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Inhibition of a Human Fibrinolytic System by Normal and Pathologic Sera. (27765)

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The potential importance of fibrinolytic mechanisms in health and disease has been suggested(1). However, our knowledge of fibrinolysin inhibitor concentration of serum in pathologic states is meager(2). In previous studies streptokinase was usually used in the assay system. This practice imposes a limitation since sera contain variable levels of streptokinase antibody(3).

The purpose of this study was to construct a lysing medium from human fibrinogen-plasminogen, activated with human urokinase and clotted with thrombin, and to investigate the inhibition of such a system by sera from normal human controls and from patients with various diseases. Several inhibitors to fibrinolysis have been described(2). The assay medium organized for this study was believed to contain all essential human fibrinolytic components. It was, therefore, thought that any inhibitor response measured would reflect the total inhibiting capacity of serum on all the fibrinolytic reactions, under the conditions employed. Variations in the proportions of individual inhibitors would not, of course, be observed. Measurement of the summation of serum inhibitor activity might mimic physiological conditions more realistically than would the use of purified entities. Herein is reported the fibrinolytic inhibitor activity of 16 normal human control sera and sera from 32 patients with a variety of clinical conditions.

Materials and methods. Human fibrinogen†: The solids from several vials of lyophilized, partially purified human fibrinogen were mixed intimately in a closed container. The composite analyzed 11% moisture and 80% protein; 30% of the protein was clottable. The fibrinogen was assayed for plasminogen using streptokinase as an activator and contained 0.12 caseinolytic units/mg clottable protein. The composite was stored at 4°C.

Urokinase‡: A stock solution was prepared

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† Courtesy of Dr. K. B. McCall, Biologic Products Section, Michigan Dept. of Health, Lansing. Outdated material from the American Red Cross blood plasma fractionation program.

‡ Leo Pharmaceutical Products, Denmark.

by dissolving in 2 ml of water the contents of a vial labeled to contain 10,000 Ploug units. It was stored frozen. A working dilution containing 500 units per ml was made with water for each assay.

Thrombin (bovine)§: A stock solution, prepared by dissolving in 10 ml of water the contents of a vial labeled 1,000 NIH units was stored frozen. A working dilution of 10 units per ml was made for each assay using veronal-saline as a diluent.

Veronal-saline buffer: Nine grams NaCl were dissolved in 1:1 of .04 M veronal containing 16 ml of 0.2 N HCl, (pH = 7.3); 20 ml of 1:1000 aqueous merthiolate solution were added as preservative.

Human sera: Ten ml of venous blood were drawn pre-prandially and held at room temperature for an hour. The sponaneously formed clot was gently broken up during this period. Clots were removed by centrifugation at approximately 4000 rpm for 30 minutes at 4°C and the serum used at once. The serum did not show fibrin precipitation upon addition of human fibrinogen, indicating absence of residual thrombin. Nor did any further fibrin precipitate upon the addition of thrombin to an aliquot of the serum demonstrating the absence of fibrinogen.

An assay model which was arbitrarily controlled to give a fibrinolytic time of 13 ± 1 minutes was constructed as follows. Into a serological tube (75 × 10 mm) was introduced 0.1 ml of a 5% aqueous solution of the fibrinogen composite (1.2 mg of clottable protein and 0.14 unit of plasminogen). To this was added 0.7 ml of veronal-saline followed by 0.1 ml of urokinase. A stopwatch was started, 0.1 ml (1 unit) of thrombin was blown in, and the reagents mixed by upending against a disposable cork stopper. The tube was immediately placed in a water bath at 37°C. A firm, visible fibrin clot formed in about 30 seconds. Lytic times were recorded to the nearest minute when the contents of a tube ran as free-flowing fluid upon gentle tipping and no fibrin strands could be seen. The urokinase concentration was controlled to give a lytic time of 13 ± 1 minutes; this was usually attained by adding 50 units calculated from the label. A clot formed without urokinase showed no lysis within 48 hours.

§ Upjohn Co.

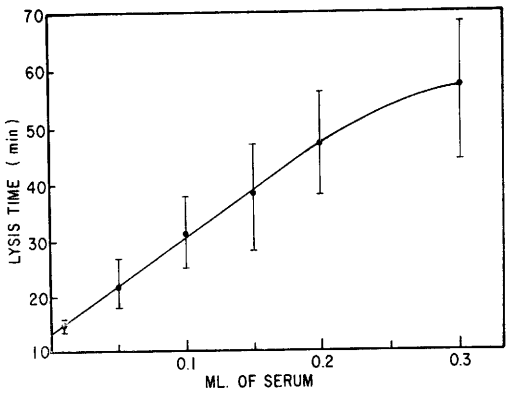

FIG. 1. Fibrinolysis inhibitor activity of 16 normal human sera. Each point is a mean value with range indicated by brackets.

Control sera obtained from 16 normal male and female hospital personnel were assayed in duplicate or triplicate by adding .01 to 0.3 ml to the system at the expense of veronal-saline. Sera from hospitalized patients were tested only in a volume of 0.2 ml. Each study of pathologic sera included control tubes without serum, and tubes containing 0.2 ml of normal serum.

Results. The inhibitor activity of normal serum on fibrinolysis in this system is evident from Fig. 1. The individual lysis times with 0.2 ml normal serum were: for 8 males—53, 52, 50, 38, 48, 54, 42, 48 minutes; and for 8 females—53, 38, 38, 56, 39, 54, 41, 48 minutes. Statistical analysis‖ showed no significant difference between the mean lysis times of males and females, nor in the variations within these groups. The data were, therefore, treated as a single group. The average time for all 16 was 47 minutes with a standard deviation of 6.6 minutes. Further analysis established that at least 95% of the population would yield lysis times within the range of 28 to 66 minutes at a 95% level of confidence(4). Any values obtained outside the 95% probabilities might reasonably be suspected of being abnormal.

Of 32 patients studied, significantly enhanced serum fibrinolysis inhibitor activity was observed for 8 patients representing 5

‖ We are indebted to J. I. Northam, Upjohn Co., for analyzing the data.

TABLE I. Patients with Significantly Enhanced Serum Fibrinolysis Inhibitor Activity.

Case No.	Age	Sex	Major condition(s)	Lysis time* (min)
1	62	♂	Acute pulmonary edema	92
2	53	♂	Hemorrhage (following cardiac valvuloplasty)	70
3	12	♀	Familial hyperlipemia	110
4	30	♂	Hyperlipemia (lipid storage disease, unknown type)	87
5	41	♀	Intestinal malabsorption syndrome	83
6	25	♀	Thyrotoxicosis	80
7	15	♀	"	71
8	37	♀	"	85

* Longer than 66 min considered significant; see text for details.

different primary disorders (Table I). The remaining 24, who demonstrated lysis times within the normal range, had the following diseases: active thrombophlebitis with cirrhosis and hypertension; recurrent idiopathic thrombophlebitis—2 cases; migratory thrombophlebitis and metastatic carcinoma—2 cases; pheochromocytoma; pre- and postoperative pheochromocytoma; malignant hypertension; essential hypertension—5 cases; Marfan's syndrome; hypercholesterolemia, diabetes, nephrosis; arteriosclerotic heart disease; hypercholesterolemia; uremia, hereditary nephropathy; urticaria pigmentosa; protein-losing enteropathy; psychoneurosis; pregnancy, last week—2 cases; and thyrotoxicosis.

The ability of serum to inhibit fibrinolysis was destroyed by heating at 56°C for 30 minutes, or at 37°C for 20 hours; there was an appreciable loss after 8 weeks' storage at 4°C. There was no loss of activity when serum was dialyzed against saline at 4°C for 24 hours. However, dialysis against water until the 1000-ohm resistance of whole serum was increased to about 13,000 ohms resulted in a precipitate with complete loss of inhibitor activity in the supernatant.

In some ancillary studies a few substances were tested which had been reported to have *in vivo* effects on fibrinolysis. A lysis time at least twice that of the 13-minute control value was considered to represent inhibition. Epsilon-amino-n-caproic acid inhibited the urokinase activated human fibrinogen-plasminogen system at a concentration of 2×10^{-3} mols; this is approximately 10 times the amount required to double the lysis time of a streptokinase activated bovine fibrinogen-human plasminogen system(5). Histamine (5×10^{-2} mols) and 5-hydroxytryptamine (10^{-2} mols) also showed inhibition while 1-butyl-3-(p-tolylsulfonyl) urea (tolbutamide, 10^{-3} mols) had no effect.

Discussion. The paucity of studies on serum fibrinolytic inhibitor activity can be attributed to the yet unresolved need for an uncomplicated, sensitive assay technic. Under the conditions of the assay used in this work, serum inhibitor concentration was in the normal range in a variety of pathologic conditions including such "thrombogenic conditions" as thrombophlebitis, multiple thrombosis, arteriosclerosis, and hypercholesterolemia. The results were not unexpected, since even in non-pathologic serum the inhibitor capacity greatly exceeds the potential for fibrinolysis(6). Attractive hypotheses(1,7) have been offered suggesting that fibrin deposition in these syndromes may result from an imbalance in the fibrinolytic mechanism. The present failure to observe elevated systemic inhibitor activity in these conditions seems to strengthen the argument that if such an imbalance exists it is a local phenomenon (1).

Eight pathologic sera showed inhibitor activity well above the normal range. Two of these were lipemic sera and the results agree with previous reports(8) concerning the *in vivo* effect of lipemia on fibrinolysis. It is not clear why fibrinolytic inhibitor was elevated in the serum of a patient with pulmonary edema, one with intestinal malabsorption, a case of hemorrhage immediately following cardiac surgery, or in 3 of 4 patients who presented thyrotoxicosis. None of these patients had manifest thrombosis, which again suggests that an enhanced systemic fibrinolytic inhibitor activity may have no relationship to fibrin deposition *in vivo*.

The effect of 5-hydroxytryptamine was investigated because of a continued interest in a possible role of serotonin in blood coagula-

tion(9). Inhibition was seen, which could be in the direction supporting the hypothesis that serotonin has a function in hemostasis; however, the minimum effective concentration of 10^{-2} molar was greater than would be expected physiologically.

The interrelationship of serotonin, histamine, and mast cells(10) and anaphylaxis in which fibrinolytic activity increases(11), stimulated exploration of the possible effect of histamine in the *in vitro* assay. In dilute solutions histamine had little effect; inhibition sufficient to double the control lysis time was seen at a histamine level of 5×10^{-2} M which would be a toxic concentration. Thus, this *in vitro* technic did not demonstrate any role for histamine in the fibrinolytic response observed in anaphylaxis. Oral administration of sulfonylureas has been said to increase the *in vivo* fibrinolytic activity of blood(12), but tolbutamide in high concentration had no effect on the *in vitro* system described here.

Summary. A standardized, urokinase activated, human fibrinogen-plasminogen fibrinolytic system with an arbitrarily controlled lysis time of 13 ± 1 minutes is described. Normal human serum inhibited this fibrinolytic complex. Sera from 8 of 32 patients with various diseases had inhibitor activity significantly above normal. None of these had diseases with abnormal accumulations of fibrin. In ancillary studies epsilon-amino-n-caproic acid inhibited the assay system as, to a lesser degree, did serotonin and histamine; tolbutamide had no significant effect.

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Regeneration of the Liver in *Triturus viridescens.** (27766)

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The livers of amphibia such as newts(1), frogs(2), and toads(3) were until recently considered to undergo compensatory hypertrophy but not cellular proliferation following partial hepatectomy. Recent work based on observations of mitosis has shown that amphibian liver undergoes at least some degree of hyperplasia after partial removal(4-6). Mitotic figures are few and difficult to find in normal and regenerating newt liver; consequently, tritiated thymidine (H^3-thymidine) and autoradiography were used in this study to detect cells synthesizing deoxyribonucleic acid (DNA). As will be shown, this gives information for study of times and quantitative aspects of cell proliferation.

Materials and methods. Approximately 90 newts obtained from ponds in western Massachusetts, and from commercial suppliers in Massachusetts and Wisconsin, were used. Animals were housed in large aquaria at room temperature (24°C). Versonate,

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