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INDUCTION OF SEROTONIN SECRETION BY CROSS-LINKING OF SURFACE RECEPTORS OF A DERIVATIVE OF WHEAT GERM AGGLUTININ ON HUMAN PLATELETS

Pankaj Ganguly and Nancy G. Fossett

From the Department of Hematology, St. Jude Children's Research Hospital and the Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38101

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SUMMARY: A nonagglutinating derivative of wheat germ agglutinin has been prepared that binds to platelets and precipitates an antibody to the lectin. Platelets treated with this inactive derivative released serotonin when exposed to bivalent $F(ab')_2$, but not monovalent Fab, fragments of the lectin antibody. Bridging of platelet-bound Fab by an antibody again induced secretion. The $F(ab')_2$ or Fab fragments plus IgG, without the derivative, did not induce secretion. This secretion was not affected by indomethacin showing a direct activation of platelets. Platelets treated with con A followed by $F(ab')_2$ to con A did not secrete. In addition, lentil lectin failed to release platelet serotonin. The receptors of the lectin derivative are mobile on the platelet surface and their redistribution may lead to secretion.

Recent studies have shown that the initial step in platelet activation is the binding of a ligand to specific receptors on the cell surface (1). In platelets, the nature of the events following the binding of the ligand remains unknown. An important observation in this connection is that some lectins bind to platelets and cause cell aggregation and secretion (2,3) in many ways similar to that of the physiological agent thrombin. Since lectins are thought to act on the target cell by cross-linking of its receptors, these observations suggest the possibility that a similar phenomenon may be involved in the generation of the transmembrane signal leading to platelet stimulation by physiological agents. Wheat germ agglutinin (WGA) is known to bind to platelets and induce cell activation (3). We have prepared a nonagglutinating derivative of WGA by selective cleavage of a methionine residue of the lectin which competitively inhibited platelet stimulation by thrombin while aggregation induced by ADP, collagen, ristocetin, trypsin or WGA was not significantly affected. In addition, the membrane receptor of the derivative, a

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74,000 dalton glycoprotein, specifically blocked platelet stimulation by thrombin (4). We show that platelets treated with this inactive derivative will secrete serotonin independent of cell aggregation when exposed to specific bivalent, but not monovalent, antibodies to WGA.

MATERIALS AND METHODS: Blood was collected from volunteers in plastic syringes utilizing citrate as the anticoagulant (9:1 v/v). Platelets were washed with oxalate-EDTA (6) and resuspended in phosphate buffered-saline (PBS), pH 7.2 containing 5% plasma. These platelets routinely agglutinated with WGA or con A. Wheat germ agglutinin (U.S. Biochemicals) showed a single band in SDS gel electrophoresis (7). Conconavalin A and lentil lectin were purchased from Sigma. The WGA derivative was prepared by treating the lectin (1 mg/ml) in 65% formic acid with 500 mg of cyanogen bromide for 20 hr. The solution was dried under vacuum, redissolved in water and lyophilized. The dry powder was reconstituted and dialyzed extensively against PBS (5).

Antibody to WGA was prepared in rabbits. Quantitative precipitation studies showed that each ml of this antiserum contained 1.6 mg of antibody. Antiserum to con A was purchased from Miles Laboratories and contained 1.9 mg of antibody/ml of serum. Bivalent $F(ab')_2$ fragments were prepared by sodium sulfate precipitation of the peptic digest of the IgG fraction of the antiserum followed by gel filtration through a column of Sephadex G-200 (8). Monovalent Fab was isolated by affinity chromatography of the papain digest of the IgG on a protein A - Sepharose column. The final products were dialyzed against PBS and concentrated by ultrafiltration. Immunoelectrophoresis of WGA and the derivative was carried out in 1% agar as described (9).

Secretion and agglutination assays were carried out routinely at room temperature in microtiter plates (10). Platelets (1×10^9/ml), loaded with [^{14}C] serotonin or control, were incubated with the WGA derivative for 15 to 30 min at room temperature. The suspension was centrifuged in the presence of EDTA and the platelets were resuspended in the original volume of buffer. The same procedure was followed for preparing platelets with lentil lectin or con A and appropriate control samples were always maintained. Aliquots of 100 µl of platelets were then exposed to antibodies in the microtiter wells in a final volume of 200 µl. After overnight (~15 hr) incubation, the plates were examined for cell agglutination. Platelet secretion was determined at the same time by measuring the radioactivity in the supernatant (100 µl) which was expressed as percent of the control (3).

RESULTS: In this study, we have utilized an immunological procedure to explore the concept that a redistribution of membrane receptors may be involved in platelet secretion. For such an approach to be successful, the derivative must have the capability to precipitate the lectin antibody and to bind to platelets without causing any significant cell stimulation. The immunoelectrophoretic pattern of WGA against the anti-lectin serum showed a single precipitin arc (Fig. 1). The WGA derivative produced a similar pre-

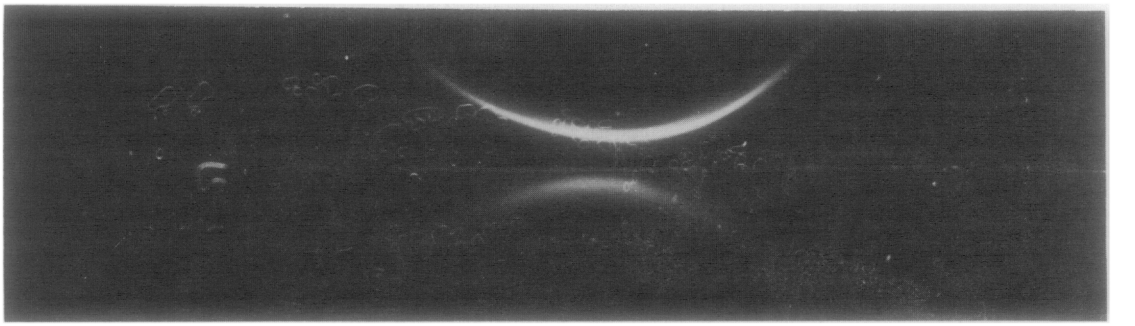


Fig. 1. Agar gel immunoelectrophoretic pattern of wheat germ agglutinin (top well) and its derivative (bottom well). The central channel contained an antiserum to the lectin. The pattern was recorded after overnight incubation and did not change after 3 days.

cipitin line in the same position as WGA. These results show that the WGA derivative can precipitate the WGA antibody. Platelets were incubated with the derivative and the unbound derivative was removed by centrifugation. These platelets when exposed to the WGA antibody (IgG) agglutinated in a dose-dependent manner while the control (minus IgG) did not agglutinate (Fig. 2). The antibody at different concentrations did not agglutinate platelets in the absence of the WGA derivative. These results show that the lectin derivative retains the capacity to bind to platelets. Direct determination of binding with [^{125}I] derivative showed that there are about 1.5 x 10^5 lectin binding sites/platelet with an apparent dissociation constant of 1.7 μM.

Platelets preincubated with the inactive WGA derivative released serotonin when exposed to the bivalent antibody fragments (Fig. 3). The amount of serotonin secreted increased with increasing concentrations of the antibody reaching a plateau at about 30 μg of $F(ab')_2$. There was no detectable lactate dehydrogenase released showing that the serotonin secretion was not due to platelet lysis. Neither the lectin derivative alone nor in conjunction with the antibody fragment caused platelet agglutination under these conditions. In the absence of the derivative, the antibody fragment had no effect on platelets. Indomethacin did not have any significant effect (Fig. 3) showing

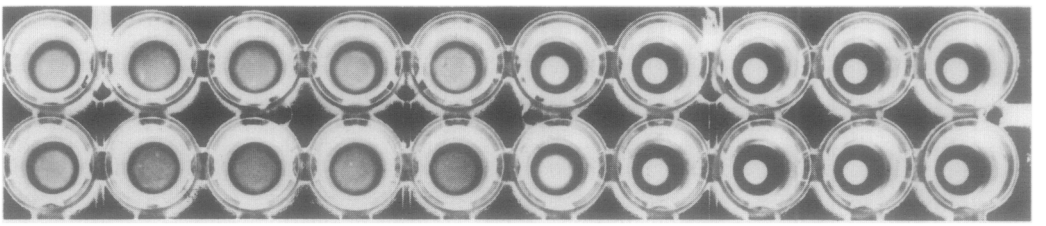
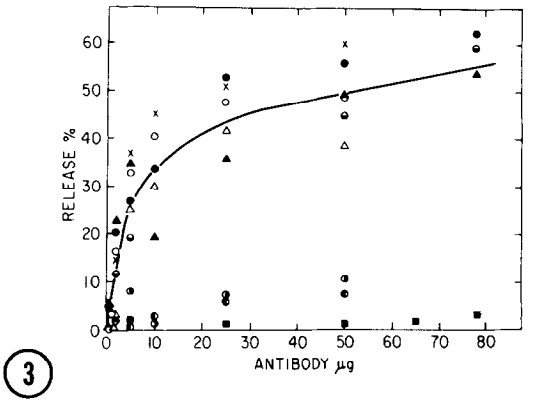


Fig. 2. Agglutination of platelets pretreated with the lectin derivative by the IgG fraction of lectin antiserum. Platelets (1 x 10^9/ml) in PBS were incubated with the derivative (75 µg/ml) for 15 min. The sample was centrifuged and platelets were resuspended in the original volume of buffer. Aliquots (100 µl) of these platelets were then tested for agglutination in duplicates (top and bottom rows). From left to right, antibody concentration (µg/200/µl) in well 1 to 7 were 7.8, 4.8, 3.6, 2.4, 2.4, 1.2, 0.6. Wells 8-10 were controls of derivative-treated platelets without antibody, antibody-treated platelets without lectin derivative and platelets in buffer respectively.

that the secretion of serotonin induced by derivative-$F(ab')_2$ represents direct activation of platelets (11). Studies on the time dependence of this release process showed that the platelets treated with the lectin derivative started to secrete serotonin between 1 and 2 hr after exposure to $F(ab')_2$ which attained a plateau between 4 and 5 hr (Fig. 4). The amount of serotonin released did not change significantly after overnight incubation. In contrast, the derivative-treated platelets did not release serotonin when exposed to monovalent Fab fragments of the antibody. Bridging of the platelet-bound Fab fragments with a goat antibody to rabbit IgG caused secretion of serotonin (Fig. 3) without platelet agglutination indicating the requirement of cross-linking of the lectin receptors for platelet secretion. The goat antibody alone or with the Fab fragments did not secrete serotonin in the absence of the derivative. Concanavalin A (3.3 µg/ml) followed by bivalent antibody fragments to con A failed to induce secretion (Fig. 3). Platelets are known to be agglutinated by con A showing that this lectin does bind to platelets, presumably to GP-III (12). In addition, lentil lectin which binds mainly to GP-II did not stimulate platelets at saturating concentrations (2,13). Thus, it appears that there is some specificity in the action of the WGA derivative-$F(ab')_2$ on platelets to elicit a secretion response.



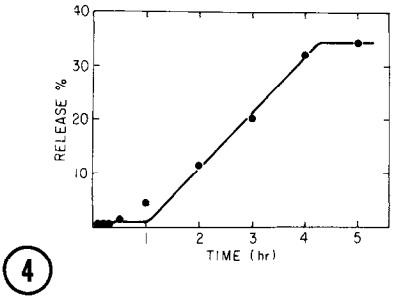


Fig. 3. Serotonin-loaded platelets (1 x 10^9/ml) were incubated with lectins and platelets processed as in Fig. 2. Aliquots (100 µl) of these platelets were then exposed to different concentrations of the antibody fragments. 0,● (50 µg/ml) and Δ,▲ (100 µg/ml) of the derivative followed by $F(ab')_2$ fragments of the antibody to WGA. Data were obtained in experiments on different days with different preparations. ◍ 50 µg/ml of derivative-treated platelets exposed to $F(ab')_2$ antibody in the presence of indomethacin (70 µM). ◐,◑ 75 µg/ml of WGA derivative-treated platelets exposed to monovalent Fab antibodies. Data from two different experiments are shown. X Platelets treated with 50 µg/ml of WGA derivative followed by 50 µg/ml of Fab antibody to WGA. These platelets were then exposed to different concentrations of a goat antibody to rabbit IgG. ■ Platelets incubated with 3.3 µg/ml of con A followed by bivalent antibody fragments to con A. Data shown are representative of six different experiments.

Fig. 4. Dependence of serotonin secretion from platelets treated with the lectin derivative (33 µg/ml) on the time of incubation with $F(ab')_2$ fragments of the lectin antibody (33 µg/ml). These experiments were carried out following the same procedure as described in Fig. 3. Platelets released serotonin between 1 and 2 hr which reached a plateau between 4 and 5 hr and did not significantly change up to 16 hr.

DISCUSSION: The results of this study suggest that cross-linking of the receptors on platelets by ligands may be necessary to elicit a biological response. Since cross-linking requires lateral movement, the receptor of the lectin derivative must be mobile in the plane of the platelet membrane. Such receptor cross-linking has been demonstrated in other cell-ligand systems (14). Secretion of serotonin occurred approximately 2 hr after the addition of $F(ab')_2$ to platelets. Since antibodies bind to cells in a few minutes, it appears that cross-linking of the receptors following antibody binding is involved in signal generation. Whether a similar mechanism is involved in the physiological activation of platelets remains unknown. However, with physiological agents, ligand-induced receptor aggregation may occur in a few

seconds. The diffusion coefficient of insulin- and epidermal growth factor-receptor complexes in living fibroblasts has been estimated to be 4×10^{-10} cm^2/sec. Thus, assuming an initial homogeneous distribution of the receptors and that they move in a random walk, two receptors can encounter each other within 50 msec after complex formation (14).

The platelet secretion may be aggregation-dependent (e.g. epinephrine or ADP-induced) and abolished by indomethacin or it may be independent of aggregation (e.g. collagen or thrombin-induced) (11). Indomethacin, a cycloxygenase inhibitor, did not affect platelet secretion brought about by redistribution of the lectin receptors. Thus, this pathway of platelet stimulation might be involved in the action of those agents which do not depend on the products of arachidonate metabolism for their effects. It has been shown that monomeric collagen is ineffective and fibril formation is essential for platelet secretion (15) clearly suggesting a multivalent type of interaction which may involve a redistribution of the platelet receptors for collagen. The mechanism by which thrombin activates platelets has remained controversial (1,5). One reason for the paucity of information is the inability to separate the specific thrombin-induced changes from the general metabolic effects which are also produced by other agents. The WGA derivative as well as its membrane receptor both blocked platelet activation by thrombin under physiologic conditions with a high degree of specificity. These results suggested that this protein may be a physiologic receptor of thrombin in human platelets (5). Since cross-linking of this protein appears to cause platelet secretion, it is possible that a redistribution of the receptors might be a step in platelet activation by thrombin.

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