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SPECIFIC FUNCTIONAL AND IMMUNOLOGIC ASSAY OF PLASMA PLASMINOGEN IN HEREDITARY ANGIOEDEMA, IN HEREDITARY ANGIOEDEMA TREATED WITH TRANEXAMIC ACID, AND IN NORMAL SUBJECTS[1]

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Plasma plasminogen levels were determined by a specific esterolytic assay and a radial immunodiffusion assay, both of which were standardized on a weight basis by using highly purified plasminogen diluted in plasminogen-free plasma. In thirteen normal individuals the functional and antigenic levels were 275 ± 61 μg/ml and 285 ± 61 μg/ml, respectively (r = 0.95), whereas in 58 plasmas from individuals with hereditary angioedema the levels were 272 ± 58 μg/ml and 265 ± 64 μg/ml, respectively (r = 0.89). In two patients treated with tranexamic acid either acutely or chronically, both the functional and antigenic plasminogen levels were diminished.

The plasma proenzyme, plasminogen, can be converted to plasmin by activators from plasma (1, 2), tissue (3), or urinary (4, 5) sources, as well as by a complex formed between the bacterial product, streptokinase, and plasminogen (6). Once formed, plasmin plays a role in fibrinolysis, feedback activation of Hageman factor (7), and formation of the Hageman factor fragment which appears to be more active than the parent molecule in converting prekallikrein to kallikrein in the fluid phase (8). Plasmin may also interact with the C system both at the level of activation of the first component (9) and in direct release of C3 anaphylatoxin from the third component of C (C3) (10).

In individuals with hereditary angioedema, who lack the inhibitor of the first component of C (11) or have an inactive form of the inhibitor (12), the possible role of plasmin activation of the C system as well as the recent finding that a peptide formed by the interaction of the first, fourth, and second components of the C system requires cleavage by plasmin to become vasoactive (13) provide a rationale for the use of the inhibitor of plasminogen activation, ε-aminocaproic acid (14), or the analogue tranexamic acid (AMCA)[3] (15) in the treatment of this disease. However, monitoring plasma plasminogen levels during the course of such therapy has been subject to the limitations of the assay systems employed.

As previously reviewed (16), the methods used to measure plasminogen functionally depend upon the conversion of plasminogen to plasmin and require quantitative removal or inactivation of plasmin inhibitors before the interaction of the plasmin with any of several substrates (for examples, see 17 to 23). In the case of the whole plasma streptokinase-activated clot lysis assay, a normal capacity to generate the plasmin substrate (i.e., the ability to form a clot via the intrinsic coagulation pathway) from the test plasma is also required (24). A method independent of functional activity, a radioimmunoassay for plasminogen, has been reported (25) with a standard whose weight was defined by absorbance, and purity and function by ultracentrifugal pattern and caseinolytic assay, respectively (26). A radial immunodiffusion assay that also obviated the problems associated with functional assays has been described. It employed antiserum shown to be monospecific for plasmin(ogen) by several immunochemical criteria and standard plasminogen antigen quantitated both on a weight basis and with the active site titrant, p-nitrophenyl guanidinobenzoate (16).

Although shown to be a sensitive and specific assay for plasma plasmin(ogen), the radial immunoassay alone would not yield sufficient information in situations where the limited clearance of inactivated plasmin or of nonfunctional but antigenically intact plasmin metabolites would elevate the assay values. The report of a new synthetic plasmin substrate N^{α}-carbobenzoxy-L-lysine-*p*-nitrophenyl ester (CLN), which sustained the same cleavage in whole or acidified plasma and in euglobulin or ammonium sulfate precipitates of plasma (27, 28) has permitted quantitation of plasminogen in whole plasma on a weight basis. This functional assay has been used together with the radial immunodiffusion assay to determine the correlation between the two techniques in whole plasma from normal individuals and from individuals with hereditary angioedema. In two instances, the temporal relationship between the changes in the two assays after institution of AMCA therapy was examined.

MATERIALS AND METHODS

Materials. Enzodiffusion plasminogen-free human fibrin plates and streptokinase (Hyland Div., Travenol Labs, Inc., Costa Mesa, Calif.), Sephadex G100 (Pharmacia Fine Chemicals, Piscataway, N. J.), collodion bags (Schleicher and Schuell, Inc., Keene, N. H.), and N^{α}-carbobenzoxy-L-lysine-*p*-nitrophenyl ester · TFA (CLN; Vega-Fox Biochemicals, Tucson, Ariz.) were purchased as indicated. Highly purified streptoki-

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[2] Established Investigator of the American Heart Association, Inc. Mailing Address: The Seeley G. Mudd Building, Room 625, 250 Longwood Avenue, Boston, Massachusetts 02115.
[3] Abbreviations used in this paper: AMCA, tranexamic acid; CLN, N^{α}-carbobenzoxy-L-lysine-*p*-nitrophenyl ester; EDTA, ethylenediaminetetraacetic acid.

nase was obtained from Dr. P. H. Bell, Lederle Laboratories, Pearl River, New York.

Plasminogen and plasmin. Plasminogen was purified from fresh human plasma by a modification (16) of the procedure of Deutsch and Mertz (29), in which the plasminogen eluted from lysine-Sepharose was further purified by Sephadex G100 gel filtration. Plasminogen was assessed for purity by alkaline disc gel electrophoresis, for protein content by Folin analysis (30), and for functional integrity by quantitating fibrinolytic activity after conversion to plasmin.

To convert plasminogen to plasmin in experiments for fibrinolytic assay, plasminogen in concentrations of approximately 200 μg/ml was incubated for 30 min at 37°C with 1/10 volume of a 1 mg/ml solution of highly purified streptokinase to yield an approximate molar reaction ratio of 1:1. Ten microliters of the reaction mixture were placed in a fibrin plate and incubated at 37°C. After 24 hr, the diameter of the lysed area was measured and converted to plasmin content with a standard curve prepared with highly purified plasminogen activated with streptokinase (16).

Plasminogen-free plasma. Normal blood and blood from an individual documented as having the variant form of hereditary angioedema with nonfunctional $C\bar{1}$ inhibitor were collected without glass contact in 1/10 volume of 3.8% sodium citrate. Five milliliters of each plasma were passed separately over lysine-Sepharose (29) packed in a 5-cc plastic syringe and previously washed with 0.1 M phosphate buffer at pH 7.4 and containing 0.003 M Na_2 EDTA.[3] The column effluents were collected, and the fractions containing protein, determined by absorbance at 280 nm, were concentrated to the original plasma volume by negative pressure ultrafiltration in collodion bags. The resulting plasminogen-free plasma contained neither plasminogen functionally, as assessed in the CLN assay, nor antigenically, as determined by radial immunodiffusion analysis.

Plasminogen functional assay. Plasminogen in whole plasma was assayed functionally, after activation with streptokinase, by a modification of a synthetic substrate cleavage technique (28). Blood was collected in 1/10 volume 3.8% sodium citrate and the separated plasma was either assayed immediately or frozen at −70°C and thawed once just before assay. One-tenth milliliter of plasma previously diluted 5-fold with saline, or 0.1 ml of a plasminogen source was mixed with 2.0 ml of 0.1 M potassium phosphate buffer, pH 6.0 containing 0.1 M NaCl and warmed to 30°C. Streptokinase was diluted with the potassium phosphate buffer to contain 33,000 units/ml and 0.05 ml was added to the buffered plasma. The mixture was incubated for 10 min at 30°C, transferred to a spectrophotometer cuvette, and the optical density at 340 nm was adjusted to zero. A 0.05 ml volume of CLN, containing 2 mg/0.56 ml of acetonitrile, was added with mixing, and the change in optical density was recorded at intervals over a 5-min period. A duplicate sample, incubated with buffer instead of streptokinase before the addition of CLN, served as a control. The change in absorbance per minute between 1 and 5 min was linear and was calculated for both sample and control, and the net change in absorbance per minute was converted to plasminogen concentration by using a standard curve developed with highly purified plasminogen.

Plasminogen/plasmin immunoassay. Radial immunodiffusion analysis was performed by a modification of the technique of Mancini *et al.* (31), with monospecific rabbit anti-human plasminogen serum (16). A 1.7-ml volume of 2% agar in Veronal buffer, pH 8.6, ionic strength 0.1, containing 0.01% sodium azide, was mixed with an equal volume of the same Veronal buffer containing 0.025 ml of antiserum and poured into a 60 x 15 mm Petri dish placed on a level surface. Wells were cut with a 1-mm punch and filled with: varying dilutions of purified plasminogen; varying dilutions of purified plasminogen mixed with a 1/5 volume of plasminogen-free plasma; or sample plasmas and standard reference plasma each diluted 5-fold with saline. After incubation for 48 hr at 25°C in a humid environment, the plates were washed with saline and distilled water and stained with 1.0% tannic acid. The diameters of the precipitin rings were determined with a measuring magnifier (Bausch and Lomb) and converted to micrograms of plasminogen by using a standard curve developed with highly purified plasminogen which related the square of the diameter (31) to the plasminogen concentration.

RESULTS

Functional plasminogen assay. The functional plasminogen assay depends upon the quantitative conversion of plasminogen to plasmin during the 10-min interaction with streptokinase. To determine the dose range of streptokinase required for this effect, 0.1-ml samples of 1:5 dilutions of plasma from five individuals were mixed with 2.0 ml of CLN assay buffer and incubated with 1 to 100 μl of a streptokinase solution containing 33,000 units/ml for 10 min at 30°C. Cleavage of CLN was then performed and measured as usual. Under these conditions, the rate of CLN cleavage, as reflected by the mean increase in absorbance per minute, was 0.018 absorbance units in the absence of streptokinase, and increased 3-fold to 0.055 when 30 μl of streptokinase were used (Fig. 1). Between 30 and 100 μl of streptokinase, the mean change in absorbance per minute increased from 0.055 to 0.069. For assay of plasma samples, a streptokinase dose within the flatter portion of this curve was chosen, and 50 μl were routinely used. None of the plasmas assayed demonstrated net esterolytic activity in the absence of streptokinase, i.e., CLN cleavage greater than the spontaneous hydrolysis seen with buffer alone.

Because the functional plasminogen assay is performed in diluted whole plasma, it was necessary to determine the possible effect of other plasma components on the assay. Highly purified plasminogen in concentrations of 10 to 40 μg/ml was added to 0.1 ml of assay buffer or to 0.1-ml samples of 1:5 dilutions of plasminogen-free plasma from a normal individual or from an individual with hereditary angioedema of the variant type. After dilution to a final volume of 2.0 ml, streptokinase activation and CLN cleavage were performed as usual. Under these conditions, the rate of CLN cleavage, expressed as the net change in absorbance per minute, was the same when known

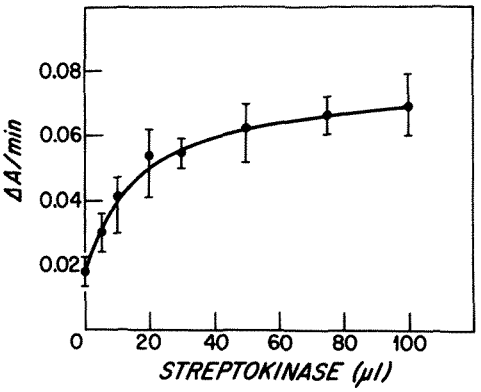

Figure 1. Cleavage of CLN in plasma after incubation with varying concentrations of streptokinase. Points plotted represent the mean values obtained with five different plasmas. The range of values for each streptokinase concentration is also indicated.

quantities of plasminogen were measured in buffer, in diluted normal plasminogen-free plasma, or in diluted plasminogen-free plasma obtained from an individual lacking the functional inhibitor of the first component of C (Fig. 2). The standard curve used for this assay was therefore derived from results obtained with plasminogen diluted in the CLN assay buffer.

Radial immunodiffusion assay of plasminogen. The effect of the presence of plasma constituents on the quantitation of plasminogen by radial immunodiffusion assay was also examined. Twenty microliters of varying dilutions of plasminogen were mixed with 5 μl of plasminogen-free plasma so as to reproduce the customary 1:5 dilution of normal plasma, and the radial immunoassay was performed as usual. It is apparent in Figure 3 that the presence of plasma in the radial immunodiffusion assay mixture increased the slope of the dose response curve as compared to that obtained with plasminogen in buffer. This effect required that the standard curve for the plasminogen radial immunodiffusion assay be prepared in the presence of 20% plasminogen-free plasma, and a curve similar to the upper one depicted in Figure 3 was routinely employed.

Assay of plasminogen in human plasma samples. When plasma samples obtained from 13 normal adults were examined

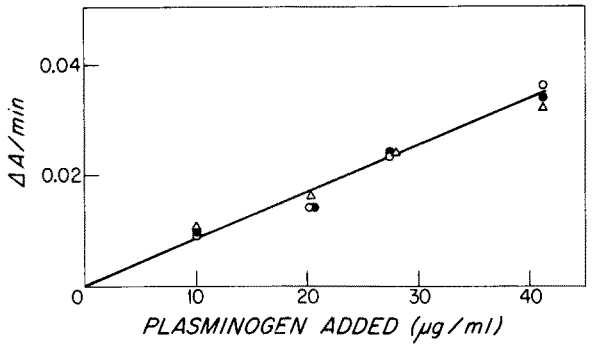

Figure 2. Cleavage of CLN, expressed as change in A_{280}/min, as a function of plasminogen added to either buffer (●), normal plasminogen-free plasma (○), or plasminogen-free plasma from an individual with hereditary angioedema (△).

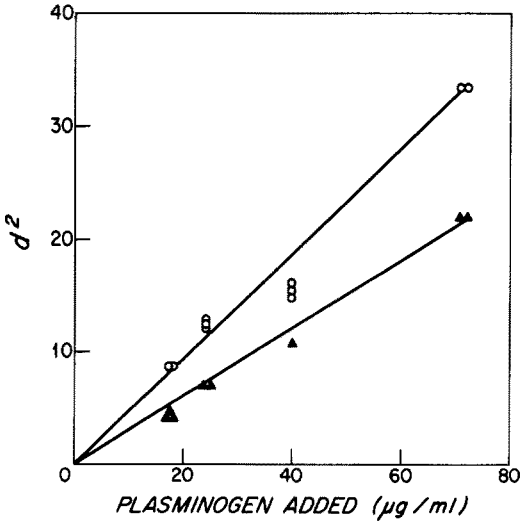

Figure 3. Plasminogen radial immunodiffusion values, expressed as the square of the precipitin ring, as a function of the plasminogen added to buffer (▲), or the normal plasminogen-free plasma (○). Multiple points represent replicate assay values. Plasminogen added to plasminogen-free plasma from an individual with hereditary angioedema gave the same results as those obtained with normal plasminogen-free plasma.

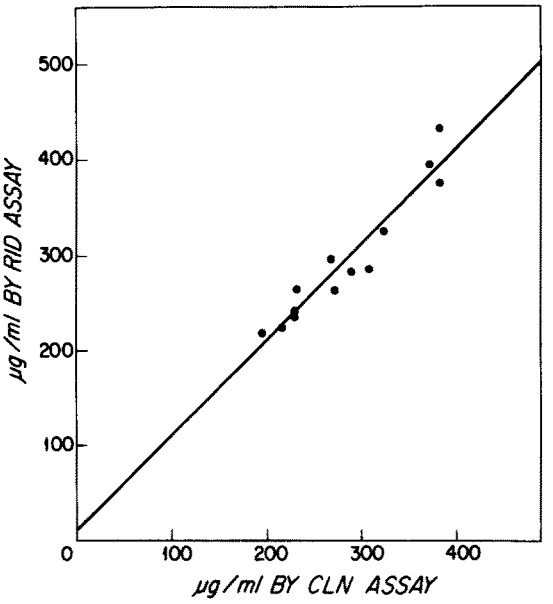

Figure 4. Correlation between functional (CLN cleavage) and immunologic plasminogen assays in normal plasma (n = 13; r = 0.95; p < 0.001).

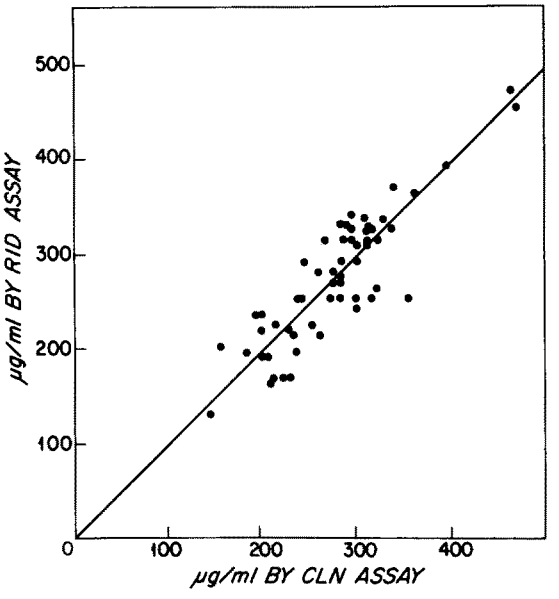

Figure 5. Correlation between functional (CLN cleavage) and immunologic plasminogen assays in plasma from individuals with hereditary angioedema (n = 58; r = 0.89; p < 0.001).

in both the CLN and radial immunodiffusion assays, the mean plasminogen levels were 275 ± 61 μg/ml and 285 ± 61 μg/ml, respectively, with a correlation between the assays of r = 0.95 at p < .001 (Fig. 4). When esterolytic activity of a single plasma specimen was determined in replicate assays, the net absorbance per minute varied by less than .005 units, resulting in a variation in plasminogen level of less than 4% in the midrange values. In the radial immunodiffusion assay, the diameter of the ring produced by a standard reference plasma, included in each assay, varied by no more than 0.1 mm.

Plasma samples from individuals with hereditary angioedema, documented by functional and immunologic assay for the inhibitor of the first component of C, were also examined by both plasminogen assays. In a series of 58 plasma samples the mean plasminogen level in the CLN assay was 272 ± 58 μg/ml, and in the radial immunodiffusion assay, it was 265 ±

64 μg/ml (Fig. 5). The correlation between the two assays was $r = .89$ with $p < .001$.

Because several points in the lower end of the curve depicted in Figure 5 represented plasminogen levels in individuals receiving prophylactic AMCA therapy, the rate of change in esterolytic and antigenic plasminogen content was examined in a single individual with hereditary angioedema who received only AMCA for 5 days in anticipation of a major surgical procedure (32). Previous plasminogen levels in the absence of therapy had averaged 272 μg/ml in the CLN assay and 252 μg/ml in the radialimmunoassay (Table I). Assays of samples drawn approximately 24 hr after the 0 day values indicate a drop in both the functional and antigenic values, and by 72 hr, the values were 67 and 68% of the initial values, respectively. Twenty-four hours after cessation of AMCA therapy, both functional and antigenic values had returned to the initial plasminogen levels. Later values were not obtained due to the reinstitution of attenuated androgen therapy.

In a second individual followed over a period of 11 months during which three doses of AMCA were administered prophylactically, both the functional and antigenic plasminogen levels appeared to vary with the AMCA dose (Table II). In the absence of therapy (Sample 7), or when treated with the synthetic androgen oxymethalone (Sample 8), the mean plasminogen levels were 281 and 290 μg/ml in the functional and antigenic assays, respectively. Plasminogen levels obtained during treatment with 2 g/day AMCA (Sample 4) indicate no change in the functional value and a 10% change in the radial immunodiffusion value. In duplicate samples obtained while the patient was on 3 g of AMCA/day (Samples 1 and 2), the mean levels were 195 and 165 μg/ml, whereas during therapy with 4 g/day (Samples 3, 5, and 6), the mean levels were 152 and 160 μg/ml, respectively, representing 54 and 55% of the untreated values.

TABLE I

Effect of acute AMCA administration on plasma plasminogen levels measured by functional and antigenic analysis.

Therapy	Plasminogen Concentration	
	CLN assay	RID assay
	μg/ml	
Mean control values	272	252
Day 0, before AMCA	255	240
Day 1, 1 g AMCA/6 hr	210	180
Day 3, 1 g AMCA/6 hr	186	170
Day 5, AMCA stopped		
Day 6	255	245

TABLE II

Effect of AMCA dose on plasma plasminogen levels measured by functional and antigenic analysis

Day	Therapy[a]	Sample	Plasminogen Concentration	
			CLN assay	RID assay
			μg/ml	
1	AMCA, 3 g/day	1	200	165
1	AMCA, 3 g/day	2	190	165
10	AMCA, 4 g/day			
14	AMCA, 4 g/day	3	130	125
203	AMCA, 2 g/day			
210	AMCA, 2 g/day	4	290	260
244	AMCA, 4 g/day			
259	AMCA, 4 g/day	5	188	160
330	Stop AMCA	6	140	195
337	Begin oxymethalone	7	282	290
345	Oxymethalone	8	280	290

[a] Plasma samples obtained on a day new therapy was instituted are considered to reflect the previous drug dose.

DISCUSSION

In order to quantitate both the total functional and antigenic plasminogen levels present in plasma from normal and treated individuals, specific esterolytic and radial immunodiffusion assays have been standardized on a weight basis with highly purified plasminogen under conditions similar to those of plasma sample assay. With 50 μl of a standard streptokinase preparation (Fig. 1), the esterolytic assay was linear between 10 and 40 μg/ml of plasminogen, and the data were superimposable when plasminogen was assayed in either buffer or plasminogen-free plasma (Fig. 2).

However, although linear between 18 and 75 μg/ml of plasminogen, the slope obtained in the radial immunodiffusion assay was dependent on whether the plasminogen standard was diluted in buffer or in plasminogen-free plasma (Fig. 3). This effect could not be attributed to the incomplete removal of plasminogen from the plasminogen-free plasma, since the discrepancies in plasminogen content between the two curves represent considerably more plasminogen (e.g., 15 μg/ml at $d^2 = 10$ and 28 μg/ml at $d^2 = 20$) than the lower limits of sensitivity of the assays used to determine the completeness of its removal. These limits are approximately 3 μg/ml, 5 μg/ml, and 5 to 10 μg/ml for the fibrinolytic, esterolytic, and radial immunodiffusion assays, respectively. The effect of plasminogen-free plasma was probably not attributable to the formation of a complex with plasma constituents, since the effect of plasma was to increase the diffusion rather than retard it as would be expected with the formation of a larger molecule bearing the antigenic sites, unless such sites are protected from the antibody. Because the assay was performed in agar rather than agarose, the most likely explanation was a nonspecific facilitating effect of plasma constituents by diminishing the negative charge on the agar in the region of the plate through which they and the plasminogen diffused.

The finding that plasminogen diffuses further in the presence of other plasma constituents has required that the earlier radial immunodiffusion assay (16) be restandardized. The present mean level obtained with a series of 13 normal plasmas (285 μg/ml) is in excellent agreement with the esterolytic assay (275 μg/ml; $r = .95$) as seen in Figure 4. Similar data were obtained when both assays were employed to screen a series of patients with hereditary angioedema (Fig. 5).

The observation that individuals with hereditary angioedema who were receiving AMCA either acutely (Table I) or chronically (Table II) showed a significant decrease in their functional plasminogen levels is consistent with the report (33) that normal individuals receiving two grams of AMCA three times daily demonstrated an increased turnover of injected, iodinated plasminogen. However, in this earlier study the total plasma plasminogen level was shown to decrease when examined functionally (caseinolysis), but not by immunoassay, suggesting the possibility that the AMCA had reached an *in vivo* concentration capable of interfering with the subsequent functional assay or with its activation step. Thus, it is not clear in the earlier study whether labeled plasminogen and native unlabeled plasminogen were both cleared more rapidly in the presence of AMCA. With the related drug, ε-aminocaproic acid, diminished blood levels of immunoreactive plasminogen have been reported in a series of five hemophiliacs (34) and in an individual with hered-

itary angioedema (35). In the present report, both functional and antigenic plasminogen levels were diminished during AMCA therapy. Since AMCA has no effect in the immunoassay, this finding suggests that the drug increased the rate of removal of both the functional and antigenic entity from the circulation.

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