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Regulation of Platelet Function in the Acute Phase: A Review of the Biology of the C-Reactive Protein

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ABSTRACT

Earlier investigations had demonstrated that CRP inhibited platelet aggregation and release reactions, activation of platelet factor 3 and platelet-dependent clot retraction, and suggested that CRP exerted these effects by interfering with an aspect of prostaglandin metabolism. Recent studies pointed to a role for an accessory molecule in the platelet inhibition mediated by CRP, and led to the conclusion that the platelet inhibitory properties of CRP result entirely from its association with a low-molecular weight factor (LMF) of approximately 8,300-12,500 daltons. The removal of LMF resulted in a CRP which could be thermally modified (H-CRP) to induce platelet aggregation/secretion in isolated systems and enhance the platelet activation in plasma induced by ADP, collagen or arachidonic acid. The activation of platelets by H-CRP was sensitive to nonsteroidal anti-inflammatory drugs and metabolic inhibitors as well as the ADP-removing enzyme system creatine phosphate/creatine phosphokinase. Thin-layer chromato-

graphic (TLC) analysis of prostanoate end-products following platelet activation with H-CRP revealed the formation of thromboxane A_2, an important endogenous platelet activator and contractor of vascular tissue; bioassay on rabbit aorta strips of supernatants obtained from platelets undergoing challenge with H-CRP supported the TLC analysis. Complexes formed between CRP and certain of its polycationic ligands were found to share platelet activating properties with H-CRP. These data imply an agonist role for CRP in platelet physiology, suggest that the interaction of modified CRP with the platelet at sites of vascular damage could have pathological significance, and support the concept that CRP can initiate biological activities similar to those mediated by immunoglobulin. In experiments directed to determine whether CRP and IgG might share a receptor on the platelet membrane, neither plasmin nor phospholipase C altered the platelet response to aggregate CRP (H-CRP) or aggregate IgG (AHGG), although these reagents enhanced the platelet expression to acid-soluble collagen (ASC). Conversely, chymotrypsin treatment of platelets resulted in an elevated response to each H-CRP and AHGG, but not to ASC. These data suggested that the H-CRP and AHGG platelet receptors share characteristics which contrast with those of the receptor for collagen. However, monomeric IgG, which can bind with the platelet and inhibit the response to AHGG, exerted no effect on the platelet response to H-CRP. Further, a functional receptor for thermally modified human or rabbit CRP was detected on rabbit platelets in the absence of a demonstrable Fc receptor for aggregated IgG. These data indicate that the platelet receptors for the modified forms of CRP and IgG are distinct. Thus, CRP seems to differentially regulate platelet function in the acute phase depending upon its physicochemical state and/or its association with other molecules.

OVERVIEW

In 1930, Tillett and Francis observed that sera obtained from patients during acute febrile illnesses have the ability to precipitate with an extract of the pneumococcus, designated Fraction C (C-substance) and later C-polysaccharide. Tillett, Goebel and Avery (1930) termed this serum material, "C-precipitin" and Ash (1933) showed that it occurred in non-febrile illnesses caused by both Gram-positive and Gram-negative bacteria. This material also was found in the sera of patients with rheumatic fever and other diseases of bacterial origin, and Abernethy and Francis (1933) observed that the C-substance could induce cutaneous reactions selectively in patients who had the C-precipitins in their sera. Abernethy and Avery (1941) presented evidence that the C-precipitin was a protein and demonstrated that calcium was required for it to react with the C-substance. They proposed that the C-reactive substance was distinct from antibody because it occurred only during the acute stages of infection, occurred in a variety of infections and non-infectious illnesses independent of the inciting agent, and was present in the albumin rather than in the globulin fractions during precipitation with ammonium sulfate.

MacLeod and Avery (1941a, b) extended these observations and purified the protein, demonstrating its immunologic specificity by both precipitation and complement fixation tests with antisera raised in rabbits to the purified material. Now termed C-reactive protein, or CRP, it can be detected and/or quantified by

precipitation with specific antisera, precipitation with the C-substance, or by calcium-dependent capsular swelling reactions with appropriate strains of pneumococcus (Lofstrom, 1944); of these, precipitation with specific antibody is most sensitive, rapid, and convenient. The widespread use of these tests extended the range of clinical conditions during which serum CRP was readily measurable, and revealed that a feature common to these conditions was the presence of reactions of acute inflammation or tissue destruction, particularly in inflammatory and malignant diseases (Anderson and McCarty, 1950; Shetlar et al., 1955). Thus, an elevated CRP level has served as a nonspecific indicator of the presence of these processes, rather than as an indicator of a specific group of diseases. As such, CRP has become known as the prototypic acute phase reactant.

The elevated levels of CRP detected in the circulation during the acute phase has justifiably raised the question of its role during inflammation and tissue destruction. The numerous functional similarities between CRP and immunoglobulin including the ability to initiate reactions of precipitation, agglutination, capsular swelling, opsinization and activation of complement (reviewed in Gewurz et al., 1982), suggests that CRP may serve as a non-immune effector in analogy to immune-mediated reactions. Such a role for CRP would be supportive of early participation in host defense mechanisms in the absence of immune protection. In contrast, CRP and immunoglobulin are distinguishable on the basis of nonspecific induction, antigenicity and lack of heterogeneity. Therefore, CRP may have additional biological functions whose purpose(s) are just now being appreciated; these may pertain to a role for CRP in the modulation of mechanisms involved in specific host resistance to infection, immune induction, tissue repair and homeostasis.

A single detailed discourse into all CRP biology is beyond the scope of this review. I have, however, collected our investigations into the effects of CRP upon platelet reactivity. The work of this laboratory and Department, which have as a major commitment the study of the acute phase response, has demonstrated that the interactions between CRP and the platelet are the most promising approach to the study of CRP-cellular interactions. This has become evident over the past few years as the effects of CRP upon platelet function have been the most consistent and sensitive indicator of CRP-cellular biologic function. It is possible that platelets represent the most important of the formed elements of the blood whose activity is in some manner regulated by CRP. Platelets are known to function in numerous capacities including maintenance of endothelial integrity, blood clotting, hemostasis and thrombosis, fibrinolysis, wound-healing and immune and inflammatory reactions. The functions attributed to platelets have in common the aegis of being a host response to injury or a defense mechanism. The modulatory effects that CRP exerts upon the platelet suggest that the acute phase in general may play a major role in regulating host responsiveness to insult and may, indeed, be a homeostatic response in the body economy. Moreover, since the internal events that precede the biologic expression of platelet function are in many ways similar to those of other cell types (e.g. lymphocytes, polymorphonuclear neutrophils, monocytes, basophils, and mast cells), knowledge of how CRP and other reactants modulate the response of the platelet to normally active stimulatory agents may yield information applicable to other cellular systems.

MATERIALS AND METHODS

(Fiedel et al. 1982a-d and Simpson et al. 1982)

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.). The column was washed with 20 mM Tris-buffered saline containing 10 mM $CaCl_2$ (TBS^{+}; pH7.5) 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, 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 dialyzed 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). The purity of CRP was assessed by polyacrylamide gel electrophoresis (PAGE) in SDS and urea, 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, 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, fibrinopepitdes 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. Similarily, 50-100 ul (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 Pharamacia Fine Chemicals.

Heat modification of CRP

CRP (500 μg/ml in TBS) was heat-aggregated (H-CRP) by incubation at 63° for 2.5 - 3.0 min and the aggregates collected by centrifugation (1000 g; 22°). 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. Between 47% and 53% of CRP was removed from solution during heat-modification.

Preparation of CRP-ligand complexes

Complexes were formed between CRP and two of its ligands, C-polysaccharide (CPS) and the synthetic polycation, poly-L-lysine (PLL; 15,000 mol. wt). 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 formed at ratios of 10:1 to 2:1 CRP:CPS (w/w) during incubations at either 22° or 37° for 15-120 min, with an occasional further overnight incubation at 4°. All precipitate complexes were collected by centrifugation at 2000 g (22°; 10 min), the supernates removed and the pellet resuspended in 50 μl TBS. CRP:PLL complexes (25:1 to 10:1, w/w) were similarly prepared 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. CRP:CPS complexes, which form only in the presence of calcium, were prepared in TBS containing 1.2 mM $CaCl_2$; CRP:PLL complexes were prepared in TBS as their formation is markedly inhibited by the presence of calcium. Latex-aggregated CRP (Latex-AggCRP) was prepared by incubating 40 μl of a 1/10 dilution of washed latex beads (Bacto Latex 0.81, Difco, Detroit, Mich.; used as supplied) in 500 μl CRP (540 μg/ml) for 30 min at 37°. The beads were washed once and resuspended in 250 μl saline. Latex-aggregated IgG (Latex-AggIgG) was similarly prepared. In some instances it was necessary to adjust the number of latex beads or the stock concentration of CRP in order to maximize the platelet response. Calcium (1.2 mM) was present during the formation of Latex-AggCRP (or IgG). As native CRP does not interfere with the activation of platelets by Latex-AggCRP, the washing of these beads need not be routinely employed. Washing of Latex-AggIgG was required as native IgG interfered with the platelet activation stimulated by Latex-AggIgG.

Isolation of platelets and platelet activation

Washed human platelets were prepared from platelet-rich plasma (PRP) by sequential centrifugations and washing in Tris-EDTA buffer, pH 7.5, and resuspended at 3-5x 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 NaCl and 0.8 mM $CaCl_2$, adjusted to pH 7.5. Platelet aggregation was monitored in a Model 300 BD 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. The simultaneous measurement of platelet aggregation and secretion was performed in a Model 400 Lumi-aggregometer (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 cuvettes (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.

Conversion of platelet [^{14}C]-arachidonic acid to [^{14}C]-TXB_2.

Blood was collected into 50 ml syringes, mixed with bisodium citrate-dextrose-

phosphate (9:1 v/v) and centrifuged at 200 g for 20 min to obtain PRP. The PRP was adjusted to 3×10^8/ml and incubated with TLC rechromatographed [^{14}C]-arachidonic acid (0.05 μCi/ml) at 37° for 60-90 min. The labeled platelets were then isolated by sequential centrifugations at 2000 g for 15 min and suspended in TBS at a concentration of 5×10^8/ml.

To assay the generation of [^{14}C]-TXB_2 (thromboxane B_2), the hydrated end-product of TXA_2 (thromboxane A_2), 1 ml of platelets was stirred (1000 r.p.m.) in the aggregometer, 10-100 μl of the agonist added and the reaction allowed to proceed for 5 min. Five microliters of [^{3}H]-TXB_2 (125 mCi/mmol) were added as an internal standard to determine the recovery of [^{14}C]-TXB_2 generated during the reaction. The reaction was terminated by addition of an aliquot of 1 M citric acid (10-20 μl) sufficient to reduce the pH to 3.5. The platelet mixtures were decanted into 10 ml of ethyl acetate (4°) in 13 × 125 mm acid washed glass culture tubes. The aqueous and organic layers were intermixed for 5 min using a paint shaker (Dayton Electric Co., Chicago, Ill.) and the mixtures centrifuged at 2000 g for 10 min (4°), resulting in upper-organic and lower aqueous phases. The extraction was repeated, organic layers pooled and evaporated under nitrogen at 32°, and dissolved in 1 ml of methanol.

For thin layer chromatography (TLC), the methanolic solution was evaporated to dryness and dissolved in 25-30 μl of methanol. The extracted lipids were applied to 250 micron silica gel G-60 plates and chromatographed at 22°. TLC plates were run in a first solvent of chloroform:acetic acid (90:1 v/v) to remove arachidonic acid and the hydroxyacids from the origin. The plates were then rotated 90° and chromatographed in a second solvent of diethylether:methanol:acetic acid (90:2:2 v/v) to separate prostaglandins E_2, $F_{2\alpha}$ and TXB_2. To assess the location of radioactive [^{14}C]-TXB_2, the thin-layer plates were scanned on a radiochromatograph scanner (Packard Model 7230); non-radioactive standards of prostaglandin E_2 and D_2 were chromatographed in the second solvent to determine the location of these prostaglandins (detected with iodine vapor). For a quantitative determination of TXB_2 generation, 1 × 1 cm areas were partitioned on the silica gel plates and those radioactive areas containing the appropriate lipids were scraped and eluted into scintillation fluid and counted. The total radioactivity of [^{14}C]-TXB_2 was corrected for background, percentage recovery of [^{3}H]-TXB_2, and the final results were expressed as the percentage of total platelet [^{14}C]-arachidonic acid converted to [^{14}C]-TXB_2. Total platelet [^{14}C]-arachidonic acid incorporation was determined by removing a 1 ml aliquot of the final platelet suspension, washing it in TBS and counting the pellet. The production of radioactive prostaglandins (PGE_2 and $PGF_{2\alpha}$) was qualitatively assessed.

Generation of thromboxane A_2 (bioassay).

Spirally cut strips of thoracic aorta, approximately 2 × 0.2 cm, were removed from anaesthetized male rabbits. Male rabbits were used because of their greater sensitivity to PGG_2/PGH_2 (prostaglandins G_2 or H_2) and TXA_2. Rabbits were anaesthetized by injection of 2-3 ml of a 50 mg/ml solution of Sodium Nembutal (Abbott Laboratories, N. Chicago, Ill.) into the ear vein using a 21 gauge butterfly. The aorta strips were suspended from an isotonic force transducer (Gould UC2) by surgical thread. The strips were continually superfused with Krebs-Bicarbonate buffer (37°) at a constant flow rate of 10 ml/min and aerated with 95% O_2- 5% CO_2. Contractions were recorded with a polygraph, Model RPS 79C (Grass Instruments, Quincy, Mass.). A resting tension of 2 gm/cm was applied to strips

according to the calibration of the isotonic force transducer before the experiment. To increase the specificity of the tissue response, various pharmacological antagonists were included in the superfusate to inhibit tissue contractions to acetylcholine, serotonin, histamine, epinephrine, norepinephrine and arachidonic acid. These included 0.33 μM phenoxybenzamine, 0.25 μM mepyramine maleate, 0.9 μM indomethacin and 1.4 μM atropine (Sigma Chemical Co.; Sandoz Co., East Hanover, N.J.).

Platelet incubations were carried out in a final reaction volume of 0.5 ml in a Payton aggregation module. Precise kinetic analysis of the generation of TXA_2 activity for the platelet suspensions was done for each agonist. A dose response for platelets challenged with arachidonic acid was determined and a final concentration of 100 μM was used as a positive reaction control. Platelets, stirring in a cuvette, were preincubated for 1 min and then challenged with an activator, the contents of the cuvette drawn into a 1 ml tuberculin syringe and immediately administered into the injection portal superfusing the aortic strip. The time lag between removal from the reaction mixture and tissue perfusion was approximately 15 sec.

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

Pneumococcal C-polysaccharide (CPS) was the generous gift of Dr. Carolyn Mold-DuClos, 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.).

Radiochemicals

Radioactive materials were obtained as follows: [^{14}C]-5HT (serotonin), Amersham-Searle Inc., Arlington Heights, Ill.; [^{3}H]thromboxane B_2 (100-150 mCi/mmol), [^{14}C]-arachidonic acid (40-60 mCi/mmol) ([^{14}C]-AA) both from New England Nuclear, Boston, Mass. A β-thromboglobulin radioimmunoassay kit was obtained from Amersham-Searle, Inc. Nonradioactive prostaglandins E_2, D_2, $F_{2\alpha}$ and thromboxane B_2 (TXB_2) were the generous gift of Dr. J.E. Pike, Upjohn Pharmaceutical Co. Kalamazoo, Mich.

RESULTS

1. Description of the inhibitory effects of certain CRP preparations on platelet function.

As early as 1976 (Fiedel and Gewurz, 1976a, b) we had demonstrated that CRP could modulate certain platelet reactivities. CRP preparations were first found to inhibit the aggregation of washed platelets stimulated by both heat-modified human immunoglobulin (AHGG) and by thrombin. The inhibition was dose-dependent, not due to chelation of calcium or cytotoxicity, and characterized by reductions in the rate and maximal extent of aggregation and in the size of individual aggregates. Inhibition was not seen in the presence of known CRP ligands phosphorylcholine or C-polysaccharide, suggesting that it was mediated by CRP. Inhibition also was overcome by larger amounts of AHGG or thrombin, indicating an agonist-antagonist relationship dependent upon the relative concentrations of both the stimulating agent and CRP. The amounts of CRP required to inhibit platelet aggregation induced by thrombin (by 90%) was 10-fold greater than the amount required to inhibit AHGG. CRP preparations similarly inhibited

the generation of platelet factor 3 (PF3) activity by AHGG and thrombin and routinely reduced the release of β-glucuronidase following platelet challenge with thrombin, although extraordinarily high amounts of CRP were required to observe this latter effect. The inhibition of CRP was evident, though reduced, following preincubation of the platelets with CRP and subsequent washing of the platelets; CRP could not be found to interact with AHGG or thrombin as determined by sucrose density sedimentation profiles of ^{125}I-CRP alone or in combination with each agent and CRP failed to affect the thrombin-fibrinogen clotting time. Further study of the effects of CRP upon thrombin-induced platelet activation revealed that the inhibition of platelet aggregation coincided with the decreased release of ADP, serotonin (5HT) and a non-prostanoate degradative product of PGH_2, malondialdehyde. This inhibition occurred in the absence of an effect upon basal levels of platelet cAMP.

CRP was subsequently found to inhibit platelet response in citrated plasma stimulated with ADP, epinephrine or collagen (ASC). The inhibitory effects of CRP following platelet challenge with ADP or epinephrine were associated solely with second wave aggregation and concomittant with decreased secretion of 5HT (Fig. 1) and the generation of second-wave associated PF3 activity. CRP preparations also inhibited collagen-stimulated platelet aggregation in citrated plasma (data not shown) in the absence of any observable effect upon collagen-platelet adhesion (Table 1). CRP did not alter platelet shape change following collagen addition and greater amounts of CRP were required to effectively inhibit collagen induced aggregation than were required to effectively inhibit second-wave ADP- and epinephrine stimulated platelet responses.

CRP was found not to inhibit indomethacin-insensitive platelet responses stimulated with the calcium ionophore A23187 (Fig. 2) nor the indomethacin and 5,8,11, 14 eicosatetraynoic acid (ETYA)-insensitive secretory response initiated by semi-purified platelet activating factor. By contrast, CRP readily inhibited platelet aggregation stimulated with arachidonic acid (Fig. 3) (Fiedel, Simpson and Gewurz, 1977). However, there existed an inconsistancy in the biologic effects of CRP upon the platelet. Although the majority of CRP preparations (87%) inhibited platelet activation, this effect was often short-lived being lost upon storage; such loss was associated with the generation of insoluble material in the CRP preparations, previously suspected to be aggregates of CRP. A putative role for an accessory molecule in these reactions now has been obtained.

2. Evidence for a low molecular weight factor in CRP-associated inhibition of platelet function.

CRP isolated from ascites or pleural fluids by PC (phosphocholine)-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 acid soluble collagen (ASC) in both the absence and presence of C-polysaccharide (CPS), a known ligand of CRP (Fig. 4). Platelet inhibitory activity generally was observed in two eluant zones, the first involving a factor migrating as a species of 115,000 molecular weight (mol. wt.) coincident with CRP antigenicity (main peak) and the second involving a low molecular weight factor (LMF) migrating as an apparent species of 8300-12,500 mol. wt. Only the inhibition observed with the former was abrogated in the presence of CPS sug-

gesting 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, reflective of a close relationship between the two molecules.

In order to more critically assess the presence of LMF, portions of the PC-affinity eluates were passaged over a mini-column of Sephacryl S-200 and CRP antigenicity and platelet aggregation inhibitory capacity were evaluated for each fraction (Fig. 5) (Fiedel et al., 1982a). A single large broad protein peak and a second smaller area of absorbance cooresponding to the molecular weight of the ribonuclease A marker were observed. CRP antigencity was present throughout the first peak and anomalously present in the second peak, corresponding to a molecular weight slightly less than 12,700. When this low molecular weight region containing LMF activity was analyzed by SDS-PAGE (following concentration), both an apparent 23,000 dalton 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 mol. wt. in SDS-PAGE) as well as a large molecular weight peak free of CRP antigenicity eluting at the column Vo (»200,000) were observed; little low molecular weight material was visualized. This suggested that under appropriate conditions (concentration?) the low molecular weight component might assemble into aggregates. The high molecular weight component did not inhibit platelet activation.

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 (Fig. 6). Chromatography of the PC-affinity eluate over DE-52 yielded material eluting at 0.24-0.27 RSC (relative salt concentration) which migrated as a single 115,000 mol. wt. peak over Sephacryl S-200, gave a 23,000 dalton component in SDS-PAGE and had an A_{280}:A_{260} ratio of 1.9-2.1; these data are representative of 34 of 40 preparations. In 6 of 40 preparations (Fig. 7), the PC-affinity eluate resulted in an eluate from the DE-52 anion exchange resin at 0.17 RSC (peak 1) as well as at 0.27 RSC. The peak 1 material contained CRP antigenicity, had an A_{280}:A_{260} ratio of $\sim$1.1 and after concentration 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, one containing and the other lacking CRP antigenicity; the latter peak had an extremely low A_{280}:A_{260} ratio (0.4), 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 (after concentration) and precipitated from solution within 3-6 h of isolation. The material which eluted from the ion exchange column at 0.24-0.27 RSC (peak 2), when resolved over Sephacryl S-200, chromatographed identical to the 0.24-0.27 RSC material described previously 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 each CRP-LMF and of CRP free of LMF.

The materials eluted from these columns were tested for their ability to modulate platelet activation stimulated by ADP, ASC, and arachidonate in both

the absence and presence of C-polysaccharide. As expected, CRP with A_{280}:A_{260} ratios of 1.9-2.1 did not inhibit platelet activation in amounts of 200 μg/ml. However, in the presence of LMF (whether isolated as CRP-LMF [A_{280}:A_{260} ratio of ~1.1] or obtained by recombination of the separated components), inhibition of platelet activation stimulated by ADP, ASC and arachidonate was observed in a manner consistent with previous reports (Fiedel and Gewurz, 1976a, b; Fiedel, Simpson and Gewurz, 1977). Morever, this inhibition was not evident in the presence of the CRP ligand, CPS. These data imply that association with LMF was responsible for the platelet inhibitory activity attributed to native CRP, and that this association accounts for the sensitivity of this inhibition to the presence of the CRP ligand, CPS.

In summary, this material (LMF) often co-elutes (co-purifies) 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. 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. 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 (Fig. 8) suggests that if LMF is a protein it is either lacking or relatively poor in tyrosine and tryptophan residues, or that such residues participate little in its absorption spectra.

LMF is not generally detectable by immunological analyses against a wide variety of antisera, although the association of LMF with CRP reduces the overall negative charge of the CRP molecule and isolated CRP-LMF exhibits a different antigenic array than purified CRP as judged by Ouchterlony and Mancini analyses. On occasion, we observe separated LMF to react with anti-sera against native CRP; the meaning(s) of this remains unclear. Collectively, these data supported our contention that CRP could serve to down-modulate platelet reactivity during inflammation and tissue destructive conditions, but that this modulatory capacity requires CRP to be associated with LMF.

3. Definition of the platelet modulatory capacity of purified CRP when in complexed form.

CRP shares many functional similarities with immunoglobulin including the ability to initiate reactions of complement consumption and phagocytosis and the capacity to bind with mononuclear peripheral blood cells *in vitro*. These reactivities each require CRP to bind with one of its multivalent ligands or to be heat-modified or chemically aggregated (modified) (reviewed in Gewurz et al., 1982). We questioned whether CRP, once separated from LMF, could be re-complexed (or altered) by physical means or by association with ligand to bring about a biological effect upon the platelet. The functional similarities of CRP and IgG suggested that such might be the case, and further, that this association might stimulate the platelet unlike CRP (LMF). Thermally aggregated CRP (H-CRP), but not

unmodified CRP, induced reactions of aggregation and constituent release from isolated platelets; maximum responses occurred with 40-80 μg/ml H-CRP and were similar to responses mediated by thermally aggregated human IgG (AHGG) (Figs. 9, 10) (Fiedel, Simpson and Gewurz, 1982b). Platelet aggregation was monophasic in nature and activation was sensitive to the presence of EDTA and dibucaine, required metabolic energy and was inhibited by increased levels of cAMP.

H-CRP participated with other platelet activators to synergistically 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. 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 (Fig. 11).

Complexes formed between CRP and one major ligand class, the polycation (e.g. PLL and/or protamine), like H-CRP, also activated the platelet in an isolated buffer system (Fig. 12 and Table 2) (Fiedel, Simpson and Gewurz, 1982b, c). Since platelets are activated by various polycations (reviewed in Fiedel et al., 1976b), it is 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 complexes between CRP and another ligand, CPS, 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 on CPS. Alternatively, 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 which shows that CRP, like IgG, activates platelets when absorbed onto latex beads (see below), illustrates that CRP has an intrinsic ability to be cross-linked or altered in a manner suitable for platelet activation, and suggests that the polycation in CRP-PLL complexes may, in part, serve to appropriately 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 (or more?) allosteric forms of CRP, it is possible that the cross-linking of CRP molecules by heat, absorption onto latex beads or with an appropriate polycation (performed in the absence of calcium) might permit formation of a CRP aggregate which is in an allosterically favored 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 nor will it form aggregates with polycation to form a complex capable of activating platelets. We 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; they clearly can (Fig. 13) (Fiedel, Simpson and Gewurz, 1982c).

Immunoglobulin G, aggregated by absorption to latex or polystyrene particles, has been found capable of initiating reactions of aggregation and secretion from isolated human platelets. We therefore determined whether CRP absorbed to latex beads, like H-CRP and CRP:PLL (or protamine), would initiate platelet reactivities. Not unlike IgG, CRP-coated latex particles activated the platelet to

reactions of aggregation and the secretion of dense body constituents (Fig. 14). Dilution experiments revealed that a minimum of 20 μg/ml CRP (coated on latex) was required to activate the platelet with maximal aggregation occurring at 40 μg/ml latex-aggregated CRP. Unlike H-CRP and AHGG (in which H-CRP was a 10-20 fold more effective platelet activator on a weight basis than AHGG), latex aggregated CRP and IgG were of approximately equal ability in the induction of a platelet response.

Continued studies of the interaction between CRP and the platelet now reflect that platelet activation stimulated by an aggregated CRP is sensitive to nonsteroidal anti-inflammatory drugs as well as the ADP-removing enzyme system creatine phosphate/creatine phosphokinase. Thin-layer chromatographic analysis of prostanoate endproducts following platelet activation with H-CRP revealed the formation of thromboxane B_2, the hydrated endproduct of thromboxane A_2, an important endogenous platelet activator and contractor of vascular tissue; bioassay on rabbit aorta strips of supernatants obtained from platelets undergoing challenge with H-CRP supported the TLC analysis (Fig. 15) (Simpson et al., 1982).

If one defines the relative potency of platelet activators into a hierarchy (Holmsen, 1977) dependent on their ability to stimulate the platelet responses of shape change, aggregation, endoperoxide and thromboxane production, dense granule and alpha granule secretion, then H-CRP has been previously shown to stimulate four of five basic responses. We have now determined that H-CRP stimulates the release of alpha granule secretion (Fig. 16) (Simpson et al., 1982), which is consistent with a platelet stimulatory potency equivalent to thrombin and collagen and superior to that of ADP or epinephrine (Holmsen, 1977). These data imply an agonist role for this acute phase reactant in platelet physiology and suggest that the interaction of modified CRP with the platelet at sites of vascular damage could have pathological significance.

4. Elucidation of the binding reaction between CRP and the platelet.

As stated before, CRP and immunoglobulin share many functional similarities including reactions of agglutination, opsonization, activation of complement and complement-dependent hemolysis and opsonic mechanisms (reviewed in Gewurz et al., 1982). Further, we have demonstrated that CRP as well as IgG, in their heat-aggregated or latex absorbed forms, can activate human platelets. This litany of overlaps between the ligand-binding and effector properties of the respective molecules led us to consider whether aggregated CRP might in fact utilize the IgG Fc receptor to initiate platelet activation.

We first approached this question by evaluating whether the expression of the platelet receptor for H-CRP and AHGG could be functionally separated; and established the comparative enzymatic sensitivities of the respective platelet receptors (CHY = chymotrypsin; PLC = phospholipase C; PLS = plasmin). Both the H-CRP and AHGG platelet receptors exhibited increased expression following treatment of platelet with CHY, but not following similar treatment with PLS or PLC (Fig. 16) (Fiedel et al., 1982d). The expression of platelet receptors for collagen (ASC) exhibited a different sensitivity profile, as do the platelet receptors for thrombin (Tam et al., 1980). These data illustrated a categorical similarity between receptors for H-CRP and AHGG as compared to receptors for ASC or thrombin; moreover, as CHY treatment of platelets releases much of the major glycoproteins from the platelet surface (Pfueller et al., 1977), it seems possible

that these glycoproteins mask platelet reactivity to both H-CRP or AHGG. However, differences in the sensitivity of the platelet response to H-CRP or AHGG in the presence of column fractions containing various plasma components revealed a distinction between these receptors, as did evidence that monomeric IgG substantially interfered with the platelet response to AHGG but not to H-CRP. Our recent descriptions of an H-CRP receptor on the surface membrane of rabbit platelets whose expression is also CHY sensitive and that is presently characterized by its ability to initiate both platelet aggregation and the formation of metabolic products from platelet arachidonate in the absence of demonstrable IgG Fc receptor activity (Fiedel et al., 1982d), further indicates that the platelet receptors for aggregated CRP and IgG are distinct.

CONCLUDING REMARKS

Data spanning the last eight years concerning the effects of CRP upon platelet function have been presented herein. In our opinion, CRP is a multi-functional molecule with respect to its ability to alter platelet behavior, being an agonist (or synergist) as well as an antagonist dependent upon is physical state and/or association with secondary molecules. There are clearly areas in which intensive research must be directed in order to ascertain the *in vivo* physiological significance of the CRP-platelet interaction: we must better define LMF and its distribution in the various disease states and evaluate its associative reaction with CRP; we must search *in vivo* and find complexed or physically altered forms of CRP with biologic potential directed towards the platelet, and we must describe them biochemically; we must also describe the platelet-CRP interaction at the membrane, fully understand its stimulus-response coupling, and map the platelet surface for CRP receptors; and lastly, as there is little reason to suspect that other acute phase reactants do not inter-play with one another to produce biologic phenomena, we must better understand these complex interactive relationships in the context of not only platelet function but, more generally, in terms of multiple biologic response modifiers during inflammatory and tissue destructive states. The future remains challenging.

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Table 1. Effect of CRP on collagen-platelet adhesion[a]

Expt. No.	CRP	Marker	% Mean Adherence ± S.D.		p
			Buffer	CRP	
	μg/ml				
1	45	^{14}C-5HT	8.1 ± 3.4	8.1 ± 3.1	> 0.2
2	45	^{51}Cr	11.9 ± 4.2	10.3 ± 3.4	> 0.2
3	100	^{51}Cr	8.3 ± 1.4	8.3 ± 1.3	> 0.2

[a] Appropriately labeled platelets (10^9) were incubated for 15 min with varying concentrations of CRP in 1.0 ml and added to collagen-coated tubes; these were rotated 15 rpm for 15 to 20 min at room temperature, decanted, and washed four times with modified Tyrodes solution. The bound ^{51}Cr was counted directly in a well-type gamma counter, while aliquots of solubilized platelets were assayed for ^{14}C-5HT by liquid scintillation. The results represent the percentage of counts bound compared to the total radioactivity of the platelet suspensions and are presented as the percent mean adherence of eight replicate samples ± one standard deviation (S.D.); p values were determined by the paired t-statistic.

Table 2. Aggregation of platelets by CRP:PLL and H-CRP complexes or by PLL

Reactant	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 g/ml)	75	< 5	79	—

[a] 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 of 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.

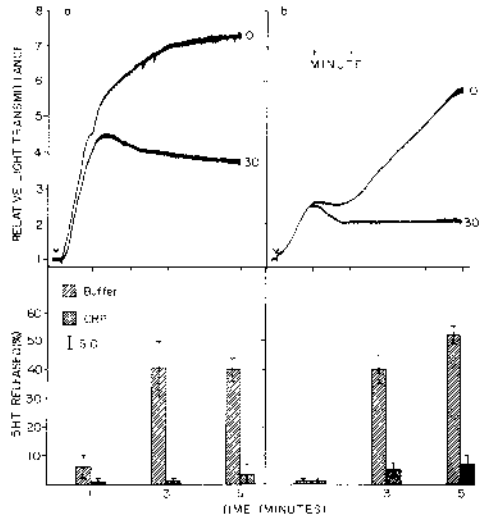

Fig. 1. Effects of certain CRP preparations (30 μg/ml) on the aggregation of and release of 5HT from platelets in PRP in response to: a) 10^{-5}M ADP and b) 10^{-5}M epinephrine.

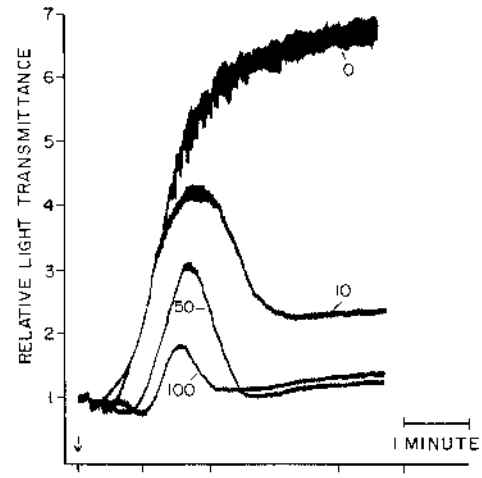

Fig. 2. Effects of certain CRP preparations on the aggregation of and release of 5HT from platelets in PRP in response to A23187: a) no CRP and b) 150 μg/ml CRP. The concentrations of A23187 are expressed as μM final concentration, and the percent release of 5HT is shown in parentheses.

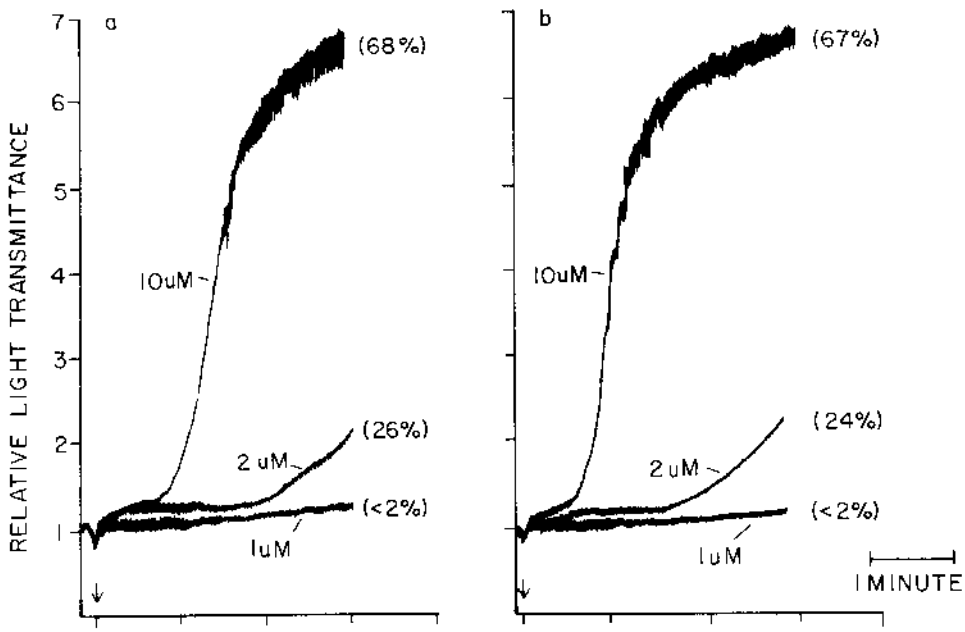

Fig. 3. Effect of certain CRP preparations on the aggregation of platelets in PRP in response to 0.5 mM arachidonic acid. The concentrations of CRP are expressed as μg/ml.

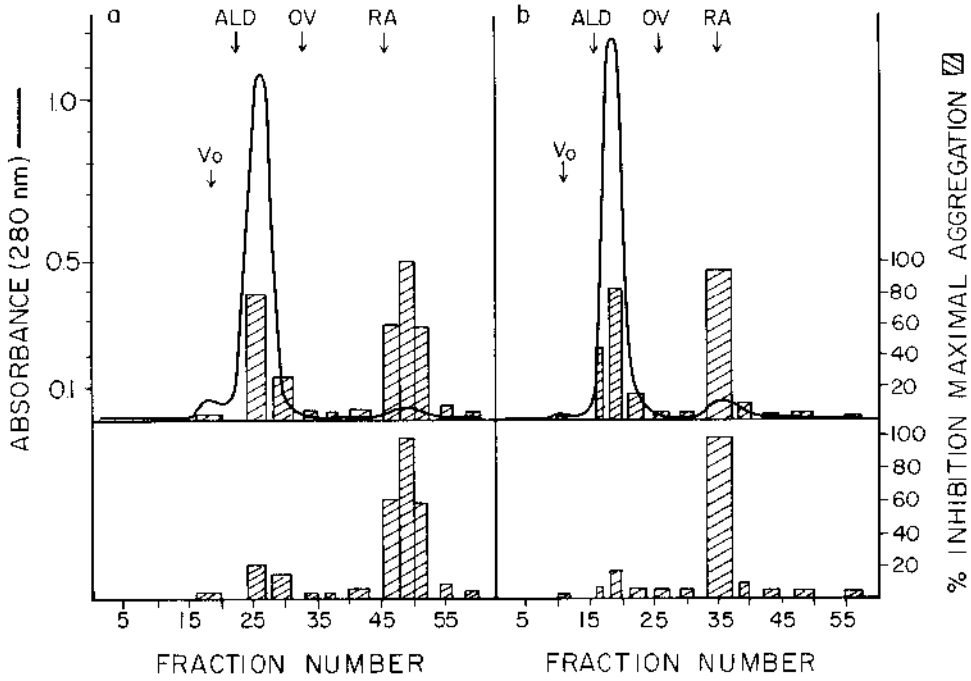

Fig. 4. Passage of CRP prepared by PC-affinity chromatography through Sephacryl S-200 and the inhibitory action of the various pooled fractions on platelet aggregation stimulated by ADP (a) or acid soluble collagen (b). Upper panels reflect the inhibition in the absence and lower panels in the presence of C-polysaccharide (CPS). 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 (▨).

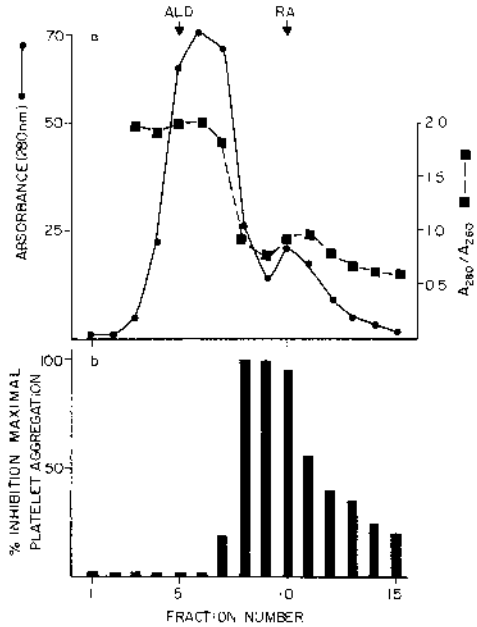

Fig. 5. Chromatographic separation of the PC-affinity eluate over Sephacryl S-200 resin (a) and inhibition of platelet aggregation by the various fractions (b). The A_{280}:A_{260} ratios are shown (■). ADP was used as the platelet stimulator at a concentration yielding 50% maximal irreversible aggregation; similar data was obtained with collagen as the platelet activator. The column was calibrated with ALD and RA. Absorbance: 280 nm (—).

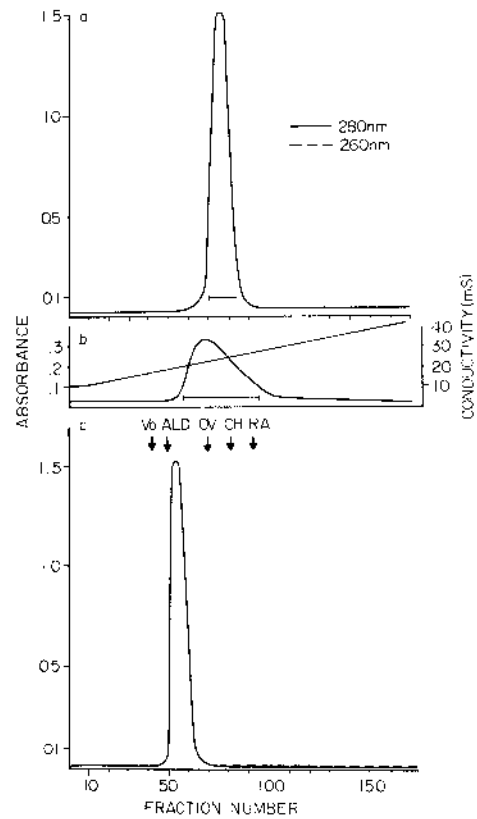

Fig. 6. Isolation of CRP by PC-affinity column chromatography (a), with further purification using DE-52 (b) and Sephacryl S-200 (c). Pooled fractions are designated (—) and DE-52 material was concentrated by Amicon ultrafiltration. The gel sieve column was calibrated with blue dextran 2000, ALD, OV, chymotrypsinogen (CH) and RA. Absorbance: (—) 280 nm; (---) 260 nm.

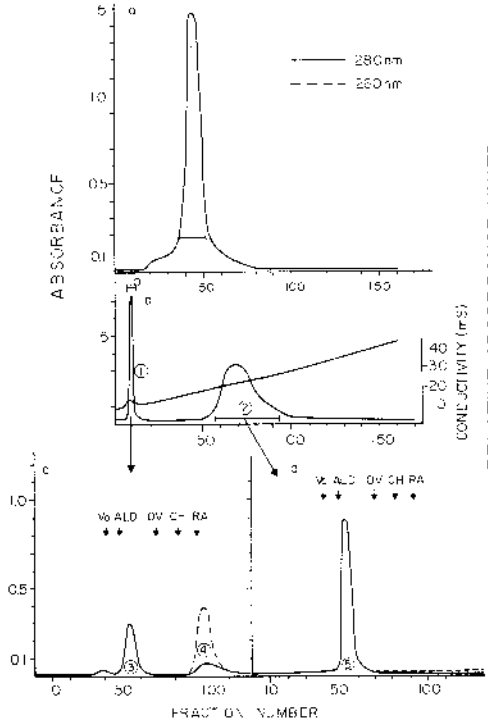

Fig. 7. Isolation of CRP by PC-affinity column chromatography (a), with further purifications using DE-52 (b) and Sephacryl S-200 (c,d). Pooled fractions are designated (—) and all DE-52 material was concentrated by Amicon ultrafiltration before gel sieve chromatography; the gel sieve column was calibrated as in Fig. 4. Absorption: 280 nm (—); 260 nm (---).

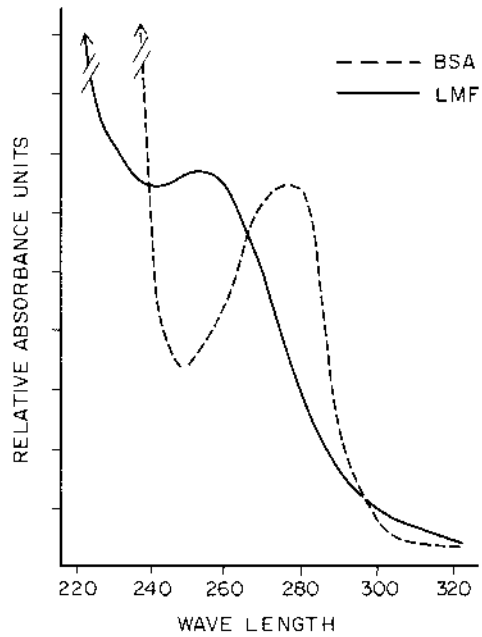

Fig. 8. Ultraviolet absorption spectra of the low molecular weight factor (···) and a reference protein, bovine serum albumin (---).

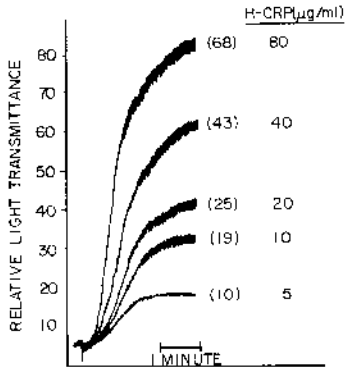

Fig. 9. Aggregation of human platelets following addition of various concentrations of heat-modified CRP (H-CRP) in buffered saline. The aggregation response was monophasic and similar to that observed with heat-modified human immunoglobulin G. The percent secretion of 5HT is shown in ( ).

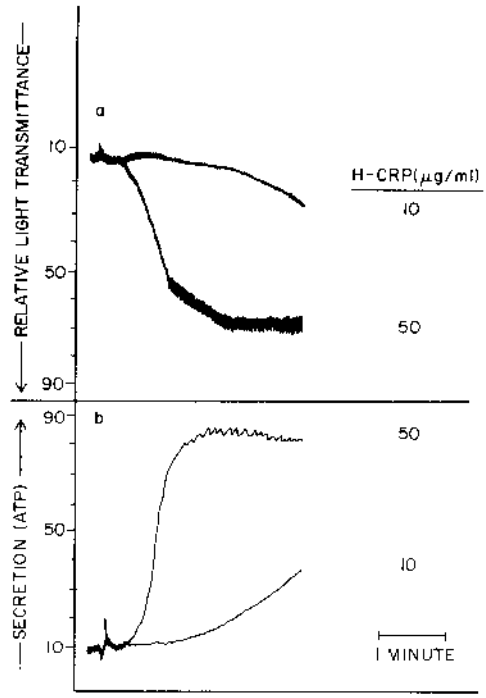

Fig. 10. Simultaneous comparison of platelet aggregation (a) and relative secretion of ATP (b) stimulated by heat-modified CRP (H-CRP) in buffered saline.

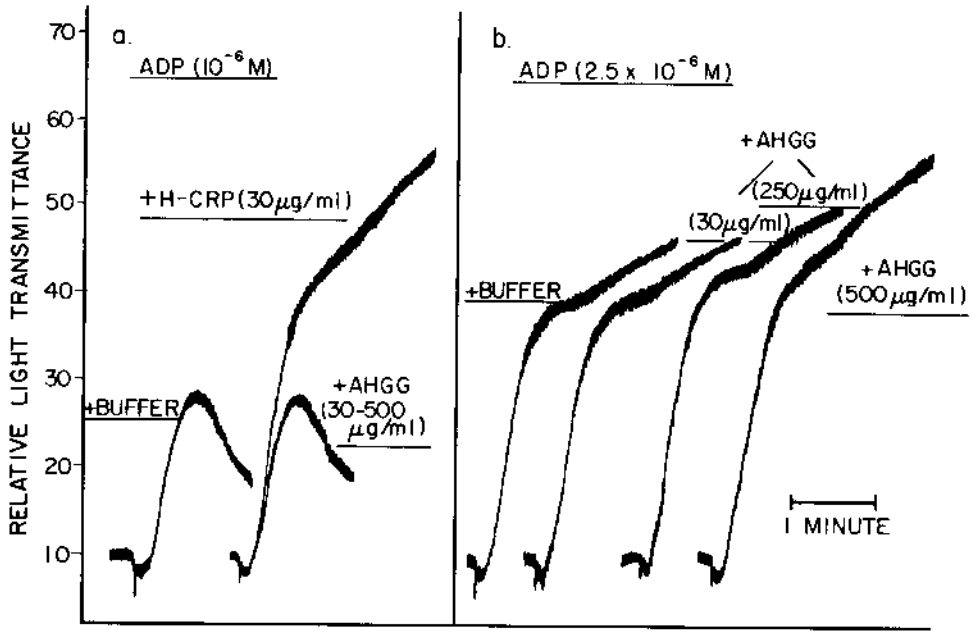

Fig. 11. Comparison of the ability of heat-modified CRP (H-CRP) or human immunoglobulin G (AHGG) to act synergistically with ADP in platelet-rich plasma. In (a) ADP was used at 10^{-6}M and gave a minimal level of activation while in (b), ADP was used at 2.5×10^{-6}M and yielded a more substantial level of platelet aggregation. H-CRP and AHGG were used at the indicated concentrations. H-CRP was, on a weight basis, at least tenfold more effective than was AHGG and required a lesser degree of basal platelet stimulation.

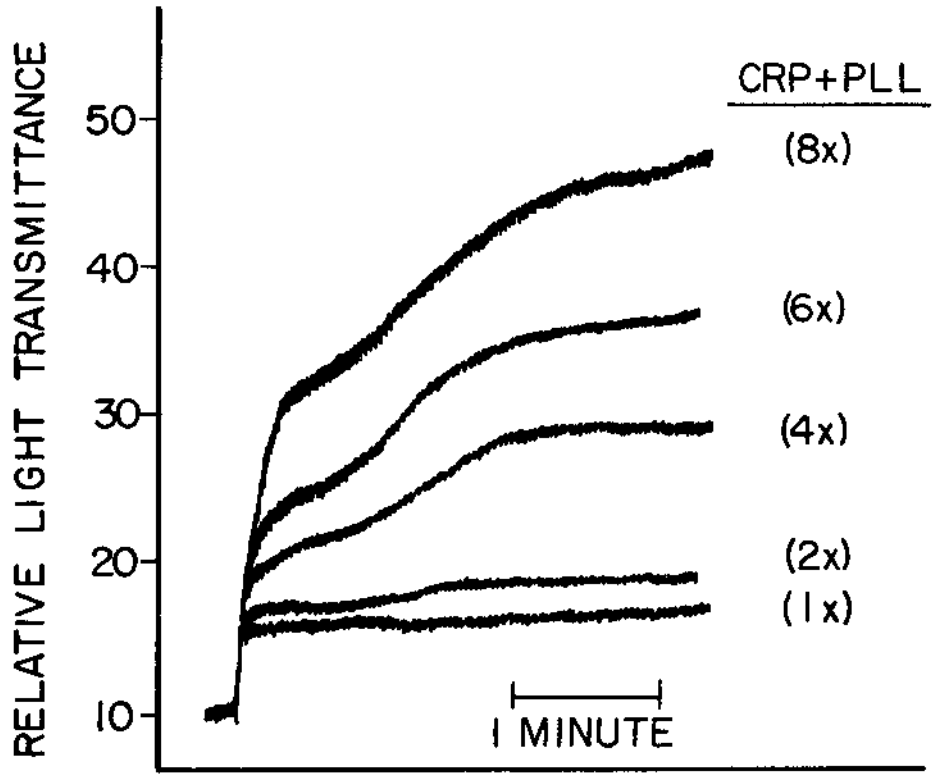

Fig. 12. Aggregation of isolated human platelets by complexes formed between CRP and poly-L-lysine (PLL; 15,000 daltons) at a 70:1 CRP:PLL (w/w) ratio. CRP and PLL were incubated at 37° for 15 min and aliquots of the washed precipitate were brought to appropriate concentrations (parenthesis) in TBS. PLL alone at the concentrations used in these experiments did not induce platelet aggregation.

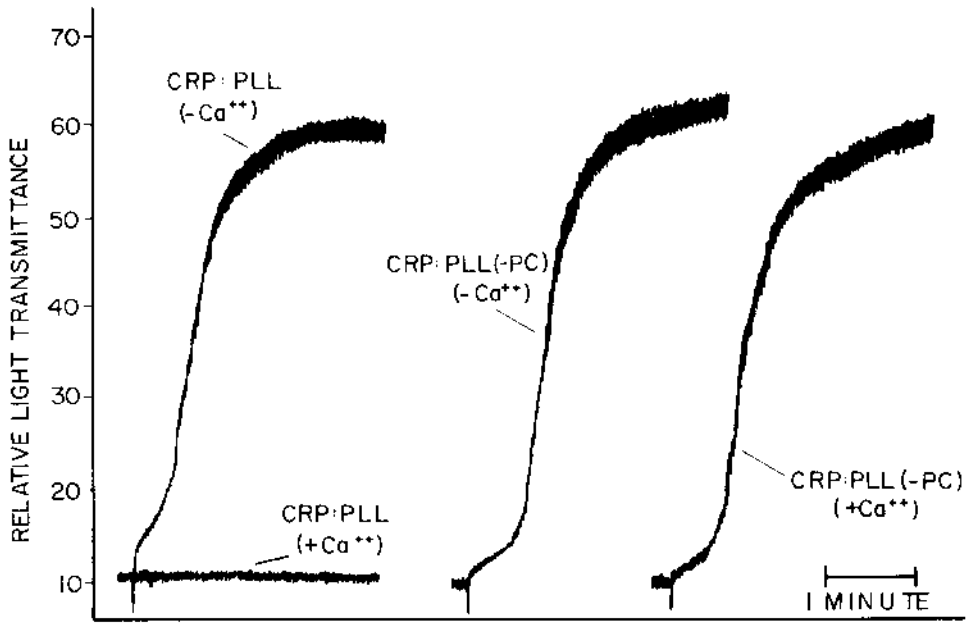

Fig. 13. Effect of the covalent attachment of PC to PLL on the inhibitory influences of Ca^{++} during CRP-poly-L-lysine complex stimulated platelet aggregation. Such attachment negated the depressive influence of calcium ions. (See also Fiedel et al., 1982c).

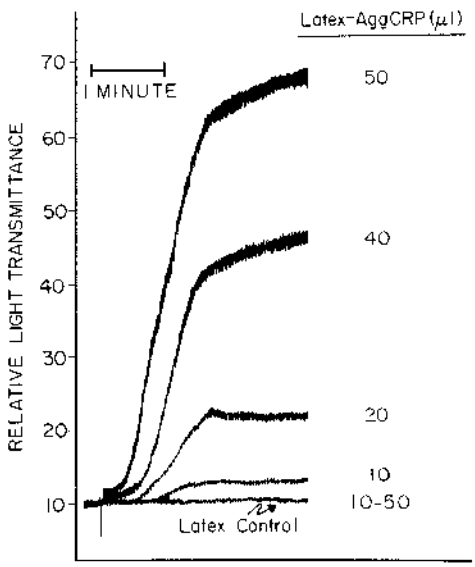

Fig. 14. Activation of isolated platelets by latex-aggregated CRP (Agg CRP). In this experiment each 10 μl addition of latex AggCRP represented approximately 9.6 μg/ml of CRP offered. These values were determined by antigenic analysis of the CRP solution before and following incubation with latex.

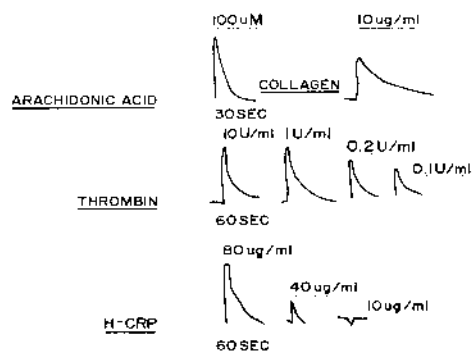

Fig. 15. A comparison of the ability of arachidonic acid, collagen, thrombin or H-CRP to generate TXA_2 activity from platelets. Platelets suspensions were challenged with the concentrations shown and the reaction mixtures assayed. Aortic strips were suspended from transducers at a constant tension of 2 gm/cm and calibrated using a polygraph with recorder.

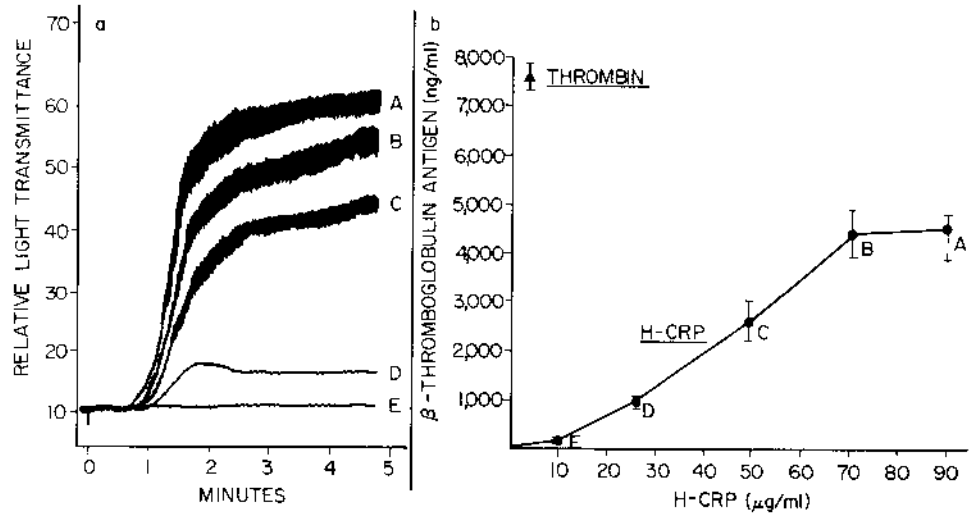

Fig. 16. In (a), the aggregation response of isolated platelets to increasing amounts of H-CRP. In (b), the release of β-thromboglobulin antigen from platelets challenged in (a) using five different H-CRP concentrations marked A-E and corresponding to the H-CRP concentrations used in (a). β-Thromboglobulin antigen was measured using a radioimmunoassay with platelet activating conditions as described in Materials and Methods. For comparison, the β-thromboglobulin antigen released following challenge of platelet suspensions with a concentration of thrombin (▲)yielding a maximal platelet aggregation event is depicted in (b). Data are given as the mean and range of three independent determinations for each H-CRP or thrombin concentration used.

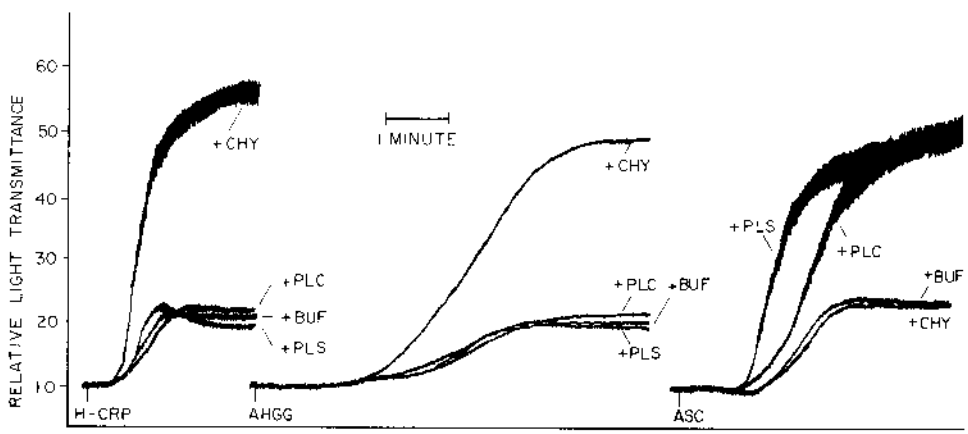

Fig. 17. Effect of enzyme treatment on the platelet response to H-CRP, AHGG and ASC. CHY = chymotrypsin; PLC = phospholipase C and PLS = plasmin.