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Inhibition of Rabbit Platelet Aggregation and Clot Retraction by Rabbit and Human C-Reactive Proteins[1] (39741)

ROBERT J. MARDER,[2] BARRY A. FIEDEL, ALEXANDER P. OSMAND, AND HENRY GEWURZ[3, 4]

Department of Immunology, Rush Medical College, 1725 West Harrison, Chicago, Illinois 60612

C-reactive protein (CRP) is a trace serum constituent which has been shown to increase in concentration as much as 1000-fold during reactions of inflammation and tissue destruction (1–3). CRP has been reported to initiate reactions of agglutination (4, 5), precipitation (1, 6), and capsular swelling (7), to enhance phagocytosis (8, 9), and to activate the complement system and promote complement-dependent reactions of adherence and phagocytosis involving B lymphocytes and monocytes (5, 10–13); it binds selectively to T lymphocytes and inhibits certain of their functions (14–16). Human CRP recently was shown to inhibit aggregation, activation of platelet factor 3, and release of both serotonin and β-glucuronidase in reactions involving human platelets, and these effects were shown to be independent of cytotoxicity and the chelation of calcium (17, 18).

CRP has been demonstrated in several species in addition to man, including the monkey (19), rabbit (20), and dog (21). Rabbit CRP precipitates only with a less degraded form of the C polysaccharide than does human CRP (20), and has a molecular weight of 115,000–120,000 daltons as compared with a molecular weight of 120,000–140,000 daltons for human CRP (22, 23). However, rabbit CRP resembles that of man in amino acid composition and sequence, binding affinity for phosphocholine, and ultrastructural appearance by negative-stain electron microscopy (24, 25). The present studies were directed to test whether the rabbit could serve as a suitable experimental model for further studies of the platelet–CRP interaction, and to test the effects of CRP upon an additional platelet function, that of clot retraction.

Materials and methods. C-Reactive protein (CRP). Human CRP was isolated from ascites and pleural fluid pools by affinity chromatography utilizing pneumococcal C polysaccharide (CPS) linked to Bio-Gel A-50m agarose beads (Bio-Rad Laboratories, Richmond, Calif.), followed by passage through Bio-Gel A-0.5m as previously described (5). Rabbit CRP was isolated from pooled sera of New Zealand White rabbits in which an acute inflammatory reaction had been induced 36–48 hr earlier by five separate subcutaneous injections of 1% croton oil; the exact procedure used for isolation of human CRP was followed for isolation of rabbit CRP as previously described (24). CRP at the concentrations used in this study did not affect platelet viability as assessed by functional assays described elsewhere (17).

Diluents. Platelet aggregation experiments were performed using the diluents described by Pfueller and Lüscher (26). Diluent A consisted of 0.15 *M* NaCl (8 vol), 3.8% trisodium citrate (1 vol) and 5% glucose (1 vol). Diluent B contained 0.5% glucose in 0.15 *M* NaCl. Diluent C consisted of diluent B (3 vol) and a solution of 0.09 *M* Tris, 30 m*M* KCl, 2 m*M* $CaCl_2$, and 30 m*M* NaCl (5 vol). In clot retraction experiments, the buffer of Widmer and Moake (27), which was modified to contain 0.14 *M* NaCl, 3 m*M* KCl, and 10 m*M* Tris, was used. Each solution was adjusted to pH 7.5.

Isolation of rabbit platelets. Platelet-rich plasma (PRP) was prepared from rabbit arterial blood (9 parts) drawn into 50-ml plastic tubes containing 0.1 *M* citrate buffer (1 part), pH 5.2, which were centrifuged at

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[2] Holder of a Boothroyd Foundation Medical Student Fellowship.

[3] Holder of the Thomas J. Coogan, Sr. Chair of Immunology established by Marjorie Lindheimer Everett.

[4] To whom reprint requests should be sent.

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180*g* (20 min, 22°); the PRP was removed with a siliconized Pasteur pipet. Washed platelets for aggregation procedures were prepared by the method of Walsh (28). PRP (9.0 ml) was layered onto 1 ml of 40% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 2500*g* (15 min, 22°). The platelets were removed, washed in diluents A and B, respectively, reisolated by BSA gradient centrifugation, and resuspended (3×10^9 platelets/ml) in diluent C. Platelet-poor plasma (PPP) was prepared by centrifugation of fresh rabbit blood at 2500*g* (30 min, 22°).

Platelet aggregation. Washed suspended platelets (0.3 ml) were placed in siliconized glass cuvettes, and buffer or CRP (0.1 ml) was added. The mixtures were equilibrated (37°, 1 min), and 20 μl of bovine topical thrombin (Parke–Davis Co., Detroit, Mich.) at a concentration selected to induce a 50% maximum aggregation rate was added. Aggregation was recorded in a platelet aggregometer (Chrono-Log Corp., Havertown, Pa.) as increase in light transmittance with time for 4 min or until maximal aggregation was achieved. CRP was tested as an aggregating stimulus by immediate recording upon its addition to the platelet suspensions at 37°.

Dilute clot retraction (DCR). The DCR assay described by Widmer and Moake (27), presented as a method to quantitate the retraction of dilute human clots, was modified to allow assay of the effects of CRP upon the retraction of dilute rabbit clots. The platelet concentration was adjusted by diluting rabbit PRP with PPP to maintain a constant fibrinogen concentration; 0.25 ml of the adjusted PRP was added to 1.65 ml of DCR buffer containing various concentrations of CRP. The suspensions were incubated (37°, 5 min) with continuous mixing, a wooden applicator stick was introduced, 0.1 ml (5 units) of bovine topical thrombin was added, and clotting and clot retraction were allowed to proceed at 37°. Clot retraction was quantified by careful removal of the clot via the applicator stick and measurement of the residual fluid expressed. The appropriate platelet concentration, determined prior to each experiment, was that which produced a clot expressing 70% of the original volume at 30 min and all of the original volume at 1 hr. This platelet concentration allowed detection of inhibition of DCR at 30 min, and the kinetics of clot retraction correlated well with those obtained when 2×10^4 human platelets/ml were used (27). Inhibition of DCR at 30 min was calculated by the following formula:

$$\% \text{ inhibition of DCR} = \left(1 - \frac{\text{residual ml test}}{\text{residual ml control}}\right) \times 100$$

Results. Inhibition of rabbit platelet aggregation. The kymogram tracing (Fig. 1) demonstrates the ability of rabbit CRP to inhibit thrombin-induced aggregation of washed rabbit platelets. CRP concentrations of 25, 50, 100, and 200 μg/ml inhibited the rate of aggregation by 33, 57, 78, and 85%, and the extent of aggregation by 45, 62, 73, and 83%, respectively. Rabbit CRP alone at concentrations as high as 200 μg/ml did not induce aggregation. Human CRP inhibited both the rate and extent of rabbit platelet aggregation with approximately the same effectiveness on a weight basis as did rabbit CRP, e.g., 100 μg/ml of human CRP inhibited the rate of rabbit platelet aggregation by 82% and its extent by 79%.

Inhibition of clot retraction. The assay of

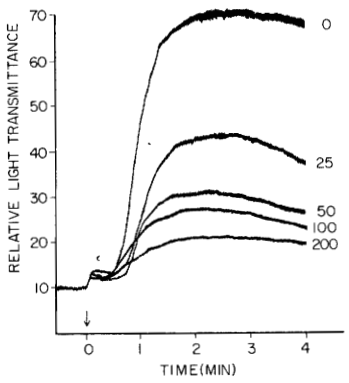

FIG. 1. Inhibition of thrombin-induced aggregation of rabbit platelets by rabbit CRP. The final CRP concentrations (0–200) are expressed as μg/ml. Both the rate and maximal extent of aggregation were significantly inhibited at each CRP concentration (25, 50, 100, and 200 μg/ml) tested.

Widmer and Moake (27) was adapted for study of the effects of CRP upon the retraction of rabbit clots. For these experiments, Tris replaced phosphate as the buffer, because the latter was found to block or diminish the ability of CRP to inhibit both platelet aggregation and clot retraction. This was presumably due either to inhibition of CRP binding or calcium sequestration by phosphate; the buffer used contained no added calcium or magnesium, so that the concentration of these metals available reflected the amounts present in citrated PRP during activation with thrombin. The kinetics of clot retraction were identical to those previously observed using human platelets (27).

CRP was found to inhibit the clot retracting ability of rabbit platelets (Table I). Both human and rabbit CRP at concentrations of 50 and 100 μg/ml, respectively, induced marked inhibition of dilute clot retraction, and CRP of both species was significantly inhibitory even at 25 μg/ml.

Discussion. The results of these studies indicate that physiologic concentrations (25–200 μg/ml) of both rabbit and human CRP are capable of inhibiting the aggregation of rabbit platelets and the retraction of rabbit clots. Such cross-species inhibition of aggregation and the inhibition of clot retraction are previously undescribed properties of CRP, and emphasize both the suitability of the rabbit as an experimental model for further studies of the platelet–CRP interaction and the potential role of CRP as a significant regulator of platelet function.

TABLE I. INHIBITION OF DILUTE CLOT RETRACTION BY CRP.[a]

CRP (μg/ml)	Percentage of inhibition				
	10	25	30	50	100
Rabbit	12%	14%	20%	34%	47%
	8%	7%	20%	20%	47%
Human	N.D.[b]	21%	ND	30%	37%
		17%		21%	30%

[a] Results of duplicate points in a representative assay of the effects of human and rabbit CRP upon the retraction of dilute clots. The assay was performed at 37° by adding 0.25 ml of rabbit platelet-rich plasma to mixtures containing various concentrations of CRP and 5 units of thrombin in 1.75 ml of Tris buffer. Clot retraction was quantified at 30 min by measurement of the residual volume after removal of the clot, and is expressed as percentage of inhibition of clot retraction. The platelet concentration was adjusted such that 70% of the residual volume was released in control suspensions in the absence of CRP.

[b] N.D., not determined.

Numerous other agents have been shown to affect platelet function, including adenosine, cyclic AMP, the methylxanthines, salicylates, sulfhydryl inhibitors, cytochalasin B, and the prostaglandins (29, 30). Recently, Widmer and Moake (27) demonstrated that prostaglandin E_1(PGE_1), which is known to inhibit platelet aggregation and release reactions and to influence the contraction of smooth muscle (30), can inhibit thrombin-induced clot retraction of PRP in the absence of observable effects upon platelet aggregation (27). A similar experience was observed with CRP: a thrombin concentration of 2.5 Um/ml overcame CRP (100 μg/ml)-mediated inhibition of platelet aggregation but could not overcome the inhibition of clot retraction mediated by a similar amount of CRP. Perhaps this difference between these two platelet functions reflects only varying threshold requirements for activation of the appropriate platelet elements, but other explanations are possible. CRP and PGE_1 shared this and other properties such as an ability to inhibit ADP- and epinephrine-induced platelet aggregation. However, they differ in that PGE_1 has a pronounced inhibitory effect upon the primary wave of aggregation and also inhibits the adhesion of platelets to collagen while CRP does not (18, 29, 31).

The mechanism(s) by which CRP inhibits platelet function remains unclear. Human CRP appears to interact via a phosphocholine moiety on the platelet surface (32), to preferentially suppress the secondary ADP-mediated wave of aggregation and to interfere with the release reaction (17, 18). Platelet shape change, adherence, aggregation, release, and clot retraction may all be dependent upon the contractile process (29, 30), and whether CRP affects platelet contractility directly, as do cytochalasin B and sulfhydryl inhibitors (29, 30, 31, 33), or mediates its effect through another mechanism(s) such as alteration of intracellular calcium sequestration, cyclic AMP levels, and/or prostaglandin metabolism, is presently under investigation.

Clearly platelets have an important role in

inflammation. They adhere to sites of vascular damage, have receptors for immune complexes (human platelets) and complement components (rabbit platelets), release vasoactive amines, and synthesize and release the prostaglandins PGE_2 and $PGF_{2\alpha}$ which are potent inflammatory agents (29, 30, 33, 34). The elevation of CRP levels during reactions of inflammation and tissue destruction thus may serve to limit platelet aggregation, release, and contractility, and thereby to limit potentially damaging effects by platelets in association with the host response to injury.

Summary. Human C-reactive protein (CRP), whose concentration in serum is increased during acute inflammation, previously was shown to inhibit aggregation, activation of platelet factor 3, and release of both serotonin and β-glucuronidase in reactions involving human platelets. In the present study, rabbit as well as human CRP was found to dramatically inhibit both the rate and extent of thrombin-induced aggregation of washed rabbit platelets. Similarly, rabbit and human CRP both were found to inhibit the retraction of dilute rabbit clots, using an assay involving stimulation with excess thrombin and measurement of fluid volume expressed. These effects were observed using CRP concentrations (25–200 μg/ml) well within the range characteristically seen during reactions of inflammation and tissue destruction. Inhibition of clot retraction and cross-species inhibition of platelet aggregation are previously unreported properties of CRP. Thus, these observations further implicate CRP as a significant regulator of platelet function and emphasize the potential value of the rabbit as an experimental model in further studies of CRP.

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