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Activation of platelets by modified C-reactive protein

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Summary. The functional similarities between C-reactive protein (CRP) and immunoglobulin raised the possibility that modified CRP might resemble immunoglobulin in its activating effects upon the human platelet. Thermally-aggregated CRP (H-CRP), but not unmodified CRP, induced reactions of aggregation and secretion from isolated platelets; maximum responses occurred with < 50 μg/ml H-CRP and were similar to responses mediated by thermally-aggregated human IgG (AHGG). Platelet activation induced by H-CRP was sensitive to the presence of EDTA and dibucaine, required metabolic energy and was inhibited by increased levels of cAMP. Like AHGG, H-CRP acted synergistically with other platelet stimulators, although on a weight basis H-CRP appeared approximately ten- to twenty-fold more effective than AHGG. Complexes formed between CRP and certain of its polycationic ligands (PLL and protamine) shared platelet activating properties with H-CRP, whereas complexes of CRP and CPS did not. These data point to the ability of appropriately modified CRP to stimulate or enhance platelet responsiveness, and taken together with those reactivities described previously between modified CRP and certain lymphocytes, phagocytes, and the complement system, support the concept that CRP can initiate biological activities similar to those mediated by immunoglobulin.

Abbreviations: AHGG, thermally-aggregated human IgG; CRP, C-reactive protein; LMF, low molecular weight factor; PRP, platelet-rich plasma; ASC, acid soluble collagen; CPS, pneumococcal C-polysaccharide; TBS, tris-buffered saline; PLL, poly-L-lysine; PC, phosphocholine.

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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 (Kushner & Kaplan, 1961; Claus, Osmand & Gewurz, 1976). CRP has an M_r of 115,000, consisting of five identical, non-covalently bound subunits (Osmand, Friedenson, Gewurz, Painter, Hofmann & Shelton, 1977; Oliveira, Gotschlich & Liu, 1979), and shares multiple functional similarities with immunoglobulins. These include the ability to initiate reactions of complement consumption and phagocytosis and the capacity to bind with mononuclear peripheral blood cells *in vitro* (Kaplan & Volanakis, 1974; Siegel, Rent & Gewurz, 1974; Mortensen, Osmand, Lint & Gewurz, 1976; James, Hansen & Gewurz, 1982a, 1982b). These reactivities each require CRP to bind with one of its multivalent ligands or to be heat-modified or chemically-aggregated (Kaplan & Volanakis, 1974; Siegel *et al.*, 1974; Mortensen *et al.*, 1976; James *et al.*, 1982a, 1982b; Claus, Siegel, Petras, Osmand & Gewurz, 1977). We previously

demonstrated preparations of CRP to inhibit platelet activation through a mechanism critical to secondary-wave ADP-mediated platelet aggregation (Fiedel & Gewurz, 1976b); subsequently, a low molecular weight factor (LMF) was identified which in combination with CRP conferred this inhibitory activity (Fiedel, Potempa, Frenzke, Simpson & Gewurz, 1982). We now report that in the absence of LMF, CRP can be thermally modified (H-CRP) such that it initiates platelet responses in isolated systems, and enhances platelet activation in plasma stimulated by various platelet agonists. Moreover, complexes formed between CRP and one of its primary ligand classes, the polycation, were found to share platelet activating properties with H-CRP. These observations further support the concept that CRP can initiate biological activities similar to those mediated by immunoglobulin.

MATERIALS AND METHODS

Isolation of CRP

CRP was isolated from human pleural or ascitic fluids using sequential phosphocholine (PC)-Sepharose 4B affinity chromatography, DE-52 ion exchange, and molecular sieve chromatography on superfine Sephacryl S-200 (Fiedel *et al.*, 1982). The final CRP preparations were dialysed into tris-buffered saline (TBS, pH 7·4) with purity assessed as described (Fiedel *et al.* 1982); these preparations were shown to be free of the low molecular weight factor which often copurifies with CRP isolated using CPS or PC affinity chromatography.

Isolation of platelets and platelet activation

Washed human platelets were prepared from platelet-rich plasma (PRP; Fiedel & Gewurz, 1976b) by sequential centrifugations and washing in tris-EDTA buffer, pH 7·5 (Bills, Smith & Silver, 1976), and resuspended at $3–5 \times 10^8$/ml in a diluent consisting of three parts 0·5% glucose in normal saline and five parts 0·09 M tris, 30 mM KCl, 30 mM NaCl and 0·8 mM $CaCl_2$, adjusted to pH 7·5. Platelet aggregation was monitored in a Model 300BD aggregometer (Payton Associates, Buffalo, N.Y.) by equilibrating TBS (300 μl) with 450 μl of PRP or the isolated platelet suspension for 1 min at 37° (1000 r.p.m.), adding the test agent, and monitoring aggregation responses for a minimum of 4 min (Bills *et al.*, 1976; Fiedel, Simpson & Gewurz, 1977). Platelet activators 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), and complexed CRP. The simultaneous measurement of platelet aggregation and secretion was performed in a Model 400 Lumiaggregometer (Chrono-Log Corp., Havertown, Pa.); the instrument utilizes the luminescent firefly luciferase system to detect secreted ATP with aggregation measured by turbidometric techniques. Reactions were performed at 37° in siliconized aggregation curvettes (Chrono Log Corp.) with a stirring speed of 1200 r.p.m. The mixture consisted of 450 μl PRP or the isolated platelet preparation and 50 μl Chronolume reagent (luminescence assay mixture; Chrono-Log Corp.) prepared as directed; the platelet agonist (in TBS) was added and aggregation/secretion monitored on a dual channel recorder. In some experiments either CRP or H-CRP was included in the reaction mixture before challenge with the platelet stimulator.

Pneumococal C-polysaccharide and poly-L-lysine (PLL)

Pneumococcal C-polysaccharide (CPS) prepared as described by Liu & Gotschlich (1963) was the generous gift of Dr Carolyn Mold, Rush University, Chicago, Ill. CPS was prepared at 0·5 mg/ml in isotonic saline and stored at −70°. PLL (15,000 Daltons) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Heat-modification of CRP

CRP (500 μg/ml in TBS) was heat complexed (H-CRP) by incubation at 63° for 2·5–3·0 min and collecting the aggregates by centrifugation. The concentration of H-CRP was established by antigenic analyses of the CRP solutions before and following removal of the aggregates after heat modification and by Lowry protein analysis of the washed aggregate with virtually identical results. Heat-modified human immunoglobulin G (AHGG) was prepared as previously described (Fiedel & Gewurz, 1976a).

Preparation of CRP-ligand complexes

Complexes were also formed between CRP and two of its known ligands, CPS and PLL. These complexes were generally formed with CRP at a final concentration of 500 μg/ml in a total reaction volume of 200 μl. Complexes of CRP–CPS were prepared at ratios of 100:1 to 2:1 CRP:CPS (w/w) during incubation either at 22° or 37° for 15–120 min, with an occasional further overnight incubation at 4°. All precipitated complexes were collected by centrifugation at 2000 *g*

Table 1. Aggregation of platelets by CRP–PLL and H-CRP complexes or by PLL*

Reactant	Maximum platelet aggregation (%)			
	Unseparated complex (%)	Supernate alone (%)	Precipitate alone (%)	PLL alone (%)
CRP:PLL (w/w)				
100:1	6	<5	<5	<5
50:1	28	<5	32	<5
25:1	88	<5	83	<5
10:1	75	<5	68	<5
H-CRP (50 μg/ml)	75	<5	79	—

* CRP–PLL complexes were formed by addition of 20 μl of varying amounts of PLL to 1·0 ml of CRP (500 μg/ml) in TBS followed by incubation at 37°/15 min. PLL controls were prepared by adding equivalent amounts of PLL to 1·0 ml saline and represents the maximum PLL concentrations used to prepare the CRP–PLL complexes (1–10 μg/assay). H-CRP was prepared as described previously. These CRP aggregates were either used unseparated or the precipitates were collected by centrifugation (22°; 10 min) and resuspended in 1·02 ml (CRP–PLL) or 1·0 ml (H-CRP) saline, respectively. Two hundred microlitres of each CRP–PLL or PLL control preparation was then tested for the ability to aggregate platelets.

(22°; 10 min), the supernates removed for testing and the pellet resuspended in 50 μl TBS. CRP–PLL complexes were similarly prepared (or as described in Table 1) with the exception that incubation was carried out at 37° for 15–30 min. When individual complexes were pooled, the precipitate from a number of reaction tubes was transferred to a single tube and resuspended in a total of 50 μl TBS (as in Fig. 6). CRP–CPS complexes, whose formation is calcium-dependent, were prepared in TBS containing 1·0 mM $CaCl_2$; CRP–PLL complexes were prepared only in TBS as their formation is inhibited by the presence of calcium. Other salient conditions are described in the text.

Other reagents

Protamine, fibrinogen, dibutyryl cAMP, 2-deoxy-D-glucose, antimycin A and dibucaine were obtained from Sigma Chemical Co.

RESULTS

Activation of isolated platelets by H-CRP

The functional similarities between CRP and immunoglobulin raised the possibility that modified CRP might resemble aggregated immunoglobulin in its activating effects upon the human platelet. We therefore tested the ability of H-CRP to influence platelet responsiveness in an isolated buffer system, as shown in Fig. 1. In concentrations between 5–40 μg/ml, unlike native CRP, H-CRP stimulated a platelet response. This was characterized by an initial lag period followed by rapid monophasic aggregation, and was similar to that observed with heat-modified IgG (AHGG). The platelet aggregation stimulated by H-CRP was sensitive to the presence of a dibutyryl analogue of cAMP (0·5 mM), EDTA (1 mM) or dibucaine (0·1mM), or the combination of 2-deoxy-D-glucose and antimycin A (50 mM and 1 μM, respectively), indicating this activation event to be under metabolic control and not a simple agglutination process. As measured by the secretion of ATP, H-CRP also generated a platelet release reaction with aggregation and secretion occurring coincidentally (Fig. 2). These activation processes were not affected by the addition of up to 0·4 gm% human fibrinogen, illustrating an independence from this plasma protein for initiation of the aggregation event. Moreover, these reactivities were unaffected in the presence of certain

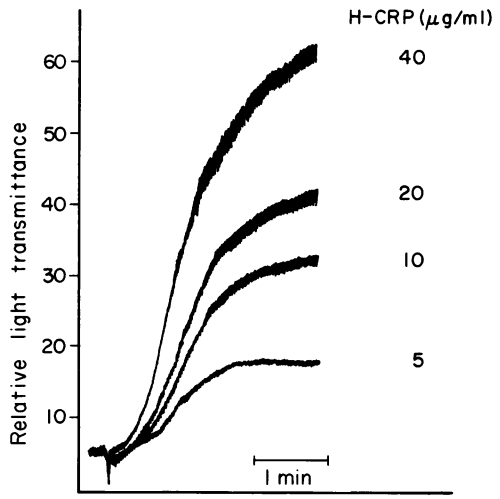

Figure 1. Aggregation of human platelets following addition of various concentrations of heat-modified CRP (H-CRP) in buffered saline. The aggregation response was monophasic and similar to that observed with heat-modified human immunoglobulin G.

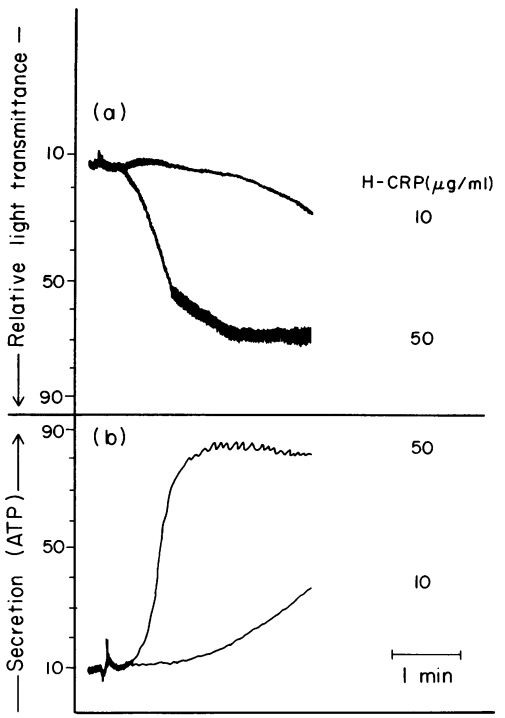

Figure 2. Simultaneous comparison of platelet aggregation (a) and secretion of ATP (b) stimulated by heat-modified CRP (H-CRP) in buffered saline.

CRP ligands including up to 50 μg/ml CPS or 10^{-4} M phosphocholine, or up to 500 μg/ml native CRP. The activating principle of H-CRP resided entirely in the precipitated aggregate (see Table 1).

Synergism between H-CRP and other platelet activators in a plasma-free system

Most platelet activators can act synergistically with each other to stimulate the platelet (Kinlough-Rathbone, Packham and Mustard, 1977). We next attempted to ascertain in a washed platelet system whether H-CRP had this capacity; data for the platelet activator ADP is presented in Fig. 3. H-CRP worked in synergy with suboptimal amounts of ADP to produce a platelet response. This was evident regardless whether the H-CRP concentration used was itself sufficient to induce platelet aggregation. The generation of these data in the absence of added fibrinogen suggests that modified CRP might be capable of bypassing the usual fibrinogen requirement for ADP to participate substantially in platelet aggregation. H-CRP also operated synergistically with other platelet activators including acid soluble collagen (ASC) and arachidonate. Unmodified CRP did not have this synergistic capacity and (in amounts up to 500 μg/ml) did not interfere with the platelet stimulating activity observed in the presence of H-CRP plus another platelet activator. When similar experiments were performed using AHGG, it became clear that suprathreshold concentrations of this activator often were required to elicit synergistic responses. This made the direct assessment between H-CRP and AHGG difficult since H-CRP could synergistically co-operate with other platelet activators at subthreshold concentrations of 3–5 μg/ml. Nonetheless, a nominal estimate is that H-CRP, on a weight basis, is at least ten- to twenty-fold more effective than AHGG in participating in a synergistic platelet response. This difference was easier to demonstrate in platelet-rich plasma and is detailed in the next section.

Participation of H-CRP in platelet activation in plasma

Antibody aggregates are well known to activate isolated platelets but fail to have a direct agonist role in many experimental plasma systems (Pfueller, Weber & Lüscher, 1977). Therefore, we examined the effect of

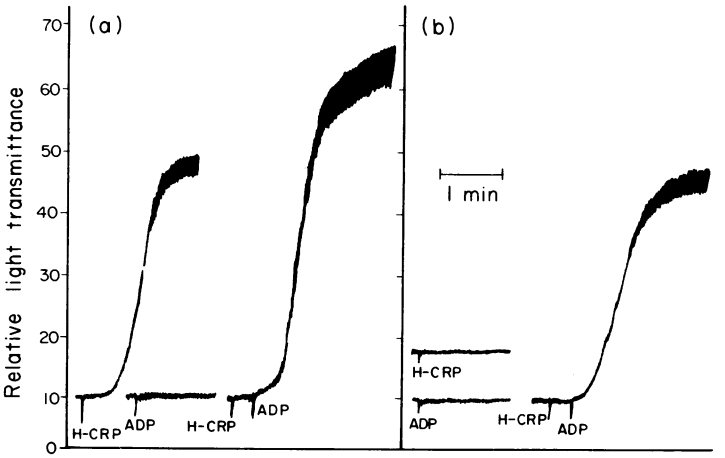

Figure 3. Synergism between heat-modified CRP (H-CRP) and ADP in the induction of platelet aggregation in buffered saline. In (a), H-CRP was used at 20 μg/ml and in (b) H-CRP was used at 3·0 μg/ml. ADP was used at 10^{-6} M. Fibrinogen was not added to these reaction mixtures.

H-CRP on platelet responses in PRP with data presented in Fig. 4. H-CRP alone was ineffective as a platelet activator. However, as observed previously in the isolated platelet system, H-CRP added to PRP in combination with a suboptimal concentration of ADP

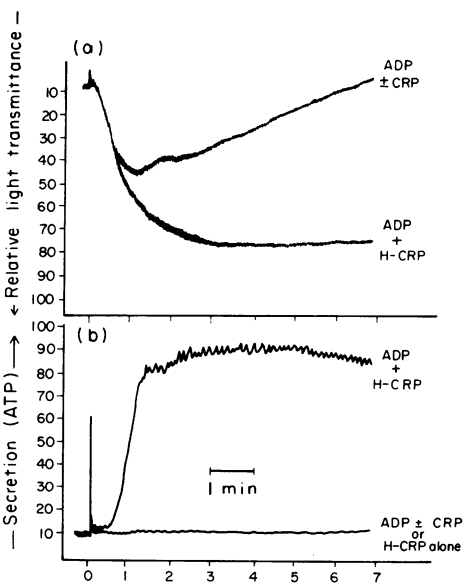

Figure 4. Comparison of the synergistic effects of native or heat-modified CRP (H-CRP) upon platelet aggregation (a) and secretion of ATP (b) stimulated by 10^{-6} M ADP in PRP. CRP was used at concentrations up to 200 μg/ml; H-CRP was used at 50 μg/ml.

greatly enhanced platelet reactivity, stimulating the platelet to irreversible aggregation and a simultaneous secretory event. Native CRP had no effect on the ADP response nor did it interfere with the platelet response to the combined presence of H-CRP and ADP. As seen previously in the isolated platelet system, synergy was also observed between CRP and the platelet activators ASC and arachidonate. AHGG also gave evidence of synergism with other platelet activators in platelet-rich plasma but, on a weight basis, again was approximately ten- to twenty-fold less effective than H-CRP (300–500 μg/ml AHGG to 30 μg/ml H-CRP for comparable activity) and required a greater basal level of platelet stimulation than did H-CRP (Fig. 5).

Activation of platelets by complexes formed between CRP and PLL or CPS

As CRP is reactive with polycations (exemplified by PLL) and phosphocholine-containing compounds (exemplified by CPS), we attempted to determine whether CRP-ligand complexes of each of these types would share platelet-activating properties with H-CRP. Complexes formed between CRP and PLL resulted in activation of the platelet when tested in the buffered system (Fig. 6). CRP–PLL complexes were formed at varying ratios, and those prepared at a CRP:PLL ratio of 25:1 (w/w) were the most effective for platelet activation (Table 1). As with H-CRP, the activating principle resided entirely in the precipitated aggregate. PLL alone, in the maximum concentrations

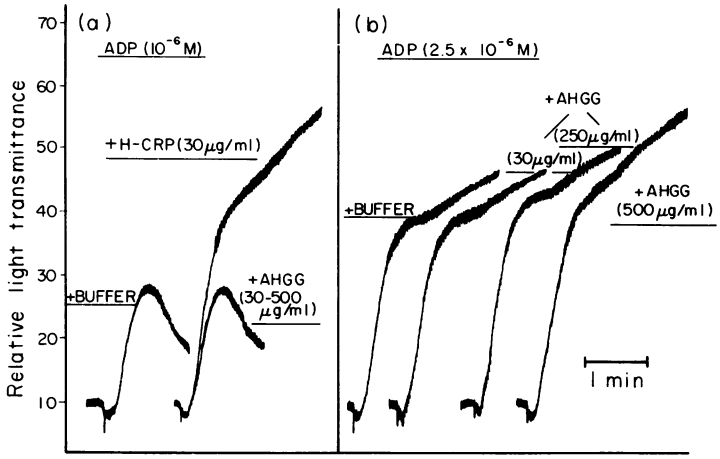

Figure 5. Comparison of the ability of heat-modified CRP (H-CRP) or human immunoglobulin G (AHGG) to act synergistically with ADP in platelet-rich plasma. In (a), ADP was used at 10^{-6} M and gave a minimal level of activation while in (b), ADP was used at $2{\cdot}5 \times 10^{-6}$ M and yielded a more substantial level of platelet aggregation. H-CRP and AHGG were used at the indicated concentrations. H-CRP was, on a weight basis, at least ten-fold more effective than was AHGG and required a lesser degree of basal platelet stimulation.

used to prepare the CRP–PLL complexes described in Table 1 (1–10 μg/assay), did not significantly activate the platelet. Like H-CRP, CRP–PLL induced platelet aggregation was not inhibited in the presence of CPS or phosphocholine. Similar results were obtained with complexes formed between CRP and another polycationic ligand, protamine. Complexes of CRP and CPS preformed at 37° or 22° (with or without overnight incubation at 4°) or formed in the presence of platelets at combining ratios ranging from 100 to 2:1 CRP:CPS (w/w) using maximum CRP concentrations in excess of 1 mg/ml elicited no activation of platelets, nor did they interfere with platelet-activation stimulated with H-CRP or CRP–PLL complexes. Thus, complexes formed between CRP and CPS did not share platelet-activating properties with H-CRP or the CRP-polycation complex.

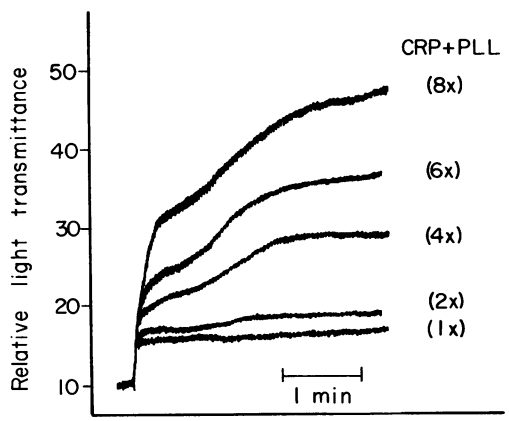

Figure 6. Aggregation of isolated human platelets by complexes formed between CRP and poly-L-lysine (PLL; 15,000 Daltons) at a 70:1 CRP:PLL (w/w) ratio. CRP and PLL were incubated at 37° for 15 min and aliquots of the washed precipitate were brought to appropriate concentrations (parenthesis) in TBS. PLL alone at the concentrations used in these experiments did not induce platelet aggregation.

DISCUSSION

Associated with the acute inflammatory response is an elevation in the circulating level of CRP, a prototypic acute phase reactant which is one member of a recently described superfamily of proteins termed pentraxins (Osmand *et al.*, 1977). CRP differs from immunoglobulin in antigenicity, tertiary structure, homogeneity, stimuli required for formation and release, and binding specificities which for certain reactivities of CRP require calcium and seem to be directed to phosphate esters and polycations generally. However, numerous functional similarities between CRP and immunoglobulins have been appreciated, including the ability to initiate reactions of precipitation, agglutination, complement consumption, capsular swelling, and en-

hancement of phagocytosis (reviewed in Gewurz, Mold, Siegel & Fiedel, 1982). Immunoglobulin G aggregated by heating, antigen, chemical treatment, or coating to inert particles has been found to react with isolated human platelets to initiate reactions of aggregation and constituent release (Movat, Mustard, Taichman & Uruihara, 1965; Pfueller & Lüscher, 1972). Thus, the functional similarities between CRP and immunoglobulin led us to investigate its effects upon the platelet. The presence of CRP in the circulation and deposited at tissue sites during host injury, along with the known role of the platelet in inflammation and haemostasis, speak to the potential importance of such effects.

Heat-modified CRP (H-CRP), like its immunoglobulin G counterpart (AHGG), activated isolated human platelets to reactions of aggregation and constituent release. Aggregation was monophasic in nature, as previously observed for AHGG (Fiedel & Gewurz, 1976a), and occurred coincidently with the secretory event. This activation process required metabolic energy (ATP) as evidenced by sensitivity to the combined presence of 2-deoxy-D-glucose and antimycin A; required calcium (both external and internal) as demonstrated by sensitivity to EDTA and dibucaine, respectively; and was inhibited in the presence of increased levels of cAMP. These observations indicate that the platelet response stimulated by H-CRP reflects an actual activation process and not a simple agglutination reaction.

H-CRP participated synergistically with other platelet activators to bring about a platelet response *in vitro*. This is conceptually important since it has been hypothesized that *in vivo* platelet reactivity rarely is promulgated by any single activator but rather reflects the interactive involvement of multiple activators (Huang & Detwiler, 1981). AHGG also exhibited the capacity to operate synergistically with other platelet stimulators but, compared with H-CRP, was much less effective and required a greater basal level of platelet stimulation in order to be effective.

Complexes formed between CRP and one major ligand, the polycation (e.g. PLL), like H-CRP, also activated the platelet in an isolated buffer system. Since platelets are activated by various polycations, including poly-L-lysine (reviewed in Metcalf & Lyman, 1974; Fiedel & Gewurz, 1976b), we consider it possible that polycations cross-linked by CRP might present a more positively-charged particulate surface to the platelet to induce activation than does the soluble polycation alone. The inability to generate CRP–CPS complexes that activate and/or interfere with activation of the platelet by H-CRP or CRP–PLL complexes may be the result of the absence of appropriately presented positive charges, or indeed, the highly negative nature of CPS may itself render the complex inactive. However, the ability of H-CRP alone to activate the platelet and data (not presented) which show that CRP, like IgG, activates platelets when adsorbed onto latex beads (Mueller-Eckhardt & Lüscher, 1968), illustrate that CRP has an intrinsic ability to be cross-linked in a manner suitable for platelet activation, and suggest that the polycation in CRP–PLL complexes may, in part, serve appropriately to cross-link CRP. Moreover, since CRP shows a distinctly different circular dichroism spectrum dependent upon whether calcium is present or absent suggesting the existence of two allosteric forms of CRP (Young & Williams, 1978), it is possible that the cross-linking of CRP molecules by heat, adsorption onto latex beads or with an appropriate polycation in the absence of calcium might permit formation of a CRP aggregate which is in an allosterically favoured form to activate the platelet. Those CRP aggregates formed in the presence of calcium or reacting with the phosphocholine (PC) moiety (e.g. with CPS) may not be in an allosterically suitable form. It is thus of interest to note that in the presence of calcium, CRP does not suitably heat aggregate to form a complex capable of activating platelets. We have synthesized bifunctional PC-substituted cationic homopolymers (e.g. PC-PLL) in order to test whether CRP complexes formed in the presence of calcium can activate the platelet given a sufficient distribution of positive charges.

The data presented herein are consistent with and support the concept that CRP can initiate biological activities similar to those mediated by immunoglobulin. However, we do not yet know whether these similarities indicate an overlap in the mechanism by which modified CRP or AHGG activate the platelet or whether both react through the Fc receptor (Movat *et al.*, 1965; Pfueller & Lüscher, 1972; Pfueller *et al.*, 1977). In any case, complexes of modified CRP could serve to act with other platelet agonists (e.g. ADP, collagen and arachidonate) released during acute inflammatory episodes. In this regard, we have recently communicated (Simpson, Gewurz & Fiedel, 1981) that stimulation of platelets by modified CRP can generate and release into the environment thromboxane A_2, an important platelet activator and contractor of vascular tissue. These considerations all

point to the capacity of modified CRP to activate and support the inflammatory and haemostatic processes, a role in keeping with its reported activating effects upon both the complement and phagocytic systems.

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