Modification of acyl-plasmin-streptokinase complex with polyethylene glycol

Reduction of sensitivity to neutralizing antibody

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Acyl-plasmin-streptokinase complex has advantages as a 'site' directed fibrinolytic agent with the active site protected from the plasma protease inhibitors. But, in clinical use, the fibrinolytic potential of this acyl-enzyme complex is modified or abolished by the presence of streptokinase antibodies in the patients. Therefore, better therapeutic agents are required. In this work, chemical modification of the acyl-plasmin-streptokinase complex with polyethylene glycol was found to result in marked resistance to neutralization with streptokinase antibodies.

Streptokinase *Plasmin* *Antibody* *Fibrinolysis* *Polyethylene glycol* *Chemical modification*

1. INTRODUCTION

The treatment of deep vein thrombosis with currently available thrombolytic drugs, streptokinase and urokinase, is not very satisfactory. The major problem with streptokinase as a thrombolytic agent is the induced hemostatic defect leading to severe and life-threatening or fatal hemorrhage and a new approach to this problem has been reported [1].

BRL 26921 is a novel thrombolytic agent in which streptokinase-plasminogen complex is chemically modified by acylation at the catalytic center of its plasmin moiety. Because the fibrin-binding site of the complex and its catalytic center are functionally separate, the acylated complex can bind to fibrin and is protected from the normal plasma protease inhibitors [1]. This complex has been shown to be a potent and specific thrombolytic agent in rabbits and dogs with experimental venous thrombosis [1].

In patients with acute myocardial infarction, Walker et al. [2] found that the complex was well tolerated and produced no serious bleeding when administered into a coronary artery to patients with doses of BRL 26921 ranging 5 to 25 mg, although it appeared less 'selectively' thrombolytic in patients than had been expected from animal models. They also found that the degree of induced systemic fibrinolytic activation was modified by the presence of streptokinase antibodies.

In healthy volunteers [3] and in patients with deep vein occlusion [4], it has been shown that administration of acyl-plasminogen-streptokinase complex (or plasminogen-streptokinase complex) induces anamnestic rise in streptokinase antibodies which neutralize the action of enzyme complex. Matsuo et al. [5] investigated the influence of streptokinase antibodies on the fibrinolytic and fibrinogenolytic activity of BRL 26921 and reported that high titers of streptokinase antibodies abolished the effect of the agent.

In clinical use, therefore, it is important to take account of the patient's pretreatment streptokinase resistance.

This study was designed to prepare a new class

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of thrombolytic agents, acyl-plasmin-streptokinase complex derivatives, of which the fibrinolytic potential is not influenced by streptokinase antibodies present in the patients.

2. MATERIALS AND METHODS

2.1. *Reagents*

Purified streptokinase (EC 3.4.99.22; kabikinase) and human fibrinogen (grade L) were purchased from Kabi Vitrum AB (Stockholm). Monomethoxypolyethylene glycol (M_r 5000) was from Aldrich (USA). Synthetic fluorogenic substrate, Boc-Val-Leu-Lys-MCA, was from Peptide Institute, (Osaka, Japan).

2.2. *Acyl-plasmin-streptokinase complex*

Human plasminogen was activated by incubation with a catalytic amount of streptokinase. Plasmin was further purified by affinity chromatography on a soybean trypsin inhibitor-immobilized Sepharose 4B column. Purified plasmin was acylated with *p*-nitrophenyl-*p'*-guanidinobenzoate. Acyl-plasmin (31 mg) was absorbed on an L-lysine-Sepharose column and washed with 0.1 mM *p*-nitrophenyl-*p'*-guanidinobenzoate/50 mM sodium phosphate buffer (pH 7.4). Streptokinase (26 mg) was applied to the column and washed with the same buffer. Acyl-plasmin-streptokinase complex was obtained by elution with the same buffer containing 0.2 M ϵ-aminocaproic acid. This acyl-plasmin-streptokinase complex was shown to consist of equimolar amounts of acyl-plasmin (M_r 85 000) and streptokinase (M_r 47 400) by SDS-polyacrylamide gel electrophoresis (not shown).

2.3. *Modification with monomethoxypolyethylene glycol*

Acyl-plasmin-streptokinase complex was modified as in [6]. Monomethoxypolyethylene glycol (15 g) was coupled with cyanuric chloride (0.55 g) to form 2-*o*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine. To an acyl-plasmin-streptokinase complex solution (5 mg) in 0.5 M tetramethylammonium hydroxide/0.1 mM *p*-nitrophenyl-*p'*-guanidinobenzoate/50 mM sodium phosphate buffer (pH 7.4), activated polyethylene glycol was added. The mixture was incubated for 30 min at 4°C. Then excess activated polyethylene glycol was removed by ultrafiltration with an XM-50 membrane (Amicon). Acyl-plasmin-streptokinase complexes with various degrees of modification were prepared using different molar ratios of activated polyethylene glycol (PEG) to amino groups in the acyl-enzyme molecule (PEG/-NH_2: 1.0, 3.0 and 10.0). The extents of modification of amino groups in the molecule were determined by measuring the amounts of free amino groups with trinitrobenzenesulfonate [7].

Protein concentrations were determined by the biuret method [8].

2.4. *Deacylation of the acyl-plasmin-streptokinase complex*

The native (50 μg) and modified complexes (50 μg) were incubated in 1 ml of 100 mM L-lysine/100 mM NaCl/50 mM Tris-HCl (pH 8.0) at 37°C.

2.5. *Plasminogen activator activity*

Plasminogen activator activities of the deacylated native and modified enzyme complexes were assayed with a synthetic fluorogenic substrate, Boc-Val-Leu-Lys-MCA, as follows [9]: enzyme complexes (0.5 μg) were preincubated with 50 μg human plasminogen in 1 ml of 20 mM L-lysine/100 mM NaCl/50 mM Tris-HCl (pH 8.0) for 10 min at 37°C. Then, 1 ml of 0.1 mM Boc-Val-Leu-Lys-MCA was added and incubated for 20 min at 37°C. The liberated 7-amino-4-methylcoumarin (AMC) was measured fluorometrically with excitation at 380 nm and emission at 460 nm. Under these conditions, streptokinase showed a specific activity of 10 μmol AMC/min per mg.

2.6. *Neutralization of plasminogen activator activity*

After incubation for 8 h under the above conditions to generate enzyme activity, the native and modified enzyme complexes (0.5 μg) were incubated with streptokinase antibodies (0–0.4 mg) in 1 ml of 20 mM L-lysine/100 mM NaCl/50 mM Tris-HCl (pH 8.0) at 37°C for 20 min. Then, remaining plasminogen activator activity was measured with the synthetic substrate as described above.

2.7. *Fibrinolytic activity*

The fibrin clot lysis activities of native and modified acyl-plasmin-streptokinase complexes were determined by incubating them (220 μg) in 5

ml normal human serum (streptokinase antibody titer 25 units per ml) at 37°C with a fibrin clot, which was prepared in a silicon-coated glass tube (ϕ 2.4×20 mm) from human fibrinogen and human thrombin. After incubation for 6 h, lysis of the fibrin clot was determined by measuring the length of the fibrin clot. A standard curve was prepared with various amounts of streptokinase.

Streptokinase antibody titers were determined as described by Amery et al. [10].

2.8. *Streptokinase antibodies*

Anti-streptokinase serum was obtained from rabbits immunized 5 times by subcutaneous injection of streptokinase (5 mg×5). Streptokinase antibodies were obtained from antiserum by $(NH_4)_2$-SO_4 precipitation and DE-52 chromatography.

3. RESULTS AND DISCUSSION

The acyl-plasmin-streptokinase complex has the proposed advantages as a site directed fibrinolytic agent with the active site protected from normal plasma protease inhibitors of plasmin. Since the active site of the acyl-enzyme complex is blocked, there is little residual enzymic activity. But the acyl moiety can be released by deacylation. Thus, if an acyl-plasmin-streptokinase complex is applied to an assay of fibrinolytic activity, the release of plasmin-streptokinase complex by deacylation would be expected to lead to fibrinolysis. Fig.1 shows the deacylation of the native and modified complexes after incubation at 37°C. The degree of generation of plasminogen activator activity was influenced by polyethylene glycol modification. The generated activator activity of native enzyme complex rapidly decreased with time of incubation (curve A), while the activities of the modified enzyme complexes were more stable (curves B–D). These results suggest that polyethylene glycol protects the complexes from proteolytic inactivation. This conclusion is consistent with the findings that modification of catalase [11] and L-asparaginase [12] with polyethylene glycol result in their resistance to proteolytic digestion.

These generated plasminogen activator activities of the modified complexes showed marked resistance against neutralization with streptokinase antibodies (fig.2). After incubation for 8 h at 37°C to generate enzyme activity, the native (curve A) and

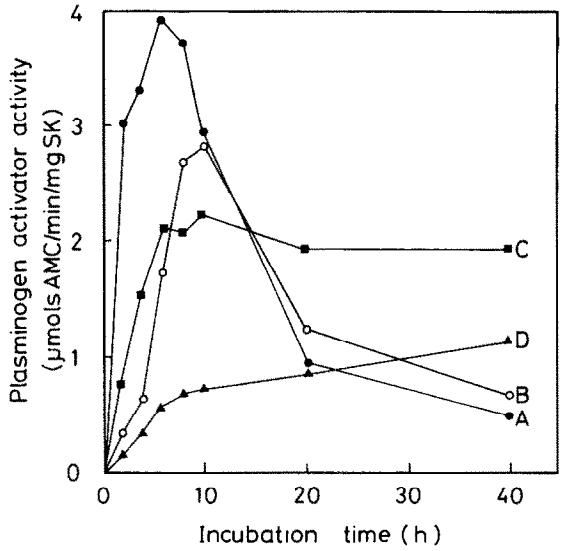

Fig.1. Deacylation of native and polyethylene glycol-modified acyl-plasmin-streptokinase complexes after incubation. Curves: A (●), native complex; B (○), modified complex (26.2% modification); C (■), modified complex (57.5%); D (▲), modified complex (68.6%).

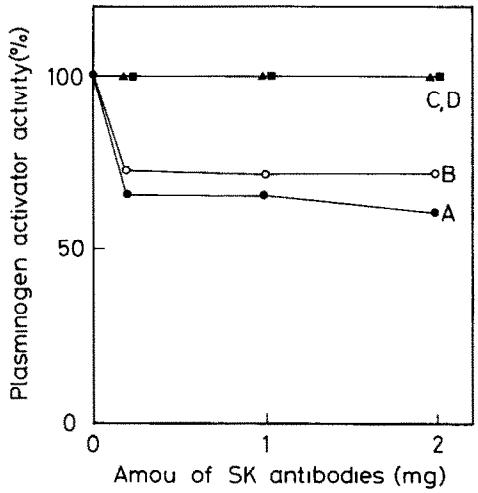

Fig.2. Effect of streptokinase antibodies on plasminogen activator activities of native and polyethylene glycol-modified acyl-plasmin-streptokinase complexes. Curves: A (●), native complex; B (○), modified complex (26.2% modification); C (■), modified complex (57.5%); D (▲), modified complex (68.6%).

modified (curves B–D) complexes were incubated with various amounts of streptokinase antibodies

at 37°C for 20 min and then remaining plasminogen activator activity was measured with the synthetic substrate. The incubation with antibodies resulted in the reduction of the activator activity of native complex (40%), while the polyethylene glycol-modified complexes, in which 57.5% (curve C) and 68.6% (curve D) of the amino groups in the native complex had been modified, showed complete resistance to neutralization with streptokinase antibodies. Streptokinase showed complete loss of enzymic activity on incubation with 0.4 mg streptokinase antibodies (not shown).

The fibrin clot lysis activities of the polyethylene glycol modified acyl-plasmin-streptokinase complexes were determined in human serum (streptokinase antibody titer 25 units per ml) (table 1). Increasing the degree of modification of amino groups with polyethylene glycol resulted in the reduction of fibrinolytic activity. The modified acyl-plasmin-streptokinase complex in which 57.5% of the amino groups in the native complex were substituted showed 40% of the activity of the native complex. Although, we have not assessed the fibrinolytic potential of this material in human serum which had high titers of streptokinase antibodies, it is suggested that this material should show no reduction of the fibrinolytic activity with antibodies in human serum or plasma, from the results described in fig.2.

The fibrinolytic activity of acyl-plasminogen-streptokinase complex, BRL 26921, has been shown to be abolished in human plasma with high titers of streptokinase antibodies [5]. They had investigated the fibrinolytic activity of BRL 26921 at a final concentration of 100 units per ml in normal plasma (streptokinase antibody titer 25 units per ml) and plasma obtained from a patient 3 weeks after treatment with streptokinase (streptokinase antibody titer 610 units per ml) and reported that high titers of streptokinase antibodies abolished the effect of BRL 26921 [5].

Table 1

Fibrinolytic activities of the polyethylene glycol modified acyl-plasmin-streptokinase complexes

Sample	Modification degree (%)	Fibrinolytic activity (%)
Native complex	0	100
Modified complex	26.2	71
Modified complex	57.5	40
Modified complex	68.6	10

In healthy volunteers [3] and in patients with deep vein occlusion [4], it has been shown that administration of acyl-plasminogen-streptokinase complex (or plasminogen-streptokinase complex) induces anamnestic rise in streptokinase antibodies which neutralize the action of these agents. In clinical use, therefore, it is essential to take account of the patient's pretreatment neutralizing antibody, because until this neutralizing antibody is exceeded no fibrinolysis will take place in the patient.

The preparation of polyethyelene glycol modified acyl-plasmin-streptokinase complex reported here showed: (i) generation of plasminogen activator activity by deacylation; (ii) fibrinolytic activity in human serum; (iii) complete resistance to neutralization with streptokinase antibodies. Because of its low sensitivity to the neutralizing antibody, we believe that the polyethyelene glycol-modified acyl-plasmin-streptokinase complex should be useful for treating patients who have high titers of streptokinase antibodies, in particular, those who had previous streptokinase treatment.

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