

ULTRASTRUCTURAL LESIONS ON THE SURFACE OF PLATELETS ASSOCIATED WITH EITHER BLOOD COAGULATION OR WITH ANTIBODY-MEDIATED IMMUNE INJURY*

BY MARGARET J. POLLEY‡ AND RALPH L. NACHMAN

(From the Department of Medicine, the New York Hospital-Cornell Medical Center, New York 10021)

Evidence has been presented which implicates the complement (C) system as a potentially important effector of blood coagulation (1). In studies on rabbit whole blood, it was found that agents which promoted blood coagulation by the C-dependent mechanism activated the alternate mechanism of C rather than the classic pathway (2). Further, it was found in experiments with human platelets that zymosan-induced release of serotonin and platelet aggregation were dependent on activation of the C system by the alternate mechanism (3).

It has been known for some time that activation of C on a cell membrane leads to production of ultrastructural lesions on the membrane surface (4). These lesions are approximately 100 Å in diameter surrounded by a white rim 20 Å in thickness. More recently (5) it was shown that activation of the C system by the alternate mechanism in the presence of red cells led to the appearance of ultrastructural lesions on the red cell membrane which were morphologically distinct from those previously described. These lesions were the same shape, however, they were larger, approximately 150 Å in diameter and were surrounded by a white rim 60-80 Å in thickness. These lesions were referred to as type II lesions in order to distinguish them from those previously described (type I).

The purpose of the present communication is to present evidence indicating that during blood coagulation, ultrastructural lesions similar to type II were produced on the platelet membrane. On the other hand, as would be expected, antibody-dependent activation of the C system on the surface of the platelet by antibody directed to a membrane-bound antigen led to the production of ultrastructural lesions of type I.

Materials and Methods

Platelet Suspensions. Platelet-rich plasma (PRP)¹ was prepared from sodium citrate (3.2%) anticoagulated whole blood obtained from normal volunteers. One part anticoagulant was used for nine parts of blood. PRP was prepared by centrifugation at 150 g for 15 min at 37°C in a Sorvall RC-3 centrifuge (Ivan Sorvall, Inc., Newtown, Conn.). The platelet count of the PRP was adjusted to

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

200,000/ μ l with platelet poor plasma. Washed platelets were prepared using the Ardlie buffer system as previously described (6).

Induction of Clot Formation in Platelet Preparations. Three methods were employed. In the first, EDTA was added to a preparation of PRP to a final concentration of 0.01 M. Increasing amounts of a solution of $\text{Ca}^{++}\text{Mg}^{++}$ in saline were added to the EDTA-treated PRP over the range from 1/20 of the EDTA-neutralizing amount to two times the concentration needed to neutralize the chelating effect of the EDTA. 1 ml of the PRP containing EDTA and $\text{Ca}^{++}\text{Mg}^{++}$ at varying concentrations was incubated at 37° for 1 h. Electron microscope studies were performed on preparations in which small clots had formed.

In the second method PRP was centrifuged to remove the platelets. To the platelet-poor plasma thus obtained, EDTA and $\text{Ca}^{++}\text{Mg}^{++}$ were added at concentrations identical to those used in the first method. 1 ml of the EDTA-containing plasma with varying amounts of $\text{Ca}^{++}\text{Mg}^{++}$ was incubated at 37°C for 1 h with 1 ml of a suspension of washed platelets in buffer at a final concentration of 100,000/ μ l. Ultrastructural analysis was performed on the platelets subsequent to this incubation procedure and control studies were performed on the initial platelet suspension which had not been exposed to the EDTA-containing plasma.

In the third method, in order to avoid the use of EDTA and its possible ultrastructural effect on the platelet membrane, 1 ml of washed platelets suspended in buffer at 200,000/ μ l was incubated at 37°C for 1 h with 1 ml of a 1/2 dilution of fresh human serum.

Absorption of Serum with Aluminum Hydroxide. In some experiments, fresh human serum was absorbed with aluminum hydroxide to remove prothrombin and interfere with thrombin generation (7). Initially the absorption of serum with aluminum hydroxide was performed at 0°C, room temperature, and at 37°C. In order to ascertain whether thrombin generation in the serum had been blocked, 0.2 ml of each preparation was incubated at 37°C for 24 h with 0.5 mg fibrinogen. No clot was formed in the mixture containing fibrinogen and serum absorbed with aluminum hydroxide at room temperature or at 37°.

C Assays on Serum Absorbed with Aluminum Hydroxide. The serum which had been absorbed with aluminum hydroxide at room temperature and at 37°C was tested for its C activity by assays designed to measure both the activity of the classic mechanism and alternate mechanism of C activation. Assays were performed to measure whole C and C2 activity by standard hemolytic assays (8, 9). Activity of the alternate mechanism was measured by an assay for hemolysis of glutathione-treated red cells under acid conditions and by inulin-induced lysis of these cells at neutral pH (10-12). Further tests for alternate mechanism activity were assays to measure the electrophoretic conversion of C3 proactivator (C3PA) to the active fragment C3 activator (C3A) and of C3 to its hemolytically inactive form (C3i) (13).

It was found that the absorption of serum with aluminum hydroxide at 37°C had removed 40-60% of the C activity. However, in the serum absorbed at room temperature, no decrease in C level was demonstrable by any of the tests employed. Since serum treated in this manner did not clot when incubated with fibrinogen, this reagent was employed as the source of thrombin-free-C containing serum throughout the present study.

Incubation of Platelets with Thrombin. 1 ml of washed platelets (230,000/ μ l) in buffer was incubated at 37°C for 60 min with 1, 0.1, or 0.01 U of purified human thrombin, kindly supplied by Dr. Peter Harpel, New York Hospital-Cornell Medical Center, New York, (100 U/ml of sterile saline) either alone or subsequent to the addition of 0.5 ml of fresh human serum as a source of C.

Preparation of C3-Depleted Human Serum. The γ -globulin fraction was prepared from a rabbit serum containing monospecific antibody to human C3 by ammonium sulfate (50%) precipitation followed by TEAE-cellulose chromatography utilizing phosphate buffer at pH 8.0, 0.0175 M. This preparation was conjugated to sepharose 4B by the method of Cuatrecasas (14). 1 ml of the anti-C3-conjugated Sepharose was mixed with 1 ml of fresh human serum and allowed to stand at 0°C for 1 h. After centrifugation, the absorbed serum was tested for its content of C3. It was found that approximately 80% of the original C3 contained in the serum had been removed by this procedure.

Platelet-Antiplatelet Interaction. The serum containing antiplatelet antibody was obtained from a woman who had been isoimmunized to one or more antigenic determinants on her husband's platelets. In addition to reacting with her husband's platelets, the antibody was found to react with platelets from all of six individuals tested (M. J. Polley, unpublished observations). No attempts were

made to determine the antigenic specificity of this antibody. 0.5 ml of serum containing the platelet antibody and 0.5 ml fresh human serum as a source of C were incubated at 37°C for 60 min with 0.5 ml of the washed platelet preparation.

Preparation of Platelets for Electron Microscopy. Platelets from each method of treatment were washed once with normal saline and then three times with a 1/10 dilution of saline buffered to pH 6.5. After the first wash in diluted saline, the platelet suspension was frozen at -70°C for 15 min before the washing steps were continued.

The platelet stroma was stained with 2% sodium silicotungstate and applied to grids coated with collodion and carbon. The grids were viewed with a Philips 301 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) at 80 kv.

Results

C-Induced Morphologic Changes in Platelets during Coagulation. When washed platelets which had not been incubated in vitro with plasma or serum were viewed under the electron microscope, no ultrastructural lesions were seen. Similarly, lesions were not seen in platelets after exposure to nonrecalcified EDTA plasma. However, as shown in Fig. 1, ultrastructural lesions were found on the platelet membrane subsequent to incubation with EDTA plasma to which Ca^{++} had been added in an amount sufficient to neutralize the chelating activity of the EDTA resulting in clot formation. These lesions were few in number and were the same shape and size as those produced in red cells by activation of C by the alternate mechanism (type II) (5). The lesions were 150 Å in diameter across the dark central areas surrounded by a white rim approximately 60 Å in thickness (Fig. 1 b). Occasionally a lesion was seen in which the rim was not at a constant thickness all around the lesion but appeared to be much thickened in one area. An example of a lesion of this type is shown in Fig. 1 c.

When washed platelets were incubated with fresh untreated serum, lesions morphologically similar to those produced by the recalcified plasma were produced on the platelet membrane. That these lesions were thrombin dependent was demonstrated in an experiment in which thrombin generation was prevented by absorption of the serum with aluminum hydroxide. When platelets were incubated for 1 h at 37°C with the "thrombin-free" serum and then processed for electron microscopy in the usual way, no ultrastructural lesions were seen (Table I).

In order to ascertain whether thrombin alone would induce morphologic changes in the platelet membrane, washed platelets were incubated in buffer with purified human thrombin and then viewed ultrastructurally. Platelets which had been treated with thrombin alone were morphologically indistinguishable from those that had had no such treatment. However, as was expected, ultrastructural lesions were visible in those platelets which had been incubated with thrombin together with C (provided as aluminum hydroxide absorbed serum).

When platelets were incubated with serum which contained thrombin but was 80% depleted in its C3 content, the number of ultrastructural lesions visualized were much fewer than when serum containing a normal amount of C3 was employed. In fact, it was found necessary to view several areas of platelet membranes in order to observe a single lesion when the serum used for incubation of the platelets had been largely C3 depleted. As shown in Table I only two type II lesions were visualized in 10 areas of platelet membrane, each 0.45 μm^2 .

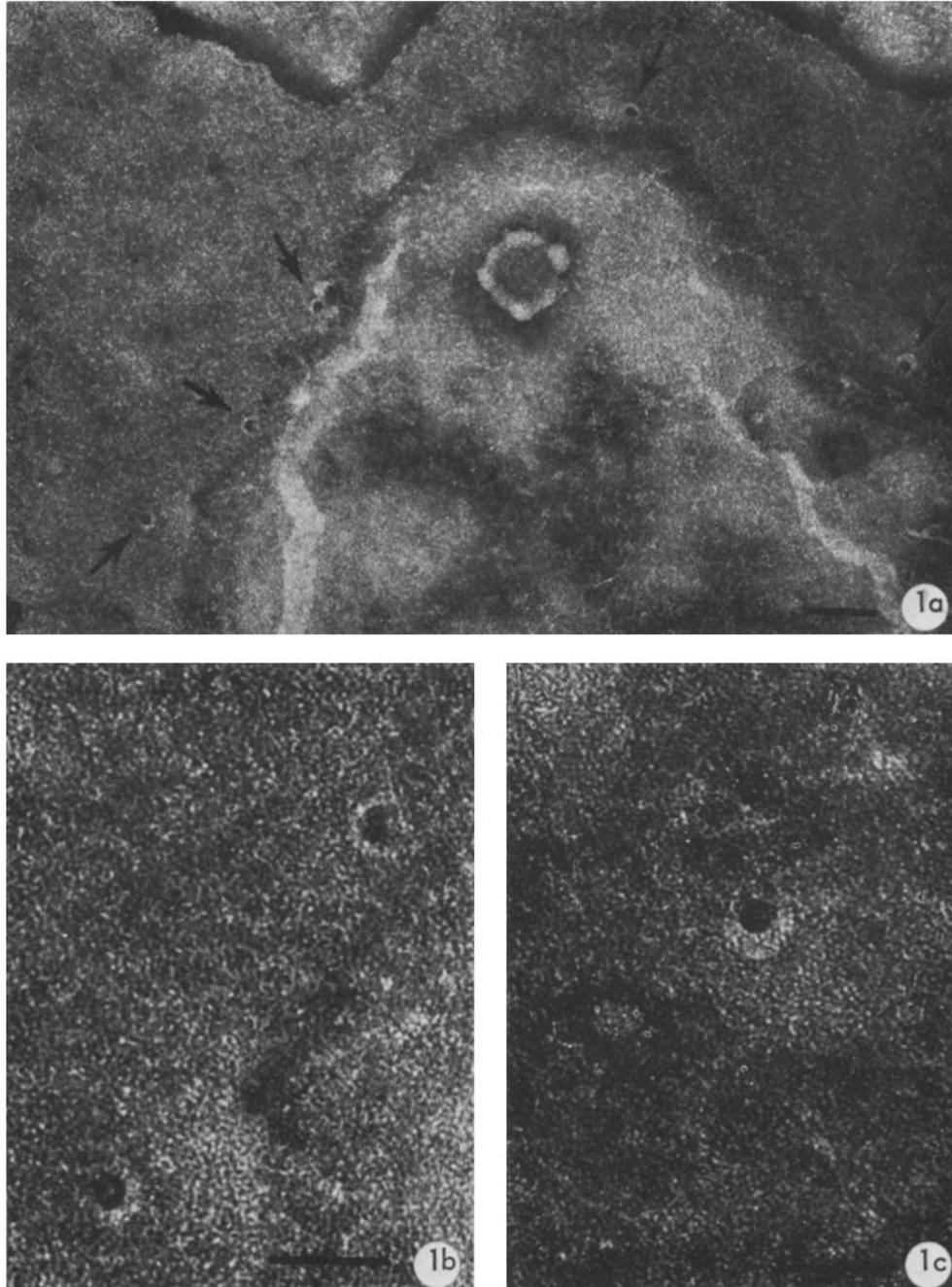


FIG. 1. Platelet membrane subsequent to incubation with EDTA-containing plasma to which Ca^{++} and Mg^{++} had been added (see text). (a) $\times 102,000$. Solid bar represents 1,000 A. Arrows indicate ultrastructural lesions. (b and c) Selected areas of a $\times 336,000$. Solid bars represent 500 A.

TABLE I
*Quantitation of Ultrastructural Lesions in Membranes of Platelets**
Incubated with Various Reagents

Reagent	No. of type II‡ ultrastructural lesions
EDTA-containing plasma	0
EDTA-containing plasma + Ca ⁺⁺ Mg ⁺⁺	21
Serum	17
Thrombin-depleted serum	0
C3-depleted serum	2
Thrombin	0
Thrombin + C	27

* Platelets utilized were a preparation of "washed platelets." For details of this preparation and of all other reagents listed in this table, see Materials and Methods section.

‡ The number of ultrastructural lesions visualized in 10 arbitrarily selected areas of platelet membrane, each approximately 0.45 μm^2 .

C-Induced Ultrastructural Changes in Platelets Sensitized with Antibody. As shown in Fig. 2, ultrastructural lesions of identical morphology to type I seen in red cells were demonstrated on platelets subsequent to their interaction with antiplatelet antibody and C. Since the source of both antibody and C was serum, in addition to type I lesions, some type II lesions were also seen. As is seen in red cells (5) the number of type I lesions was far greater than the number of type II lesions.

Morphological Comparison of Type I and Type II Ultrastructural Lesions seen in Platelets. Fig. 3 demonstrates both visually and graphically the comparative size of type I and type II lesions. Type I lesions were approximately 100 Å in diameter surrounded by a rim 20 Å in width which are similar in size to those lesions produced in red cells by immune lysis (4). In comparison, type II lesions were approximately 150 Å in diameter surrounded by a rim 60 Å in width which are identical in dimension to lesions produced in red cells by activation of the alternate mechanism (5). Table II lists the mean of multiple measurements of both types of lesions and the calculated standard deviations.

Discussion

Most studies of the effect of C on membranes have been performed with red cells. Some years ago (4) it was shown that activation of C produced distinct ultrastructural changes on the membrane surface. More recently (5) it has been shown that the morphology of these ultrastructural changes varies depending on whether C is activated by the classic or by the alternate mechanism.

To date, C-induced ultrastructural changes in the platelet membrane have not been described. In our present studies we have found that during blood coagulation ultrastructural changes appeared on the surface of the platelet.



FIG. 2. Platelet membrane subsequent to incubation with antiplatelet antibody and C. $\times 336,000$. Solid bar represents 500 Å. Arrows indicate representative lesions.

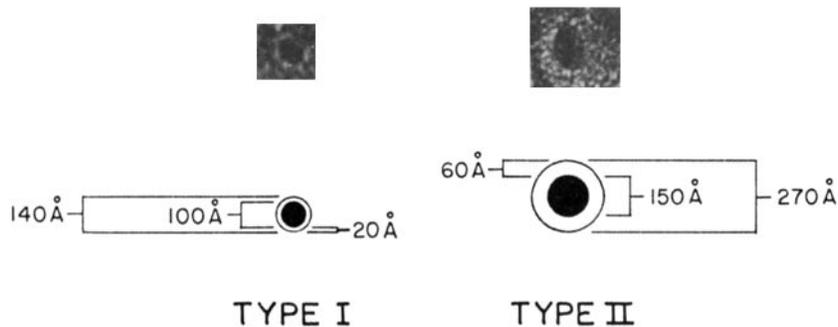


FIG. 3. Visual and graphic comparison of type I and type II lesions. Areas selected from electron micrographs both $\times 336,000$.

These changes were found to be dependent on the presence of both thrombin and C3. The changes seen electron microscopically in the platelet membrane were morphologically identical to those seen and described as type II lesions which are produced in red cells by the activation of C by the alternate mechanism.

On the other hand, when antibody-mediated activation of C occurs with platelets, ultrastructural changes take place on the membrane surface and lesions are produced which are identical in morphology to the type I lesions produced in red cells by activation of C by the classic mechanism.

These studies raise the intriguing possibility that thrombin-platelet inter-

TABLE II
*Dimensions of Two Morphologically different Ultrastructural Lesions
 Visualized in Platelet Membranes*

Type of ultrastructural lesion	Total diameter	Diameter of dark central area	Thickness of white rim
	Å	Å	Å
Type I*	139 (13)‡	96 (9)	23 (9)
Type II§	270 (21)	149 (12)	58 (10)

* Lesions seen in platelets treated with antiplatelet antibody and C (for details see text).

‡ Figure represents mean of at least 20 measurements of lesions in electron micrographs at five different magnifications. The figure in parenthesis is the calculated standard deviation.

§ Lesions seen in platelets incubated with recalcified plasma (for details see text).

actions are mediated at least in part by the activation of the alternative C pathway.

Summary

During blood coagulation ultrastructural lesions were produced on the platelet membrane. They were dependent on the presence of both thrombin and complement (C) and were morphologically identical to the C-dependent ultrastructural lesions (type II) seen in red cells subsequent to activation of C by the alternate mechanism. Though similar in shape they differed in size from ultrastructural lesions (type I) produced by activation of C via the classic mechanism. Lesions of type I were seen on the platelet surface subsequent to antibody-dependent activation of the C system.

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