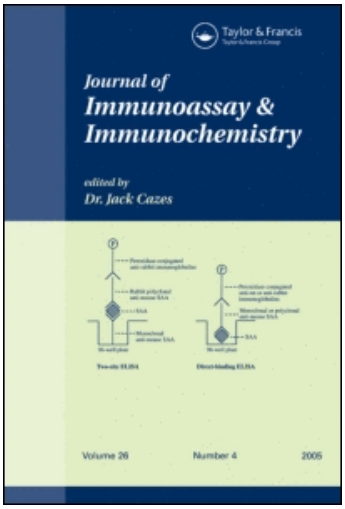

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE MEASUREMENT OF ACTIVATED FACTOR XII (HAGEMAN FACTOR) IN HUMAN PLASMA

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ABSTRACT

A direct enzyme-linked immunosorbent assay (ELISA) employing 2/215 mouse monoclonal hybridoma antibody is described. The assay is capable of detecting activated factor XII in human plasma and can be used to assess early detection of the intrinsic blood coagulation pathway. No cross reactivity with human factor XII zymogen has been found. The assay was used to assess activation of factor XII in patients with renal failure, pregnancy and diabetes compared to a control group.

(KEYWORDS: human activated factor XII, monoclonal antibody, enzyme-linked immunosorbent assay).

INTRODUCTION

Factor XII or Hageman factor circulates in plasma as a zymogen and on activation initiates the intrinsic pathway of blood coagulation, as well as participating in kinin formation and fibrinolysis (1). However the precise physiological role of factor XII is unclear which is partly due to the observation that individuals with inherited factor XII deficiency are usually asymptomatic (2-3). In an attempt to more

accurately define the role of factor XII in normal haemostasis and defined pathological conditions, a sensitive and specific monoclonal antibody based ELISA has been developed for the measurement of activated factor XII in human plasma.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Factor XII deficient plasma was from Immuno, Sevenoaks, Kent, UK.

Preparation and Purification of Factor XII, XIIa and ßXIIa

Human Factor XII was isolated from fresh frozen human plasma by a combination of ammonium sulphate precipitation and anion exchange chromatography (4). In some batches of plasma a proportion of the factor XII was recovered as a XIIa. This was separated from the zymogen by adsorption onto a column of monoclonal antibody 2/215 - Sepharose in 50mM Tris HCl buffer pH 7.5 containing 100mM sodium chloride and eluted with the same buffer containing 3.5M sodium thiocyanate. ßXIIa was prepared by a modification of the method of Fujikawa and McMullen (5). Murine monoclonal antibodies to human ßXIIa were prepared by a modification of the method of Kohler and Milstein (6). Female Balb/C mice were immunized by intraperitoneal injection of 20µg of ßXIIa conjugated to purified protein derivative of tuberculin (7). Clones were selected on the basis of their production of monoclonal antibodies which exhibited minimal

cross-reactivity with factor XII, but high affinity for ßXIIa. The selected monoclonal antibody (clone 2/215) was purified from ascitic fluid by precipitation at 4°C of the immunoglobulin fraction obtained by adding an equal volume of saturated ammonium sulphate. The precipitate was centrifuged, dissolved in a volume of 50mM Tris-HCl buffer pH 7.5, equal to the original ascites volume and then dialysed against the same buffer. The monoclonal antibody was then applied to a Mono-Q anion exchange column and eluted using a salt gradient. The salt gradient was applied using 50mM Tris-HCl pH 7.5 (Buffer A) and the same buffer containing 1M sodium chloride (Buffer B). Buffer B was increased from 0-20% over 60ml, then 20-35% over 20ml and 35-100% over 20ml. 100% of Buffer B was maintained for 10ml. The flow rate was 2ml per minute. Using this gradient the antibody eluted at a salt concentration of 150-200mM sodium chloride.

Preparation of Polyclonal Antibody - Alkaline Phosphatase Conjugate

Polyclonal antisera against human factor XIIa were raised in sheep using standard procedures. The purified immunoglobulin fraction was then conjugated to alkaline phosphatase using the thiol-maleimide method (8).

Preparation of Standards

XIIa standards (1-20ng/ml) were prepared by addition of purified ßXIIa to 50mM sodium phosphate buffer pH 7.4 containing 0.05% (w/v) bovine serum albumin. The purified ßXIIa was quantitated by measurement of the absorbance at 280nm

using an extinction coefficient $E^{1\%}_{280}$ of 15.3 (5) and by measurement of the enzyme activity (see below). The use of plasma as a standard matrix was also investigated.

Specificity of the Monoclonal Antibody 2/215

The cross-reactivity of the monoclonal antibody with factor XII and fibronectin was determined by radioimmunoassay using ^{125} I-radiolabelled ßXIIa prepared by the chloramine T method. Dose response curves against non-labelled ßXIIa, factor XII and fibronectin were generated by addition of 50µl of monoclonal antibody 2/215 at an appropriate dilution in assay buffer (50mM Tris-HCl pH7.4, containing 0.15M sodium chloride, 0.25% (w/v) bovine serum albumin, 10mM EDTA, 3mM sodium azide and 0.1% (w/v) Triton) to 50µl of ^{125}I-radiolabelled ßXIIa and 100µl of either unlabelled ßXIIa, factor XII or fibronectin, to 4ml volume polystyrene tubes. All tubes were mixed and incubated at 21± 1°C for 19 hours with shaking. After this period 50µl of suspension containing an optimal amount of sheep anti-mouse IgG coupled to Sephacryl S-1000 was added to each tube and the tubes incubated at 21±1°C for 1 hour with shaking. After this step 1.5ml of assay buffer containing 10% (w/v) sucrose was layered underneath the reaction mixture in each tube. The sheep anti-mouse-Sephacryl was allowed to sediment at 21±1°C for 30 minutes after which the supernatant was removed from each tube, leaving approximately 0.3ml Tubes were then counted for 60 seconds in a gamma counter. Results were expressed as percent radioactivity bound.

Total radioactivity added was determined by addition of 50µl ^{125} I-radiolabelled ßXIIa to 150µl of assay buffer which was also counted for 60 seconds in a gamma counter. ßXIIa was added at concentration 0-100ng/ml, factor XII at 0-2000ng/ml and fibronectin at 0-2000ng/ml. Cross-reactivity was determined by calculating the ratio of the weight of unlabelled ßXIIa required to achieve 50% maximum binding compared to the weight of the potential cross-reactant required to achieve 50% maximum binding. The ratio was expressed as a percentage.

The specificity of monoclonal antibody 2/215 was further characterised by determination of the dissociation constants for antibody-factor XII, antibody-factor XIIa and antibody-factor ßXIIa, using an indirect antibody consumption assay (9) The monoclonal antibody was first incubated in solution with the relevant antigen until equilibrium was reached and the proportion of antibody which remains unsaturated at equilibrium was measured by an indirect ELISA (9). Factor XIIa was incubated with antibody at concentrations of 0-480ng/ml whilst factor XII was incubated at concentrations of 0-104µg/ml.

ELISA

The wells of a Maxisorb microtitre plate (Nunc, Kamstrup, Denmark) were coated with monoclonal antibody 2/215 by addition of 100µl of a 5µg/ml solution of antibody in 0.1M sodium phosphate buffer, containing 0.15M sodium chloride, 0.1% (w/v) sodium azide, pH 7.4 to each well. After incubation for 16-20 hours at 20°C the wells were decanted and rinsed with wash buffer (10mM sodium

borate buffer containing 50mM sodium chloride, 0.1% (v/v) Triton-X-100 and 0.05% (w/v) sodium azide, pH 7.4). 100µl of either standards or samples were added to the wells and incubated at 18-25°C for 1 hour. The wells were decanted and washed 5 times with wash buffer by addition of a minimum of 200µl per well, per wash cycle. 100µl of sheep polyclonal antihuman XIIa conjugated to alkaline phosphatase in 0.1M Tris:HCl buffer pH7.4, containing 0.1M sodium chloride, 1mM magnesium chloride, 0.1mM zinc chloride, 0.1% (w/v) sodium azide, 0.1% (v/v) Triton-X-100 and 1% (w/v) bovine serum albumin at a 1:500 dilution of a stock solution was added. The plates were incubated for 1 hour at 18-25°C and the wells were decanted and washed as before. 100µl of phenolphthalein monophosphate substrate (0.1% (w/v) phenolphthalein monophosphate in 0.5M diethanolamine:HCl buffer pH 8.6 and 0.02% (w/v) Bronidox) was added to each well and incubated at 18-25°C for 15 min. The enzymatic reaction was stopped by addition of 100µl of stop solution (0.4M sodium carbonate, 0.1M 3-(cyclohexylamine)-1-propanesulphonic acid, 0.1M ethylene diamine tetraacetic acid tetrasodium salt and 0.4M sodium hydroxide). The absorbance was measured at 550nm with a Titertek Multiscan microplate plate reader.

Samples

Blood samples were collected by venepuncture into vacutainers containing 3.8% citrate anticoagulant (Becton Dickinson, Cowley, Oxford, UK), at a ratio of 9 volumes of blood to 1 volume of anticoagulant. Plasma was obtained by

centrifugation at 2,000g for 10 minutes and either assayed on the day of collection or frozen rapidly. Plasma was then stored at -20°C or below. Prior to assay plasma samples were thawed at 37°C and then allowed to equilibrate to room temperature.

Sample Stability

To determine sample stability plasma samples were assayed on the day of collection or stored for 30 days in 500µl aliquots.

Assay of XIIa Enzyme Activity

The enzyme activity of factor ßXIIa was assessed by the measurement of amidolytic activity using the synthetic substrate S2302 (Quadratech, P.O. Box 167, Epsom, UK). 200µl of S-2302 (2mM) in 65mM Tris/HCl buffer pH 8.0 containing 135mM sodium chloride and 0.1% (w/v) bovine serum albumin was added to 50µl of sample in a well of a microtitre plate. The rate of change of absorbance per minute at 37°C was determined at 405nm. 1ng of ßXIIa was equivalent to the amount which generates 25 pmol of p-nitroaniline per minute at 37°C.

Statistical Analysis

The results obtained in different patient groups were compared using Student's t-test.

RESULTS

Specificity of Monoclonal Antibody 2/215

Cross-reactivity with human fibronectin and factor XII was found to be less than 0.1%. The dissociation constants for factor XII and the two active forms αXIIa and ßXIIa were > 10^{-5}M, $1.8x10^{-9}$M and $6.7x10^{-10}$M respectively, indicating the high degree of specificity of monoclonal antibody 2/215 for the active forms of factor XII. In the ELISA assay, no cross-reactivity with factor XIIa in samples of citrated plasma from Cynomolgus monkey, cow or guinea pig, was observed. Assay of human factor XII deficient plasma and purified factor XII were also found to give a negative response.

Standard Curves, Recovery and Assay Performance

A typical standard curve for ßXIIa in buffer is shown in Fig.1. Problems with preparation of standard curves in plasma were apparent and this is due to loss of added XIIa due to complex formation with plasma proteinase inhibitors. Further problems are associated with long-term instability at 2-8°C of factor XII and factor ßXIIa in plasma, which leads to an apparent increase in the XIIa concentration of the standards. Storage of plasma at 2-8°C results in 'cold activation' of factor XII which leads to a slow but gradual increase in the factor XIIa measured in the plasma. Addition of ßXIIa to plasma also results in a gradual increase in the XIIa concentration due to activation of endogenous factor XII. This is more evident if plasma is stored at 2-8°C and is largely due to the

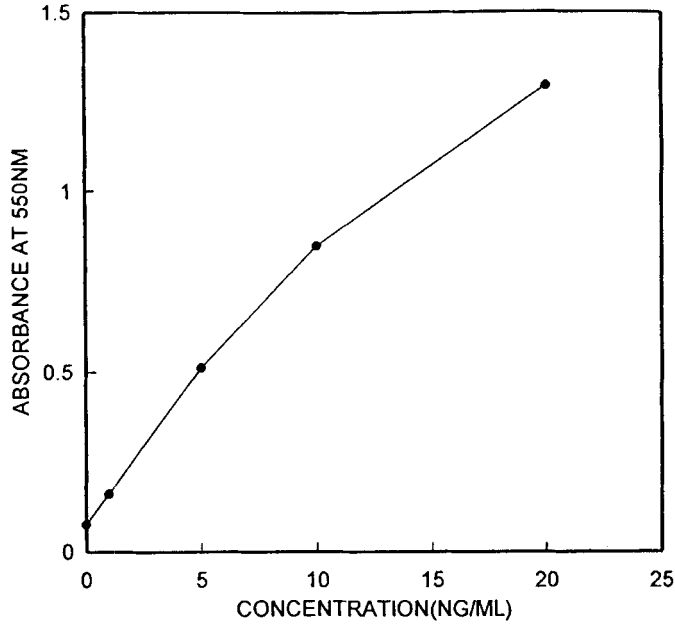

FIGURE 1. A Typical Dose Response Curve for XIIa Standards in Buffer.

decrease in the effectiveness of the plasma proteinase inhibitor Cl-esterase inhibitor, which is the major plasma inhibitor of factor XIIa.

The validity of results obtained on patient plasma is therefore dependent on how plasma samples are stored. Storage of plasma samples at 2-8°C prior to assay results in artificially high values. If plasma samples are not assayed immediately after processing they must be frozen and prior to assay thawed at 37°C. This procedure avoids 'cold activation' and allows reliable results to be reported. The standards of choice are therefore purified ßXIIa in buffer. These standards are stable at 2-8°C for up to 68 weeks. This approach was further validated by

dilution of three human plasma samples containing a high concentration of factor XIIa with normal plasma. The observed results for each dilution were compared to the theoretical values. Recovery was 101% with a range of 98-107%. Inter- and intra-assay imprecision were evaluated in order to assess the reproducibility of the ELISA. The intra-assay coefficient of variation obtained on two plasma controls in six separate assays was 4.6% (2.79ng/ml) and 2.89% (5.85ng/ml). The inter-assay coefficient of variation obtained on the same plasma controls in six separate assays was 5.02% and 6.91% respectively. Sensitivity of the assay was determined on 20 separate estimations of the zero standard. The sensitivity or detection limit of the assay, defined as the mean absorbance value of the zero standard, plus three standard deviations was equivalent to 0.3ng/ml.

Stability of Stored Plasma Samples

The concentration of factor XIIa in stored plasma samples was determined and compared to the results obtained on the day of collection. No significant difference in results (Table 1) were obtained on these plasma samples following storage at -20°C.

Concentration of XIIa in Human Plasma

The concentration of factor XIIa was determined in the plasma of patients with renal failure, diabetes and pregnancy compared to a control population (Table 2). The results clearly indicate that the intrinsic blood coagulation pathway, as determined by measurement of XIIa, is activated in these patient groups.

TABLE 1

The Effect of Storage of Plasma Samples at -20 C on the Plasma Concentration of Factor XIIa

	Concentration of Factor XIIa (ng/ml)	
	DAY 0	DAY 29
SAMPLE 1	5.41	5.15
SAMPLE 2	1.91	2.31
SAMPLE 3	2.53	2.27
SAMPLE 4	3.42	3.19
SAMPLE 5	5.14	5.22
SAMPLE 6	1.93	1.86

TABLE 2

The Concentration of Factor XIIa in Plasma From Different Patient Groups Compared to a Control Population of Healthy Volunteers

PATIENT GROUP	n	MEAN ng/ml	STANDARD DEVIATION ng/ml
CONTROL	71	3.54	2.19
DIABETES MELLITUS	98	8.71*	6.74
RENAL FAILURE	98	9.98*	5.01
PREGNANCY	52	6.95*	3.11

Significant increases in the plasma concentration of XIIa were observed in the different patient groups compared to the control group(*$p<0.0005$)

DISCUSSION

Our ability to investigate the role of factor XII in normal haemostasis and different disease states has been impaired by the lack of a sensitive specific assay for activated factor XII. A specific microtitre plate based direct ELISA has been developed which utilizes a monoclonal antibody with excellent specificity for factor XIIa and can be used to detect activation of factor XII in human plasma. A potential drawback of the assay is that a standard curve cannot be prepared in a human plasma matrix due to the presence of plasma protease inhibitors and activation of endogenous factor XII during storage of plasma at 2-8°C. However, the results of dilution studies, stability studies and the imprecision data on the assay indicate that reliable results can be obtained. A stable standard solution has been prepared which allows a reproducible comparison of factor XIIa in patient samples. The data presented clearly indicates that factor XIIa is elevated in patient groups known to be at risk of thrombotic events. It is possible that the observed increases in the plasma concentration of factor XIIa are due to alterations in the normal concentration of the plasma proteinase inhibitors. However we have demonstrated that the assay can be used to investigate the early activation of blood coagulation via the intrinsic pathway. Further studies will be required to investigate the significance of factor XII activation in different disease states.

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