FIBRINOLYTIC ACTION OF AN ENZYME PREPARATION CONJUGATED WITH SPECIFIC ANTIBODIES

V. P. Torchilin, A. V. Maksimenko,
G. V. Ignashenkova, E. G. Tishchenko,
G. A. Ermolin, and V. N. Smirnov

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The use of systems of targeted drug transport in clinical practice can result in a sharp increase in the effectiveness of treatment of several diseases. The general principles of creation of such systems, with the use of vector (recognizing) molecules have frequently been discussed in the literature [4, 7, 9, 10]. Synthetic polymers, repeatedly modified with molecules of the therapeutic agent and the vector compound [9], or microcontainers of liposome or cell type, containing the drug inside them, and the vector compound outside [4, 8], have been suggested as the basic transporting vehicle. However, concrete examples of such systems are very few in number. One such example is the use of a complex in which the antibiotic daunomycin is linked through a dextran bridge with monoclonal antibodies against rat α-fetprotein [11], as an immunoselective chemotherapeutic agent. Such an approach may be promising also for the use of thrombolytic (fibrinolytic) preparations. In this case, the thrombolytic agent can be bound with antibodies against any component of the thrombus.

In the investigation described below, the model proteolytic enzyme chymotrypsin and polyclonal antibodies against fibrinogen, which are known [6] to take part in immunochemical reactions with degradation products of fibrin and fibrinogen, by interacting with their common antigenic determinants, were chosen as test objects.

EXPERIMENTAL METHOD

Preparations of stabilized chymotrypsin were obtained by binding the enzyme with dextran, with a molecular weight of 35,000-50,000 daltons, previously activated by partial oxidation, as described in [3]. The enzyme, bound with the carrier, was separated from native enzyme by gel chromatography on a column with Sephadex G-75. As a result, enzyme preparations were obtained which did not differ significantly from the native product in their kinetic parameters of hydrolysis of the specific substrate — N-acetyl-L-tyrosine ethyl ester (ATEE, method of determination of initial rates of hydrolysis by titration on a pH-stat), but which surpassed it in stability [3]. The number of aldehyde groups in the oxidized dextran (20-24 aldehyde groups per 100 glycoside members) was found to be sufficient both for binding with chymotrypsin and also for subsequent addition of antibody molecules to the resulting chymotrypsin—aldehyde-dextran. Antibodies against fibrinogen were obtained by affinity chromatography on BrCN-Sepharose from a pool of sera from rabbits immunized with fibrinogen (from "Sigma," USA) in an initial concentration of 10 mg/ml, in accordance with the scheme developed previously [1].

Pure antibodies in 0.1 M phosphate buffer (pH 8.3) were mixed in a volume of 7 ml (concentration as protein 10 mg/ml) with 70 mg of the aldehyde-dextran—chymotrypsin preparation in a concentration of 10 mg/ml, and the mixture was incubated overnight with constant mixing. The conjugate thus formed was dialyzed against phosphate buffer (pH 7.4) and kept at −20°C until required for use. Determination of the catalytic activity of the enzyme showed that 5-10% of the initial quantity of dextran—chymotrypsin was bound with antibodies against fibrinogen. The esterase activity preserved by this conjugate (substrate 0.01 M ATEE in 0.1 M KCl, 20°C, pH 7.5) was 5-7% of the initial activity of the native enzyme. Determination of the fibrinolytic activity of preparations of native chymotrypsin, aldehyde-dextran—chymotrypsin, and conjugates of antibody and aldehyde-dextran—chymotrypsin *in vitro* was carried out by the

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method suggested by the writers previously [2]. To 0.5 ml of a solution (10 mg/ml) of fibrinogen 0.2 ml of a solution of 4 mg/ml of thrombin was added and the mixture was kept at room temperature. During this time a fibrin clot formed. It was placed in a reinforced plastic tube 1.0-1.5 cm in diameter (Fig. 1), with a bottom made of fine-mesh Kapron gauze, bathed in a solution of 0.1 M phosphate buffer (pH 7.4). Experiments with native or modified enzyme were carried out parallel to each other, using chymotrypsin derivatives in quantities ensuring their identical esterase activity ($8.75 \cdot 10^{-8}$ M). Lysis of the clot was monitored by measuring the increase in optical density of the bathing solution at 280 nm on a spectrophotometer ("Perkin-Elmer-Coleman-55," USA). Dependence of the increase in optical density of the solution (as a result of destruction of the insoluble fibrin clot and transfer of its fragments into solution) on time characterized the rate of lysis. The experiments to determine the relative affinity of the enzyme derivatives for the fibrin clot were carried out in accordance with the same scheme, except that the fibrin clot was incubated briefly (10 min) beforehand with a $2.2 \cdot 10^{-7}$ M solution of the corresponding enzyme preparation in 0.1 M phosphate buffer (pH 7.4), after which the tube containing the model clot was transferred into a solution of the same buffer, but without chymotrypsin, and the change in optical density (280 nm) of the solution was monitored for 4 h. In this case, lysis must have taken place only under the influence of the adsorbed enzyme preparation. In control experiments the model clot was bathed throughout the duration of the experiment in a solution of 0.1 M phosphate buffer (pH 7.4).

EXPERIMENTAL RESULTS

An increase in the affinity of the fibrinolytic preparations for thrombin is an inportant reserve for increased effectiveness of thrombolytic therapy [5]. Targeted application of drugs in microcontainers of liposome [4] or erythrocyte [8] type requires some sort of system for releasing the thrombolytic. Polymer derivatives possessing stable thrombolytic activity and increased affinity for thrombin may prove to be simpler and more promising in use. The general scheme of preparation of such products is by addition of molecules of antifibrinogenic antibodies to the stabilized thrombolytic enzyme preparation through the matrix of the polymer carrier, with the formation of a triple enzyme–carrier–antibody conjugate. This method is realized sufficiently easily experimentally, and enables binding of antibodies without any significant disturbances of the structure of their active centers. This results in increased absorbability of the resulting conjugates on the fibrin clot compared with ordinary enzyme preparations and, as a result of the increase in the local concentration of the fibrinolytic, it causes more rapid lysis of the thrombus. The use of a stabilized form of the enzyme to modify antibodies also enabled active preparations, not distinguished by appreciable stability, to be obtained on the basis of fibrinolytic enzymes.

A study of the fibrinolytic process in a model system *in vitro* showed (Fig. 2) that the rate of fibrinolysis was highest when a preparation of antibody–aldehyde-dextran–chymotrypsin conjugates was used. Since the concentration of active enzyme in all the bathing solutions was the same, it can be postulated that the increased rate of fibrinolysis in the case when a preparation of chymotrypsin covalently bonded through aldehyde-dextran with antibodies was used was due to increased affinity of the preparation for the thrombus. It will be recalled that relative to the kinetic parameters of enzymic hydrolysis of ATEE preparations of native and modified chymotrypsin are closely similar, and modification of the enzyme increases only its stability, but not the effectiveness of its catalytic action.

This hypothesis was confirmed by experimental comparison of the affinity of the chymotrypsin derivatives for the fibrin clot. In experiments with transfer of the clot from a solution of enzyme into pure buffer, the increased rate of lysis of the fibrin clot in the case of the triple complex could be due only to the action of the enzyme preparation specifically adsorbed on the clot. The rate of the fibrinolytic process recorded in this experiments is proportional to the quantity of enzyme preparation firmly bound with the substrate. It was in fact shown (Fig. 3) that it is indeed conjugates of antibodies with aldehyde-dextran–chymotrypsin that have increased affinity for the material of the thrombus, as shown by the more effective lysis of the fibrin clot. This fact is further confirmation that antibodies against fibrinogen do not lose their specific activity after conjugation.

Covalent binding of antibodies with aldehyde-dextran–chymotrypsin thus makes it possible to obtain preparations of stabilized proteolytic enzyme covalently bound through a molecule of high-molecular-weight activated carrier (aldehyde-dextran) with molecules of antibodies against fibrinogen. Addition of the latter under these conditions takes place without any significant loss of their specific activity, and it results in increased affinity of the en-

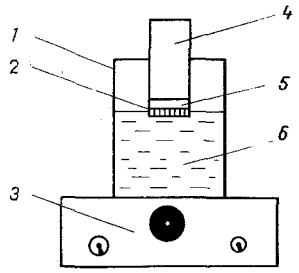

Fig. 1

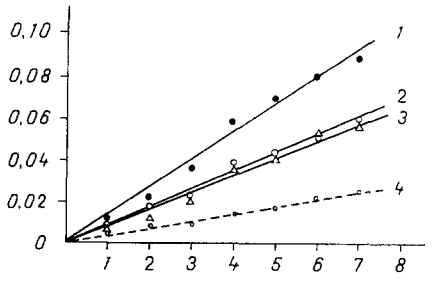

Fig. 2

Fig. 1. Schematic drawing of experimental system for measuring parameters of the thrombolytic process. 1) vessel, 2) fine-mesh gauze, 3) magnetic mixer, 4) tube, 5) fibrin clot, 6) bathing solution.

Fig. 2. Change in rate of lysis of fibrin clot under the influence of preparations of native and variously modified α-chymotrypsin. Abscissa, time (in h); ordinate, optical density at 280 nm. 1) antibody–aldehyde-dextran–chymotrypsin conjugate, 2) aldehyde-dextran–chymotrypsin; 3) native α-chymotrypsin, 4) control (0.1 M phosphate buffer, pH 7.4).

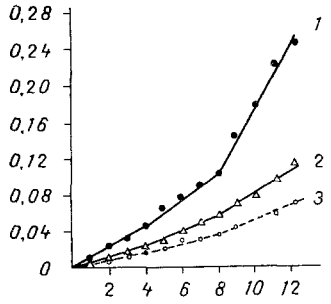

Fig. 3. Change in rate of lysis of model fibrin clot under the influence of modified α-chymotrypsin specifically and nonspecifically adsorbed by substrate. 1) antibody – aldehyde-dextran–chymotrypsin conjugate, 2) aldehyde-dextran–chymotrypsin, 3) control (0.1 M phosphate buffer, pH 7.4). Point of inflection corresponds to transfer of clot from enzyme solution into pure buffer. Remainder of legend as to Fig. 2.

zyme preparation for the fibrin clot. It may accordingly be hoped that derivatives of thrombolytic enzymes synthesized in this way may prove to be an effective means of systemic thrombolysis.

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