize during or shortly following isolation. It is not yet clear whether LMF is comprised of protein, nucleic acid and/or nucleoprotein lipid and/or (apo-) lipoprotein; its ultraviolet absorption fingerprint with peak absorbance at ~254 nm suggests that if LMF is a protein it is either lacking or relatively poor in tyrosine and tryptophan residues. Investigations to more clearly identify the source and nature of LMF should permit assessment whether the presence of LMF in CRP preparations represents a physiological association.

Other molecules have been reported to be physiologically associated with CRP, including albumin (Tillett & Francis, 1930), lipid (MacLeod & Avery, 1941), β-lipoprotein (Wood, McCarty & Slater, 1954), sialic acid-containing mucopolysaccharides (Hokama, Coleman & Riley, 1967), and the complement component Clq in the presence of β-lipoproteins (Bieber, Fuks & Kaplan, 1977). Rabbit CRP has been reported to bind with low density lipoprotein Pontet, Ayrault-Jarrier, Burdin, Gelin & Engler (1979), while Cabana, Gewurz & Siegel (1981) report rabbit CRP to interact with very low density lipoproteins. The relationship of LMF to these molecules remains to be fully assessed, as does the capacity of CRP to initiate or modulate platelet function when complexed with these materials or its known ligands. In this latter respect, we have recently communicated (Fiedel, Frenzke, Simpson & Gewurz, 1980; Simpson, Gewurz & Fiedel, 1981; Fiedel, Simp-

son & Gewurz, 1981) that CRP purified free of LMF, like IgG, could be thermally modified such that it enhanced platelet activation in plasma stimulated by ADP, acid soluble collagen and arachidonate, and induced reactions of aggregation and secretion from isolated platelets. Moreover, complexes formed between CRP and one of its primary ligand classes, the polycation, shared certain of these activities.

In summary, these data explain the platelet inhibitory capacity of CRP preparations as contingent upon the presence of a co-isolating, low molecular weight accessory factor. Moreover, these studies provide further insight into the manner in which CRP can serve to modulate platelet reactivity perhaps dependent upon the physicochemical form of CRP and its association with other substances.

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