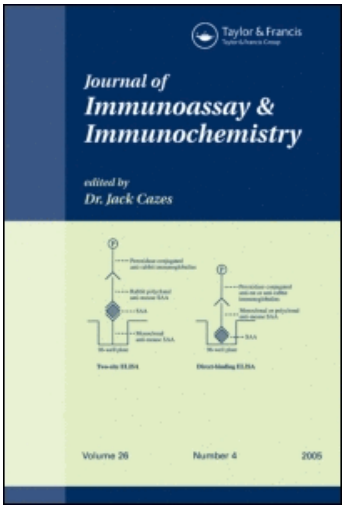

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Monoclonal Antibodies Against Human Angiotensinogen, Their Characterization and Use in an Angiotensinogen Enzyme Linked Immunosorbent Assay

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MONOCLONAL ANTIBODIES AGAINST HUMAN ANGIOTENSINOGEN, THEIR CHARACTERIZATION AND USE IN AN ANGIOTENSINOGEN ENZYME LINKED IMMUNOSORBENT ASSAY.

(KEYWORDS: Human angiotensinogen, monoclonal antibodies, enzyme linked immunosorbent assay, radioimmunoassay, normal sera, pregnant sera).

(ABBREVIATIONS: ELISA, RIA, HA, A1, SDS-PAGE, HPLC, BNHS, DMF).

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ABSTRACT

Monoclonal antibodies were produced against human angiotensinogen.

An enzyme linked immunosorbent assay (ELISA) was developed using a high affinity monoclonal antibody as catching antibody and a polyclonal rabbit anti human angiotensinogen antibody as detecting antibody in a "sandwich" ELISA.

Linear range of the ELISA was 15-450 pmol/l of human angiotensinogen. Intra- and inter- assay variation coefficients were in the range of 2% to 8 %.A correlation coefficient, r = 0.97, (n=20), with values obtained by radioimmunoassay. This correlation coefficient, obtained by using both normal and pregnant sera, confirmed that the ELISA fullfill the requirements for clinical useful assay. Characterization of the antibodies were performed with respect to affinity constant and epitopes.

INTRODUCTION

Measurements of angiotensinogen concentration in human plasma are performed by enzyme linked immunosorbent assay, ELISA, and radioimmunoassay, RIA.

Angiotensinogen is a plasma protein that serves as substrate for the enzyme renin and is synthesized and secreted by the liver. Renin cleaves one single peptide bond in angiotensinogen to release the N-terminal decapeptide, angiotensin I, A1.

Angiotensinogen is usually measured as a constituent of plasma by addition of exogenous renin and measurement of the product, angiotensin I, by RIA. As the ratio angiotensinogen/angiotensin I is 1:1, the amount of angiotensinogen can be measured, when the total amount present has been degradated by renin.

This method is reliable but cumbersome and time consuming, due to the narrow range of the RIA and the extremely slow reaction rate for renin/renin substrate.

The aim of the study was to produce high affinity monoclonal antibodies, which could be used in an ELISA for quantitative measurements of the concentration of human angiotensinogen in untreated plasma and plasma preparations.

MATERIALS AND METHODS

1. Antigens

1.1. Plasma

Human blood samples were obtained by venipuncture from asymptomatic, non-hypertensive male and female on a normal sodium intake. Blood from pregnant women (1.trimester) was obtained by the same criteria as mentioned above. The blood was collected into 10 ml tubes containing 0.5 ml 0.2 mol/l EDTA and centrifuged for 15 min at 2000 g at 4°C, to seperate cells from plasma. The plasma samples were kept at -25°C until tested.

1.2. Preparation of antigen

Sera from pregnant women were used to prepare human angiotensinogen, HA. The procedure comprised

ammoniumsulfate precipitation, chromatography on S-200 Sephacryl, Con-A Sepharose (Pharmacia, Sweden) and DE-52 cellulose (Whatman, USA) table 1 (1). The purified HA was used as antigen for immunization. All steps were carried out at 4°C and chemicals were of reagent grade if not otherwise indicated.

1.3. Measurement of antigen

HA was measured by RIA of AI generated after incubation with an excess of human renin (2,3). 5 µl (duplicate) of angiotensinogen in appropriate dilution was mixed with 25 µl human renin, diluted in 0.2 mol/l TRIS/HCl buffer pH 7.5 with 0.3% human serum albumin, (Behringer Werke, West Germany) (RIA-buffer). The human renin was prepared as described by Hass et al (4).

Each vial contained rabbit anti-AI and 1.2 x 10^{-3} G.U. of human renin. The reaction was allowed to continue for 2 h at 37°C and stopped by cooling to 0°C (icebath). ^{125}I-AI was added in 1 ml RIA-buffer and equilibrium was obtained after 18 h at 4-5°C. Free AI was removed with charcoal coated with Dextran T7o (Pharmacia, Sweden) and centrifugation. The antigen-antibody complex was counted in a gamma-counter (Hydrogamma, USA).

2. Polyclonal antibodies

2.1. Rabbit anti-human angiotensinogen

Antisera to pure HA which were a generous gift from D. Tewksbury, were produced in rabbits by intradermal injection of 100 µg/injection, emulsified in Freund's complete adjuvant. The injections were repeated each month. High titre antibodies were obtained after 3 months. Antisera to antigen prepared according to 1.2 were raised in the same way.

3. Monoclonal antibodies

3.1. Immunization and fusion

RBF/Dd strain mice containing the RB (8.12) 5Bnr Robertsonian translocation chromosome were immunized 3

TABLE 1

FOUR-STEP PARTIAL PURIFICATION OF HUMAN ANGIOTENSINOGEN

	ml	substrate mg H.A.	protein g	mg H.A. g protein	Yield %
Serum from pregnant women	420	88.4	31.5	2.8	100
$(NH_4)_2SO_4$ fractionation between 1.3-2.3 mol/l sec. precipitate, dialyzed	97	29.6	4.6	6.4	33
S-200 Sephacryl	159.5	26.6	3.3	8.1	30
Con-A Sepharose	100	5.3	0.153	34.6	6
DE-52 cellulose	87	1.33	mg 45	295.6	1.5

times at 2 weeks interval with HA, specific octivity 300 μ HA/mg protein. The antigen (10 μg/mouse, in 10 mmol phosphate buffer/l, 0.15 mol NaCl/l, pH 7.4 (PBS), 100 μl) was emulsified 1:1 in Freund's incomplete adjuvant and 200 μl was administered subcutaneously. For fusion, mice were boosted intravenously with 10 μg of HA 100 μl PBS at least four weeks following the last subcutaneous immunization. After another 3 days, the mice were sacrified and their spleens removed for fusion with myeloma cells. Spleen cells from a RBF/Dn mouse were fused with myeloma cells of the FOX-NY myeloma line deficient in the selectable enzyme marker loci adenosine phosphoribosyl transferase ($ARPT^-$) and hypoxanthine phosphoribosyl transferase ($HRPT^-$). Thus the exposure of cell fusion mixtures to medium requiring $ARPT^-$activity, ($ARPT^+$ selection) eliminates both the unfused $ARPT^-$ myeloma cells and $ARPT^-$ hybridomas (5). The fused cells were seeded on BALB/c strain macrophage feeder layers.

The clones were grown in ten 96-well microtiter plates (NUNC, Roskilde, Denmark) in a medium consisting of RPM 1640 (GIBCO, UK) with 15% v/v fetal calf serum (GIBCO, UK) supplemented with adenine, 7.5×10^{-5} mol/l, hypoxanthine, 8×10^{-4} mol/l, aminopterine, 8×10^{-7} mol/l, and thymidine 1.6×10^{-5} mol/l, (AHAT). Cultures were incubated for 12 days at 37°C in air containing 5% CO_2 and then subjected to primary screening.

3.2. Screening

For screening cultures immunoplates I (NUNC. Roskilde, Denmark) were coated with the semipure antigen using 100 μl of 1 μg/ml HA in PBS per well for 16 h at 4°C. After blocking the plate for 1 h with 1% Triton X-100 and 1% bovine serum albumin in PBS (blocking buffer) the wells were emptied, 100 μl of hybridoma supernatant was added to each well and incubated for 1 h. The wells were washed three times with PBS and 100 μl peroxidase-labeled goat anti-mouse immunoglobulin (TAGO, USA), (diluted 1:1000 in blocking buffer) was added to each well and the plates were incubated for 1 h at room temperature. Following 4 wash cycles, 100 μl of 1,2-phenylen diaminehydrochloride (2.2 mmol/l) in a sodium citrate buffer (0.1 mol/l, pH 5.7) 6.5 mmol/l hydrogen peroxide (substrate buffer) was added to each well. The enzyme reaction was allowed to continue for 10 min at room temperature, then stopped by addition 100 μl 1 mol/l sulphuric acid. Optical density was determined at 492 nm.

The specificity of all reactive clones were tested in an ELISA using pure HA (6), which was a generous gift from Dr. D. Tewksbury. This ELISA was performed as described above.

3.3. Electrophoresis and immunoblotting

The angiotensinogen specificity of positive clones was further confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-PAGE was run in a 10% slab gel according to Laemmli (7). High and low molecular weight standards were used according to manufacturers instruction (Bio-Rad, USA). Each sample, contained 5-15 µg protein in 25 µl, were boiled for 3 min after addition of 25 µl 0.5 mol/l TRIS buffer pH 7.8, containing 4% SDS (w/v), 4% mercaptoethanol (v/v), 10% glycerol (v/v) and 0,015% bromphenol blue (w/v). Protein staining of polyacrylamide slab gels were performed with Commasie Brilliant Blue R 250.

Electrophoretic transfer of proteins to nitrocellulose was adapted according to Towbin (8) and Lauritzen (9). The slab gel/nitrocellulose was inserted between 8-9 sheets of Whatman no 1 filterpaper in a semi-dry electroblotter (JKA-Biotech, Denmark), all soaked in 0.05 mol/l TRIS/0.15 mol/l NaCl buffer, pH 7.6, TBS, containing 20% methanol (v/v) and run for 2.5 h at 200 m amps (e.i. 0.8 m amp/cm^2). Staining of marker proteins on nitrocellulose was performed for 3 min with amidoblack 5 g/l in methanol/acetic acid/water (45:10:45) and destaining in the same solvent without amidoblack. The nitrocellulose was type HAHY, pore size 0.45 (Millipore, USA).

Immunological detection of HA on nitrocellulose was obtained by incubation with antibody overnight after blocking for 3 min in 2% Tween 20 (Sigma, USA) in TBS. Monoclonal antibody was diluted to 50 µg/ml in TBS, while polyclonal antibody was diluted 1:1000 in TBS. After three washes in TBS/0.05% Tween 20 and once in TBS alone, horse radish peroxidase conjugated anti mouse immunoglobulin from rabbit (DAKO PATTS, Denmark) was used in 1:1000 dilution in TBS. Color development was achieved after 2-10 min using reactants according to Lauritzen (9)

3.4. Cloning and large scale cell cultivation

Selected, positive cultures were cloned by limiting dilution followed by growth in microtiter plates on washed peritoneal macrophages to ensure monoclonality of the cell lines. Large scale production of hybridoma antibodies was carried out in 800 ml NUNC culture flasks.

3.5. Purification of monoclonal antibodies

Protein A-Sepharose (Pharmacia, Sweden) was used for the purification of monoclonal antibodies of the IgG class from hybridoma cell culture supernatants according to Ey et al. (10).

3.6. Angiotensinogen determination

Immunoplates were coated with monoclonal antibody 3 µg/ml in PBS using 125 µl well. The plates were incubated at 4°C overnight. The wells were blocked with 1% bovine serum albumin, BSA (Behringer Werke, West Germany) in PBS 200 µl, for 1 h at room temperature.For the construction of standard curve the semipurified HA was used: its content of angiotensinogen was calculated from RIA. Human sera were added at appropriate dilutions in PBS/0.1% BSA. 100 µl of calibrators and samples were added and incubated for one hour. After washing, the plates were incubated with rabbit anti-HA diluted 1:9000 or 1:500.000 in PBS/0.1% BSA for 1 h. Following washing, wells were incubated for one hour with 100 µl peroxidase labelled goat anti rabbit immunoglobulin (TAGO, USA) diluted 1:6000 in PBS/0.5% BSA. After addition of 100 µl substrate buffer, enzyme reaction was allowed to continue for 10 minutes. The reaction was stopped by addition of 100 µl 2.5 mol/l sulfuric acid. Optical density was read at 492 nm. Washing between each step were performed using PBS/0.5% Tween 20. All incubations were performed at room temperature on a rocking platform, 4 cycles/sec.

3.7. Immunoglobulin class determination

The IgG and subclass of hybridoma antibodies were determined by a microimmunodiffusion technique (11) against panels of class- and subclass-specific antisera (Celltech, UK).

3.8. Epitope analysis of human angiotensinogen

The epitope analysis was performed as a competition analysis between a biotinylated monoclonal antibody

and increasing amounts of an unlabelled monoclonal antibody. Biotinylation was performed according to (12). 50 µl of a biotinylated monoclonal antibody (1 µg/ml) was incubated with 50 µl samples of each of the 7 unlabelled monoclonal antibodies in concentrations from 0.1 to 30 µg/ml. 50 µl of each of the incubation mixtures were transferred to an immunoplate coated with HA 1 µg/ml. After 1 h, the immunoplates were washed and incubated with avidin-peroxidase (VECTOR, USA) diluted 1:10,000 in PBS + 0.5 mol/l NaCl. After 20 min the plates were washed four times and the enzyme reaction was developed as described above.

4. Affinity determination

4.1. Association constant measured by RIA

Direct angiotensinogen RIA (13,14), using both monoclonal and polyclonal antibodies, were employed to determine the association constants for the interaction of HA with the antibodies.

Pure HA which was a generous gift from D. Tewksbury was labelled with ^{125}I by the iodogen method (15,16).

The RIA technique used for association constant determination was adopted from the procedure of Clauser et al (14). 200 µl of ^{125}I-angiotensinogen 7.500 cpm in 0.1 mol/l sodiumphosphate buffer, pH 7.5, containing 5 mmol/l EDTA, was added at the same time to 200 µl of monoclonal or polyclonal antibodies in the same buffer with or without 10 µl unlabelled angiotensinogen, and incubated for 24 h at 4°C. Separation of free and bound angiotensinogen was carried out by addition of 1000 µl of 20 % polyethylen glycol 6000 (Koch-Light Lab., UK) in 10 mmol/l TRIS/Hl buffer, pH 8.0 containing 1 mg rabbit gamma-globulin X903 (DAKO PATTS, Denmark). After centrifugation at 3000 g for 20 min at 4°C, the precipitate was counted. The respective antibody association constants K_a were determined by using concentrations of pure angiotensinogen ranging from 1-24 x 10^{-9} mol/l. The K_a was calculated according to Scatchard (17). Fig. 3.

4.2. Association constant measured by ELISA

The association constant, K_a, was determined using an ELISA, as outlined by Friguet et al. (18)

Immunoplates were coated with HA, for 20 h at 4°C, 150 µl/well, 0.1 µg HA/ml, in PBS with 10 µg BSA/ml, and blocked with 175 µl/well 1% Tween 20 in PBS for 1 h at 20°C. Antibody at a constant concentration of 70 ng/ml was mixed with HA, and incubated for 20 h at 20°C. HA was present in concentrations from 20 to 2000 ng/ml, in PBS/0.5% BSA, 150 µl was added to each well. After 1 h each supernatant was transferred to a second well and incubated for 1 h The reaction was visualized as described above, after incubation with peroxidase labelled goat anti mouse immunoglobulin (TAGO, USA), diluted 1:1000 in PSB/0.5% BSA. Washing between each step were performed using PBS/0.5% Tween 20. The fraction $r = \frac{Ao-A}{Ag}$ was calculated from the actual absorbance (A) the absorbance with out antigen (Ao). The association constant was determined by a Scatchard plot ($r/[A_g]$ns r).

RESULTS

Antigen

Angiotensinogen was purified to 20-40% purity as shown in Table 1. This preparation has been used to raise monoclonal antibodies in mice and polyclonal antibodies in rabbits.

Monoclonal antibodies, class specificity and affinity

The two fusions performed, resulted in 90 and 200 hybridoma clones, respectively. One clone from the first fusion and six clones from the second fusion produced monoclonal antibodies against human antiotensinogen. All 7 were positive in immunoblotting.

The first colony, 4E11, produced IgM antibody. The next six colonies (F1A1, F2A1, F5A1, F6A1, F7A1 and F8A1,) produced monoclonal antibodies of the IgG_1 subclass as determined using class- and subclass-specific antisera by micro immunodiffusion. Epitope analysis showed that the monoclonal antibodies reacted with three different epitopes on the angiotensinogen molecule. The above mentioned antigen preparation was used for identification of monoclonal antibody in immunoblotting. Moreover, the identity of the monoclonal antibodies were shown in ELISA by pure human angiotensinogen (Tewksbury). Dr. U. Hilgenfeldt (personal

communication) has shown that two of the monoclonal antibodies, F1A1 and F5A1 reacts in immunoblotting with pure human angiotensinogen, separated on high pressure liquid chromatography, HPLC, from the contaminating' alanin-protein' (19).

Cross-reactivity with angiotensin I and tetradecapeptide in ELISA was not detectable, while cross reactivity with des-angiotensin I angiotensinogen was 100%. The antibodies did not recognize angiotensin I in RIA, indicating that their epitopes are located apart from the N-terminal angiotensin I sequence of the angiotensinogen molecule.

The epitope analysis showed that the monoclonal antibodies reacted with 3 different epitopes on the angiotensinogen molecule.

ELISA results

Using 5 to 1000 x 10^{-12} mol/l of human angiotensinogen, the binding curve showed a region of the curve closely fitting a straight line from 10 to 500 x 10^{-12} mol/l or 10^{-15} mol/well. This was selected as the primary standard curve (fig 2). For both normal human sera (n=8) and for pregnant women's sera (n=18) the results obtained correlated closely with RIA, R = 0.97 (Fig. 1). In Fig.1 the solid line represents the linear regression of the ELISA, while the dotted line represent ± 1 standard deviation, S.D. The intra assay variation was 2%. Inter assay variation was 8%, Table 2.

Association kinetics

The monoclonal antibodies raised against human angiotensinogen were used in solid-phase ELISA and assessed by checkerboard titration to estimate their capacity to bind angiotensinogen in serum and in purified preparations. Three antibodies showed high sensitivity as mentioned above, being able to detect 10^{-11} mol/l or 10^{-15} mol HA/well.

The affinities of the monoclonal and polyclonal antibodies were determined in a direct RIA of angiotensinogen. Their association constants being calculated from a Scatchard plot. The K_a value for the monoclonal antibodies possessing the highest affinity were in ranges from 0.5 x 10^{10} to 0.7 x 10^{10} 1/mol. The polyclonal antibody had a K_a value of 0.8 x 10^{10} 1/mol.

In the determination of the association constant, K_a, using ELISA, the absorbance at 492 nm was the basis for the measurements and calculations, as outlined by Friguet et al. (18).

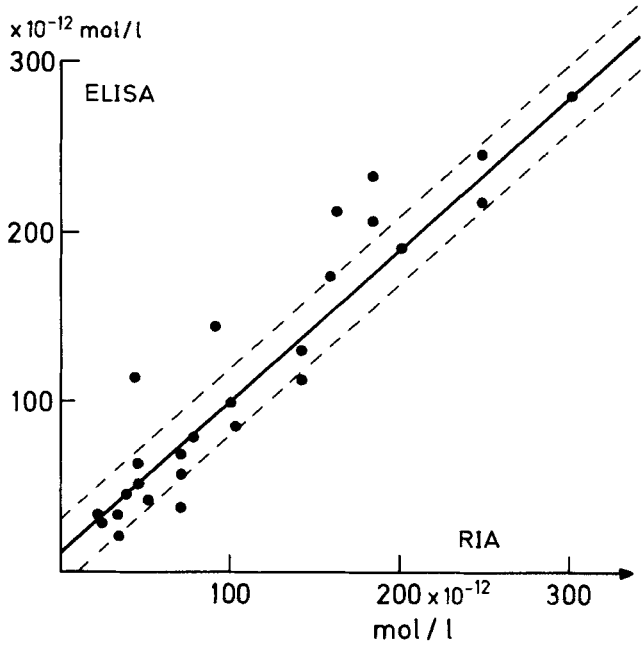

Fig. 1.
Relationship between plasma angiotensinogen concentration determined by ELISA and by RIA (converting angiotensinogen to angiotensin I) 1 SD (dashed line).
Coefficient of correlation; R = 0.95; p = 0.001
y = 0.909 · x + 8.82 showing the linear regression for ELISA. RIA has earlier been determined to have an CV of 8-9%.

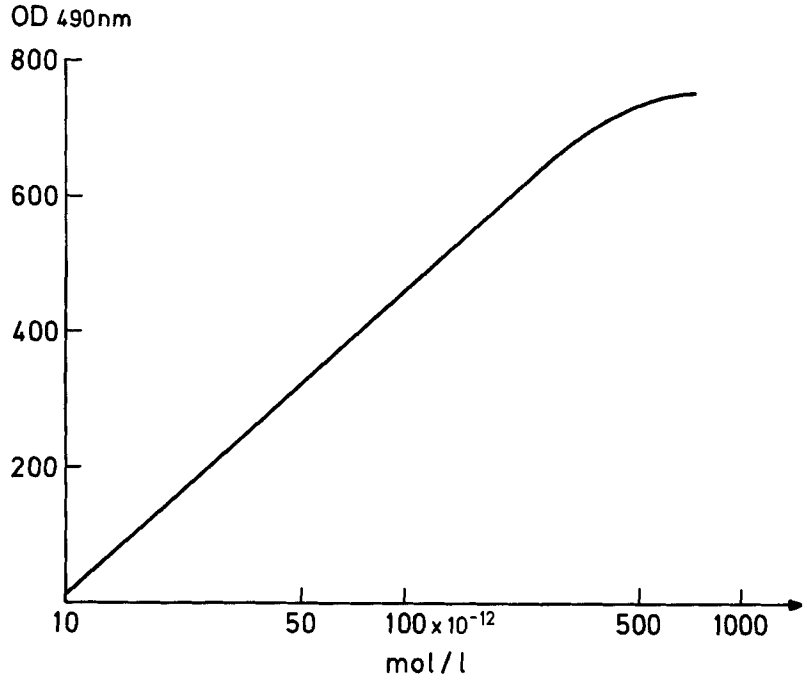

Fig. 2.
Calibration curve for renin substrate. Relationship between optical desity (OD) and increasing amount of angiotensinogen in ELISA.

In the present study K_a was calculated to be 1.67×10^{10} 1/mol ± 0.02 (n=5) from regression analysis. As a control, all the r-intercept were found to be close to 1.

The difference in the K_a's measured by two methods reflects the difficulties experienced in conserving the antigenicity while iodinating the antigen.

DISCUSSION

The non competitive "Sandwich" ELISA for the quantification of human angiotensinogen, described above, is precise, specific and less time consuming than RIA.

The ELISA has been developen using two monoclonal and two polyclonal antibodies, one of these had been

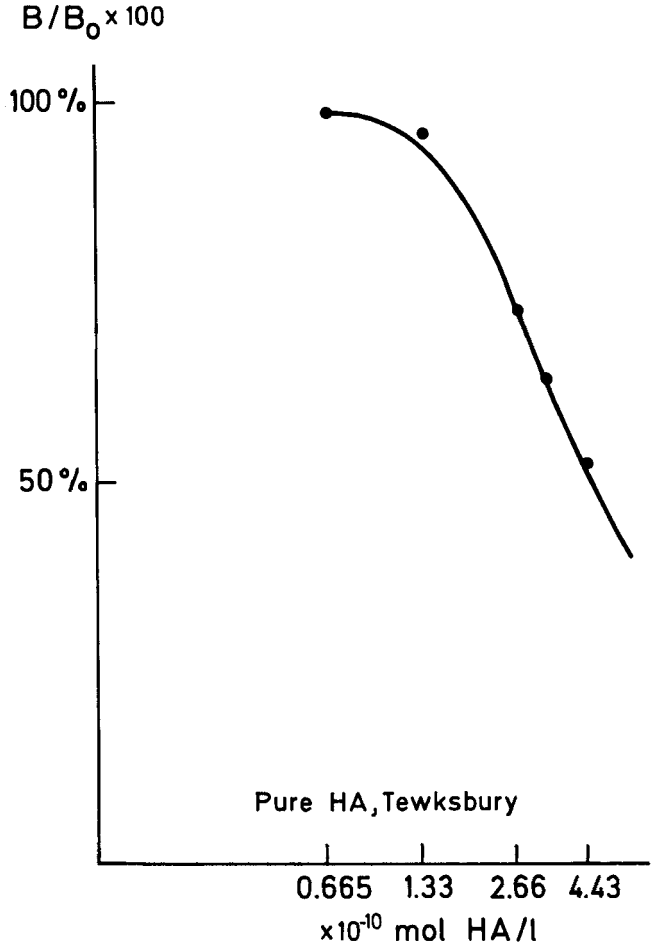

Fig. 3.
Scatchard plot for a monoclonal antibody, F1A1, displacement of ^{125}I-labelled HA (Tewbsbury). B/B_o x 100: is the binding of tracer expressed in per cent.

TABLE 2

Intra- and Inter-Assay Precision

High concentration of human angiotensinogen. Pregnant women's sera. 3×10^{-10} mol/l	Intra assay precision Mean mol/l	SD	CV %	Inter assay precision Mean mol/l	SD	CV %
	3.08×10^{-10}	0.06	1.9	3.08×10^{-10}	0.20	6.5
Medium concentration of human angiotensinogen. Pregnant women's sera. 7.7×10^{-10} mol/l	0.77×10^{-10}	0.02	2.3	0.77×10^{-10}	0.06	7.6
Low concentration of human angiotensinogen. Pregnant women's sera. 15.4×10^{-12} mol/l	0.15×10^{-10}	0.003	1.8	0.15×10^{-10}	0.01	6.5

raised against electrophoretical pure HA (Tewksbury). We could thus be sure of the specificity of our assay using an antibody raised against pure antigen. The assay we have developed could be used to measure pure HA (Tewksbury), HA in preparations of purification and in plasma from pregnant and non-pregnant individuals.

The range of the ELISA is from 15 to 450 pmol/l which is 5 times more than the indirect RIA, having a range from 40 to 240 pmol/l (3). This implicates that less dilutions is necessary in order to be within measuring range. The precision of the ELISA is stated in table 2. The intra- and interassay variation is between 2 and 7%, which is lower compared to the indirect RIA, having 7% intra- and 9% interassay variation (3). The specificity is demonstrated by the fact that the correlation coefficient between the ELISA and the RIA is 0.97 and no cross reactivity against angiotensin I, angiotensin II, or tetradecapeptide is demonstrated. Plasma samples from normals and pregnants (low and high concentrations of angiotensinogen) demonstrated superimpossability with standardcurve.

Determination of the association constants was performed in RIA and ELISA. The two methods used gave almost identical K_a-values. The K_a-value determined by ELISA was a factor 2 higher than the determination by angiotensinogen by iodination, because the maximum binding of the monoclonal antibodies with iodinated angiotensinogen did not exceed 72%.

The monoclonal antibodies recognized angiotensinogen as well as its metabolic product, des-angiotensin I-angiotensinogen. The absence of cross reactions with angiotensin I, angiotensin II or tetradecapeptide indicate that the epitope on the angiotensinogen molecule recognized by each antibody is distant from the N-terminal region. This is in agreement with Mizrahi et al, (20) finding the same lack of differentiation between angiotensinogen and its inactive residue des-angiotensin I angiotensinogen.

The monoclonal antibodies directed against three different epitopes were not able to inhibit the renin-angiotensinogen enzymatic reaction, supporting the conclusion that the epitopes are placed distant to the N-terminal region. The polyclonal antibody directed against pure HA (Tewksbury) did not exhibit any inhibitory capacity against the renin angiotensinogen enzymatic reaction in dilutions form 100-10,000.

Plasma samples contains endogenous rening, which is able to convert angiotensinogen. However, as the plasma samples have to be diluted 3 to 4 orders of magnitude before beeing measured on the ELISA, the degradation is reduced beyond the detection limit of the assay, e.i. 6 to 8 orders of magnitude. The degradation has thus been reduced to levels of no significance.

Under certain conditions the ELISA overestimate the concentration of genuine angiotensinogen because of

abnormal high concentration of des-angiotensin I-angiotensinogen (2). This is the case with high renin concentration, e.i., in severe hypertension with low sodium diet; converting enzyme inhibition and adrenal insufficiency. In plasma samples taken from normal individuals or gregnants, there were no significant difference between the values obtained by the ELISA and the values obtained by the indirect RIA, which has been depicted in Fig. 1. This is the case when renin activity is normal an unstimulated.

With this new and highly sensitive technique we are able to measure HA in very low concentrations, as the detection limit is 6 orders of magnitude lower than the serum concentration. This enables us to dilute the plasma samples in order to avoid non specific plasma protein interaction, and be able to perform the assay using 0.02 - 0.6 µl of human plasma/well.

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