An Ultrastructural Investigation of the Fibrin Clot Utilizing Ferritin-Labelled Anti-Human Fibrinogen Antibody

By JAMES G. WHITE, WILLIAM KRIVIT AND ROBERT VERNIER

The primary purpose of the complex mechanism of coagulation is to convert soluble fibrinogen molecules into a stable meshwork of fibrin. Appreciation of the macro-molecular architecture of fibrin is essential to an understanding of how clot formation fulfills its role in hemostasis.

An electron microscopic study, combining the use of immune methods, has been applied to the ultrastructural evaluation of fibrin formation. Ferritin-labelled anti-human fibrinogen antibody (fer-A.F. antibody) incubated with plasma during clot formation specifically stains the developing fibrin strands. Periodic bands, indicative of a high degree of order, are produced by the ferritin conjugated antibody on the intrinsic cross striations of fibrin. These observations suggest the mode by which fibrinogen molecules unite to form the fibrin strand.

Materials and Methods

Seven large male rabbits were immunized with crude human fibrinogen. Squibb human fibrinogen, fraction I, 70 per cent clottable, was dissolved to a concentration of 50 mg. per ml. in sterile water, and emulsified with Freund’s adjuvant 1:3. Each animal received 0.1 ml. of this suspension, amounting to 5 mg. of total fraction I, intradermally. A second injection of 7.5 mg. of fraction I without adjuvant was given each animal intravenously 23 days after the first. A third injection of 20 mg. was given intravenously a day after the second, and a fourth shot containing 30 mg. of fraction I was administered two days after the third, also by the intravenous route. Blood was obtained from the rabbits after each of the intravenous injections. The antihuman fibrinogen antibody titer of the pooled sera of all of the blood samples determined by the micro Ouchterlony gel diffusion technic was 1:320. Micro-immune electrophoresis of this antisera is shown in figure 1.

The antisera was then adsorbed with normal human serum to remove non-specific antibodies. Four ml. of rabbit antisera were mixed with 2 ml. of normal aged human serum, and the mixture incubated at 37 C. for one hour. The mixture of sera was then kept at 4 C. for 24 hours, and, at the end of that time, was centrifuged at 2500 rpm at 4 C. for 30 minutes. The supernatant serum was carefully separated, and the precipitate discarded. An equal volume of saturated ammonium sulfate was added to the serum mixture to precipitate the gamma globulin. The mixture was centrifuged at 17,000 rpm for 30 minutes at 4 C. The supernatant was removed and discarded, and the precipitate was redissolved in distilled water to ¼ the original volume. Dialysis of the antibody globulin against normal saline for 24 hours at 4 C. was then performed. Micro-immune electro-
phoresis after adsorption of the antibody demonstrated a single precipitin band to the fibrinogen of human plasma (fig. 1).

Subsequently, the antibody globulin was conjugated to ferritin according to the method of Singer,\textsuperscript{2} using toluene 2,4-diisocyanate as the coupling agent. This chemical procedure results in covalent bonds between the isocyanate groups of toluene 2,4-diisocyanate, and reactive amine groups on the proteins of ferritin and antibody.

Following the coupling procedure, the ferritin conjugated antibody was again submitted to micro-immune electrophoresis. A precipitin band between the antibody and plasma fibrinogen, identical to that of the antisera prior to the coupling procedure, could be identified. When the immune electrophoresis of the labelled antibody was stained for iron\textsuperscript{6} rather than protein, the identical band of the antigen-antibody reaction was clearly demonstrated (fig. 1).

Fresh plasma was separated from whole human blood using 0.6 per cent EDTA 1:9 as the anticoagulant. Aliquots of the plasma and ferritin labelled antihuman fibrinogen antibody were incubated, and then recalcified with 0.1 M CaCl\textsubscript{2}. A clot formed in the several preparations between 70 and 200 seconds. After solid clot formation, the clotting reaction was stopped by plunging the tube containing the recalcified plasma-antibody mixture into ice water, and immediately adding gluteraldehyde. After a two hour fixation in gluteraldehyde, the clots were post fixed in 1 per cent osmic acid for 1 1/2 hours, and embedded in vestopal-w. Thin sections cut with an L.K.B. ultramicrotome were counterstained with uranyl acetate, and examined in an RCA-EMU-3D electron microscope.

Controls consisting of plasma incubated with ferritin alone, or ferritin conjugated with toluene 2,4-diisocyanate, but without antibody, were recalcified, and the clots prepared for electron microscopy in an identical manner.

**Results**

Examination of the clots formed from control samples of recalcified plasma containing ferritin, but not fer-A.F. antibody, revealed distinct features of the structure of fibrin (fig. 2A). The long filaments comprising the fibrin strand are related in a manner similar to wires placed side by side to form an electrical cable. Along the length of the strand periodic intrinsic cross striations are apparent.

Ferritin can be observed adjacent to the strands of fibrin in the control clots, and a few ferritin molecules are distributed randomly throughout the length of the strand. These features suggest nonspecific incorporation of the ferritin into fibrin during clot formation.

In marked contrast to the fibrin strands developing in control preparations, are those forming in recalcified plasma containing the ferritin labelled antihuman fibrinogen antibody (fig. 2B). The fibrin strands are periodically banded by the fer-A.F. antibody. Ferritin appears on the intrinsic cross striation of the fibrin strand, and has a periodic interval of $195 \pm 10 \, \text{A}^\circ$ (fig. 3). At the terminal ends of these strands, and in adjacent areas, the formation of the ferritin labelled fibrin filaments into long strands can be observed. This takes place by apposition of the filaments in the long axis of the fibrin strand.

**Discussion**

The action of thrombin on fibrinogen results in the cleavage of four small peptides from the molecule. This limited proteolysis does not significantly
alter the molecular weight of fibrinogen, but the chemical change is sufficient to initiate the polymerization of altered fibrinogen molecules into the insoluble fibrin clot.

Experimentally, if the fibrinogen molecule could be attached by an electron dense marker, without inhibiting the response to the action of thrombin, a means of investigating the structural formation of fibrin would be provided. To accomplish this, ferritin was conjugated to antihuman fibrinogen antibody, utilizing the technic of Singer. Micro-immune electrophoresis of
Fig. 2.—Fibrin strand from central clot containing fibrinogen. (a) Fibrinogen interface, fibrinogen molecules arrayed in the fibrin strand. The fibrinogen molecules are deposited on the filamentous structure of the fibrin strand. Original magnification X 35,000.

The fibrin strand from central clot containing fibrinogen. (b) Fibrinogen interface, fibrinogen molecules arrayed in the fibrin strand. The fibrinogen molecules are deposited on the filamentous structure of the fibrin strand. Original magnification X 35,000.
the antibody complex after the coupling procedure demonstrated the retention of immune specificity for fibrinogen, and the deposition of ferritin at the site of antigen-antibody reaction. These preliminary studies indicated the suitability of the fer-A.F. antibody for use as an electron dense stain of fibrinogen.

Addition of the fer-A.F. antibody to human plasma permitted its attachment to the antigenic binding sites on the fibrinogen molecule. Recalcification of this mixture of plasma and conjugated antibody resulted in clots whose fibrin strands were uniquely delineated. The ferritin-labelled anti-human fibrinogen antibody periodically banded the intrinsic cross striations of the fibrin strands. This striking regularity of the fer-A.F. antibody deposition further demonstrated the specificity of the ferritin tagged antibody, and suggested fundamental mechanisms of fibrin formation.

Fibrin strands of control clots, as well as the experimental preparations, have a filamentous structure. The appearance of single rows of closely apposed ferritin molecules separated by periodic intervals, indicated the high order of orientation of the long filaments to one another in forming the fibrin strand. Furthermore, the presence of a single, rather than a double row of ferritin molecules forming each periodic band, suggested that each fibrinogen molecule, or fibrin monomer, had the ferritin-labelled antibody attached at only one end. The association of monomer units during polymerization of the long filaments must also be highly ordered, since an indifferent incorporation of the units would not have resulted in fibrin strands having periodic-cross striations composed of single rows of ferritin molecules.

These results are in accord with the model of the fibrin strand proposed by Hall and Slayter. These investigators suggested that fibrinogen molecules, converted to fibrin monomer by thrombin, line up end to end. The resultant filaments become laterally associated in the long axis of the developing fibrin strand just as wires are laid side by side to form an electrical cable. A major periodic band, demonstrated by phosphotungstic acid staining, occurs every 240 Å, and represents the site of end to end association of the individual fibrin monomers.

The periodic interval of 195 ± 10 Å which we observed in thin sectioned, fer-A.F. antibody banded fibrin strands is not widely divergent from the value reported by Hall and other workers. The difference is conceivably due to the different methods of clot preparation and examination which we have used.

The data we are reporting do not support the model of the wide fibrin strand suggested by Laki. He has proposed that the wide strands develop by a side to side aggregation of monomer units in a plane perpendicular to the long axis of the fibrin strand. The major periodic interval of 240 Å he considered to be due to attachment of the phosphotungstic acid only on every fourth monomer of this "picket fence" arrangement.

In our results the formation of filaments, the filamentous structure of large fibrin strands in thin sectioned clots, and the periodic interval of the ferritin bands, mitigate against a "picket fence" association of fibrin monomers in
Fig. 3—The periodic banding of the fibrin strand by fer-A.F. antibody at higher magnification. The interval between bands is 195 ± 10 Å. Original magnification X 58,500.
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the fibrin strand. Had every fibrinogen molecule been attached by ferritin-labelled antibody, a period of 60 Å would have been expected in the arrangement of monomers Laki suggested. The wider interval we have observed would fit the model of Laki only if every fourth monomer incorporated in the wide fibrin strand carried the ferritin antibody tag, and the other three were without it. It is extremely unlikely that such an event could occur.

The immune methodologies developed by Singer and his co-workers for electron microscopic investigations have been useful in the study of fibrin clot development. The application of ferritin-labelled antihuman fibrinogen antibody to the investigation of abnormal clot formation, fibrinolysis, platelet-fibrin relationships, and other problems related to fibrinoid deposition will be of value.

SUMMARY

A study of fibrin ultrastructure utilizing ferritin-conjugated antihuman fibrinogen antibody is reported. The structure of fibrin strands in thin section, and the periodic banding of the fibrin by ferritin conjugated antibody permits conclusions regarding the structural formation of the clot. The observations are in accord with the model of fibrin strand formation suggested by Hall and Slayter. Further areas of investigation utilizing this technic are suggested.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Ben Pollara who provided the antihuman fibrinogen antibody; to Dr. Robert Bridges and Grace Anderson for the micro-immune electrophoresis; and to Marian Collander and Shirley Steinlicht for technical assistance.

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