The Effect of Macroglobulins and their Dissociation Units on Release of Platelet Factor 3

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Recent work (1) has shown that macroglobulins prepared from patients afflicted with macroglobulinemia of Waldenström have the capacity to "coat" the platelet surface and prevent the normal release of the prothrombin activation component, platelet factor 3. On the basis of coagulation, fluorescence and electron microscopical studies the authors proposed that the hemorrhagic diathesis, noted in some two-thirds of all cases of primary macroglobulinemia, might frequently be accounted for on the basis of this mechanical blockade. The possibility of a combination of an external effect plus entrance of the macroglobulin into the platelet with resultant deleterious action on the contained elements has also been postulated (2). Despite the ability to abolish the "coating phenomenon" by use of specific rabbit-anti-macroglobulin serum the exact relationship that exists between the macromolecule and the platelet surface has remained obscure. It is the purpose of the present report to analyse the relationship of molecular size with respect to the prevention of release of platelet factor 3 activity by use of the dissociation units of the native macroglobulin.

Materials and Methods

Source of Macroglobulins. Serum was obtained from D. M. W., 58-year-old white male, diagnosed in our laboratory by use of electrophoretic, ultracentrifugal and immunochemical techniques as having macroglobulinemia of Waldenström.

Preparation of Rabbit Anti-Macroglobulin Serum. Rabbits were immunized according to the procedure described by Pachter et al. (1) following the technique of Habich (3).

Preparation of the Macroglobulin Fraction. Fractionation was carried out, using the serum obtained from patient D. M. W., by diluting 200 ml serum 1 : 20 with distilled water and removing the insoluble macroglobulins by centrifugation. These were redissolved in 25 ml of 0.2 M sodium chloride.

Physical Studies. Electrophoretic patterns were carried out by the paper electrophoretic method. Sedimentation velocity was carried out in the Spinco model E ultracentrifuge.

Dissociation of Macroglobulin. The method of Deutsch and Morton (4) was followed. As in the above work, the unmodified isolated macroglobulin is classified as native, the depolymerized material dialysed against phosphate buffer at pH 7.4 as reaggregated and the depolymerized material dialysed against 0.02 M iodoacetamide in potassium phosphatase buffer at pH 7.4 as monomer.

Platelet Factor 3 Assay. This test was performed according to the method of Alkjaersig, Abe and Seegers (5). The method involved the activation of purified prothrombin by platelet extract and Benadryl in an environment containing calcium and imidazole. The thrombin thus formed was measured quantitatively by the clotting of fibrinogen by the method of Seegers and Smith (6). It was found by Murray, Johnson and Seegers (7) that platelet extracts containing platelet factor 3 activated purified prothrombin when coupled with one of several antihistamines in place of the antihemophilic factor. Previous to this, Johnson, Smathers and Schneider (8) showed that platelets and the antihemophilic factor (platelet cofactor 1) activated purified prothrombin to thrombin. The ability to replace a protein, for example the antihemophilic factor, by a synthetic compound such as Benadryl was advantageous for purposes of assaying platelet factor 3. It has been found necessary to seed this reaction mixture with thrombin before activation of purified prothrombin was begun. The reaction tube contained the following:

Prothrombin 3000 units per ml	1.0 ml
Platelet extract	0.5 ml
Calcium in imidazole	0.5 ml
Benadryl 0.1 gms%	0.5 ml
Thrombin 50 units per ml	0.5 ml
	3.0 ml

The resulting thrombin was measured by clotting fibrinogen. Platelet suspensions were assayed in this procedure.

Normal Intact Platelets with added Native Macroglobulins

The blood was drawn into plastic bags containing potassium oxalate as anticoagulant. The platelets were separated immediately by differential centrifugation, washed, suspended in physiological saline and counted. The following components were incubated together at 28° C for 18 hours.

	Platelets ml	Normal rabbit serum ml	Macro-globulins ml	Rabbit anti-macroglobulin serum ml
1	0.5	1.0	—	—
2	0.5	0.5	0.5	—
3	0.5	—	0.5	0.5

The final concentration of platelets in these tubes was 1 000 000 cmm^3. The platelets after incubation were washed once and studied for platelet factor 3 activity.

Normal Intact Platelets with added Monomer

Platelets prepared as above were incubated with the following components at 28° C for 18 hours.

	Platelets ml	Normal rabbit serum ml	Monomer ml	Rabbit anti-macroglobulin serum ml
1	0.5	1.0	—	—
2	0.5	0.5	0.5	—
3	0.5	—	0.5	0.5

The final concentration of platelets in these tubes was 1 000 000/cmm³. The platelets after incubation were washed once and studied for platelet factor 3 activity.

Normal Intact Platelets with added Reaggregated Macroglobulins

Platelets prepared as above were incubated with the following components at 28° C for 18 hours.

	Platelets ml	Normal rabbit serum ml	Reaggregated macroglobulin ml	Rabbit anti-macroglobulin serum ml
1	0.5	1.0	—	—
2	0.5	0.5	0.5	—
3	0.5	—	0.5	0.5

The final concentration of platelets in these tubes was 1 000 000/cmm³. The platelets after incubation were washed once and studied for platelet factor 3 activity.

Results

Dissociation of Macroglobulins. The ultracentrifugal pattern of the native macroglobulin, as shown in Figure 1 a, has sedimentation coefficients of 30 S, 24 S and 16 S.

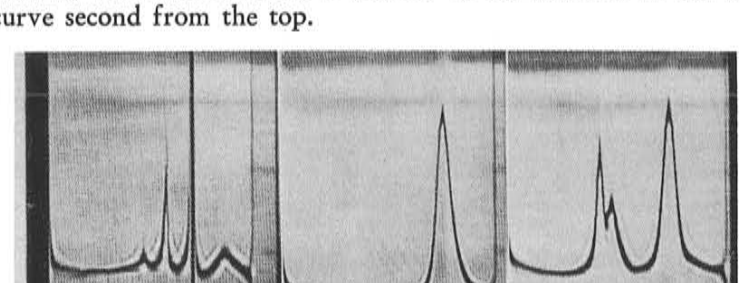
The monomer form, as shown in Figure 1 b, has a sedimentation coefficient of 6 S.

The reaggregated macroglobulin, shown in Figure 1 c, has sedimentation coefficients of 17 S, 15 S and 8 S. The values are notably smaller than those of the native macroglobulin.

Platelet Factor 3 Activity of Incubated Platelets

Incubation with Monomer. In a substrate of 800 units per ml purified prothrombin, all of the prothrombin was converted to thrombin by the platelet factor 3 activity of the platelets. The platelets had been incubated in iodoacetate buffer as a control, as can be seen in Fig. 2, top curve. When the monomer was

added to the platelets and incubated, all of the prothrombin was converted to thrombin by the platelet factor 3 activity of the platelets as can be seen in Fig. 2, curve second from the top.



a b c

Fig. 1a: Original macroglobulin of patient D. M. W. at a concentration of 1.7 gms% in 0.2 M sodium chloride, studied in a Spinco Model E ultracentrifuge, speed 52,640 r.p.m. The photograph was taken at 19 minutes after reaching speed.

Fig. 1b: Monomer resulting from treatment of the original macroglobulin with 0.1 M thioethanol in 0.05 M phosphate buffer pH 7.5. The thioethanol was removed by dialysis against iodoacetate 0.02 M in phosphate buffer. The study was carried out at a concentration of 1.2 gms% in a Spinco Modell E. ultracentrifuge, speed 56,100 r.p.m. The photograph was taken at 38 minutes after reaching speed.

Fig. 1c: Reaggregated material resulting from treatment of the original macroglobulin with thioethanol, removal of thioethanol by dialysis against phosphate buffer at a concentration of 1.2 gms% in a Spinco Model E ultracentrifuge, speed 52,640 r.p.m. The photograph was taken at 39 minutes after reaching speed.

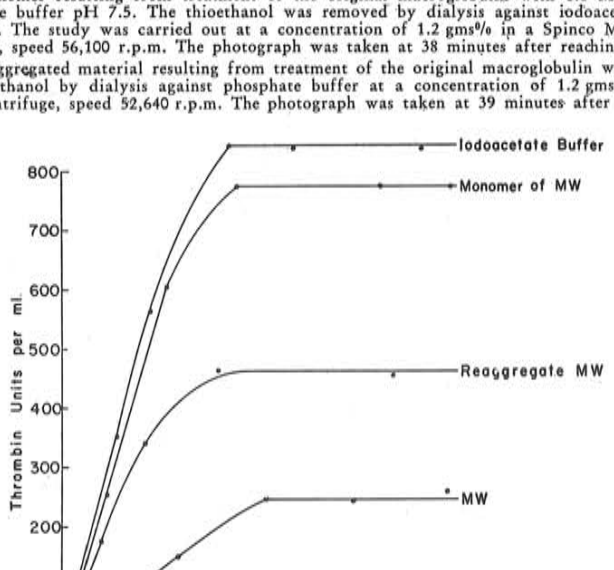


Fig. 2: The top curve represents the activation of purified prothrombin to thrombin by platelets in a controlled environment with iodoacetate buffer. The second curve represents the activation of purified prothrombin to thrombin by platelets in the presence of a monomer of macroglobulins. The third curve represents the activation of purified prothrombin to thrombin by platelets and the reaggregate of the monomer from macroglobulins. The lower curve represents the activation of purified prothrombin to thrombin by platelets and macroglobulins.

Incubation with Reaggregated Macroglobulin. Reduction of platelet factor 3 activity can be seen in Fig. 2 when the platelets were incubated in the reaggregated macroglobulin. In this instance only about 450 units of thrombin formed from the 800 units per ml of purified prothrombin substrate.

Incubation with Macroglobulins. Only 200 units per ml thrombin formed from the purified prothrombin substrate by the platelet factor 3 activity of platelets incubated in macroglobulins. This treatment resulted in the greatest reduction of platelet factor 3 activity (Fig. 2).

Effect of the Addition of Anti-Serum

Incubation with Macroglobulins and Rabbit Anti-Macroglobulin Serum. All of the purified prothrombin was converted to thrombin, 800 units per ml, when the platelets were incubated with rabbit anti-macroglobulin serum and macroglobulins (Fig. 3).

Incubation with Saline and Normal Rabbit Serum. All of the purified prothrombin was converted to thrombin, 800 units per ml, by the platelet factor 3 activity of platelets incubated with saline and rabbit serum (Fig. 3).

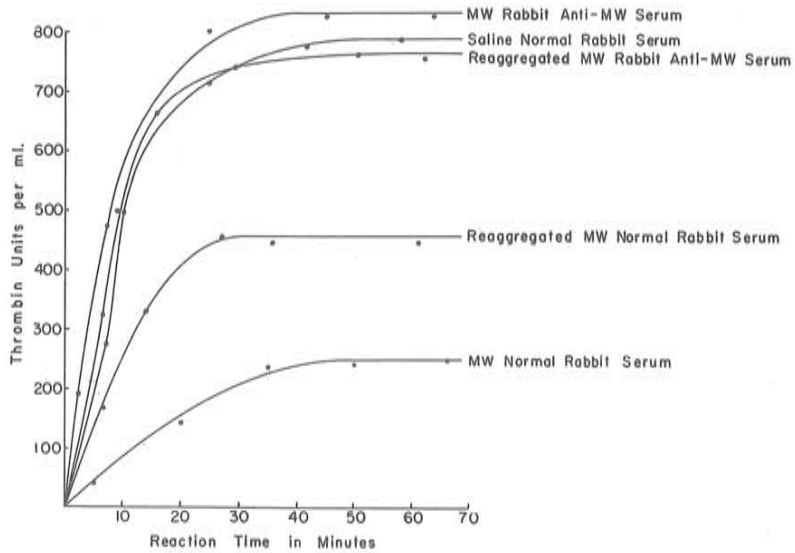


Fig. 3: The top curve represents the activation of purified prothrombin to thrombin by platelets, macroglobulins and rabbit anti-macroglobulin serum. The second curve represents the activation of purified prothrombin to thrombin by platelets, saline and normal rabbit serum. The third curve represents the activation of purified prothrombin to thrombin by platelets, reaggregated monomer and rabbit anti-macroglobulin serum. The fourth curve represents the activation of purified prothrombin to thrombin by platelets, reaggregated monomer and normal rabbit serum. The lowest curve represents the activation of purified prothrombin to thrombin by platelets, macroglobulin and normal rabbit serum.

Incubation with Reaggregated Macroglobulin and Rabbit Anti-Macroglobulin Serum. All of the purified prothrombin was converted to thrombin, 800 units per ml, by the platelet factor 3 activity of platelets incubated with rabbit anti-macroglobulin serum and reaggregated macroglobulin (Fig. 3).

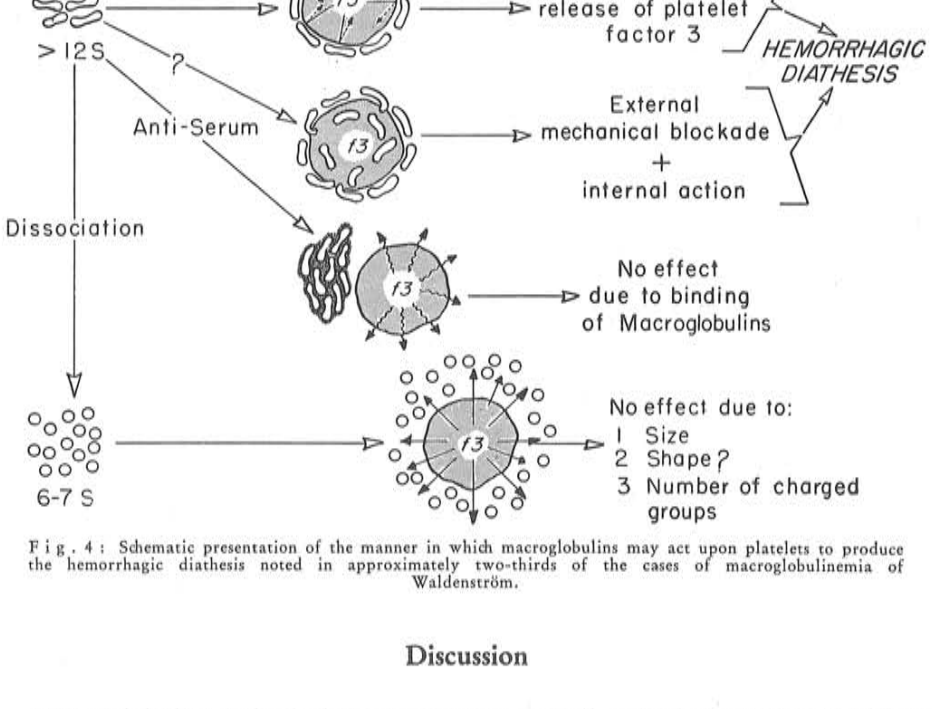


Fig. 4: Schematic presentation of the manner in which macroglobulins may act upon platelets to produce the hemorrhagic diathesis noted in approximately two-thirds of the cases of macroglobulinemia of Waldenström.

Discussion

Macroglobulins in both their native state as well as in their reaggregated form have been shown capable of preventing release of platelet factor 3. The monomer, to the contrary, fails to demonstrate any adverse effect. Deutsch and his associates (9) have failed to demonstrate new antigenic determinants in the monomer and state that the reaggregated and monomer forms have the same antigenic determinants as the native macroglobulin. Thus, if the "coating-phenomenon" was based on an antigen-antibody reaction, irrespective of molecular size, one might reasonably expect that all three forms would exert some adverse, if not a relatively similar effect, in a similar experimental system, upon platelets. That this is not the case for the dissociation units appears to be presumptive evidence that the inhibitory effect of macroglobulins is directly dependent upon the size of the molecule. In this respect, several workers (10, 11) have shown

that large dextran molecules, as averse to the smaller-sized ones, exert a greater deleterious effect on platelet factor 3 release.

At present there is no direct evidence to support an antigen-antibody interaction, rather than a physicochemical one, to explain the macroglobulin-platelet surface affinity. It is, of course, possible that in the process of dissociating and reaggregating the molecules the spatial arrangement is altered and a loss of some of the normal antigenic groupings transpires. If this did occur, and led to an alteration in biological activity, it might serve to explain the variation in action of the native, reaggregated and monomer units in respect to the prevention of platelet factor 3 release. However, the fact that the rabbit anti-macroglobulin serum, prepared by use of the native form, demonstrated the capacity to prevent a blockading action by the reaggregated molecule (despite the marked difference in sedimentation coefficients) is evidence that very little, if any, alteration in antigenic identity occurred.

Preliminary work with interference microscopy to determine possible lytic-antibody activity of macroglobulins against normal platelets has been carried out. These prefatory studies using platelets as the antigen complement and macroglobulins as suspected antibody failed to reveal lysis in a three-hour observation period. It would, at least, appear that macroglobulins posses no lytic-type antibody. At present experiments are being undertaken with rabbit anti-human platelet serum and macroglobulins in an attempt to clarify the problem of an antigen antibody relationship being active in the "coating phenomenon."

The graphs clearly indicate that although the native and reaggregated forms significantly prevent platelet factor 3 release the latter has a less complete effect. This fact may relate to the observation that the reaggregated molecule is not identical with the original native macroglobulin. The reaggregated product reveals lower sedimentation coefficients than the native one. Thus, the smaller size of the reaggregate may lessen the physicochemical capacity to "coat" the platelet surface. In the same regard A d e l s o n et al. (10) and J a c o b a e u s (11) observed that as the molecular weight of the dextran molecule, used in their experimental system, increased a more markedly pernicious effect was exerted on the bleeding time and prothrombin consumption time determinations.

Finally, it is conceivable that not only the actual size of the molecule but also its hypothetical asymmetrical configuration and difference in charged groups may militate for a greater "coating" capacity than is the case with the monomer. In this vein D e u t s c h (9) noted that the monomer had a greater number of charged groups than the native form. I s l i k e r (12) has opined that in dissociating the macromolecule there is a shift from an asymmetrical molecule to a more symmetrical form. Thus, all of the factors of size, shape and number of

charged groups may interact in the physicochemical affinity of the macroglobulin to the platelet surface.

Summary

The effect of the macroglobulins on the release of platelet factor 3 has been reviewed (Fig. 3). It has been shown that dissociation units of native macroglobulins fail to exert any deleterious effect upon platelets. Evidence is presented to support the theory that molecular size is of paramount importance in preventing platelet factor 3 release.

Résumé

Etude de l'influence des macroglobulines sur la libération du facteur 3 plaquettaire (fig. 3). Il a été démontré que les produits de dissociation des macroglobulines natives sont sans effet nocif pour les thrombocytes. Certaines observations de cette étude confirment la théorie que les dimensions moléculaires sont d'une importance primordiale pour empêcher la libération du facteur 3 plaquettaire.

Zusammenfassung

Es wird die Wirkung von Makroglobulinen auf die Freisetzung von Thrombozytenfaktor 3 besprochen. Dissoziation des nativen Makroglobulins in seine Monomere beseitigt den hemmenden Einfluß auf die Thrombozyten. Die Ergebnisse sprechen dafür, daß die Molekulargröße von hervorragender Bedeutung für die Blockierung der Freisetzung von Thrombozytenfaktor 3 ist. Die Möglichkeit einer Antigen-Antikörper-Beziehung wird besprochen.

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