Antiplasmin activity of electrophoretically separated human serum fractions

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SYNOPSIS The antiplasmin which migrates electrophoretically with the α_2 globulins preponderates in effect over that of the α_1 migrating antiplasmin. This preponderance persists at physiological pH value *in vitro* and the significance of this finding is discussed.

No evidence has been obtained of the existence of anti-urokinase activity in antiplasmin-free serum fractions.

The fibrinolytic activity of the enzyme plasmin is inhibited by two different plasma components which migrate electrophoretically with the α_1 and α_2 globulins respectively. The α_1 migrating antiplasmin combines relatively slowly and irreversibly with plasmin in a manner which is temperature dependent. The α_2 migrating antiplasmin produces an immediate, temperature-independent inhibition which is more readily reversible (Norman, 1958, 1960). In addition, it has been suggested that antiplasmin activity resides largely in the α_2 globulin fraction (Jacobsson, 1955).

Plasmin neutralization may, therefore, be predominantly due to an antiplasmin fraction which combines in a rapid and reversible manner with plasmin. As this concept is of importance, we have undertaken studies of the antiplasmin and anti-urokinase activity of each fraction of normal human serum separated by curtain electrophoresis. In this paper, we report the antiplasmin activity of each such fraction, at the pH of the electrophoretic separation and at the physiological pH value, and the anti-urokinase activity of the fractions at the pH of separation, over a range of urokinase concentrations.

It has been found that, in this experimental system, the potency of the α_2 antiplasmin preponderates over that of the α_1 antiplasmin at both pH values examined. We consider, therefore, that plasmin neutralization is likely to be predominantly rapid and reversible in the circumstances in which these characteristics are typical of the α_2 antiplasmin.

MATERIALS

The following were used:—Fibrinogen, human, grade L (bottles of 1 g.), Kabi Pharmaceuticals Ltd., plasminogen, human, grade B (120 casein units/vial), Kabi Pharmaceuticals Ltd; plasmin, human, grade B (28 casein units/vial), Kabi Pharmaceuticals Ltd.; urokinase reference standard, human (2,400 Ploug units/vial), Leo Laboratories Ltd.; thrombin reagent, bovine (2,500 units/vial), Leo Laboratories Ltd.; veronal buffer pH 7.4, 0.154 M.: sodium diethyl barbiturate 11.745 g., sodium chloride 14.67 g., hydrochloric acid 0.1 N 430 ml., distilled water to 2 litres; and Tris citrate buffer pH 8.6: 0.076 M. Tris 9.21 g./litre, 0.005 M. citric acid 1.05 g./litre.

METHOD

Fresh human serum was obtained on three separate occasions from a male subject without evidence of arterial disease or altered fibrinolytic activity and each specimen was individually separated by curtain electrophoresis into 47 fractions using Tris citrate buffer pH 8.6. On each occasion, serum was applied at a rate of 38 ml./day. A potential of 2,100 volts was used for a period of 48 hours on two occasions and 24 hours during the third separation. A temperature of 5 to 7°C. was maintained during the electrolysis with the buffer pump delivering 80 ml./hour.

The optical density of each fraction was determined at 280 m μ using a Unicam SP.500 spectrophotometer. The fractions were of equal size and the pH of each was determined using a direct reading pH meter (Electronics Instruments Ltd.).

Each fraction was then tested for interference with the plasmin-substrate interaction, the following reagents being added, in the order given, to assay tubes contained in an ice water bath at 4°C. in which each reagent had been assembled.

1 Human fibrinogen, 0.6 ml. (5 mg.), in veronal buffer pH 7.4

2 Serum fraction, 2 ml., or, in the control tubes, 2 ml. of the Tris citrate buffer used in the electrophoretic separation

3 Plasmin, 0.1 ml. (0.5 c.u.), in veronal buffer pH 7.4

4 Calcium (0.296 mg.) 0.2 ml., thrombin (5 units) mixture in veronal buffer pH 7.4.

The lysis time was commenced the moment the plasmin was added and the tubes were agitated as the calcium-thrombin addition was made. Each tube was then retained in the ice water bath for exactly five minutes to permit clotting to become complete, before the test clots and controls were transferred to a water bath at 37°C. to permit lysis to occur.

The end point was recorded when air bubbles trapped in the gel were suddenly released and the opaque gel became totally transparent. In addition, the development of the sol phase was obvious when the tubes were inclined at an angle of 30°, and was associated with the development of a typical meniscus in place of the previously irregular gel surface.

On the third occasion, the fractions were concentrated by dialysis to double their previous strength and half their previous volume. The test of antiplasmin activity was then undertaken on each fraction both before and after careful titration to pH 7.4 using 0.3 M citric acid.

On the second occasion aliquots, each of 2 ml. serum fraction, were tested for anti-urokinase activity, the plasmin in the above experiments being replaced by the following reagents:—

1 Plasminogen, 0.1 ml. (2.5 c.u.), in veronal buffer pH 7.4.

2 Serial concentrations of urokinase, 0.1 ml., in veronal buffer pH 7.4.

The experiments to detect anti-urokinase activity were undertaken with the consecutive serum fractions pooled in batches of four. Five clots were then prepared from each batch and the clots were individually activated with log. concentrations of urokinase between the range 0.0005 to 1.0 Ploug units. Controls were prepared as in the previous experiments.

The concentrations of plasminogen and plasmin used in these studies were derived from consideration of preliminary experiments in which they were found to be optimal. Greater concentrations did not accelerate further the rate of fibrinolysis and lesser concentrations resulted in prolonged lysis times progressively dependent upon the increasing plasminogen or plasmin deficiency.

RESULTS

EFFECT OF PLASMINOGEN AND PLASMIN CONCENTRATION The results of the preliminary experiments to determine the effect of increasing plasminogen and plasmin concentrations in the experimental system used are illustrated in Figure 1. Concentrations of 2.5 c.u. plasminogen or 0.5 c.u. plasmin were considered to be optimal.

ANTIPLASMIN ACTIVITY The antiplasmin effect, that is, the specific inhibitory effect of each serum

FIG. 1. Serial concentrations of plasminogen in a standard 2 ml. clot comprising 5 mg. human fibrinogen, human plasminogen, 200 Ploug units, urokinase, 0.296 mg. (0.001 M.) CaCl₂ and 5 units bovine thrombin all in veronal buffer pH 7.4. Also serial concentrations of plasmin in a similar standard clot in which the plasminogen and urokinase were replaced by human plasmin.

FIG. 2. The pH and optical density at 280 mμ of each fraction of human serum separated by certain electrophoresis in Tris citrate buffer pH 8.6; also antiplasmin effect, or interference with the plasmin-fibrin interaction of each fraction.

fraction on the plasmin-fibrin interaction, was calculated as the lysis time of the clot containing the serum fraction less the lysis time of the control clot.

The optical density, original pH, and antiplasmin effect of each serum fraction are shown in Figure 2. Within the limits of minor experimental error, identical results were obtained on each of the three occasions and it will be observed that the potency of the α_2 migrating antiplasmin greatly exceeded that of the α_1 migrating antiplasmin.

The pH and antiplasmin effect of each serum fraction, after 50% concentration, both at the pH of separation and after titration to pH 7.4 is shown in Fig. 3, from which it will be noted that the greater potency of the α_2 antiplasmin persists at the physiological pH value. The potency of both antiplasmin fractions is greater at pH 8.6 than at pH 7.4.

From these graphs, it will be noted that an inhibitory effect on the plasmin-substrate interaction was detected in the post gamma-globulin region. This effect coincided with increasing alkalinity of these fractions in the original separation and was eliminated by titrating these fractions to pH 8.6 as shown in Figure 3. It was also observed that this effect could be stimulated with buffers of increasing alkalinity up to and including pH 10, and it is concluded, therefore, that the interference with the plasmin-substrate interaction in the post-gamma-globulin region is an artefact due to alkaline pH changes.

ANTI-UKINASE EFFECTS When the plasmin in the

above experiments, which were undertaken to detect antiplasmin activity, was replaced by plasminogen and urokinase, so that the experiments would also reveal anti-urokinase activity, it was found that no fraction exhibited an inhibitory effect which did not show antiplasmin activity.

The inhibitory activity of the fractions previously shown to exert an antiplasmin effect was demonstrated with each concentration of urokinase used. The inhibitory effect on the plasmin-substrate interaction, which was previously demonstrated in the post-gamma globulin region, was found to cause inhibition of urokinase-activated fibrinolysis when the urokinase concentration was 0.0001 Ploug units. This effect was less when the urokinase concentration was 0.001 Ploug units and the system was insensitive to this interference when greater concentrations of urokinase were used.

The possibility remains that an anti-activator, if present in the fractions which inhibited the lysis of fibrin by plasmin, would be masked by this specific antiplasmin effect.

DISCUSSION

Jacobson (1955), using filter-paper zone electrophoresis, found human serum to contain two trypsin inhibitors which migrated with the α_1 and α_2 globulin fractions respectively; the α_2 fraction inhibited both plasmin and trypsin while the α_1 fraction inhibited only trypsin. Norman (1958) and Norman and Hill (1958), using

FIG. 3. The pH and antiplasmin effect of each fraction of human serum before and after titration to pH 7.4. The interference with the plasmin-substrate interaction originally present in the post-gamma-globulin region, and associated with increasing alkalinity of these fractions, was eliminated by an intermediate titration to pH 8.6.

starch zone electrophoresis, reported one antiplasmin migrating with the α_1 globulins and another migrating with the α_2 globulins.

Rybak and Rejnek (1959) studied the antiplasmin activity of plasma fractions, separated by preparative electrophoresis, ethanol fractionation, and fractionation by metal salts, and found activity to be restricted to the α -globulins. Nilsson, Krook, Sternby, Söderberg, and Söderström (1961), using preparative paper electrophoresis, found that in normal serum antiplasmin activity was mainly recovered from the α_2 globulin fraction, whereas most of the antitrypsin activity existed in the α_1 globulin region. Finally, Riding and Ellis (1964) confirmed the previous observations of Norman and Hill (1958) that antiplasmin activity is found in the Cohn ethanol fractions IV-4 and IV-1, corresponding to the α_1 and α_2 migrating antiplasmins. In addition, these authors found inhibitory activity in fraction III-O, the lipoprotein-containing fraction of human plasma.

In our own experiments, it has been found that the potency of the α_2 antiplasmin consistently preponderates over that of the α_1 antiplasmin and this effect persists at physiological pH range. As the α_2 antiplasmin neutralizes plasmin in a rapid and reversible manner (Norman, 1960), it appears reasonable to infer that plasmin neutralization is predominantly rapid and reversible in nature.

It has been shown that fibrin can actually partly reverse the combination of plasmin with the α_1 antiplasmin (Ambrus, Back, and Ambrus, 1960) and that an increase in the concentration of the protein

substrate can readily reverse the neutralization due to the α_2 antiplasmin (Norman, 1960).

These considerations explain how a fibrin clot can be lysed by plasmin even in the presence of potent inhibitors, and Ambrus and Markus (1960) have suggested that plasmin-antiplasmin complex exists as a reservoir of fibrinolytic enzyme which dissociates in the presence of fibrin. In addition, it has been demonstrated that, when the plasmin concentration is increased in a plasmin-fibrin inhibitor system, a portion of the inhibitor which was previously free becomes involved in the reaction (Rybak, 1962). However, increasing concentrations of the inhibitor do not completely inactivate plasmin, part at least of which can be made available to lyse fibrin (Taylor, Allen, and Bickford, 1964).

The concept that plasmin-antiplasmin complex may form a circulating reservoir of plasmin which is innocuous to other circulating proteins but which forms a ready source of plasmin when this is needed for the lysis of fibrin is, therefore, of interest. The extent of such plasmin binding may be appreciated from the work of Nanninga and Guest (1964) who have shown that bound plasmin is present in a hundred-fold greater concentration than free plasmin at plasmin concentrations below 10^{-7} M. The fact that a failure of fibrinolysis may occur in the presence of high levels of inhibitory activity is relevant and has been established by Maki and Nagasawa (1963).

Clearly, thrombolysis is dependent upon activation of plasminogen adsorbed on to the surface of fibrin (Fearnley, 1953, 1961; Sherry, Fletcher, and

Alkjaersig, 1959b). However, the presence of the fibrin polymer is not a physiological event and it may be that, in the physiological functioning of the fibrinolytic system, plasmin exerts an indirect effect by the repletion of a predominantly dynamic plasmin-antiplasmin complex or equilibrium.

Finally, it may be noted that the experimental studies reported have shown no evidence of anti-urokinase activity in antiplasmin-free serum fractions, although this evidence does not exclude the possibility that specific anti-activator activity may exist in serum fractions which inhibit the lysis of fibrin by plasmin.

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