

Reduced Thrombin Binding and Aggregation in Bernard-Soulier Platelets

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ABSTRACT Platelets from two patients with Bernard-Soulier disease showed a reduction in their ability to bind human thrombin. Thrombin binding studies in the high affinity range showed 1,500 sites for the Bernard-Soulier platelets as against 4,000 for normal controls. However, the dissociation constant was the same for both normals and patients (4.4 nM) indicating identical affinity for thrombin at the available sites. In the low affinity range, the Bernard-Soulier platelets showed 8,800 thrombin binding sites as against 24,000 for the controls, but again with identical values for K_d (37 nM). In addition, platelets from these Bernard-Soulier patients showed a decreased rate of aggregation with thrombin at both optimal (300 mU/ml) and suboptimal (60 and 120 mU/ml) thrombin concentrations. The decreased amount of thrombin which can bind to Bernard-Soulier platelets and the decrease in thrombin-induced aggregation may partly explain the hemostatic defect in these patients. In addition, the identical ratios of high affinity and low affinity binding sites in normals and in patients (0.37 and 0.36, respectively) supports the idea of a single class of binding sites for thrombin on the platelet surface.

INTRODUCTION

Platelets from patients with Bernard-Soulier Disease (Hereditary Giant Platelet Syndrome) have been reported to lack a membrane glycoprotein (glyco-

protein I) of mol wt 150,000 (1, 2). Other studies, directed toward an understanding of the topomolecular anatomy of the platelet, have shown that this high molecular weight glycoprotein exists in two forms, one of which remains bound to the membrane after platelet homogenization whereas the other is released from the platelet surface and appears in the soluble fraction (3). This soluble glycoprotein, which has been termed glycocalicin because of its origin in the platelet glycocalyx, has been purified to homogeneity (4). It has been shown to inhibit platelet aggregation induced both by thrombin and by ristocetin (5) and to be a competitive inhibitor of the binding of thrombin to the platelet surface (6). Because Bernard-Soulier platelets do not aggregate with ristocetin (7), these observations have led to the suggestion (5) that they might also show a deficient response to thrombin. The results reported here support this suggestion in the two patients examined.

The nature of the interaction between platelets and thrombin has also been the subject of extensive investigations (8–10). These studies have suggested that there are ~500 thrombin receptor sites of high affinity (K_d 2 nM) on the platelet surface and about 30,000 sites of low affinity (K_d 30 nM). Evidence has been presented indicating that the sites are of a single molecular class which exhibits negative cooperativity (11) or, alternatively, that the sites are of different molecular species (12).

In this paper we report on deficiencies in thrombin binding at both the high and low affinity sites and in thrombin-induced aggregation in two patients with Bernard-Soulier syndrome.

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METHODS

Clinical and laboratory data on the two patients examined in this study have previously been reported by others (13). Briefly, the two patients are first cousins, one (A. H.) is a 14-yr-old female, the other (T. H.) is a 17-yr-old male who manifests more active symptoms of his disease. More limited studies were carried out on this patient, as detailed in the text, due to the undesirability of repeated phlebotomy.

Because difficulties have previously been encountered in the isolation of homogenous platelet preparations from Bernard-Soulier patients, the following procedure was developed: blood was collected in one part of 3.8% sodium citrate to nine parts of whole blood and allowed to sediment at 1 g. After ~4 h, supernatant platelet-rich plasma (PRP)¹ (40 ml) was removed and diluted with an equal volume of Hanks' buffered saline solution (without Ca⁺⁺ or Mg⁺⁺). Aliquots (20 ml) of this diluted suspension were then layered onto 15 ml of Ficoll-diatrizoate lymphocyte separation medium (LSM, Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, Md.) and centrifuged at 800 g for 15 min in a swing-out rotor. Contaminating erythrocytes and granulocytes sedimented to the bottom of the tube whereas the platelets and lymphocytes remained as a narrow band at the interface. This layer was removed by aspiration and diluted with Phillips buffer (14) (20 ml) and centrifuged at 800 g for 5 min to sediment the lymphocytes. The supernatant suspension was then centrifuged at 2,400 rpm for 15 min to sediment the platelets which were then resuspended in Phillips buffer. A differential Wright stain showed that platelets comprised 90% of the cells present. The yield was 10⁹ cells from 40 ml of PRP. The platelet suspension was diluted to a final concentration of 2 × 10⁹/ml. Normal control platelets were prepared in the same way and resuspended to the same concentrations.

Platelet aggregation was measured in an aggregometer (Chrono-Log Corp., Havertown, Pa.) using purified human thrombin (15) with an activity of 2,800 NIH U/mg.

¹²⁵I-Labeled thrombin was prepared by the chloramine-T method (16), and its binding to platelets was measured by the filtration technique (8) with a Millipore RAWP filter (Millipore Corp., Bedford, Mass.) and cacodylate buffer which gives an ~10fold increase in the amount of thrombin bound in the high affinity range (17).

RESULTS

Binding of thrombin. Platelets from both patients with Bernard-Soulier symptoms showed a reduced ability to bind thrombin in the high affinity range (10–60 mU) (Fig. 1a). In the case of patient A. H. the binding studies were carried out on two separate occasions whereas with patient T. H. the available sample was sufficient to carry out only one set of binding studies. However, excellent agreement was obtained in the degree of binding from the two patients in the high affinity range (Fig. 1a open and closed circles, respectively). It should be noted that the binding studies on the two patients were carried out on separate days with two different controls as represented by the open and closed squares. We have

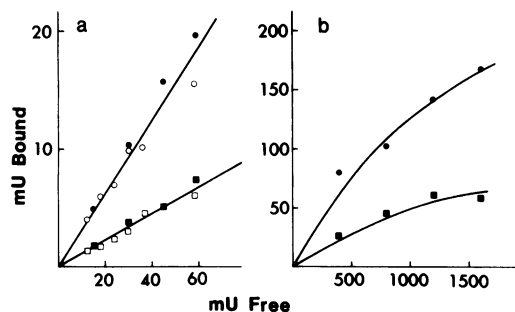


FIGURE 1 Binding of ¹²⁵I-thrombin to washed platelets. (a) High affinity binding. Filled squares and open squares are patients A. H. and T. H., respectively (13). The experiments on the two patients were run on different days and the open and closed circles represent the corresponding controls run on these 2 days. (b) Low affinity binding.

had generally good agreement in values obtained on different normal controls run on different days by this technique.

These data were converted to double reciprocal plots according to the method of Steck and Wallach (18). The intercept on the ordinate indicated 1,500 high affinity binding sites in the case of the platelets from the Bernard-Soulier patients as against 4,000 sites per platelet for the normal controls (Fig. 2). The lines intersected at the abscissa, indicating that the affinity of binding at the available high affinity sites was equal in both the normal and Bernard-Soulier platelets, and a value for K_d of 4.4 nM was calculated.

In the case of patient A. H. sufficient sample was available to examine the binding in the low affinity range (400–1,100 mU) on one occasion (Fig. 1b). This also indicated a decreased ability to bind thrombin and, when calculated from the double reciprocal plots (Fig. 2b), the Bernard-Soulier platelets appear to contain 8,800 low affinity sites per platelet compared to 24,000 sites in the normal control. Again the binding at the low affinity sites appears to be identical in both the Bernard-Soulier patient examined and in the control with a value for K_d of 37 nM in each case.

Thrombin-induced aggregation. Studies were carried out on the thrombin-induced aggregation of both platelets in plasma and of washed platelets in patient T. H. where adequate sample was available.

In the case of PRP from this patient, platelet aggregation was completely absent in the range 120–200 mU/ml although aggregation of normal PRP appeared to reach a maximum in this range (Fig. 3).

To differentiate between the direct effects of thrombin on the platelet and its effects on plasma, washed platelets were prepared as described in Methods on two separate occasions from patient A. H. who was in a clinically stable condition. Maximum aggregation of normal washed platelets prepared by this method was

¹Abbreviation used in this paper: PRP, platelet-rich plasma.

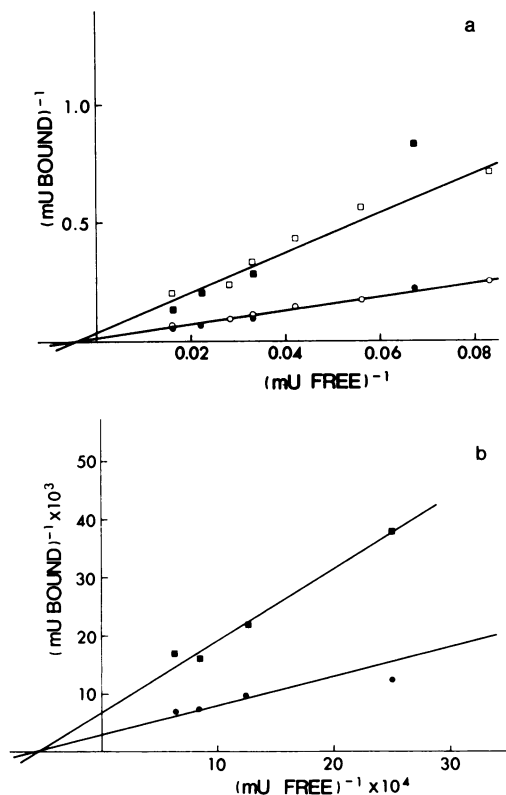


FIGURE 2 Double reciprocal plots of thrombin binding data from Fig. 1. (a) High affinity range; (b) low affinity range. The symbols are the same as in Fig. 1.

obtained at a final thrombin concentration of 300 mU/ml. At this concentration washed platelets from the Bernard-Soulier patient showed a reduced initial rate with a perceptible lag phase although the final extent of aggregation was about the same. The reduced initial rate of thrombin-induced aggregation of Bernard-Soulier platelets as compared to normal controls was considerably more noticeable at suboptimal concentrations of 60 and 120 mU/ml. Typical aggregation tracings at these three concentrations of thrombin in this patient are shown in Fig. 4.

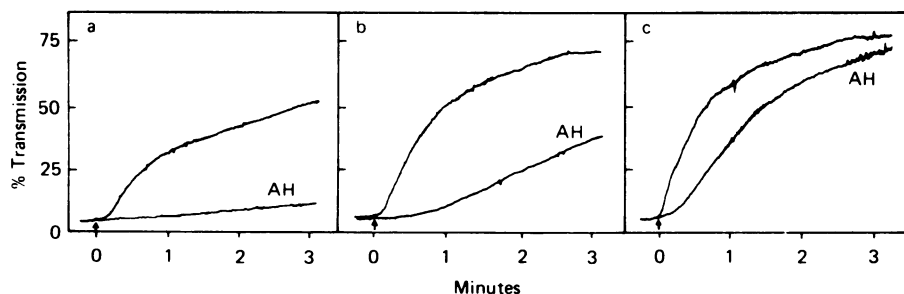


FIGURE 4 Aggregation of washed platelets (patient A. H.) at various thrombin concentrations: (a) 60 mU/ml; (b) 120 mU/ml; (c) 300 mU/ml.

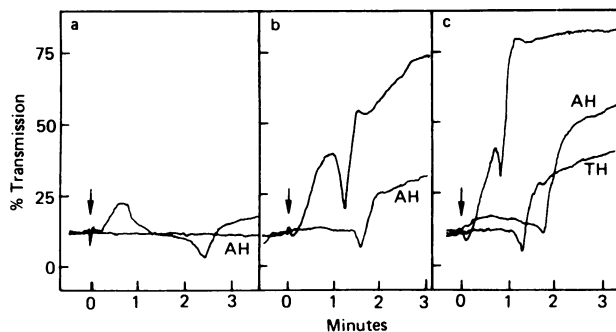


FIGURE 3 Decreased aggregation of platelets from Bernard-Soulier patients in PRP. Thrombin concentration (a) 120 mU/ml; (b) 160 mU/ml; (c) 200 mU/ml. The individual tracings are marked with the patients' initials; the unmarked tracings are normal controls.

In the case of patient T. H. only a single series of aggregation studies was carried out because of the small amount of sample available and PRP was used which had been prepared by sedimentation at 1 g. In this case also there was a markedly reduced aggregation of the platelets to thrombin (Fig. 3).

DISCUSSION

Platelets from patients with Bernard-Soulier disease, or hereditary giant platelet syndrome, not only show structural abnormalities but have also shown to be deficient in surface sialic acid (19), to lack one or more surface glycoproteins (1, 2) and surface antigens (20), and to be deficient in bound Factor XI (13).

The present work shows a further defect that can be added to this functionally complex condition; namely, the decrease in the ability of Bernard-Soulier platelets to bind thrombin and their decreased rate of aggregation in the presence of thrombin. These results confirm the previous suggestion (5) that the loss of glycoprotein I or other surface changes in Bernard-Soulier platelets, which cause a loss of susceptibility to ristocetin-induced aggregation, might also result in a decreased responsiveness to thrombin, because both

of these aggregating agents appear to react with platelets at the same surface receptor. In this respect, it may be noted that in another study platelets from these two patients were shown to have a decreased ability to bind Factor VIII (13). The affinity of the available sites for thrombin in Bernard-Soulier platelets is identical to that of normals in both the high and low affinity ranges, 4.4 and 37 nM, respectively. However, Bernard-Soulier platelets have ~one-third of the number of thrombin binding sites of normal platelets in both the high affinity and low affinity ranges, the values being 0.37 and 0.36, respectively.

Platelets from these two patients have a diameter of about 5 μ m (13) as against about 2 μ m for controls. Assuming a spherical shape, the surface area of the Bernard-Soulier platelets is about five times that of normal platelets, so that the actual density of thrombin binding sites on the surface will be only ~1/5th of that of controls.

The identity of the ratios between high affinity and low affinity sites would support the view (10) that there is a single class of receptors for thrombin on the platelet surface which exhibit negative cooperativity. In other experiments² we have found that the glycoprotein of mol wt 150,000 is not, in fact, absent in Bernard-Soulier platelets but is present in one-third of the amount in normal platelets. Thus the ratio of both high and low affinity sites is not only identical, but corresponds to the amount of putative surface receptor available.

This lack of responsiveness of the platelets to thrombin, together with their decreased adhesion to subendothelium (21), may be one of the factors in the deficient hemostasis observed in patients with Bernard-Soulier disease.

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² Okumura et al. Manuscript in preparation.