Platelet Activation by Clq Results in the Induction of αIIb/β3 Integrins (GPIIb-IIIa) and the Expression of P-Selectin and Procoagulant Activity

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Summary

Clq receptors (ClqR) have been identified on a variety of somatic and cultured cells including peripheral blood platelets. Since platelets are likely to encounter both circulating Clq multimers and Clq associated with the extracellular matrix after complement activation by the classical pathway, the present study was designed to assess the effect of fluid phase and immobilized Clq on platelet function. Platelet adhesion to Clq-coated surfaces was accompanied by the induction of fibrinogen receptors. Scatchard analysis of fibrinogen binding to adherent platelets revealed the binding of ~10,000 molecules of fibrinogen per platelet with a $K_a$ of 0.1 ± 0.03 μM (mean ± SD, n = 4). Furthermore, fluid phase Clq multimers were noted to aggregate platelets at doses > 5 μg/ml. This aggregation was preceded by a rise in inositol-1,4,5-trisphosphate (IP3) (6.9 ± 2.4 pmol/10⁹ platelets at 15 s, n = 4), and activation of GPIIb-IIIa complexes supporting fibrinogen binding. Platelet aggregation in response to Clq multimers was accompanied by the aspirin-inhibitable release of granule contents and P-selectin (CD62) expression. Platelet aggregation was inhibited by the collagenous domain of Clq (c-Clq) and a monoclonal antibody directed against Clq receptors, suggesting the direct involvement of the 67-kD platelet ClqR. Antibodies against the very late antigen 2 or CD36 collagen receptors were without effect. Platelet exposure to Clq multimers was also accompanied by the expression of procoagulant activity, as demonstrated by the dose-dependent shortening of the kaolin recalcification time of normal plasma from 108 ± 12 s in the presence of unstimulated platelets to 62 ± 14 s in the presence of platelets that had been preincubated (5 min, 37°C) with 100 μg/ml multimeric Clq (n = 3).

These data suggest that platelet interactions with Clq multimers or immobilized Clq, resulting in the activation of GPIIb-IIIa fibrinogen binding sites and the expression of P-selectin as well as platelet procoagulant activity, are likely to contribute to thrombotic events associated with complement activation and inflammation.

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Clq is a 460,000-mol wt glycoprotein present in plasma at ~75 μg/ml (1). Clq circulates as a calcium-dependent complex with Clr2 and Cls2, forming the first component of complement, C1 (2). Upon activation of C1 by circulating immune complexes or certain bacteria and viruses, Clr2 and Cls2 are disassembled by Cl-inactivator (3), leaving complex-bound Clq with its collagen-like tail exposed and potentially available for binding to Clq receptors (ClqR) (4). Whereas the extravascular localization of free Clq is limited under normal conditions, selective accumulation of this protein has been observed in inflamed and injured tissues, particularly after vascular injury or enhanced vascular permeability (5, 6). Moreover, the secretion of Clq by lymphokine-activated monocytes or macrophages has been documented (7, 8).

The interaction of Clq with its receptors on somatic and cultured cells has been reported to elicit a variety of biological responses (4). In platelets, the interaction with monomeric Clq was first reported to inhibit collagen-induced platelet aggregation (9, 10). The structural similarities between the NH₂ terminus of Clq and collagen (11) suggested that Clq and collagen binding sites were identical. Recent studies (12), however, have demonstrated the existence of distinct platelet membrane receptors for Clq and collagen. Under certain conditions, however, these sites demonstrate cross-reactivity with regard to ligand binding (12).

Since virtually all biologically important C1 activating substances, including immune complexes, RNA tumor viruses, and lipid A-rich LPS bind multiple Clq molecules, Clq is likely to circulate in multimeric or aggregated form after ac-
ativation of the classical complement pathway (13, 14). Unlike C1q monomers, C1q multimers have been suggested to support platelet aggregation (9). Because C1q has also been reported as a transient component of the extracellular matrix (15), the present study was designed to evaluate the response of platelets both to C1q multimers in the fluid phase, and to immobilized C1q on plastic surfaces.

**Materials and Methods**

**Platelet Preparation**

Blood was collected from human volunteers after obtaining informed consent. The blood was anticoagulated with 0.1 vol 3.2% sodium citrate in the presence or absence of 1 mM aspirin. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 280 g (15 min, 22°C). Washed platelets (WP) were obtained after acidifying the PRP to pH 6.5 with 1 M citric acid, centrifuging the sample (1,000 g, 20 min, 22°C), and resuspending the resulting platelet pellet in 0.01 M Hepes-buffered modified Tyrode's solution containing no added calcium, 2 mM MgCl₂, and 2 mg/ml BSA (HBMT) (Sigma Chemical Co., St. Louis, MO) (16).

**Purification of Protein Ligands**

C1q was isolated from human serum as described by Reid (17). Digestion of C1q with pepsin to obtain the collagen-like domain containing no added calcium, 2 mM MgCl₂, and 2 mg/ml BSA was performed by centrifugation of whole blood at 280 g (15 min, 22°C). Washed platelets (WP) were obtained after acidifying the PRP to pH 6.5 with 1 M citric acid, centrifuging the sample (1,000 g, 20 min, 22°C), and resuspending the resulting platelet pellet in 0.01 M Hepes-buffered modified Tyrode's solution containing no added calcium, 2 mM MgCl₂, and 2 mg/ml BSA (HBMT) (Sigma Chemical Co., St. Louis, MO) (16).

**Preparation of Protein-coated Surfaces**

Microtiter wells were exposed to purified C1q (80 μg/ml) or BSA (80 μg/ml) for 16 h at 4°C. Unreactive sites were blocked for 60 min at 37°C with 0.1% BSA. Wells were rinsed with HBMT before exposure to platelets.

**Platelet Aggregation Studies**

**Platelet Aggregation**. Platelet aggregation was monitored at 37°C in a dual channel aggregometer (Chronolog Corp., Havertown, PA) after addition of 5–100 μg/ml agg-C1q. In some experiments, platelets were preincubated (5 min, 22°C) with C1q (100 μg/ml) or mAbs, III/II, 6F1, 1OP36 (20 μg/ml) directed against the ClqR, the VLA-2 collagen receptor, or the CD36/GPIV collagen receptor, respectively. In other experiments, platelets were preincubated with mAbs (50 μg/ml), recognizing the platelet FcγRII receptor (AMAC, Inc.), before exposure to agg-C1q.

**Fibrinogen Binding to WP Suspensions**. Fibrinogen binding to WP was evaluated in an unstirred system (20) after platelet stimulation with agg-C1q in the presence of increasing amounts of 125I-fibrinogen. Nonspecific binding was assessed in the presence of 10 mM EDTA or excess unlabeled fibrinogen (20).

**Fibrinogen Binding to Adherent Platelets**. WP (1.25 × 10⁷) were added to C1q-coated microtiter wells. After 30-min adhesion (22°C), nonadherent platelets were removed, the wells rinsed three times with PBS, and platelet adhesion quantified in duplicate wells using the bichinchoninic acid protein assay (23). Additional wells were exposed to increasing concentrations of 125I-fibrinogen. After 60 min at 22°C, wells were rinsed three times, and residual radioactivity quantified using a gamma counter. Nonspecific fibrinogen binding was evaluated in the presence of excess unlabeled fibrinogen. The specificity of 125I-fibrinogen binding was further evaluated in the presence of 100 μM arginine, glycine, aspartic acid (RGDS; Peninsula Labs., Inc., Belmont, CA), or after platelet exposure to a mAb (20 μg/ml) recognizing the c5b-6 integrin, GPIb–IIa (10E5, a gift from Dr. B. Collier) (24).

**Serotonin Release**. Platelets were labeled with 14C-serotonin, 5-hydroxy(side chain-2-14C)tryptamine creatinine sulphate (Amer sham Corp., Arlington Heights, IL), in whole blood (25). 14C-serotonin release was measured at maximum platelet aggregation in the presence of 5 μM imipramine to prevent reuptake of released serotonin. Samples were fixed with an equal volume of 2% formalin, placed on ice, centrifuged for 3 min at 12,000 g, and the supernatant radioactivity evaluated in a beta counter.

**P-Selectin Expression**. The secretion of platelet α granules with concomitant expression of the platelet α granule membrane protein designated P-selectin (CD62) was assessed after stimulating platelets with saturating concentrations of agg-C1q (100 μg/ml) (26) at 37°C for 10 min. Platelet suspensions were subsequently fixed with paraformaldehyde (27), washed, and exposed to a PE-labeled monoclonal antiCD62 antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) (30 min, 22°C). Platelets were washed three times in PBS and evaluated qualitatively for fluorescence at the Center for Imaging and Analysis (SUNY at Stony Brook) using a microscope/camera (Axiophot; Carl Zeiss, Microscopy, Thornwood, NY). Resting PGE₂ (10 μM)-treated platelets, or platelets preincubated with 100 μg/ml nonimmune C1q, served as negative controls. Platelets stimulated with 50 μU/ml thrombin (a gift from Dr. John Fenton II, New York State Department of Health, Albany, NY) served as positive controls. Thrombin was neutralized with excess hirudin before platelet fixation.

**Inositol-1,4,5-Trisphosphate (IP₃) Production**. WP (2 × 10⁹/ml) were incubated at 37°C in the presence of 0.1 mg/ml apyrase and stimulated with 100 μg/ml agg-C1q. The reaction was terminated by adding 0.2 ml 1 M TCA per 1 ml of platelet suspension. After a 15-min incubation on ice, samples were centrifuged (1 min, 12,000 g, 4°C). The supernatant (1 ml) was removed, and IP₃ quantified using an IP₃ 3H-radioreceptor assay kit (New England Nuclear Research Products, Boston, MA) as described by the manufacturer.

**Platelet Procoagulant Activity**. Platelet factor 3 (PF3) availability was measured by incubating 0.1 ml pooled normal plasma (George King Biomedical Co., Overland Park, KS) with 0.1 ml kaolin (20
Table 1. Inhibition of Agg-C1q Induced Fibrinogen Binding to Platelets in Suspension and Fibrinogen Binding to Platelets Adhering to C1q-Coated Surfaces

| Inhibitor                 | Immobilized C1q | Agg-C1q |
|---------------------------|------------------|--------|
| 10E5                      | 95 ± 7           | ND     |
| RGDS                      | 92 ± 18          | ND     |
| c-C1q                     | ND               | 85 ± 12|
| II1/D1                   | ND               | 87 ± 14|
| 6F1                       | ND               | 5 ± 11 |
| Anti-Fcγ RII              | ND               | 7 ± 9  |
| Anti-CD36/GPIV            | ND               | 0 ± 8  |

Platelets were preincubated (5 min, 22°C) with inhibitors: 100 μg/ml c-C1q, 20 μg/ml 10E5, II1/D1, 6F1, or Anti-CD36, 50 μg/ml anti-Fcγ RII, or 100 μM RGDS. Platelets were subsequently stimulated with 25 μg/ml agg-C1q in the presence of 100 μg/ml fibrinogen. Values represent mean ± SD, n = 3.

Table 2. Washed Platelet Responses to Agg-C1q: Effect of Aspirin

| Response                  | Stimulated with agg-C1q | Unstimulated |
|---------------------------|-------------------------|--------------|
|                           | ASA                     | Non-ASA      | ASA          | Non-ASA     |
| Aggregation (%)           | 64 ± 24                 | >90          | 0            | 0           |
| 14C-serotonin release (%) | 32 ± 12                 | 84 ± 18      | 0.4 ± 0.2    | 0.1 ± 0.3   |
| Fibrinogen binding        |                         |              |
| (molecules/platelet)      | 27,270 ± 3690           | 41,240 ± 1648| 243 ± 124    | 285 ± 58    |

Platelet aggregation and serotonin release in response to 20 μg/ml agg-C1q were measured 5 min after stirring samples in an aggregometer at 37°C, in the presence of 100 μg/ml fibrinogen and 5 μM imipramine. Values are expressed as percent relative to maximal aggregation or total platelet 14C-serotonin, respectively. Fibrinogen binding was measured 60 min after platelet stimulation at 37°C, in the presence of 250 μg/ml 125I-fibrinogen without stirring. Unstimulated platelets served as controls. The responses of platelets pretreated with 1 mM aspirin (ASA) were compared to untreated platelets (Non-ASA). Data represent mean ± SD, n = 5.
Figure 2. \(^{125}\)I-fibrinogen binding to washed platelets stimulated with increasing concentrations of agg-C1q or aggregated IgG (agg-IgG). WP were incubated with agg-C1q or agg-IgG for 60 min at 22°C in the presence of 250 μg/ml \(^{125}\)I-fibrinogen. Fibrinogen binding was quantified as described in Materials and Methods.

obtained in the presence of 30-75 μg/ml agg-C1q (Fig. 2). Fibrinogen binding to platelets in suspension was always higher than fibrinogen binding to platelets adhering to C1q-coated surfaces. This may reflect the extent of platelet stimulation and/or the potentially decreased accessibility of fibrinogen receptors on adherent platelets which bind fibrinogen only to their nonadherent surface. Platelet aggregation, fibrinogen binding, and serotonin release were all inhibited but not abrogated after pretreatment of platelets with 1 mM aspirin, confirming the involvement of cyclo-oxygenase-dependent synthesis of thromboxane A_2 (29) and release of platelet granule contents. The release of α granules was inferred from qualitative analysis of cell surface P-selectin expression by direct immunofluorescence (Fig. 3). Interestingly, the extent of P-selectin expression by 100 μg/ml agg-C1q (Fig. 3 E) appeared similar to that noted after platelet stimulation with a standard dose of thrombin (Fig. 3 F).

To rule out platelet activation via occupancy of FcγRII receptors (30) by potential trace agg-IgG contamination, fibrinogen binding was compared in response to agg-C1q or similar concentrations of agg-IgG. As summarized in Fig. 2, agg-IgG was without effect at 22°C in unstirred systems. Moreover, agg-C1q-induced fibrinogen binding was inhibited in the presence of the collagen-like NH2-terminal domain of C1q (c-C1q) or mAbs (II1/D1) directed against the 67-kD C1q receptor (Table 1). mAbs directed against the FcγRII receptor, the VLA-2 collagen receptor, or CD36/GPIV, also reported to function in platelet–collagen interactions (31), were without effect (Table 1).

Platelet stimulation with agg-C1q was associated with rapid IP_3 production (Fig. 4). To optimize IP_3 generation, platelets were stimulated with 100 μg/ml agg-C1q. The reaction was terminated at 2, 15, and 30 s. Maximal IP_3 production was observed within 15 s of platelet stimulation (Fig. 4). By 30 s, IP_3 levels decreased almost to baseline.

Platelet stimulation with agg-C1q was also accompanied by the expression of surface membrane procoagulant activity. As summarized in Table 3, shortening of the kaolin recalcification time of pooled normal plasma was observed in the presence of platelets activated with increasing concentrations of agg-C1q. Clotting times in the presence of un-

Figure 3. Phase contrast views of unstimulated (A) platelets and platelets stimulated with 100 μg/ml agg-C1q (B), or 50 mU/ml human thrombin (C). Immunofluorescence views demonstrating P-selectin expression on unstimulated platelets (D), platelets stimulated with 100 μg/ml agg-C1q (E), or 50 mU/ml thrombin (F) using a PE-conjugated anti CD-62 mAb. Same views as A-C. ×200.

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stimulated platelets or thrombin-stimulated platelets served as negative and positive controls, respectively.

**Discussion**

C1q receptors are present on numerous somatic and cultured cells where they mediate a variety of cell-specific reactions (4). Platelets possess ~4,000 C1q binding sites per cell and bind C1q with a $K_d$ of $3.5 \times 10^{-7} \text{ M}$ in a divalent cation independent manner (26). The present study characterizes platelet responses to agg-C1q in solution and to immobilized C1q on plastic surfaces. The data demonstrate the direct involvement of the 67-kD platelet C1qR in C1q-induced
platelet stimulation. This stimulation involves several surface membrane-associated events including IP$_3$ production, activation of GPIIb–IIIa fibrinogen receptors, cyclo-oxygenase-mediated thromboxane A$_2$ formation supporting the release of platelet granule contents, and the expression of P-selectin and procoagulant activity.

Previous studies (9, 10) have described the interaction of soluble Clq monomers with human platelets. The structural
similarity between C1q and collagen suggested a potential role for platelet C1q receptors in modulating collagen-induced platelet functions. Interestingly, studies with highly purified C1q monomers and monoclonal as well as polyclonal anti-C1qR antibodies demonstrated that whereas C1q and anti-C1qR antibodies inhibited collagen-induced platelet aggregation and secretion at low concentrations of collagen, they did not affect the adhesion of platelets to collagen fibrils or collagen-coated surfaces (32). Moreover, the effect of anti C1qR Abs and an Ab directed against the platelet VLA-2 receptor clearly indicated that platelet adhesion to Type I collagen and C1q was mediated by distinct receptors (12). This concept is further supported by observations made in the present study demonstrating specific inhibition of agg-C1q-induced platelet stim-

Table 3. Effect of Platelet Stimulation with Agg-C1q on the Kaolin Recalcification Time of Normal Pooled Plasma

| Addition            | Clotting time |
|---------------------|---------------|
| Buffer              | 114 ± 13      |
| Resting platelets   | 108 ± 9       |
| Platelets + Thrombin| 56 ± 15       |
| Platelets + Agg-C1q |               |
| 20 µg/ml            | 92 ± 12       |
| 30 µg/ml            | 78 ± 11       |
| 100 µg/ml           | 62 ± 14       |

Washed platelets were stimulated with 0.10 U/ml human thrombin or agg-IgG (20-100 µg/ml). Thrombin was neutralized with excess hirudin before adding 0.2 ml of the platelet suspension to 0.2 ml normal plasma preincubated 5 min (37°C) with 10 mg/ml kaolin. Clotting was initiated by adding 0.1 ml CaCl₂ (0.035 M). Values represent mean ± SD, n = 3.
bodies contain not only serotonin, but also ADP which is by specific and saturable fibrinogen binding. This bound contain biogenic amines that may participate in inflammatory reactions. In contrast, the release of α granule contents includes a number of adhesive proteins such as fibrinogen, fibronectin, and thrombospondin, that may serve to reinforce platelet–platelet interactions (35). Secretion of α granule contents is also accompanied by the surface expression of an α granule membrane protein, P-selectin, previously referred to as GMP 140 or PADGEM (36, 37). P-selectin has been shown to mediate the adhesion of neutrophils and monocytes to activated platelets in a calcium-dependent manner (37).

Platelet α granule secretions also contain the active form of coagulation factor V (Va) (38). This, in combination with surface membrane changes leading to the exposure of negatively charged phospholipids (phosphatidylinerine, phosphatidylinositol), encompasses PF3 procoagulant activity (39). PF3 activity is essential for efficient thrombin generation leading to fibrin clot formation. Indeed, exposure of platelets to agg-Clq in the present study lead to dose-dependent PF3 generation, as measured by the kaolin recalcification time, a standard assay for the evaluation of platelet procoagulant activity (40).

Since platelet Clq receptors were previously found to participate in immune complex localization via immune complex association with the globular head region of Clq (41), the question arises as to whether ClqR occupancy by the collagen-like NH2-terminal region of Clq is physiologically beneficial or detrimental. Based on available data, low concentrations of Clq multimers (<5–10 μg/ml), formed after binding immune complexes, certain bacteria or viruses, may participate in clearance mechanisms, and/or antigen presentation to lymphoid cells of the reticuloendothelial system. In contrast, high concentrations of agg-Clq appear likely to contribute to thrombotic and inflammatory reactions.

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