Platelet Agonist Synergy by the Acute Phase Reactant
C-Reactive Protein

By Barry A. Fiedel

C-reactive protein is the prototypic acute phase reactant. A self-complexed form (H-CRP) can induce isolated platelets to undergo aggregation, secretion of dense and α-granule constituents, and generation of thromboxane A₂, but fails to function in platelet-rich plasma (PRP) as a direct agonist. In contrast, when PRP was activated with an amount of adenosine diphosphate (ADP) that produced only reversible platelet aggregation, the presence of H-CRP resulted in irreversible aggregation and the secretion of adenosine triphosphate (ATP). Following a maximum stimulus with ADP alone, where platelet secretion occurred late during the aggregation response, the presence of H-CRP shifted and increased the secretory burst to a time simultaneous with the onset of aggregation. This hyperssecretion required H-CRP to be present prior to platelet stimulation or to be added within 15 to 30 seconds following the addition of ADP. H-CRP also potentiated platelet activation stimulated with epinephrine, thrombin, and collagen. When the synergism generated in PRP by H-CRP in the presence of ADP or epinephrine was compared to the synergism similarly produced by aggregated human IgG, collagen, or thrombin, it more closely resembled that of collagen, as reflected by the kinetics and characteristics of synergism and sensitivity to creatine phosphate/creatine phosphokinase or 5,8,11,14-eicosatetraynoic acid. These data provide a philosophically ideal niche for the acute phase (and C-reactive protein) in that a platelet-directed activity associated with this acute phase reactant is not utilized unless platelets are otherwise challenged.

Thus, the functional similarities between CRP and immunoglobulin led to the investigation of the effects of CRP upon the platelet.

In earlier studies, this laboratory demonstrated that forms of aggregated CRP (H-CRP) stimulated a response in isolated platelet systems that was characterized by an initial lag period, followed by rapid monophasic aggregation, and was similar to that observed with heat-aggregated human IgG. The platelet aggregation stimulated by H-CRP was sensitive to the presence of a dibutyryl analogue of cyclic adenosine monophosphate (cAMP), EDTA or dibucaine, the combination of 2-deoxy-D-glucose and antimycin A, and aspirin or indomethacin, indicating that this activation event was under metabolic control and was not simple agglutination. H-CRP also stimulated washed platelets to undergo a typical secretion response, as measured by released adenosine triphosphate (ATP) and β-thromboglobulin antigen. The purpose of the present study was to evaluate the effects of aggregated CRP upon platelet responses in the more natural plasma environment, the results of which uncovered a previously undescribed platelet-directed activity associated with this acute phase reactant.

MATERIALS AND METHODS

Isolation of CRP

CRP was isolated from pleural or ascitic fluids obtained from patients undergoing diagnostic/therapeutic procedures. The fluid were centrifuged at 5,000 g (15 minutes; 4 °C), further clarified by filtration through gauze, and applied by gravity drip to a phosphocholine (PC)-Sepharose 4B affinity column (2.5 x 22 cm; Pharmacia Fine Chemicals, Piscataway, NJ). The column was washed with 20 mmol/L Tris-buffered saline containing 10 mmol/L CaCl₂ (TBS; pH 7.3) at 20 mL/h for 36 to 48 hours after the A₂₈₀ reached <0.05; elution of CRP was achieved using isotonic TBS-citrate

From the Department of Immunology/Microbiology, Rush Medical Center, Chicago.

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Address reprint requests to Dr B.A. Fiedel, Department of Immunology, Rush Medical Center, 1753 W Congress Pkwy, Chicago, IL 60612.

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buffer, pH 7.2, at a flow of 20 mL/h. Fractions containing CRP, as judged by immunoassay, were pooled and were applied to a DE-52 (Whatman Ltd, Kent, England) column (1.5 × 20 cm), with elution achieved using a 0.1 to 0.4 M linear salt gradient (20 mL/h). 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) in TBS in the absence of CaCl₂ (TBS; pH 7.2). All final CRP preparations were dialyzed one to three days against 1,000 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 the presence or absence of sodium dodecyl sulfate (SDS) and urea and by an RID and double diffusion screen against a battery of nonspecific antisera selected to detect components reported to variously copurify with CRP. At a minimum, this involved testing with antiserum to IgG, IgM, and IgA, serum amyloid P component, C3, and human serum; many preparations also were tested using antiserum directed against albumin, ceruloplasmin, CIq, C1r, and C1s, fibrinogen, fibrinopeptides D and E, α- and β-lipoproteins, and orosomucoid. Reactions in RID were considered negative only after eightfold application of highly concentrated CRP material, such that, in most instances, 1 to 3 μg/mL non-CRP antigen could be detected in preparations containing 1.5 to 2.0 mg/mL CRP. With the exception of antiserum to serum amyloid P component (Atlantic Antibodies, Scarborough, Me), all antibody reagents were purchased from Cal-Biochem Behring (La Jolla, Calif). Final preparations were filter sterilized and stored at 4 °C.

Isolation of Platelets and Platelet Activation6 7

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 to 5 × 10⁹/mL in a diluent consisting of three parts 0.5% glucose in normal saline and five parts 0.09 mol/L Tris, 30 mmol/L KCl, 30 mmol/L NaCl, and 0.8 mmol/L CaCl₂, adjusted to pH 7.5. Platelet aggregation was monitored in a model 300BD aggregometer (Payton Associates, Buffalo) by equilibrating TBS (100 μL) with 450 μL of PRP for one minute at 37°C (1,000 rpm), adding the test agent, and monitoring aggregation responses for a minimum of four minutes. 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 the standard turbidometric technique. Reactions were performed at 37°C in siliconized aggregation cuvettes (Chrono-Log Corp) with a stirring speed of 1,200 rpm. The mixture consisted of 450 μL PRP, 50 μL Chronolume reagent (luminescence assay mixture; Chrono-Log Corp), and 100 μL buffer, CRP, or platelet synergists; the platelet agonist (in TBS) was added and aggregation/secretion monitored on a dual-channel recorder. Platelet activators used in these studies included ADP, epinephrine (Epi), acid-soluble collagen, aggregated human γ-globulin (AHGG), bovine thrombin, and complexed CRP (H-CRP). ADP, epinephrine, creatine phosphate/creatinine phosphokinase (CP/CPK), and collagen were purchased from Sigma Chemical (St Louis). Thrombin was obtained from Parke-Davis Co (Morris Plains, NJ) and 5,8,11,14-eicosatetraynoic acid (TYA) was a gift from Dr W.E. Scott (Hoffman LaRoche, Inc, Nutley, NJ).

Heat Modification of CRP6-9

CRP (500 μg/mL in TBS) was incubated at 63°C for 2.5 to 3.0 minutes, the aggregates collected by centrifugation, and quantitated by Lowry and Bio-Rad protein assays. The insoluble aggregates formed slowly, and under the conditions used 47% to 53% of the available CRP protein precipitated (>100 assays). That this did not represent two distinct forms of CRP was shown by concentrating the resultant CRP supernatants (following removal of the insolubilized aggregate) and performing a second heat modification; once again, only ~50% of the available protein precipitated, and this material activated platelets in an identical manner to that of the H-CRP originally prepared. The nonprecipitating protein in the CRP supernatants following heat modification had a molecular weight equivalent to native CRP (~115,000) by both gel size chromatography and sucrose density gradient centrifugation; it reacted with pneumococcal C-poly saccharide or polycationic ligands (protamine, poly-L-lysine), as reported for CRP, and was indistinguishable from native human CRP. The heat-modified form of CRP gave a single line in SDS-PAGE, with an Rf value equivalent to its native subunit counterpart, although aggregated CRP was clearly separable from native CRP by centrifugation techniques. Human IgG was heat aggregated as previously described.24

RESULTS

Synergism Between ADP and H-CRP

Aggregated human CRP (H-CRP) is a potent activator of isolated platelet suspensions, but fails in concentrations to 500 μg/mL to act in PRP as a direct platelet agonist. By contrast, H-CRP is a strong synergist in plasma; data for the platelet response to the combined presence of H-CRP and ADP is shown in Figs 1 and 2. When PRP was activated with an ADP stimulus sufficient to induce primary reversible platelet aggregation, the addition of H-CRP resulted in irreversible platelet aggregation accompanied by secretion (measured as ATP; Fig 1A). Synergism was also evident following a maximal ADP stimulus and was marked by a substantial alteration in the kinetics

Fig 1. Synergistic effect of ADP and H-CRP on platelet aggregation and secretion of ATP. (A) ADP was at 10⁻⁴ mol/L and (B) at 5 × 10⁻⁴ mol/L (a, b, and c curves). H-CRP was at 50 μg/mL (b curves) or 75 μg/mL H-CRP (c curves). In this experiment, and those following, the synergist was without effect when tested in the absence of the agonist.
and extent of platelet secretion (Fig 1B). As depicted in Fig 2, this synergism required H-CRP to be added before or within 15 seconds after platelet challenge with ADP, and though still detectable, was nearly lost 30 seconds post-ADP challenge. H-CRP did not potentiate platelet activation induced by subthreshold amounts of ADP.

**Synergism Between Epinephrine and H-CRP**

As shown in Fig 3A, H-CRP synergized with Epi, converting primary (but not subthreshold) platelet aggregation to a second phase of aggregation accompanied by secretion of ATP. H-CRP also synergized with an amount of Epi that produced both platelet aggregation and secretion of ATP (Fig 3B); however, in this instance, maximum synergism between H-CRP and Epi occurred without inducing the simultaneous onset of platelet aggregation and secretion, as was observed when using ADP (Fig 1B). The synergism of H-CRP with Epi, unlike that with ADP, was not significantly dependent on time. Under activating conditions in which the platelet response to Epi constituted primary wave aggregation, the addition of H-CRP as much as 2½ minutes post-Epi still produced substantial platelet aggregation and ATP secretion.

**Synergism Between H-CRP and Collagen, Thrombin, or AHGG**

H-CRP at concentrations to 200 µg/mL did not synergize in PRP with subthreshold amounts of collagen. When the collagen concentration was increased so that platelet aggregation was ~20% of maximum, amounts of H-CRP between 30 and 75 µg/mL increased platelet aggregation to ~35% to 55% of maximum, with a concomitant increase in ATP secretion. However, larger amounts of H-CRP (80 to 200 µg/mL) decreased rather than increased the response. The response to subthreshold amounts of thrombin was not potentiated by H-CRP, but thrombin concentrations that alone normally stimulate reversible platelet aggregation (with no ATP secretion), in the presence of H-CRP, produced irreversible (biphasic) aggregation accompanied by the formation of a plasma clot (Fig 4). This latter result was not readily observed in platelet-poor plasma (PPP). In addition, ATP secretion was marked in the common presence of thrombin and H-CRP. The ability of H-CRP to synergize with thrombin, like that with ADP, was rapidly lost if aggregated CRP was added after the thrombin. When the activating dose of thrombin was increased to generate biphasic platelet aggregation, with ATP secretion occurring during the second phase, the presence of H-CRP converted this response to one that was monophasic in nature. At thrombin concentrations that alone yielded monophasic aggregation and secretion, synergy with H-
CRP was noticeable (if at all) as a shortening of the lag time. Of the responses tested, platelet synergism between H-CRP and thrombin was the most variable and difficult to titrate. H-CRP and AHGG did not synergize in PRP at any concentration tested, which is not unexpected, as neither is a direct agonist in PRP.

**Sensitivity of Synergism to an ADP-Removing System or TYA**

The two well-defined activating pathways in the platelet are the ADP and cyclooxygenase-dependent arachidonate pathways. In the next series of experiments, agonist synergy with H-CRP was assessed in the presence of an ADP-removing system or a cyclooxygenase/lipoxygenase inhibitor (TYA). The prior addition of 4 mmol/L CP and 6 U/mL CPK (Epi) or of 0.36 mmol/L TYA (ADP, Epi) to H-CRP-treated platelets completely inhibited all synergism. This is demonstrated for ADP and TYA in Fig 5.

CP/CPK or TYA inhibited by ~50% the synergism between H-CRP and low concentrations of thrombin (those that alone yielded only reversible platelet aggregation, as in Fig 4). However, this inhibition was much reduced when the basal amount of thrombin offered was intermediate (biphasic platelet aggregation with second phase ATP secretion) or high (those giving monophasic aggregation and secretion) in strength. Thus, the sensitivity to CP/CPK or TYA followed the described sensitivities of thrombin alone to these agents, at respective levels of agonist stimulation. The limited synergism observed between H-CRP and low to intermediate platelet-activating concentrations of collagen was also abolished by CP/CPK or TYA. Like thrombin, this reflected the sensitivity of collagen alone when given in a concentration sufficient to generate an amount of platelet activation equivalent to that observed when lesser concentrations of collagen were synergized with H-CRP.

**Comparison of the Abilities of H-CRP, AHGG, Collagen, or Thrombin to Synergize With ADP or Epi**

Apparent from the above studies was that synergism between H-CRP and the weaker platelet agonists, ADP or Epi, was most substantial, perhaps even more so than what was observed with H-CRP and thrombin. As most (all?) platelet agonists synergize with ADP or Epi, it becomes possible to compare the relative efficacies of a number of these agonists/synergists to that of H-CRP. Thus, studies analogous to those undertaken using H-CRP were performed, and data presenting the summation of these experiments is offered in Table 1. AHGG, collagen, and thrombin each synergized with ADP or Epi in ways similar to H-CRP; however, only collagen matched H-CRP in all categories.

**DISCUSSION**

Associated with the acute inflammatory response is an elevation in the circulating level of CRP, the prototypic acute phase reactant that is one member of a recently described superfamily of proteins termed pentraxins. This laboratory has reported on the ability of aggregated CRP (H-CRP), but not unmodified CRP, to induce reactions of aggregation, the liberation and metabolism of arachidonate, and the secretion of dense and α-granule constituents from isolated human platelets. The present report describes the synergistic capabilities of H-CRP in PRP, where it was previously found to be an ineffective direct agonist.

H-CRP added to PRP in combination with suboptimal concentrations of ADP greatly enhanced platelet reactivity, stimulating the platelet to irreversible aggregation and a simultaneous secretory event. The effect of H-CRP on the kinetics of ADP-stimulated secretion was substantial. Even under a maximum aggregating stimulus with ADP, platelet secretion of ATP normally occurs very late in the platelet aggregation response; this characteristic was earlier described by Charo et al. However, in the presence of H-CRP, the platelet secretory burst occurred simultaneously with the onset of platelet aggregation. The ability of aggregated CRP to synergize with ADP required its presence prior to or within 15 to 30 seconds following platelet challenge with ADP.

Aggregated CRP also synergized in plasma with Epi by potentiating the onset of both platelet aggregation and secretion, although not to the extent observed during synergy with ADP. In addition, the ability of
Table 1. Comparative Platelet Synergistic Abilities of H-CRP, AHGG, Collagen and Thrombin in the Presence of ADP or Epinephrine

<table>
<thead>
<tr>
<th>Synergist</th>
<th>H-CRP</th>
<th>AHGG</th>
<th>Collagen</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
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<tr>
<td>Converts primary to secondary response</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Potentiates secondary response</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Generates coincident aggregation and ATP secretion</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Synergy when added 15 s after ADP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Epi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Converts primary to secondary response</td>
<td>+</td>
<td>-</td>
<td>*</td>
<td>+</td>
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<tr>
<td>Potentiates secondary response</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Generates coincident aggregation and ATP secretion</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Synergy when added 2.5 min after Epi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>ADP or Epi</td>
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<tr>
<td>Synergy present with:</td>
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<tr>
<td>4 mmol/L CP and 6 U/mL CPK</td>
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<td>-</td>
<td>Reduced</td>
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<tr>
<td>0.36 mmol/L TYA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduced</td>
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</table>

These experiments were performed as described in Materials and Methods and Figs 1 through 3.

ND, not determined.

*At concentrations to 600 μg/mL.

H-CRP to synergize with suboptimal concentrations of Epi did not rapidly deteriorate when added subsequent to Epi.

The ability of H-CRP to synergize in PRP with ADP or Epi was abolished by the presence of CP/CPK or TYA. This suggests that H-CRP does not produce synergism through pathway(s) independent of ADP or arachidonate metabolites. When the synergism produced in PRP by H-CRP in the presence of ADP or Epi was compared to the synergism similarly produced by AHGG, collagen, or thrombin (Table 1), it more closely resembled that of collagen, as reflected by (1) kinetics and characteristics of synergism and (2) sensitivity to CP/CPK or TYA.

Aggregated CRP also synergized with thrombin and collagen in PRP. H-CRP potentiated a reversible, nonsecreting, thrombin-induced platelet response to one of biphasic aggregation, with ATP secretion occurring during the second phase of aggregation. Often, this was accompanied by the formation of a plasma clot. Intermediate and high levels of platelet stimulation with thrombin were less well potentiated by H-CRP, and as observed in platelet systems containing ADP or Epi and H-CRP, the sensitivity of these platelet responses to the presence of CP/CPK or TYA reflected those of the platelet agonist used. Platelet synergism between H-CRP and collagen was curious in that it occurred only at low to intermediate levels of basal platelet stimulation by collagen and decreased as the H-CRP concentration was increased. Of interest is the 1978 report by Bensusan et al., in which they report evidence supporting fibronectin as the platelet receptor for collagen. Salonen et al. have recently reported CRP (in a solid phase system) to bind with fibronectin. It is thus conceivable that a competitive association between collagen and aggregated CRP exists for a platelet receptor (fibronectin?) and serves as a basis for the ability of H-CRP to inhibit (at higher concentrations) platelet activation by collagen. Further evidences supporting a shared (or closely associated) platelet receptor for collagen and aggregated CRP are the preliminary findings showing complement component Clq to inhibit each collagen- and H-CRP-induced platelet activation in an isolated buffer system and the conclusion herein that H-CRP and collagen have a similar synergism profile.

As discussed by Huang and Detwiler and Holmsen, most platelet activators can be considered as weak or strong, and most (all?) platelet agonists act in synergism. In contrast to isolated buffer systems containing platelets, where H-CRP is both a strong agonist and synergist, aggregated CRP appears not to be an agonist in PRP but only a strong synergist. It is my belief that the ability of aggregated CRP to synergize with known platelet agonists in plasma, while not acting as a direct platelet activator itself, provides a philosophically ideal niche for the prototype reactant in the acute phase. Clearly, the presence of CRP in the circulation, the wide distribution in the body of aggregating ligands for CRP, and its deposition at tissue sites during host injury, along with the known role of the platelet in inflammation and hemostasis, speak to the potential importance of such an effect.
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BA Fiedel