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Diffuse Intravascular Clotting

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18. Quantitation of Circulating Fibrinogen Breakdown Products in Intravascular Clotting

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The case which prompted the investigation reported here concerns a 31 year old multipara who developed a peculiar defibrination syndrome after having given birth to twins who died of asphyxia during delivery.

Twenty-four hours after delivery the fibrinogen content of the plasma, estimated according to Clauss (1), appeared to be 20 mg/\%0, but a rather firm clot formed after longer incubation with thrombin. Moreover, the patient did not bleed, and the thrombelastogram did not show any of the signs typical of a low fibrinogen content.

This case brought out strongly a discrepancy that is rather often seen and more often suspected in the laboratory, viz. the discrepancy between the fibrinogen content measured by the Clauss method and the amount of fibrin apparently formed by the same plasma.

It is known that many kinds of fibrin- and fibrinogen-breakdown products are formed during intravascular clotting. Proteolysis accompanying intravascular clotting may cause fibrinogen to lose its clottability and immunological properties. Some of the products formed may gain the ability to inhibit thrombin action, polymerization of fibrin, or stages in the prephase of blood-clotting (2–10). Fibrin may undergo a similar breakdown. For all practical purposes in the present paper this heterogeneous group of products can be divided into three sub-groups, viz.:

a) unaltered fibrinogen
b) antifibrinogen-precipitable but unclottable material
c) material inhibiting the formation rate of a fibrin clot.

We attempted to estimate the relative amounts and half-life times of the fibrin(ogen) derivatives in our patient. The antifibrinogen positive material in the serum and plasma can be estimated on the basis of immunologic reactions. Ferreira et al. (11, 12) have worked out a semi-quantitative method which was not, however, sensitive enough to suit our purposes. Schwick (13) has developed
a method by which the amount of precipitate is measured nephelometrically. We adapted a method based on a principle developed by Mancini et al. (14) and also independently by Rümke (15).

In this method, the gel in which the sample diffuses, already contains the antibody, in this case rabbit anti-human fibrinogen.

The immunization scheme was suggested to us by Dr. W. Hijmans (Department of Rheumatology, University Hospital, Leyden). With an interval of 1 week, we gave 3 i.m. injections of 100 mg human fibrinogen (fraction 1—4, kindly supplied by Dr. Blomberg) with 1 ml of Freund's adjuvants. Three weeks after the last i.m. injection, increasing doses of alum-precipitated fibrinogen were given i.v. at 2- or 3-day intervals (twice 25 mg; twice 50 mg; twice 100 mg; twice 200 mg). Ten days after the last i.v. injection the rabbits were bled. The serum was incubated with one-tenth its volume of normal human serum to precipitate non-specific antibodies, centrifuged, and stored in 1 ml portions at −20°C. The gel consisted of 0.8% agar in a solution containing 0.36 M NaCl, 15 mM KCl, 5 mM K-Na phosphate buffer (pH 7.4), and 1‰ sodium azide. Before solidification, 0.8% (v.v.) of anti-human fibrinogen rabbit antiserum was added, and 12 ml of the mixture poured out in a plastic Petri dish (diameter 8.5 cm). Holes with a diameter of 3 mm were punched out in the gel, and 0.01 ml of the

Fig. 1. A plate after incubation.
Quantitation of Circulating Fibrinogen Breakdown Products

solution to be tested was pipetted into them. After 3 days incubation at 37°C the dishes were photographed on a one-to-one scale, and the diameter of the precipitation rings was measured. The square of the diameter of course is proportional to the surface area.

The antigen diffuses out in the gel, and after 1—3 days of incubation at 37°C a precipitation ring shows up. The area enclosed by this ring is proportional to the amount of antigen brought into the well. Fig. 1 shows a gel-plate after incubation. Fig. 2 shows the relationship between the area and the amount of antigen added with human fibrinogen as the antigen. The standard error was less than 3%/.

It is interesting to note that with this method one precipitation ring always shows up when a mixture of antifibrinogen-positive materials is present, although it is known that fibrinogen and its breakdown products differ in their antigenic and diffusion behaviour. Because of these differences we can only express the quantity of antifibrinogen-positive material as the equivalent amount of fibrinogen giving an equal precipitation ring.

Fibrinogen itself was measured in 3 ways:

1. As the clotting of the diluted plasma with a standard amount of thrombin (Clauss method).

To 0.1 ml of an appropriate dilution (1 in 10 for normal plasma ranges) of the plasma sample in Michaelis buffer (pH 7.4), 0.1 ml of a thrombin solution (64 NIH u/ml) is added at zero time. The reaction is carried out at 37°C, the moment of clotting is assessed with a Kolle hook.

2. Gravimetrically, as the amount of fibrin recovered after clotting of the plasma.
3. As the amount of antifibrinogen-positive material in the plasma minus the amount of antifibrinogen-positive material in the serum, with the assumptions that (a) no significant amount of antifibrinogen-positive material is taken up by the clot, and (b) that this material has a similar reactivity as fibrinogen.

Of these methods, the first is sensitive to inhibiting substances, whereas the last two are not. A fairly good agreement was found between the last two methods.

Fig. 3 shows the pertinent data in our patient.

Assuming that no further intravascular proteolysis occurred after delivery, we could estimate the half-life time of the antifibrinogen-positive but unclottable material from the estimation of this material in the serum of the patient. This value proved to be 79 h, which is equal to the lower limit of the half-life of normal fibrinogen (16). It should be noted that the blood was collected in a solution containing EACA and Trasylol in order to prevent proteolysis after venipuncture. (Final concentrations: EACA 1 mg/ml, Trasylol 1 u/ml).

To estimate the half-life time of the inhibiting substances, we would first have to know how these substances act. To understand this action, we would have to know the normal kinetic behaviour of the fibrinogen-thrombin interaction; and this we do not yet know in detail. We do know, however, that a graph com-
parable to the Lineweaver-Burk plot is obtained when clotting time is plotted against the inverse of the square of the fibrinogen concentration (Fig. 5) (21).

By using this plot, the mode of inhibitory action could be assessed; it was found to be a competitive one (Fig. 6).

Fig. 4. The disappearance rate of the antifibrinogen positive breakdown products. The log of the concentration of antifibrinogen-positive material in serum, expressed as the equivalent concentration of normal fibrinogen (in mg/ml) is plotted on the y-axis against time (in days) on the x-axis.

Fig. 5. The relation between fibrinogen concentration and clotting time.
This finding is in agreement with the results of Triantaphyllopoulos (17) but it undoubtedly represents an oversimplification because we know that many different kinds of inhibition occur in these cases (9, 18, 19). In our experimental set-up, however, the inhibition can for all practical purposes be described as being of a purely competitive type. In competitive inhibition the apparent amount of substrate present (S') is related to the real amount present (S) by the formula: 

\[ S' = \frac{S}{1 + \frac{I}{K_i}} \]  

(20). This equation gives the relation between the amount of fibrinogen measured in the Clauss test (S') and the amount found by the immunological test or with the gravimetrical method (S). Since both these data are known, we can find a relative measure for the amount of inhibitor (I) (viz.: \( I \sim 1 - \frac{S'}{S} \)). This is about the best we can hope to obtain with this kind of data. The half-life time of the inhibiting substances can now be estimated (Fig. 7). This value turns out to be 19 h, which is substantially lower than that of fibrinogen or antifibrinogen-positive material. The inhibitory substances are probably the smaller molecules, and it is conceivable that these small molecules disappear from the circulation more rapidly than the larger ones do. This is consistent with the fact that in chronic defibrination we found antifibrinogen-positive material in the serum, but no — or at least remarkably little — inhibitory action by the Clauss test. Our observations are not consistent with the idea that the existence of a fibrinogen with altered reactivity is the main cause of the
slow fibrinogen conversion (17), because altered fibrinogen would behave as non-
competitively — or incompetitively — inhibited normal fibrinogen and would
have about the same half-life as normal fibrinogen has. Altered fibrinogen may,
of course, be present but it is not detectable in our tests.

![Graph showing the disappearance rate of thrombin inhibiting breakdown products.](image)

Fig. 7. The disappearance rate of thrombin inhibiting breakdown products (see text).

In conclusion, it may be said that the two-dimensional agar diffusion methods
are valuable for the investigation of the defibrination syndrome, because, to-
gether with the Clauss method and the gravimetric method of fibrinogen
estimation, they enable us to assess the fibrinogen content and to get an impres-
sion of the relative amounts of antifibrinogen-positive material and thrombin-
inhibiting breakdown products.

References

767 (1959).
(4) Niewiarowski, S., E. Kowalski: Formation of an antithrombin-like anticoagulant during


(10) Marder, V.: Personal communication. (1965).


(15) Rümkö, Ph.: Personal communication.


