SEPARATION AND IDENTIFICATION OF THYROID AUTOANTIBODIES IN PATIENTS WITH THYROID DISORDERS BY HYDROPHOBIC COLUMN

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ABSTRACT

A method has been developed to separate and identify thyroglobulin autoantibody (TgAb) and thyroid peroxidase autoantibody (TPOAb) in serum obtained from normal and autoimmune thyroid diseases using phenyl Sepharose CL-4B hydrophobic column. The protein peaks obtained from hydrophobic column were identified as TgAb and TPOAb by comparing the elution profile of commercially purified standard thyroid autoantibodies. The similarity of the inhibitory effects of eluted proteins and of standard thyroid autoantibodies on lectin concanavalin A-RBC interaction confirmed the separation of TPO-Ab and TgAb by the hydrophobic column. The eluted fractions from the hydrophobic column were estimated by the radio immunoassay (RIA) to confirm the presence of both auto-antibodies. This hydrophobic column method offers an advantage of visual inspection of this autoantibodies by graphic representation of peak height along with their estimation in autoimmune thyroid disorders.

KEY WORDS: Autoantibodies, Thyroid autoimmune diseases, Lectin, Phenylsepharose CL-4B column, Hydrophobic column.

INTRODUCTION

Autoantibodies to thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) occur in elevated levels in thyroid autoimmune diseases (1,2). Assessment of these autoantibodies is of great clinical importance in diagnosis and management of thyroid diseases like Graves' disease, Hashimoto's thyroiditis and other autoimmune thyroid diseases. These autoantibodies are currently assayed by either Radio Immuno Assay (RIA) or Enzyme Immuno Assay (EIA) method for their accuracy and sensitivity. TPOAb and TgAb are gamma-globulin in nature mainly belonging to IgG class (3). In the present communication a technique using hydrophobic resin column has been developed to separate TPOAb and TgAb and to estimate these two parameters for detection of autoimmune thyroid diseases. This report may

be considered as an alternative approach for the assay of these autoantibodies along with other conventional methods.

MATERIALS AND METHODS

Chemicals

Hydrophobic resin Phenyl Sepharose CL-4B was purchased from Sigma Chemical Co. St. Louis, USA. TPOAb and TgAb were purchased from M/s. Kronus, USA and these autoantibodies were calibrated against the international reference standard IRC 65/93.

Specimens

A total of 31 subjects (age 15 to 60 years) of both sexes were included in the present study. Control sera were obtained from 12 normal healthy subjects. Pathological sera were obtained from 19 patients (11 with Hashimoto's thyroiditis and 8 with Graves' diseases). The normal and pathological samples were collected from subjects attended in

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the out patient department and thyroid clinic of B R Singh Hospital, Calcutta. The selection of normal and diseased subjects was done by clinical examination, measurement of thyroid function tests and other laboratory investigations. None of patients included in this study were suffering from hepatic diseases, renal dysfunction, malignant disorders, rheumatic and other metabolic diseases known to cause elevated γ -globulin levels. Fasting clotted blood were taken in all cases and serum was separated by low speed centrifugation. Serum was assayed on the same day or stored at -20°C for further use. Sera from other autoimmune diseases (2 with hepatic disease, 2 with rheumatic arthritides and 1 with malignant disorder) were also investigated for comparison.

Preparation of γ -globulin from serum

γ -globulin from serum was separated according to the method of Hudson & Hay (4). Serum was diluted 1:2 with normal saline and saturated $(\text{NH}_4)_2\text{SO}_4$ was added to get 45% $(\text{NH}_4)_2\text{SO}_4$ saturation. It was stirred at room temperature for 30 minute. The precipitate obtained was centrifuged at 1000g for 15 min. in cold. Precipitate was washed with 45% saturated $(\text{NH}_4)_2\text{SO}_4$ solution and centrifuged. It was then re-dissolved in the same volume of 0.15 M phosphate buffer saline, pH 7.2 (PBS) as the original volume of serum and centrifuged to remove any insoluble material and the supernatant was reprecipitated with 40% saturated $(\text{NH}_4)_2\text{SO}_4$. Thus precipitate (γ -globulin) obtained after centrifugation (1000g) was dissolved in PBS. The soluble γ -globulin was dialysed against PBS.

Separation of autoantibodies by phenylsepharose-CL-4B hydrophobic column

Phenylsepharose CL4B column (2.5 ml bed) was washed with distilled water and equilibrated with 10 mM phosphate buffer (pH 7.3) containing 30% $(\text{NH}_4)_2\text{SO}_4$ solution. Standard TPOAb (0.1 ml of 30 unit/ml), standard TgAb (0.1 of 20 unit/ml), normal sample (0.2 ml) and experimental sample (0.2 ml) were mixed separately with 30% $(\text{NH}_4)_2\text{SO}_4$ solution and added separately to the column for hydrophobic binding to the matrix. Elution of the

sample was done with gradient mixture of 10 mM phosphate buffer, pH 7.3 and 30% $(\text{NH}_4)_2\text{SO}_4$ to decreasing concentration and then only with 10 mM phosphate buffer and lastly with distilled water. Volume of each fraction was 1 ml/10 min.

Protein estimation

Absorbance at 280 nm of the column eluate was taken spectrophotometrically. Amount of protein of the eluate was determined from standard curve drawn at 540 nm using bovine serum albumin as standard (5).

Hemagglutination

The assay of concanavalin A (ConA) lectin activity by hemagglutination was performed by a standard two-fold serial dilution technique (6) using a 2% v/v washed sheep erythrocytes suspension in PBS. Effects of autoantibodies on hemagglutination were examined by preincubation of the lectin ConA with test substance (autoantibody).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at pH 8.3 in 7.5% gel (7). Protein staining was done with Coomassie Brilliant Blue. In all cases, 50 μg protein was applied to the gel. Mixture of standard TPOAb and TgAb was run in the PAGE.

Isoelectric-focussing (IEF)

pIs of the autoantibodies (TPOAb and TgAb) were determined by Isoelectric focussing (8) using Pharmalyte of pH 3-10 and buffer 0.01 M iminodiacetic acid as anode solution and 0.01 M ethylene diamine as cathode solution.

Radio immuno assay (RIA)

Serum TPOAb and TgAb were measured by highly sensitive direct RIA methods using kits and protocols obtained from M/s. Kronus. In the present study the normal ranges for TPOAb and TgAb were considered to be between 0.3 and 1.0 unit/ml.

RESULTS

Fractionation of γ -globulin obtained from patient's serum by PAGE showed several bands (Fig. 1). Bands of TPOAb and TgAb were in the lowest and identical position, which was confirmed by the band position of standard autoimmune thyroid autoantibodies. Isoelectric Focussing (IEF) experiment was performed with standard thyroid auto-antibodies and it was observed that PI values of both TPOAb and TgAb were nearly 3.5. Protein peaks of standard TPOAb and TgAb are shown in Fig. 2A & 2B respectively after elution from activated Phenyl Sepharose CL-4B hydrophobic column. Highest protein peak for TPOAb was around fraction 20 (Fig. 2A) and that for TgAb was at around fraction 40 (Fig. 2B) under the experimental conditions.

The same column after proper washing and equilibrating with 10 mM phosphate buffer, pH 7.3 containing 30% $(\text{NH}_4)_2\text{SO}_4$ solution was used for fractionation of γ -globulin isolated from normal and patient's sera (Fig.3). Peaks I & I' and peaks II & II' in Fig.3 indicated the TPOAb and TgAb, respectively, for normal and patient's sera as compared to standard autoantibodies in Fig.2A and Fig.2B. The average amount of proteins obtained from hydrophobic column for TPOAb and TgAb of

Fig.1 Polyacrylamide gel electrophoresis of two sera of different thyroid auto-immune diseases – a) Hashimoto's thyroiditis (50 μg protein), b) Graves' disease (50 μg protein) c) Standard mixture (10 μl) of equal volume of TPOAb and TgAb.

Fig.2 Elution profile of standard thyroid autoantibodies by phenylsepharose-CL-4B hydrophobic column. A) Standard TPOAb B) Standard TgAb.

normal samples were 3 mg/ml and 2.8 mg/ml respectively (Table 1). Patient's samples showed significantly increased protein values for both the autoantibodies. In Hashimoto's thyroditis, these values were 7.2 mg/ml and 6.5 mg/ml for TPOAb and TgAb respectively and in Graves' diseases, TPOAb was 8.3 mg/ml and TgAb was 5.8 mg/ml (Table 1). The data obtained from hydrophobic column was compared to the results obtained by RIA (Table 1). Diseased samples for both autoantibodies also showed significantly higher antibody levels than normal control in RIA method (2). No protein peaks were obtained by γ -globulin of the sera of other autoimmune diseases as they did not bind in the hydrophobic column. Table 2 indicated that standard TPOAb inhibited the interaction between the lectin ConA and sheep's RBC while no such inhibition was observed with standard TgAb. This result demonstrated that glycan part of two autoantibodies are different. Peak I & I' also inhibited the reaction between ConA and sheep's RBC but peak II & II' did not show such inhibition in this lectin-RBC interaction (Table-2).

Fig.3 Elution profile of γ -globulin of normal and diseased subjects by phenylsepharose CI-4B hydrophobic column. Peaks I & II (O-O) TPOAb & TgAb of normal subjects. Peaks I' and II' (O-O) TPOAb & TgAb of diseased subjects. Quantitations by RIA method are shown in the respective peaks.

Table 1. Serum TPOAb and TgAb levels in control and in thyroid autoimmune diseases.

	TgAb		TPOAb	
	RIA (Unit/ml)	Hydrophobic method (mg protein/ml)	RIA (Unit/ml)	Hydrophobic method (mg protein/ml)
Control (12)	0.62 $\pm$ 0.14	2.8 $\pm$ 0.44	0.56 $\pm$ 0.12	3.0 $\pm$ 0.14
Hashimoto's thyroditis (11)	32.50 $\pm$ 10.80 P<0.001	6.5 $\pm$ 0.41 P<0.001	41.30 $\pm$ 12.50 P<0.001	7.2 $\pm$ 0.44 P<0.001
Graves' diseases (8)	20.60 $\pm$ 9.70 P<0.001	5.8 $\pm$ 0.40 P<0.001	58.40 $\pm$ 14.60 P<0.001	8.3 $\pm$ 0.41 P<0.001

Figures in parenthesis indicate number of samples, Results are means $\pm$ S.E.M., P<0.001 Highly significant

Table 2. Effects of standard thyroid autoantibodies and column fractions on ConA-RBC interaction.

Experiment	Agglutination
Control	Positive
Control + Std. TPOAb	Negative
Control + Peak I or I'	Negative
Control + Std. TgAb	Positive
Control + Peak II or II'	Positive

Control - Washed 2% sheep's RBC + Con A (50 μ g protein), Std - Standard
Std. TPOAb, Std. TgAb, Peak I or I' and Peak II or II'

All Concentrations are 100 μ g protein

RIA estimations of hydrophobic column fractions determined that peak I & I' and peak II & II' were TPOAb and TgAb respectively. Quantitations of peak values by RIA method were shown in the respective peaks (Fig.3).

DISCUSSION

The current conventional techniques, RIA and EIA, can estimate TPOAb and TgAb in serum accurately and these methods need sophistication and specialization. The object of our study was to develop an inexpensive alternative method for visual observation of TPOAb and TgAb in diseased serum from normal control and estimation of these two antibodies in the laboratory having modest facilities. The experimental findings indicated that both autoantibodies showed identical position of band in PAGE and in IEF. So the visual evaluation of TPOAb and TgAb by the observation of band position and band concentration in PAGE or in IEF was not possible. Investigation was then made to separate both thyroid autoantibodies according to their hydrophobic binding nature to the

hydrophobic resin Phenyl Sepharose CI-4B. Successful separations of TPOAb and TgAb from isolated immunoglobulin from sera of patient and normal subject were done and it was confirmed by the separations profile of standard thyroid autoantibodies. Proteins in peak I or I' in Fig.3 were TPOAb as they, like standard TPOAb, inhibited the activity of ConA and peak II and II' were TgAb showing the non-inhibitory effect on ConA like standard TgAb showing the non-inhibitory effect on ConA-RBC interaction. RIA estimation indicated that Peak I & I' were TPOAb and peak II & II' TgAb autoantibodies. Comparative data of autoantibodies in thyroid autoimmune diseases estimated by RIA and by hydrophobic column method in Table 1 indicated the significantly increased values of these autoantibodies in diseased persons.

The hydrophobic column method may be utilized as an adjunct to the conventional techniques of thyroid autoantibody measurement. Moreover, this method is highly sensitive for the detection of thyroid autoantibodies as it has an excellent correlation with the RIA technique.

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