THE EFFECTS OF THROMBIN ON PHYTOHEMAGGLUTININ RECEPTOR SITES IN HUMAN PLATELETS

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ABSTRACT

We have previously demonstrated that lentil phytohemagglutinin (lentil-PHA) binds to human platelet membranes without causing either aggregation or the release reaction. When platelets are treated with thrombin, there is an increase in lentil-PHA binding suggesting the appearance of new receptor sites on the cell surface. We prepared a lentil-PHA-ferritin conjugate using affinity chromatography which was used to saturate cell surface receptor sites. Studies using this conjugate suggest that thrombin causes a complex change in the platelet surface involving a decrease in the number of lentil-PHA receptor sites on the external platelet surface with a marked increase in sites within the center of the canalicular system. These increased sites may result from fusion of granule membranes with the canalicular membranes during the secretion process. There is no obvious relationship between lentil-PHA receptor sites and intramembranous particles.

INTRODUCTION

We previously have demonstrated that a variety of phytohemagglutinins bind to the surface membranes of intact human platelets (Majerus and Brodie, 1972). When the phytohemagglutinins from Phaseolus vulgaris erythroagglutinating phytohemagglutinins (E-PHA) and leukoagglutinating phytohemagglutinin (L-PHA) bind to platelets, the cells are aggregated, adenylate cyclase is inhibited, and the release reaction occurs. These compounds thus mimic, in part, the action of thrombin and other agents which cause platelet aggregation and release (Majerus and Brodie, 1972; Brodie et al., 1972; Tollefsen et al., 1974). In contrast the phytohemagglutinin from Lens culinaris (lentil phytohemagglutinin [lentil-PHA]) binds tightly to platelets but does not cause either aggregation of the cells or the release reaction. Thrombin induces platelet aggregation, adenylate cyclase inhibition, and the release reaction even when platelets are saturated with lentil-PHA. After incubation of platelets with thrombin there is an apparent increase in the number of receptor sites on platelets for lentil-PHA. Whether this increased lentil-PHA binding results from uniform exposure of new receptor sites over the platelet surface, or whether new sites appear in localized areas of the platelet surface is undetermined. Thus, new binding sites might appear due to formation of new surface membrane caused by incorporation of platelet granule membranes.
into the cell surface as a part of granule discharge and release. We herein report experiments using a lentil-PHA-ferritin conjugate to directly visualize and quantitate the lentil-PHA binding sites on the platelet membrane. Both freeze-etch and thin-section electron microscopy were utilized to detect changes in membrane binding before and after thrombin exposure. These experiments suggest that thrombin causes a complex alteration in surface binding sites for lentil-PHA involving a decrease in the number of receptor sites on the external cell surface with a coincident marked increase in lentil-PHA receptors in the central area of the canalicular system where granule discharge and release occur.

MATERIALS AND METHODS

Human platelets were collected, isolated, washed, and enumerated as described previously (Tollefsen et al., 1974). All experiments were carried out within 3 h of initial blood collection. Lentil-PHA was isolated from dried lentils (Howard et al., 1971) as modified by Kornfeld et al. (1971). This purification results in the isolation of two phytohemagglutinins which are identical in terms of both erythrocyte binding and immunogenicity for lymphocytes. Lentil-B (Howard et al., 1971) was used for all studies reported in this paper. The concentration of lentil-PHA was estimated by measuring absorbance at 280 nm assuming that 1.0 absorbance unit = 1 mg/ml. Horse spleen ferritin (recrystallized six times) was purchased from Miles Laboratories, Inc., Elkhart, Ind. The iron content of the preparation used in these experiments was 28% by weight. Thus, the ferritin concentration was estimated by measuring absorbance at 340 nm assuming that 5.85 absorbance units corresponds to 1 mg/ml apoferritin (mol wt 460,000). Bovine thrombin was purified from topical thrombin obtained from Parke, Davis and Company, Detroit, Mich. (Glover and Shaw, 1971). The final preparation was homogeneous, based on sodium dodecyl sulfate gel electrophoresis in some instances, although some preparations contained two major protein bands thought to be due to autolysis (Mann et al., 1973). The clotting activity of the thrombin was 2,000-2,500 NIH units/mg protein.

Conjugation of Lentil-PHA to Ferritin

The initial ferritin solution (~70 mg apoferritin/ml) was centrifuged at 12,000 g for 10 min to remove aggregated protein. The ferritin was then pelleted by centrifugation at 50,000 rpm in a Spincno ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 150 min. The ferritin was resuspended in 0.001 M potassium phosphate pH 6.1. [125I]Lentil-PHA was initially dialyzed overnight against 0.001 M potassium phosphate buffer pH 6.1 containing 1.0 M mannose. This [125I]lentil-PHA (3.0 µmol in 10 ml) was added to ferritin (3.0 µmol in 13.0 ml). The pH of the mixture was then adjusted to 6.1 using 1.0 N HCl (~0.08 ml), and 0.15 ml of 8% glutaraldehyde was then added. After mixing for 1 h at 25°C the coupling reaction was stopped by addition of 31 ml of 1.0 M glycine. After centrifugation at 12,000 g for 10 min to remove large ferritin aggregates, the supernatant solution contained 2.5 µmol lentil-PHA and 2.1 µmol ferritin. This solution was next dialyzed for 2 h against 0.015 M potassium phosphate pH 7.5 followed by centrifugation at 50,000 rpm (160,000 g) for 120 min. The supernate containing unconjugated lentil-PHA was discarded and the bilayered pellet was resuspended in 25 ml of the same phosphate buffer. After dialysis overnight against the same buffer, the solution contained 1.5 µmol ferritin and 1.7 µmol lentil-PHA.

Fractionation of Lentil-PHA-Ferritin

Conjugate Using Affinity Chromatography

IgG glycopeptide was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) through its single free amino group. Sepharose 4B was activated with 250 mg/ml cyanoegen bromide (Cuatrecassas, 1970) and 10 ml of the activated packed Sepharose were added to 10 µmol of IgG glycopeptide in 6 ml of H2O. After mixing overnight at 4°C the IgG Sepharose was washed extensively on a Büchner funnel with 0.1 M glycine-NaOH buffer pH 10.0. The coupling yield was 4.0 µmol of bound glycopeptide. The IgG Sepharose was then packed into a 1.5-× 6.0-cm column and equilibrated with 0.015 M potassium phosphate pH 7.5. The lentil-PHA-ferritin solution was diluted to 500 ml in the same buffer and was then poured over the column and, after washing with 10 column volumes of the same buffer, the column was eluted with 100 ml of the same buffer containing 1.0 M NaCl. After this, the column was eluted with 300-ml linear gradient of 0-0.5 M mannose in this same buffer. The column was next eluted with 2.0 M mannose in the same buffer. Fractions were assayed for lentil-PHA by counting 125I in an auto-
gamma counter and for ferritin by measuring absorbance at 340 nm. The elution profile of this column is shown in Fig. 1. A small amount of the residual conjugate remained adsorbed to the column even after elution with 2 M mannose, and this material was eluted with 7.5 M guanidine HCl and discarded without further examination. The lentil-PHA-ferritin conjugates from this column were concentrated by centrifugation and stored at 4°C under a toluene atmosphere.

Binding curves to platelets using lentil-PHA and lentil-PHA-ferritin conjugates were carried out as described previously (Majerus and Brodie, 1972). For binding studies Tris-buffered saline (0.0154 M Tris-HCl pH 7.4, 0.14 M NaCl containing 1 mg/ml glucose) was used while in studies using electron microscopy phosphate-buffered saline containing 0.5% albumin was used (Tollefsen, et al., 1974).

Electron Microscopy Methods

In experiments performed to visualize lentil-PHA-ferritin 1-2 × 10⁸ platelets were incubated with lentil-PHA-ferritin (pool C) (15 × 10⁻⁷ M) in 1 ml of phosphate-buffered saline at room temperature for 30 min; when indicated, thrombin was added (2 U/ml). After incubation the samples were diluted 20-fold with cold phosphate-buffered saline and then fixed with 1% glutaraldehyde for 5 min. Subsequent processing is outlined below. In preliminary experiments we determined that ferritin was not coupled to platelets under these fixation conditions.

Freeze-Etching Procedure

Unfixed platelets were prepared by adding glycerol in small portions over a period of 45 min to platelets in phosphate-buffered saline, pH 7.4, until the concentration of glycerol reached 25%, and incubation was continued for an additional 30 min. Fixed platelets were prepared by treating platelets with 1% glutaraldehyde for 5 min, washing three times with phosphate-buffered saline, and resuspending in 1% glutaraldehyde for 5 min. Subsequent processing is outlined below. In preliminary experiments we determined that ferritin was not coupled to platelets under these fixation conditions.

Preparation of Thin Sections

Platelets were fixed in 3% glutaraldehyde in phosphate-buffered saline, pH 7.4, for 5 min, washed three times in phosphate-buffered saline, and post-fixed in 2% osmium tetroxide for 1 h. They were dehydrated and embedded in Epon-Araldite mixture and sections were stained with uranyl acetate and lead citrate.

Preparation and Characterization of Lentil-PHA-Ferritin Conjugates

Ideally a probe used for electron microscope correlation of lentil-PHA binding to the platelet cell surface should be homogeneous and functionally identical to the original lentil-PHA. This ideal was not achieved in the present study since the final product used remained functionally heterogeneous despite extensive efforts to produce a homogeneous conjugate. The coupling reaction was carried out at high protein concentration and at a pH intermediate between the pK of ferritin (pH 4.5) and that of lentil-PHA (pH 7.4) in order to minimize intramolecular cross-links and linkages of ferritin-ferritin and lentil-PHA-lentil-PHA. In addition, mannose was added since this sugar is a haptenic ligand for the lentil-PHA receptor site and thus might decrease coupling through the lentil-PHA binding site. In addition, short periods of coupling at low glutaraldehyde concentrations were used to produce minimally acceptable coupling yields. In an attempt to isolate a homogeneous conjugate of high affinity we carried out affinity chromatography on IgG Sepharose. The IgG glycopeptide has been shown previously to have a high affinity for lentil-PHA (Majerus and Brodie, 1972; Kornfeld et al., 1971 a). The lentil-PHA-ferritin conjugates were eluted from the column using solutions of mannose as shown in Table I. The three species of conjugates eluted were pooled separately and characterized as shown in Table I. While the affinities of all three conjugated products were clearly decreased compared to native lentil-PHA, it was possible to saturate the cells at high concentrations of conjugate using pools A and C. However, these pools were them-
Fractions representing the peaks labeled in Fig. 1 were pooled and concentrated by ultracentrifugation as described in Materials and Methods. The ratio of ferritin to lentil-PHA was estimated based on extinction coefficients for ferritin of 5.85/mg at 340 nm and on [\(125\)I]lentil-PHA content. Dissociation constants and numbers of binding sites per platelet were determined as described previously (Majerus and Brodie, 1972).

**TABLE I**

**Characterization of Lentil-PHA Ferritin Conjugate**

| Item | Ratio ferritin | Yield Lentil-PHA-ferritin | \(K_{diss}\) | Sites/platelet |
|------|----------------|--------------------------|-------------|---------------|
| µmol |    | \(10^{-7}\) |     |              |
| Lentil-PHA | — | — | 400,000 |
| Pool A | 1.2 | 0.09 | 5 \(\times 10^{-7}\) | 400,000 |
| Pool B | 1.1 | 0.06 | 4.8 \(\times 10^{-7}\) | 275,000 |
| Pool C | 0.8 | 0.22 | 2.2 \(\times 10^{-7}\) | 400,000 |

Fractions representing the peaks labeled in Fig. 1 were pooled and concentrated by ultracentrifugation as described in Materials and Methods. The ratio of ferritin to lentil-PHA was estimated based on extinction coefficients for ferritin of 3.83/mg at 340 nm and on [\(125\)I]lentil-PHA content. Dissociation constants and numbers of binding sites per platelet were determined as described previously (Majerus and Brodie, 1972).

TABLE II

**Binding of Lentil-PHA-Ferritin to Erythrocytes**

| Incubation | Lentil-PHA bound | Lentil-PHA-ferritin bound |
|------------|------------------|--------------------------|
|            | %                | %                        |
| 1          | 23.5             | 5.7                      |
| 2          | 25.8             | 3.8                      |
| 3          | 24.7             | 2.9                      |
| 4          | 26.4             | 2.2                      |

Initial reaction mixtures contained 0.2 ml of a 10% suspension of erythrocytes, 5.2 nmol of [\(125\)I]-lentil-PHA or [\(125\)I]lentil-PHA-ferritin (pool A), (325,000 cpm/nmol) in a final volume of 1 ml of 0.01 M sodium bicarbonate—0.15 M sodium chloride. After incubation for 30 min at 25°C the erythrocytes were collected by centrifugation and counted in an autogamma scintillation counter, 0.2 ml of fresh 10% erythrocytes was then added to the supernate solution and the process was repeated as indicated. The values reported reflect the percentage of total lentil-PHA bound to erythrocytes based on the amount present at the start of each incubation.

Figure 1  Fractionation of lentil-PHA-ferritin conjugate by affinity chromatography with IgG Sepharose (see Materials and Methods for details). Fractions 1–140 were 5 ml each while subsequent fractions contained 2.5 ml. —, ferritin nanomoles per milliliter; ——, lentil-PHA nanomoles per milliliter.
binds after each incubation even though 70% of the lentil-PHA originally present was removed from solution by the fourth incubation. In contrast, the lentil-PHA-ferritin conjugate was markedly heterogeneous by this test since a progressively decreasing fraction of molecules bound to erythrocytes with each incubation even though 85% of the conjugate remained in solution after the fourth incubation. Similar results were obtained in experiments using pool C. Thus, it is clear that even with affinity chromatography it was not possible to produce a homogeneous conjugate. However, it was possible to saturate platelets at high concentrations of conjugate under conditions where less than 2% of the added conjugate was bound to the platelets. In all of the subsequent experiments reported, pool C was used since it was present in the highest yield. From these preliminary experiments it was clear that a relative correlation between ferritin visualized by electron microscopy and bound lentil-PHA might be obtained but precise quantitation would be impossible.

Evidence that the lentil-PHA-ferritin solution was composed mainly of 1:1 conjugates is presented in Fig. 2. Examination of negatively stained dilutions of these conjugates (pool C) disclosed many single ferritin or apoferritin molecules with occasional apparently aggregated molecules. The appearance of the conjugate was similar to that seen with intact ferritin, and the ratio of ferritin to apoferritin of 3:2 was similar in the conjugate and in native ferritin.

Effect of Thrombin on Binding of Lentil-PHA-Ferritin to Platelets

It is necessary to establish that thrombin treatment of platelets resulted in increased binding of

Figure 2  Lentil-PHA-ferritin negatively stained with 2% potassium phosphotungstate. Iron-containing ferritin molecules have dark centers and apoferritin molecules have light centers. Scale bar, 0.1 μm; × 150,000.

Figure 3  Replica of a freeze-etch gluteraldehyde-fixed platelet. The external surface (ES) is exposed by sublimation of ice and is somewhat irregular in texture. Arrows mark the junction between the external surface and inner fracture face (IF), exposed by cleavage of the membrane. The inner fracture face contains sparse intramembranous particles. Scale bar, 0.2 μm; × 75,000.
the lentil-PHA conjugate similar to results obtained using intact lentil-PHA (Majerus and Brodie, 1972). Results of a typical experiment are shown in Table III. Thus, there was an approximate twofold increase in binding of the conjugate in the presence of thrombin, a result similar to that seen with lentil-PHA. Furthermore, the lentil-PHA-ferritin could be released from the platelet surface by subsequent incubation with the haptenic IgG glycopeptide. Therefore, the increased binding after thrombin reflected increased surface binding of the probe. In eight separate experiments using different preparations of platelets and different amounts and proportions of lentil-PHA-ferritin, there was a consistent increase in binding after thrombin ranging from 1.3- to 2.3-fold (mean 1.8-fold). The increased binding after thrombin was significant as determined by a paired t test \( (P < 0.001) \). We next examined these platelets electron microscopically to determine the localization of the lentil-PHA-ferritin conjugate.

### Ultrastructural Studies

The external surface of the untreated platelet can be visualized in glutaraldehyde-fixed specimens after deep etching of surrounding ice, as shown in Fig. 3. The external surface is coarse in texture and is somewhat reticulated. The membrane still cleaves after glutaraldehyde fixation, showing sparse 75Å intramembranous particles on the inner fracture face.

#### Table III

| Item        | IgG added | Ferritin-lentil bound | Percent released |
|-------------|-----------|-----------------------|------------------|
| Control     | -         | 27                    | -                |
| Control +   | +         | 1.2                   | 96               |
| Thrombin    | -         | 49                    | -                |
| 2 U/ml      | +         | 3.5                   | 93               |

Reaction mixtures contained \( 3 \times 10^7 \) platelets, 168 pmol lentil-PHA-ferritin, thrombin, 2 U/ml, where indicated in a total volume of 0.25 ml saline-phosphate buffer pH 6.5. After incubation for 15 min, IgG glycopeptide was added where indicated and after 15 min the platelets were collected by Millipore filtration (Millipore Corp., Bedford, Mass.) as described in Materials and Methods. The glycopeptide was added to give a final concentration of \( 10^{-4} \) M.

Normal platelets labeled with saturating concentrations of lentil-PHA-ferritin and then fixed and freeze etched have the ferritin conjugate on the external surface of the platelet (Fig. 4). The membrane is densely covered with the ferritin conjugate which is distributed in a slightly reticulated or aggregated pattern. The number of ferritin molecules on the normal platelet surface is approximately 800 ferritin molecules/\( \mu \text{m}^2 \) (Table IV).

Platelets incubated with lentil-PHA-ferritin and then treated with thrombin also have numerous ferritin molecules on the platelet surface after freeze etching (Fig. 5). The conjugate is distributed in a slightly aggregated pattern similar to the nontreated platelets. The number of ferritin molecules on the external surface of the thrombin-treated platelets is decreased to approximately 500 ferritin molecules/\( \mu \text{m}^2 \) (Table IV). This decrease in lentil-PHA-ferritin was significant \( (P < 0.001) \).

Binding of lentil-PHA-ferritin to the internal canalicular system of normal and thrombin-treated platelets could not be evaluated by the freeze-etching technique since the inner surfaces of the canalicular membranes are not exposed by freeze cleaving and deep etching. Therefore, thin section microscopy was used to localize the ferritin conjugate inside the platelet. Platelets contain membrane-bound dense granules in the central cytoplasmic area surrounded and interspersed with the internal canalicular system. Platelets treated with lentil-PHA-ferritin show no apparent morphological change from untreated platelets as a result of treatment with the conjugate (Fig. 6 A). Ferritin is present on the surface membrane and along the inner aspect of the membranes of the canalicular system (Fig. 6 B). The ferritin is distributed in clusters with intervening bare areas. There is some stacking of the ferritin molecules in these clusters, as shown in Fig. 6 C, with ferritin molecules at varying distances from the plane of the membrane.

Treatment with thrombin causes the platelets to undergo striking morphologic change, as shown in Fig. 7. The granules discharge at the center of the platelet, leaving dilated canalicular membranes, amorphous material, and microtubules. Numerous long pseudopods extend from the body of the platelet. Treatment of the platelet with lentil-PHA-ferritin before exposure to thrombin does not interfere with the granule release reaction (Fig. 8 A). The granules discharge,
FIGURE 4 Replica of a freeze-etched normal platelet labeled with lentil-PHA-ferritin. The ferritin conjugate is distributed in a slightly reticulated pattern over the external surface (ES) of the platelet. Scale bar, 0.2 µm; X 93,000.

FIGURE 5 Replica of a freeze-etched thrombin-treated platelet labeled with lentil-PHA-ferritin. The ferritin conjugate is scattered over the external surface (ES) of the platelet. Openings of the canalicular system on the surface membrane are present (arrow). Scale bar, 0.2 µm; X 93,000.
TABLE IV
Density of Lentil-PHA-Ferritin on Platelet Surface

| Additions       | Mean  | SD   | SEM  |
|-----------------|-------|------|------|
| Lentil-PHA-ferritin | 805.7 | 172.3| 25.4 |
| Lentil-PHA-ferritin + thrombin | 528.9 | 99.7 | 16.4 |

Reaction mixtures contained $1.2 \times 10^8$ platelets and $13 \times 10^{-7}$ M lentil-PHA-ferritin per ml. Thrombin, 2 U/ml, was added where indicated. Platelets were fixed in 1% glutaraldehyde for freeze-etch electron microscopy as described in Materials and Methods. Ferritin molecules were counted in 2-cm$^2$ areas at a magnification of $X$ 86,500. In this experiment 10 replicas were examined, five with thrombin and five without. Counts were made on approximately eight platelets per replica. There was no great variation in density of ferritin particles between different replicas within each group.

leaving membranous channels and amorphous material at the center of the platelet. Pseudopodia extend from the body of the platelet; however, this feature is less conspicuous in the lentil-PHA-ferritin-treated platelets than in normal platelets treated with thrombin. A most striking change is observed in the localization of the lentil-PHA-ferritin as a result of thrombin treatment (Fig. 8 A and 8 B). Large amounts of ferritin are present in the center of the platelet in the region of the discharged granules. It appears that the ferritin is located within membranous channels, perhaps as a result of binding of the conjugate to the residual membranes of the discharged granules. The amount of lentil-PHA-ferritin bound to the canaliculal membranes at the periphery of the platelet and to the surface membrane does not appear markedly different from that seen in non-thrombin-treated platelets.

Relation of Lentil Receptor to Intramembranous Particles

The distribution of lentil-PHA-ferritin or the number of molecules bound to normal or thrombin-treated platelets does not appear to correlate with the distribution or number of 75-Å intramembranous particles within the platelet membrane. Inner and outer fracture faces of the surface membrane and membranes of the canaliculal system and granules contain relatively few 75-Å intramembranous particles (Fig. 9). More intramembranous particles are present on the outer fracture face of the membrane than on the inner fracture face. The outer fracture face contains approximately 225-275 particles/µm$^2$ and the inner fracture face contains 100-150 particles/µm$^2$. Thus, the total number of intramembranous particles is 350-400 particles/µm$^2$, and this is to be contrasted with the 800 lentil-PHA-ferritin molecules/µm$^2$ that were counted on the freeze-etched platelet surface. Also, no clear correlation could be made between the reticulated pattern of labeling of the lentil-PHA-ferritin on freeze-etched platelet surfaces and the distribution of intramembranous particles on the fracture faces of the membrane. Thrombin treatment of the platelets did not alter the density or distribution of the intramembranous particles.

DISCUSSION

In previous studies we showed that lentil-PHA was a useful probe for the study of the changes in the platelet surface which occur with thrombin treatment (Majerus and Brodie, 1972). This lectin does not produce the platelet-release reaction itself nor does it prevent the reaction induced by thrombin when platelets are saturated with lentil-PHA. These previous results suggested that thrombin induces appearance of new receptor sites for lentil-PHA on the platelet surface. The current study using the ferritin conjugate indicates that thrombin causes a complex change in the platelet surface, involving a decrease in the number of lentil-PHA receptor sites on the external platelet surface with a marked increase in sites within the center of the canaliculal system. These results may reflect the known cleavage of surface glycopeptide which accompanies the thrombin-induced release reaction (Phillips, 1973; Nachman, 1973). The marked increase in lentil receptors at the center of the cell could reflect exposure of large amounts of granule membranes to the surface of the platelet which could result from central discharge of granule contents plus fusion of the granule membranes to the canaliculal membrane. A similar hypothesis to explain platelet secretion has been proposed previously (White, 1970).

The total surface area of the spongelike platelet remains unknown but studies with lentil-PHA suggest that it may be larger than previous estimates. The finding of 806 lentil-PHA receptor...
Figure 6  Thin sections of normal platelets labeled with lentil-PHA-ferritin. No apparent morphologic change is observed after treatment with the conjugate (Fig. 6 A). Ferritin (arrows) is present along the surface membrane and on the inner aspect of the canalicular membranes (Fig. 6 B). The ferritin is not evenly distributed along the membranes, but appears in clusters (Figs. 6 B and 6 C), and molecules of ferritin are at varying distances from the membrane. Fig. 6 A, scale bar, 1.0 μm; × 32,000. Fig. 6 B, scale bar, 0.2 μm; × 69,000. Fig. 6 C, scale bar, 0.1 μm; × 173,000.
sites/µm² undoubtedly underestimates the true density of sites both because of "stacking" of molecules (Fig. 6 C) within the glycoprotein coat of the membrane and because of the undulating and reticulated pattern of the platelet surface leading to areas where the platinum shadowing gives poor relief. If one assumes that only 50% of the molecules are counted and further correcting for the ratio of ferritin to lentil-PHA of 0.8 in pool D, the number of lentil-PHA sites would be approximately 2,000/µm². The total number of receptors on the platelet surface in control platelets is 300,000-400,000 as measured by direct binding. Using these data we calculate a total surface area of 150-200 µm². This result would suggest that the platelet, with a volume of only 7.5 µm³, has a tremendous internal surface area. Further, the appearance of new surface sites would also reflect a tremendous increase in exposure of new surface membranes, assuming that the density of sites on the membrane remains similar. Thus, using the value of 529 lentil sites/µm² obtained in thrombin-treated cells and the same estimates outlined above, the 150-200 µm² of surface accounts for only 200,000-250,000 lentil-PHA sites per cell. Since the number of lentil receptors after thrombin reaches approximately 500,000, the new receptors internally must account for the increase. The fact that the lentil-PHA (or the lentil-PHA-ferritin conjugate) remains tightly associated with the platelet indicates that the new sites are not associated with released proteins. Further, while the electron micrograph (Fig. 8) might suggest that the conjugate is bound to amorphous debris at the center of the platelet, the fact that it is readily released by the IgG haptene indicates that it is exposed to the medium while remaining tightly associated with the platelet, thus suggesting attachment to surface membrane.

The distribution of the lentil-PHA-ferritin conjugate in clusters on the surface membranes of platelets may reflect the actual arrangement of the receptors on the membrane, since a reticulated texture is seen in the surface membranes of un-

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**Figure 7** Thin section of a thrombin-treated platelet. The granules have discharged leaving membranous material and microtubules with long pseudopods protruding from the surface. Scale bar, 1.0 µm; × 30,000.
Figure 8  Thin sections of platelets treated with lentil-PHA-ferritin and thrombin. The granules have discharged and some pseudopodia protrude from the surface (Fig. 8 A). Large amounts of lentil-PHA-ferritin are present in the region of the discharged granules (large arrows), and most of it appears to be contained within membranous channels (Fig. 8 B). Some ferritin is present on the surface membrane and within peripheral portions of the canalicular system (small arrows). Fig. 8 A, scale bar, 0.2 µm; × 46,000. Fig. 8 B, scale bar, 0.2 µm; × 72,000.
labeled glutaraldehyde-fixed platelets examined by freeze etching. However, this clustering of the marker may result from movement of membrane receptors induced by cross-linking of the lectin receptors by the lentil-PHA before fixation, similar to that seen in lymphocyte membranes labeled with ferritin-conjugate antisera to surface immunoglobulins (de Petris and Raff, 1973) or in transformed protease-treated fibroblast membranes labeled with conjugates of concanavalin A at 37°C (Rosenblith et al., 1973).

The lack of correlation either in number or distribution between lentil-PHA receptor sites and platelet intramembranous particles contrasts with results obtained studying erythrocytes in which it was suggested that these particles represented in part the receptor glycoprotein (Tillack et al., 1972) or blood group A antigen sites on erythrocyte surfaces (Pinto da Silva et al., 1971). It is possible, however, that some of the intramembranous particles bear glycoprotein receptors for lentil-PHA, or that each particle bears several glycoprotein receptor sites; however, we have not been able to demonstrate this relationship in these studies. Further, the distribution and number of these particles contrasts to results seen with either erythrocytes or lymphocytes. Erythrocytes contain approximately a 10-fold greater density of particles compared to platelets while lymphocytes have approximately twice the density of particles as platelets (Scott and Marchesi, 1972). In a variety of cells including erythrocytes and lymphocytes the cleavage of the surface membrane results in uneven distribution of intramembranous particles between the two fracture faces with a two- to three-fold greater number of particles on the inner fracture face, the fracture face nearest to the internal surface of the membrane. In contrast, we find that platelets have more particles on the outer fracture face, the fracture face nearest to the
external surface of the membrane. This distribution is similar to that found in outer mitochondrial membranes (Melnick and Packer, 1971) and sarcoplasmic reticulum (Tillack et al., 1973). This is of interest since the platelet is derived from an intracellular membrane of the megakaryocyte (Behnke, 1968).

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REFERENCES

BEHNKE, O. 1968. An electron microscopic study of megakaryocytes of rat bone marrow. I. Development of the demarkation membrane system and platelet surface coat. J. Ultrastruct. Res. 24:412.

BRODIE, G. N., N. L. BAENZIGER, L. R. CHASE, and P. W. MAJERUS. 1972. The effects of thrombin on adenylyl cyclase activity and a membrane protein from human platelets. J. Clin. Invest. 51:81.

CUATRECASAS, P. 1970. Protein purification by affinity chromatography: Derivatization by agarose and polyacrylamide beads. J. Biol. Chem. 245:3059.

DE PETRIS, S., and M. C. RAFF. 1973. Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. Nat. New Biol. 211:257.

GLOVER, G., and E. SHAW. 1971. The purification of thrombin and isolation of a peptide containing the active center histidine. J. Biol. Chem. 246:4994.

HOWARD, I. K., H. S. SAGE, M. D. SRYN, N. M. YOUNG, M. A. LEON, and D. F. DICKES. 1971. Studies on a phytohemagglutinin from the lentil. II. Multiple forms of Lens culinaris hemagglutinin. J. Biol. Chem. 246:1590.

KORNFIELD, R., J. KELLER, J. BAENZIGER, and S. KORNFIELD. 1971. a. The structure of the glycopeptide of human γG myeloma proteins. J. Biol Chem. 246:3259.