Surface sialic acid is considered to be a key determinant for the survival of glycoproteins and cells in circulation. Although a similar mechanism has been proposed for the removal of platelets from blood, there is no simple procedure that broadly indicates the amount of the sialic acid present on the surface of platelets. A routine in vitro assay of platelet function is platelet aggregability. Aggregation of platelets with reduced surface sialic acid utilizing commonly used aggregating agents is normal. Wheat germ agglutinin (WGA) is a plant lectin that is being increasingly used as a tool for the isolation and characterization of many glycoconjugates, including cell surface components. In contrast to several other lectins, which may be inactive or bring about passive agglutination of human platelets, WGA is a powerful activator of platelets. This activation is accompanied by serotonin secretion, which may be independent of cell agglutination. It is known that WGA binds to N-acetylglucosamine, N-acetylgalactosamine, and sialic acid, although the precise nature of a biologic receptor for the native lectin has been reported to be a glycoprotein. The sucinated lectin had a dimeric structure similar to the native lectin, but migrated more slowly on gel electrophoresis. The modified lectin bound to about 2.8 x 10^8 sites/cell, with an apparent dissociation constant of 2 μM compared to 5 x 10^8 sites/cell and a dissociation constant of 0.4 μM for the native lectin. The succinated lectin neither aggregated nor agglutinated platelets, but agglutination of red cells in microtiter plates was normal. Aggregation of platelets by either wheat germ agglutinin or ristocetin was not affected by the succinylated lectin. Since the native wheat germ agglutinin is a strong activator of platelets and the succinylated derivative was devoid of all activity, it appears that a sialoprotein acts as the biologic receptor of wheat germ agglutinin in human platelets. This suggestion was strengthened by the observation that platelets treated with different concentrations of neuraminidase had a decreased capacity to bind this lectin. These platelets also showed reduced aggregation and serotonin secretion when activated with the native lectin. Since sialic acid has been implicated in the removal of platelets from circulation, wheat germ agglutinin may prove to be a useful tool to explore those clinical conditions in which platelet survival is shortened.

Preparation of Lectin Derivative

Wheat germ agglutinin, isolated by affinity chromatography, was purchased from U.S. Biochemicals (Cleveland, OH). The lectin showed a single band in SDS-gel electrophoresis. Succinylated WGA was prepared essentially as described. Briefly, WGA (about 10 mg) was dissolved in 5 ml of saturated sodium acetate, and the solution was placed in an ice bath. Solid succinic anhydride (6 mg) was then slowly added to the stirred protein solution, which became increasingly clearer with time. After 90 min, the solution was dialyzed against 2 x 10^5 distilled water and then lyophilized. The sample was reconstituted in sodium acetate and the succinylation step was repeated. The protein was dialyzed first against water and then against phosphate-buffered saline (PBS, 0.025 M NaH₂PO₄/ Na₂HPO₄, 0.125 M NaCl, pH 7.2). Succinylated WGA prepared in this manner retains the capacity to bind N-acetylglucosamine with an affinity similar to that of the unmodified lectin. The concentration of both WGA and succinylated WGA was measured assuming an extinction coefficient (ε₄₉₀nm) of 14.3. The results presented suggest that sialic acid plays an essential role in platelet activation by WGA, and this lectin may be a useful probe for platelet surface sialic acid in health and disease.

MATERIALS AND METHODS

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Aggregation and Agglutination Assays

Blood was drawn from healthy volunteers into plastic syringes containing 0.1 vol of 3.8% sodium citrate as anticoagulant. The red cells were removed by low speed centrifugation and the platelet-rich plasma was collected. When desired, platelets were washed in the presence of 2 mM EDTA and resuspended in PBS. Platelet aggregation was measured in a dual channel aggregometer (Payton, Buffalo, NY). Secretion was measured with 14C-serotonin-loaded platelets as described. Platelet counts in plasma were adjusted to the desired level with PBS. Agglutinating activity of WGA and succinylated WGA was routinely measured at room temperature in microtiter plates utilizing standard procedures. Human red cells and platelets were washed and suspended in PBS at 10^8 cells/ml and 10^9 cells/ml, respectively. Fifty microliters of the test material was serially twofold diluted in each well, and then 50 µl of the cell suspension was added. The plate was agitated for a few seconds and then sealed. All assays were done at least in duplicate and read usually after overnight incubation. Immunodiffusion studies were carried out in 1% agar plates as described. The antiserum to WGA was prepared in rabbits in this laboratory, and quantitative precipitation studies showed that it contained 1.6 mg antibody/ml serum.

Lectin Binding

Wheat germ agglutinin or succinylated WGA was labeled with 125I by the chloramine-T method, and the free label was removed by gel filtration. The agglutinating activity of the labeled lectins on red cells was similar to that of the unlabeled proteins. Platelets (2–3 x 10^9/ml) in PBS containing 0.1% bovine serum albumin were incubated with different amounts of the lectin for 15 min at room temperature. The cell-bound lectin was separated from free lectin by filtration through albumin-coated Millipore filters or by centrifugation through a layer of Versilub (General Electric). Details of these procedures have been published, and the results were the same by either method. Nonspecific binding of the lectin was determined in the presence of 0.1 M N-acetylglucosamine.

Other Methods

Gel electrophoresis of the proteins was carried out in 7.5% acrylamide. Molecular weight standards were phosphorylase (94,000), serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). Platelets were treated with different concentrations of neuraminidase from Vibrio cholerae (Behring Diagnostics, Somerville, NJ) for 30 min at 37°C. Buffer was added to another aliquot of platelets and maintained as the control. The cells were then collected by centrifugation and resuspended in fresh buffer.

RESULTS

Properties of the Lectins

Wheat germ agglutinin, a pure protein with a molecular weight of 36,000, is composed of two similar subunits, each possessing two binding sites for sugars. The binding sites are thought to have an extended arrangement rather than a single pocket in the protein. The succinylated WGA preparation was characterized by remarkably high solubility in contrast to the native lectin, which is only sparingly soluble. The gel filtration pattern of WGA in the presence of N-acetylglucosamine showed a single peak. Succinylated WGA eluted slightly ahead of WGA under the...
same conditions and showed a similar pattern (Fig. 1). Thus, within the limitations of this method, WGA and succinylated WGA have a similar molecular weight under these conditions, and chemical derivatization of the lectin was not accompanied by subunit dissociation of the protein. In SDS-gel electrophoresis, WGA showed an intense band corresponding to a molecular weight of about 22,000 (Fig. 2). It is known that WGA dissociates into subunits under these conditions, and the monomers migrate slower than expected (mol wt 18,000), presumably due to retardation produced by a slight interaction of the lectin with the polyacrylamide support. A faint band, corresponding probably to dimeric WGA, could also be seen. Succinylated WGA had a slower electrophoretic migration, which could be due to the incorporation of negatively charged groups into the protein, as has also been observed with the carboxymethylated protein. No products of electrophoretic mobility intermediate between the native and succinylated lectins were observed, showing a reasonable homogeneity of the lectin derivative preparations. Both WGA and succinylated WGA showed a single precipitin line against the WGA antiserum. The lines merged with each other, indicating that succinylated WGA had antigenic properties similar to WGA (data not shown).

Agglutination Studies

The results of agglutination studies with human red cells and platelets by WGA and succinylated WGA are shown in Fig. 3. Platelets were strongly agglutinated in microtiter plates by WGA, with an end-point concentration of about 5–10 μg/ml. Succinylated WGA did not agglutinate platelets, even at a tenfold higher concentration. It is possible that the structure of the glycoconjugates on the platelet surface or the fluidity of the membrane makes these cells resistant to the agglutinating action of succinylated WGA. An alternative possibility is that our succinylated WGA preparation did not have any agglutinating activity due to chemical derivatization. To distinguish between these possibilities, we repeated the agglutination experiments with red cells from the same blood donor (A'). These red cells were agglutinated by WGA and succinylated WGA in a similar manner. Thus, the lack of agglutination of platelets by succinylated WGA is due to the properties of the membrane structures of these cells. It has been reported that succinylated WGA binds to glycoconjugates containing N-acetylglucosamine but not sialic acid, while WGA binds to both saccharides. Since succinylated WGA failed to agglutinate platelets under any conditions, it appears that a sialoglycoprotein acts as the WGA receptor, and sialic acid plays a critical role in platelet agglutination induced by WGA.

Binding Studies

It is possible that succinylated WGA fails to agglutinate platelets because of the high negative charge on the platelet surface that prevents the succinylated WGA molecules, which are also negatively charged at this pH, from binding to its receptors on the cell surface. To explore this possibility, we studied the binding of succinylated WGA to washed platelets (Fig. 4A). The amount of lectin bound increased with increasing amounts of lectin added and approached saturation around 120 μg/ml of lectin. In the presence of N-acetylglucosamine, the lectin-binding curve was linear. This nonspecific binding usually ranged between 10% and 20% of the total amount bound. The
LECTIN ADDED, \( \text{ng} / \text{ml} \)
- 100
- 80
- 60
- 40
- 20

Fig. 4. (A) Binding of \( ^{125}\text{I}\)-labeled succinylated wheat germ agglutinin to platelets. Platelets \((2.5 \times 10^8 / \text{ml})\) in phosphate-buffered saline were incubated with different amounts of the lectin for 1.6 mm at room temperature and then the cells were separated from the free ligand by oil centrifugation. (q) Gross binding of the lectin. (c) nonspecific binding in the presence of 0.1 M N-acetyl-D-glucosamine. and (\( ^{125}\text{I} \)) net binding. (B) Double reciprocal plot of the data on the binding of succinylated wheat germ agglutinin to platelets. The number of binding sites per cell may be calculated from the intercept on the ordinate and the apparent dissociation constant from that on the abscissa. (€) Data from two different experiments on different days are shown.

Fig. 5. (A) Effects of wheat germ agglutinin and its succinylated derivative on platelets \((3 \times 10^8 / \text{ml})\) suspended in phosphate-buffered saline in the aggregometer. The native lectin \((30 \mu\text{g} / 0.5 \text{ml})\) added at the arrow caused rapid clumping of platelets (curve a) while the succinylated lectin (50–200 \(\mu\text{g} \)), under the same conditions, had no effect (curve c). In curve b, platelets were first incubated for 10 min with succinylated wheat germ agglutinin \((85 \mu\text{g})\), and then native wheat germ agglutinin \((30 \mu\text{g})\) was added. The succinylated wheat germ agglutinin did not affect platelet aggregation by the native lectin. The results were the same when the platelets were in plasma. (B) Lack of effect of succinylated wheat germ agglutinin on platelet aggregation caused by ristocetin. Curve a shows the control platelet aggregation pattern induced by ristocetin \((225 \mu\text{g} / 0.5 \text{ml})\). In the experiment shown in curve b, platelets were first incubated with 85 \(\mu\text{g}\) of the succinylated wheat germ agglutinin and then the same amount of ristocetin was added.

net lectin-binding curve showed saturation kinetics, and the nature of the curve was similar to the binding of other lectins to platelets and other cells.\(^9\,23\) These data were further analyzed as double reciprocals, when a straight line was obtained (Fig. 4B). There were about \(2.8 \times 10^5\) binding sites/platelet for succinylated WGA, with an apparent dissociation constant of 2 \(\mu\text{M}\). Since double reciprocal plots tend to minimize deviations from linearity, results of two experiments were also plotted according to Scatchard. There was some indication of nonlinearity in this plot (data not shown). The number of high affinity binding sites calculated from the initial part of the curve was about \(1.4 \times 10^5\) /platelet, with an apparent dissociation constant of 0.8 \(\mu\text{M}\). It is known that unmodified WGA binds tightly to platelets, and the results of these studies have been reported.\(^9\,12\) Analysis of the binding data showed that there are approximately \(6 \times 10^5\) WGA binding sites/platelet, with an apparent dissociation constant of 0.4 \(\mu\text{M}\). Thus, compared to the native lectin, succinylated WGA binds to fewer sites on platelets with a lower affinity.

Aggregation Studies
Platelets were strongly aggregated by small amounts of WGA. Usually, 30–50 \(\mu\text{g} / \text{ml}\) of WGA induced about 60% aggregation in 2 min. Aggregation of platelets in plasma by WGA was also accompanied by secretion of serotonin.\(^9\,11\) In contrast, succinylated WGA, even at a much higher concentration, did not cause aggregation of platelets in 10 min (Fig. 5A). Succinylated WGA did not have any effect on platelet aggregation caused by the unmodified WGA (Fig. 5A), suggesting that the succinylated derivative does not compete with the native lectin for binding to the productive sites on platelets. Succinylated WGA also did not affect platelet aggregation induced by ristocetin (Fig. 5B). Thus, it is unlikely that succinylated WGA binds to any significant extent to the glycoprotein-one complex of platelets.\(^9\,24\,25\)

Studies With Neuraminidase
The above studies with succinylated WGA suggested that sialic acid may play an important role in the activation of platelets by WGA. We explored this
possibility further by studying the interaction characteristics of WGA with platelets treated with different concentrations of neuraminidase. The neuraminidase-treated platelets had a significantly lower capacity to bind WGA (Fig. 6). Approximately half of the lectin-binding sites were lost when the platelets were treated with 5 U/ml of neuraminidase, without any significant alteration in the affinity of binding. In contrast, the ability of platelets to bind thrombin is not affected by prior treatment with neuraminidase. Concomitant with the loss in the lectin-binding capacity, the neuraminidase-treated platelets also had markedly reduced ability to secrete serotonin and to aggregate in response to WGA (Figs. 7 and 8). Aggregation of the neuraminidase-treated platelets induced by several other agents was not reduced; in fact, the aggregation rate was slightly higher than the control, ruling out the possibility of any nonspecific alteration of the platelets due to the presence of a contaminating protease in the neuraminidase preparation. The data obtained (Fig. 7) indicate that the secretion of serotonin from platelets by WGA is particularly sensitive to the presence of the surface sialic acid.

**DISCUSSION**

Wheat germ agglutinin is being increasingly used for the structure-function studies of cell surface glycoproteins and glycolipids. It has been shown that WGA can bind to N-acetylglucosamine, N-acetylgalactosamine, and sialic acid, although what structure is precisely recognized by the lectin on the cell surface is not clear. Several studies have indicated that neuraminidase treatment of cells altered their binding of WGA and suggested that sialic acid residues form a part of the WGA receptor structure on these cells.

Proton magnetic resonance studies indicated a $k_{\text{rec}}$ of 238 M$^{-1}$ for N-acetylglucosamine and 560 M$^{-1}$ for N-acetylnuraminic acid. Succinylated WGA is an acidic protein with an isoelectric point (pI) of 4.0, while the native lectin is basic, with a pI of 8.5. Both the native lectin as well as its derivative have a dimeric structure at the physiologic pH, with a molecular weight of about 36,000. Succinylated WGA and native WGA agglutinate red cells from humans and rabbits, and murine thymocytes and splenocytes in a similar manner. However, horse red cells are agglutinated by native WGA but not by succinylated WGA. Similarly, the binding of WGA but not succinylated WGA to BHK 21C13 cells is drastically reduced by prior neuraminidase treatment of the cells. These observa-

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**Fig. 6.** Effect of increasing concentration of neuraminidase on the binding of labeled wheat germ agglutinin (1 µg/ml, 6 × 10$^6$ cpm/µg) to fresh platelets. Platelets (2–3 × 10$^7$/ml), control or treated with neuraminidase, were incubated with a constant amount of the lectin for 15 min at room temperature, and the amount of lectin bound was determined by oil centrifugation.

**Fig. 7.** Effect of neuraminidase treatment of platelets on the secretion of serotonin induced by wheat germ agglutinin. Platelets in plasma were loaded with $^{3}$H-serotonin, washed, and treated with different concentrations of neuraminidase. After washing, the platelets were resuspended in platelet-poor plasma, and the serotonin secretion was measured after the addition of a constant amount of wheat germ agglutinin (50 µg/ml).

**Fig. 8.** Effect of neuraminidase treatment on the aggregation of platelets (3 × 10$^7$/ml) by wheat germ agglutinin (50 µg/ml). This experiment was carried out as described in the legend for Fig. 7, except that the platelets were finally resuspended in phosphate-buffered saline. Prior treatment of platelets led to reduced aggregation of the cells by the lectin.
tions have led to the suggestion that succinylated WGA does not bind to glycoconjugates containing a significant amount of N-acetylneuraminic acid. Platelets are rich in sialic acid, and a large part of the surface sialic acid is located on the glycoprotein-one complex, which preferentially binds WGA in intact platelets. Succinylated WGA neither activated platelets nor blocked platelet aggregation by native WGA. Thus, it appears that a sialoglycoprotein acts as a biologic receptor for WGA. The glycoprotein-one complex is an attractive candidate as the WGA receptor that mediates platelet activation by the lectin, although the present study does not rule out the possible involvement of any other sialoglycoprotein in this process. Since succinylated WGA did not affect platelet aggregation either by native WGA or by ristocetin (plus vWF), it is unlikely that it binds to any appreciable extent to the glycoprotein-one complex. The present results do not exclude the possibility of native WGA also binding to the receptor of succinylated WGA on the platelet. However, this receptor is likely to be biologically nonproductive, because succinylated WGA under no conditions aggregated platelets or induced secretion of serotonin.

We have previously reported the development of a nonagglutinating lectin derivative that was prepared by cyanogen bromide treatment of WGA at acid pH. This derivative blocked platelet activation by thrombin, while aggregation induced by several other agents was not significantly affected. Then, utilizing this derivative as an affinity probe, we isolated a multiprotein complex that appears to modulate the action of thrombin on platelets. The succinylated WGA reported in this study neither caused platelet agglutination nor blocked platelet agglutination by WGA. It seems that the succinylated WGA does not compete with WGA for a biologically productive site on platelets. In contrast, succinylated concanavalin-A (Con-A) is thought to bind to the same cell receptor as native Con-A. The succinylated WGA, like the cyanogenbromide-derivatized WGA, may be a useful tool to explore the initial events in platelet activation by physiologic agents. For example, preliminary studies in this laboratory show that succinylated WGA is an inhibitor of platelet aggregation by thrombin.

Platelets are known to be rich in sialic acid. Approximately 50% of the sialic acid may be removed with neuraminidase and appears to be located on the cell surface. A large part of this sialic acid is in the glycoprotein-one complex on the platelet membrane. Human platelets survive for about 1 wk to 10 days in circulation. Although the precise nature of the changes that cause removal of the old platelets remains unclear, it is known that platelets treated with neuraminidase are rapidly cleared from circulation. Similarly, platelets from Bernard-Soulier patients, which are deficient in glycoprotein-one and sialic acid, have a shortened survival time. Thus, it is likely that the surface sialic acid is one of the factors that dictates the survival of platelets in circulation. It has been suggested that the shortened platelet survival observed in different pathologic conditions may be due to the removal of sialopeptides from the glycoprotein-one complex by the action of plasmin. Platelet aggregation studies under these conditions with conventional agents have led to ambiguous results. The data presented in this article suggest that surface sialic acid is an important factor in determining the degree of activation of platelets by WGA. Wheat germ agglutinin might be used as a sensitive probe of platelet function in those conditions in which platelet survival is altered, possibly due to a modification of surface glycoproteins. The extent of aggregation and secretion of the platelets under these conditions, compared to normals, may provide a rapid estimation of the sialic acid present on the surface of these cells.

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