

Assay of serum fibrin degradation products by agglutination-inhibition of coated erythrocytes

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SYNOPSIS An immunological method for detecting fibrin degradation products by tanned cells agglutination has been standardized. The effects of different variables, such as cell concentration, pH, and length of incubation, and the specificity of the reaction, are described. The method is compared with other immunological techniques.

Proteolysis of fibrinogen and fibrin by plasmin results in the elaboration of polypeptide fragments known as fibrinogen or fibrin degradation products. Two of the fragments, D and E, comprising about 70% of total digests (Niléhn, 1967) share an immunological identity with the parent fibrinogen (Nussenzweig, Seligmann, and Grabar, 1961), and can be detected by immunological techniques.

Boyden's (1951) technique, in which tanned red cells may be coated with human fibrinogen (Das, 1970), can be adopted as a haemagglutination inhibition reaction for detecting circulating fibrin degradation products (Fox, Wide, Killander, and Gemzell, 1965; Merskey, Kleiner, and Johnson, 1966). This assay has been successfully applied for measuring serum fibrin degradation products in normal subjects (Das, Allan, Woodfield, and Cash, 1967), in uterine arterial and venous blood (Mackay, Das, Myerscough, and Cash, 1967), in normal pregnancy (Woodfield, Cole, Allan, and Cash, 1968), and in cirrhosis (Das and Cash, 1969).

The present communication describes the standardization of this assay and also compares its sensitivity with other immunological techniques such as the Ouchterlony immunodiffusion (Ouchterlony, 1962), immunoelectrophoresis (Scheidégger, 1955), comparative immunoelectrophoresis (Niléhn and Nilsson, 1964), and antigen-antibody crossed electrophoresis (Laurell, 1966).

Materials and Methods

1 Tanned sheep red cells coated with human fibrinogen (sensitized sheep red cells) are prepared according to the method of Das (1969).

2 Purified human fibrinogen (97% clottable, Kabi Pharmaceuticals Ltd, Stockholm) is used as a reference standard.

3 Diluting fluid consisting of citrate-phosphate buffer (Das, 1970), pH 6.4, containing 0.4% absorbed horse serum and 1 mg/ml sodium-azide was used routinely as a diluent except in those experiments where the effect of pH was investigated by the use of an appropriate buffer.

4 Rabbit anti-fibrinogen serum (Hoechst Pharmaceuticals Ltd, Germany) was diluted 1:500 in this diluting fluid and stored at -40°C ; further dilutions were made from this stock solution.

PREPARATION OF SPECIMEN

Serum

Five ml blood was collected in test tubes containing 500 Kallikrein inactivator (KI) units of Trasylol (Bayer, Germany) and incubated at 37°C for four hours; the serum was separated by centrifugation at 2000 g for 10 minutes. The test serum was mixed with 1/10 of its volume

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of thrombin (100 units/ml), re-incubated at 37°C for one hour, and then centrifuged: this procedure removed any residual fibrinogen.

Absorption of serum

All serum samples were absorbed for 12 hours at 4°C with 1 volume of washed packed normal red cells to 2 volumes of test serum and either assayed immediately or stored at -40°C.

INHIBITORY TITRE TEST

Experiments were performed to investigate the ability of standard human fibrinogen solutions to inhibit the haemagglutination reaction of sensitized sheep red cells at different concentrations of an antifibrinogen serum; the optimum dilution of antiserum to be used in the inhibition reaction for the fibrin degradation products immunoassay was then calculated.

PERFORMANCE OF ASSAY OF SERUM FIBRIN DEGRADATION PRODUCTS

A 'microtitre kit' (Cook Engineering Co., USA) was used for the performance of the test which has been described in detail elsewhere (Woodfield *et al*, 1968). Haemagglutination inhibition was recorded as complete (-), weak agglutination (\pm), or frank agglutination (+). Estimation of fibrin degradation products for each test sample were calculated in the following way:

$$\begin{aligned} &\text{Fibrin degradation products } (\mu\text{g/ml}) = \\ &\text{Fibrinogen concentration of standard} \\ &\times \frac{\text{endpoint of test}}{\text{endpoint of standard}} \end{aligned}$$

Each test was performed using two dilutions of antisera (1:5,000 and 1:10,000) simultaneously and paralleled by dilutions of at least two standard human fibrinogen solutions, the mean endpoints of each test and standard being taken. Positive and negative controls were included in each plate.

Results

Two possible sources of error in endpoint reading are (1) the presence of a cold agglutinin, and (2) the incomplete absorption of non-specific agglutinins. The former can be checked by keeping the plates for 30 to 45 minutes at room temperature before reading. The latter can be avoided by pre-absorption of the samples with normal sheep red cells. Haemolysis occurring in the samples during absorption can be prevented by the addition of EDTA (2 mg/ml) to the test serum, or samples can be absorbed with sheep red cell stroma.

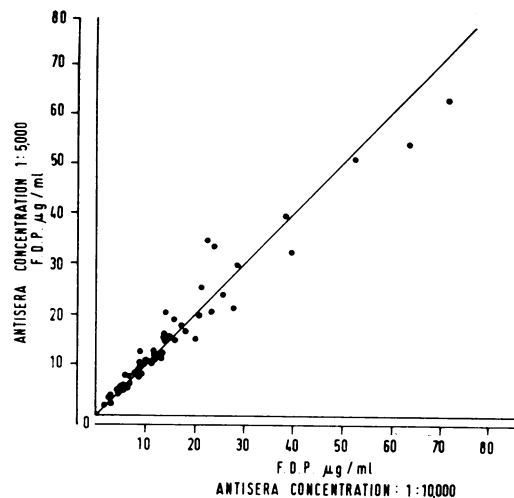


Fig. 1 Comparison of serum fibrin degradation product levels obtained by performing tests at two antisera concentrations.

Sensitivity, Precision, and Reproducibility

SENSITIVITY AND PRECISION

The inhibition titre of standard fibrinogen increased as more diluted antiserum was used. The sensitivity of the assay system, defined as the smallest quantity of antigen required to inhibit the reaction, is improved by increasing the dilution of antiserum. However, when a concentrated amount of antiserum is used in a system in which measurement depends on an endpoint reading, the difference in cell patterns is more distinct and precision therefore greater. In practice, the sensitivity and precision of the assay system for fibrin degradation products was anticipated by undertaking preliminary tests, and antifibrinogen concentrations of 1:5,000 and 1:10,000 were adopted routinely in this immunoassay.

VARIATION IN THE ASSAY

Multiple aliquots of two different standard fibrinogen solutions were assayed at two antisera dilutions. Results showed that 75-80% endpoints appeared at the same antigen concentration.

Possibly the largest variable factor is error in antigen dilution. However, precision can be improved either by carrying out parallel assays at different antiserum concentrations, or by repeating assays and calculating the mean value.

REPRODUCIBILITY

Results of serum fibrin degradation product concentrations are compared in Fig. 1; the

pH	Standard Fibrinogen (80 mg%)		Serum Sample	
	Inhibition Titre	Quantity at Endpoints ($\mu\text{g/ml}$)	Inhibition Titre	Quantity at Endpoints ($\mu\text{g/ml}$)
5.0	1/600	1.4	1/16	22.4
6.0	1/600	1.4	1/16	22.4
6.4	1/600	1.4	1/16	22.4
7.2	1/600	1.4	1/16	22.4
7.6	1/600	1.4	1/16	22.4
8.0	1/600	1.4	1/16	22.4

Table I Effect of pH on haemagglutination inhibition

standard error, analysed from duplicate observations, was 7.4%. Good agreement was obtained between the values obtained in two independent test systems (1:5,000, mean 13.13 $\mu\text{g/ml}$; 1:10,000, mean 13.11 $\mu\text{g/ml}$; $r = 0.98$, $P < 0.001$), and significant differences between the individual results were not observed ($t = 0.164$).

VARIABILITY

The presence of fibrinolytic inhibitor in the sample

Trasylol, a potent inhibitor of fibrinolysis, can be used to avoid the generation *in vitro* of fibrin degradation products without vitiating the test system. Of four specimens without Trasylol, three showed an increased haemagglutination inhibition titre and more fibrin degradation products than comparable samples containing the inhibitor.

Concentrations of sensitized sheep red cells

Parallel assays, differing only in the concentration of sensitized sheep red cells (2.0-3.5%) in the

final test system, were performed. Results showed an increase in inhibition proportional to the cell concentration.

Effect of pH

When the assay was performed at different levels of pH (5.0-8.0), neither inhibition nor the quantities of fibrin degradation products detected in the serum samples were significantly affected (Table I).

Length of incubation

Tests were set up for the inhibition reaction and sensitized sheep red cells were added after periods of time varying from one to six hours. Results summarized in Fig. 2 showed that titres for both standard and test samples increased in proportion to the time of incubation. This did not affect measurement but the finding that the sensitivity of the system could be increased in this way has a practical value.

Specificity

The specificity of the assay was studied using 1:5,000 antiserum; the results demonstrated that there was no inhibition by purified human albumin and γ -globulin. Reagents used in the test system, such as Trasylol, Ca-thrombin, or EDTA, caused no inhibition, neither did the addition of a supernatant obtained by the incubation at 37°C for 30 minutes of purified fibrinogen with thrombin in the presence of Trasylol (Table II).

In a second series of experiments plasma, human fibrinogen, and their clots were incubated

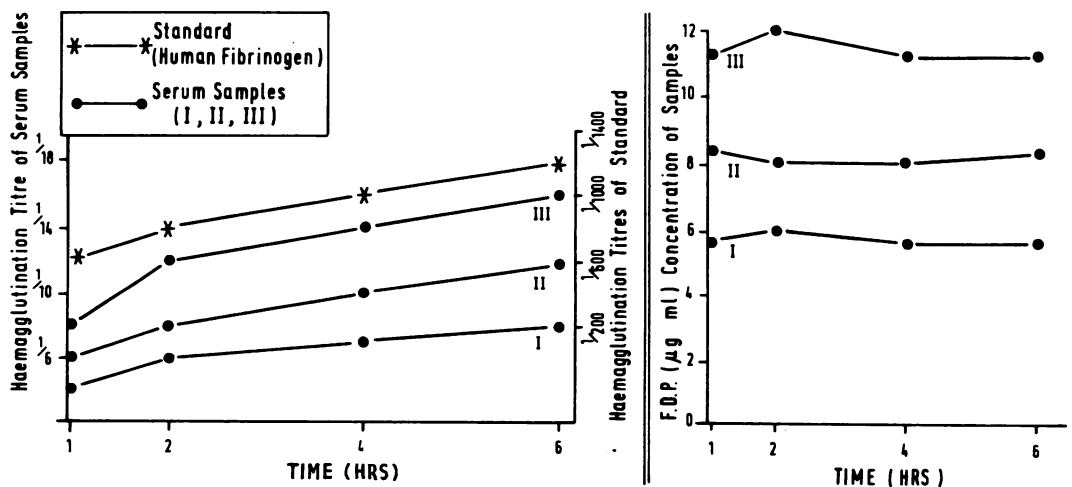


Fig. 2 Effect of increasing incubation time on fibrin degradation product (FDP) assay by haemagglutination inhibition presented as haemagglutination inhibition titre (left) and $\mu\text{g/ml}$ (right).

No.	Materials	Results
1	Trasylol (5,000 KI units/ml)	No inhibition
2	Ca-thrombin (100 units/ml)	No inhibition
3	Urokinase (5,000 units/ml)	No inhibition
4	Human albumin (500 mg/100 ml)	No inhibition
5	Human gamma globulin (50 mg/100 ml)	No inhibition
6	EDTA (2 mg/ml)	No inhibition
7	Supernatant obtained after incubation at 37°C for 30 minutes of purified fibrinogen with thrombin in the presence of Trasylol	No inhibition
8	Plasma clots incubated with urokinase and plasmin for 12 hours	
	Supernatant (a) with thrombin	Inhibition
	(b) without thrombin	Inhibition
9	Purified human fibrinogen incubated with urokinase and plasmin for 12 hours:-	
	Supernatant (a) with thrombin	Inhibition
	(b) without thrombin	Inhibition

Table II Specificity of the haemagglutination reaction

with plasmin (Kabi Pharmaceuticals) and urokinase (Leo Pharmaceuticals, Denmark). After complete digestion of the substrate, aliquots were tested before and after the addition of thrombin (100 units/ml). Results showed that haemagglutination was inhibited by the products of fibrinolysis and fibrinogenolysis (Table II).

A third series of experiments was designed to study the effect of heparin (Evans Ltd, UK) in the assay system and indicated that heparin masks the presence of fibrin degradation products

Comparison of Haemagglutination Inhibition Immunoassay with Other Immunological Techniques

Some serum samples with a high content of fibrin degradation products showed two precipitation lines on Ouchterlony plates; single lines usually appeared with lower concentrations of split products. Good separation of fibrin degradation products was obtained by immunoelectrophoresis although low concentrations frequently failed to show precipitin arcs; with higher concentrations, two arcs resembling D and E fragments were occasionally visible. The use of comparative immunoelectrophoresis allows the serological identification of precipitins and precipitinogens in addition to clarifying their mobility by combining immunoelectrophoresis and gel diffusion.

Table III shows the results of 20 serum samples in which the concentration of fibrin degradation products was measured by haemagglutination inhibition assay. In these test systems the sensitivity of Ouchterlony immunodiffusion and comparative immunoelectrophoresis are found to be similar (10-20 $\mu\text{g}/\text{ml}$). On the other hand immunoelectrophoresis was found to be less sensitive. A precipitin arc of 13 mm was obtained from a serum sample containing 56 $\mu\text{g}/\text{ml}$ fibrin degradation products on crossed electrophoresis; the length of the precipitin arc was proportional

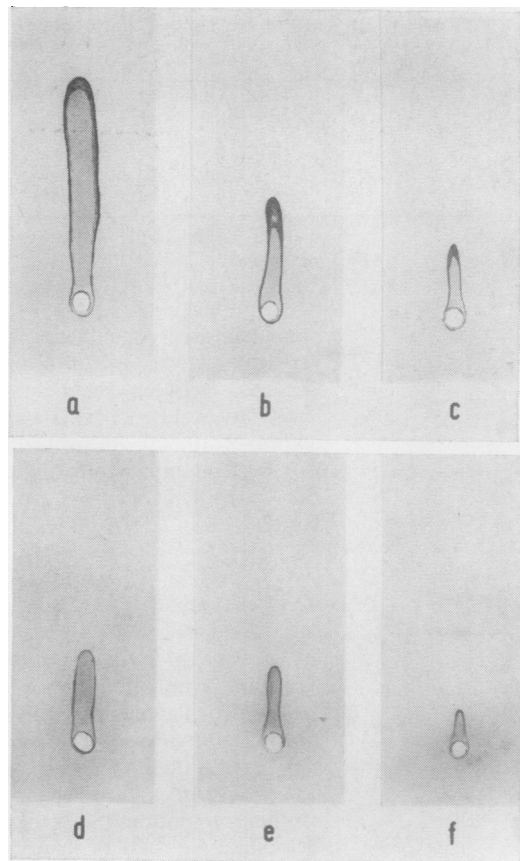


Fig. 3 Crossed electrophoresis of serum albumin and serum fibrin degradation products. Slides a, b, and c contain neat (2.5 g%), $\frac{1}{2}$, and $\frac{1}{4}$ solutions of albumin respectively; slides d, e, and f contain neat (56 $\mu\text{g}/\text{ml}$), $\frac{1}{2}$, and $\frac{1}{4}$ dilutions of serum fibrin degradation products respectively.

to the amount of fibrin degradation products present in a manner similar to that obtained using standard albumin solution (Fig. 3).

Comments

The present investigation shows that haemagglutination inhibition immunoassay is 10-20 times more sensitive than the conventional immunological techniques, thus permitting the measurement of small quantities of fibrin degradation products in normal serum (Das *et al.*, 1967). A trained technician can assay 25 samples per day. Samples may be deep frozen if necessary (Das *et al.*, 1967), and Merskey, Johnson, Kleiner, and Wohl (1967) have shown that storage for six months is quite safe. Although a little laborious at first and demanding rigorous control, the technique is rapid, simple, and economical.

Method	Fibrin Degradation Products					Total No. of Samples
	Number of Samples Giving Positive Reaction with					
	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	>100 µg/ml	
Haemagglutination inhibition immunoassay	7	4	4	3	2	20
Ouchterlony	0	3	4	3	2	12
Comparative immunoelectrophoresis	0	3	3	3	2	11
Immunoelectrophoresis	0	0	1	2	2	5

Table III Comparison of haemagglutination inhibition immunoassay with Ouchterlony, immunoelectrophoresis, and comparative immunoelectrophoresis techniques

Increased inhibition after prolonged incubation of the antigen with antibody is believed to be due to the progressive aggregation of antigen-antibody complexes and a consequent reduction in their ability to coat sensitized cells (Fulthorpe, 1959). This observation can be put to practical use by improving the sensitivity of the test system, this being less when dilute antisera are used. However, provided the standard and the unknown sera are treated identically, the length of incubation, change of cell concentration, or pH of the test medium will not affect the results significantly.

Heparin in the test system interferes with the reaction by showing an apparent decrease in the amount of fibrin degradation products; this is believed to be a manifestation of inhibition by a polyanion, having a high affinity for fibrinogen, on the interaction of fibrinogen and its antibody (Murakami, 1965).

It is of interest to note the observation by McLaughlin (1968) that *in vitro* early fibrinogen degradation products produced by plasmin form a complex with chylomicrons: lipaemic serum may interfere with the immunological techniques and further study is required.

It is possible that an assay system for measuring fibrin degradation products in serum may detect only a proportion of the total amount, since it is known that some products are incorporated in the clot structure (Bang, Fletcher, Alkjaersig, and Sherry, 1962; Latallo, Budzyński, Lipiński, and Kowalski, 1964). Moreover, the haemagglutination method gives no information on the heterogeneous molecular size and physico-chemical characteristics of fibrin degradation products. For this reason it seems that other methods of comparable sensitivity, but greater specificity and precision, must be developed, perhaps by utilizing preliminary separation procedures. Although, in the present work antigen-antibody crossed electrophoresis was carried out on only a limited scale, similar work by Laurell (1965 and 1966) seems to be encouraging and, for preliminary separation,

the use of acrylamide gel (Fisher, Fletcher, Alkjaersig, and Sherry, 1967) may be justified.

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