Labelling and immunoprecipitation of thyroid microsomal antigen

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Human thyroid microsomes have been solubilized, labelled with ^{125}I, immunoprecipitated with microsomal antibody and analysed by gel electrophoresis. The analysis indicated that two peptides of relative molecular masses 108 and 118 kDa, under reducing conditions, were specifically immunoprecipitated by microsomal antibody. Similar values were obtained under non-reducing conditions indicating that the two peptides were not linked by disulphide bridges to each other or to different peptides. These results suggest that the microsomal antigen contains two components which may be linked by non-covalent bonds to form a single protein of 230 kDa. Studies with lectin affinity columns suggested that the antigen was glycosylated.

Autoimmunity *Autoantibody* *Thyroid* *Thyroglobulin* *Thyroid disease* *Hashimoto's disease*

1. INTRODUCTION

Antibodies to an antigen associated with thyroid microsomes appear to be involved in the pathogenesis of autoimmune thyroid disease [1] but little is known about the auto-antigen's structure. Consequently we have attempted to characterise the microsomal antigen in more detail and now report ^{125}I labelling of solubilized thyroid microsomes followed by immunoprecipitation with microsomal antibody and analysis by gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Microsomal and thyroglobulin preparations*

Thyroglobulin and microsomal fractions were prepared from homogenates of human (Graves') thyroid tissue as described [2] and stored in aliquots at −70°C. Solubilization of microsomal proteins was effected by addition of 1% sodium deoxycholate (DOC) [3] in 10 mM Tris-HCl, pH 8.0 (1 ml/mg protein), incubated for 30 min at room temperature followed by centrifugation at 100000 × *g* for 1 h at 4°C. The supernatant was dialysed overnight at 4°C against 2 × 100 vols of 0.1% DOC, pH 8.0, and stored at −70°C. The activity of the solubilized preparations was assessed by using them as coating antigen in a microsomal antibody ELISA [2] and this showed that most of the microsomal antigen present in the original microsomes had been solubilized by DOC. An ELISA system was also used to measure thyroglobulin antibodies [4].

2.2. *Labelling of microsomes and thyroglobulin with ^{125}I*

The iodogen method [5] was used to label both preparations to a specific activity of about 50 μCi/μg protein. Labelled thyroglobulin was purified by chromatography on a 30 × 2.6 cm column of Sephacryl S-300 in 50 mM NaCl, 10 mM Tris, pH 7.5. Labelled microsomes were dialysed overnight against 100 vols of 10 mM Tris-HCl, pH 8.0, containing 0.1% DOC prior to further analysis.

2.3. *Patients sera*

Sera from 3 patients with Hashimoto's thyroiditis were used. Two sera (MR and HH) had

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high titers of microsomal antibody but thyroglobulin antibody levels were only just detectable by ELISA. The other serum (UK) had a high titer of microsomal antibody and thyroglobulin antibody was detectable by precipitation in Ouchterlony plates. In some experiments, all detectable thyroglobulin antibody activity (as measured by ELISA) was removed from aliquots (200 μl) of the Hashimoto sera by affinity chromatography (2 ml/h) on columns of Sepharose-thyroglobulin (2 ml, containing 10 mg/ml thyroglobulin).

2.4. *Immunoprecipitation and gel electrophoresis*

Labelled microsomes or thyroglobulin (100 μl, 30–40 μCi) were incubated overnight at 4°C with serum (5 μl) containing unlabelled thyroglobulin (0–20 μg). A 10% suspension of *Staphylococcus aureus* cells in phosphate-buffered saline (Pansorbin, Calbiochem, 100 μl) was then added and incubation continued for 1 h at 4°C. After centrifugation (14000 × *g*, 3 min) and washing in 0.1% DOC the immunoprecipitates were solubilized by heating for 5 min with an equal volume of 4% SDS with or without 2% mercaptoethanol. The solubilized materials were analysed by 5–12% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography [6]. The following molecular mass markers were used: thyroglobulin (320 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), transferrin (77 kDa), bovine serum albumin (67 kDa), IgG and component chains (150, 50 and 23 kDa), ovalbumin (44 kDa) and TSH (28 kDa). Analysis under reducing and non-reducing conditions used reduced and non-reduced standards, respectively.

2.5. *Lectin affinity chromatography*

Lentil lectin Sepharose 4B (2 mg lectin/ml gel) and concanavalin A-Sepharose 4B (10 mg/ml gel) were obtained from Pharmacia, Sweden. IgG Sepharose (10 mg/ml gel) was prepared by coupling IgG prepared from normal human serum to CNBr-activated Sepharose 4B (Pharmacia) using the conditions recommended by the manufacturers. Columns (1.5 ml) of the various gels were packed and equilibrated with 150 mM NaCl, 10 mM Tris-HCl, pH 8.2, containing 0.1% DOC (starting buffer). Aliquots of unlabelled DOC-solubilized microsomes (200 μl, 200 μg protein) or ^{125}I-labelled microsomes (200 μCi, 4 μg protein) were run (2 ml/h at room temperature) onto the columns and eluted (2 ml/h) with starting buffer followed by starting buffer containing 0.5 M α-methyl D-glucoside. In the case of unlabelled microsomes, the column fractions (0.5 ml) were diluted with an equal volume of 0.1 M sodium bicarbonate buffer, pH 9.3, and used to coat micro-titer plates for analysis of microsomal antigen content by microsomal antibody ELISA as described by Schardt et al. [2]. In the case of labelled microsomes, only lentil lectin columns were used and the peaks of radioactivity eluted by starting buffer and starting buffer containing α-methyl D-glucoside were collected, immunoprecipitated and analysed by gel electrophoresis.

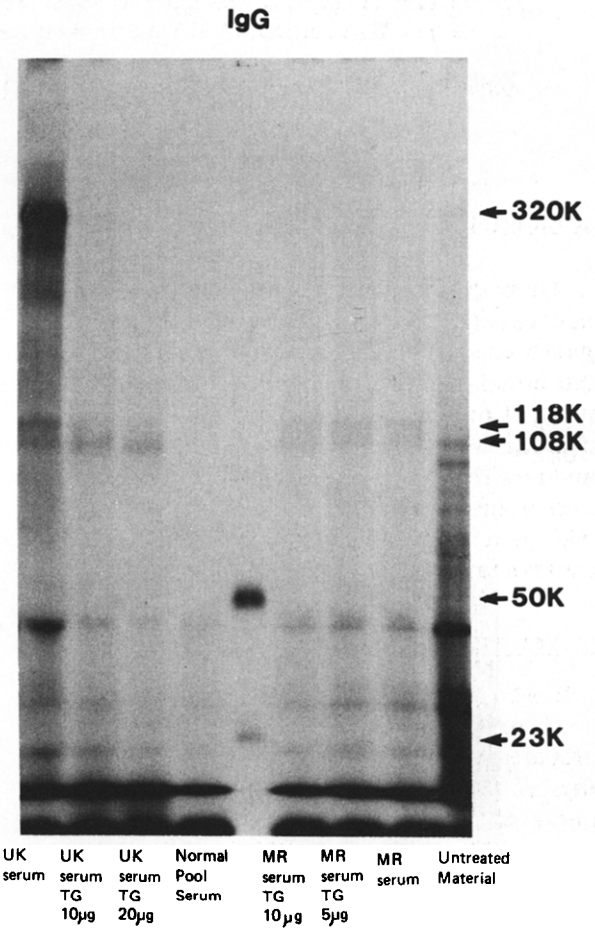

Fig.1. Analysis by SDS-PAGE under reducing conditions of labelled microsomes immunoprecipitated with anti-microsomal antibody sera (patients U.K. and M.R.).

3. RESULTS

Analysis of untreated labelled microsomes on SDS-PAGE under reducing conditions showed the presence of a major band characteristic of an intact thyroglobulin subunit (relative molecular mass 320 kDa) [7] and lower molecular mass bands (fig.1). Immunoprecipitation with the serum containing high titers of antithyroglobulin and antimicrosomal antibody (UK serum) resulted in bands of increased intensity at 320, 280, 220, 118 and 108 kDa. However, when this serum was absorbed with thyroglobulin, prior to immunoprecipitation only the bands at 108 and 118 kDa remained. Immunoprecipitation of labelled thyroglobulin with the Hashimoto sera gave bands characteristic of thyroglobulin but not the 108 and 118 kDa bands (not shown).

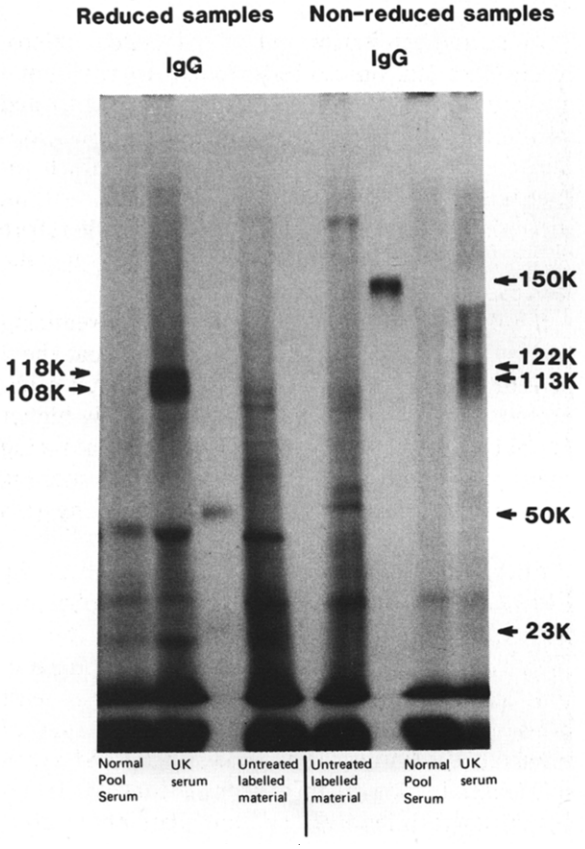

Fig.2. Analysis by SDS-PAGE of labelled microsomes immunoprecipitated with anti-microsomal antibody sera which had been absorbed using Sepharose-thyroglobulin.

Immunoprecipitation with serum MR which had only low titers of thyroglobulin antibody but high microsomal antibody titers gave the two bands at 108 and 118 kDa and immunoprecipitation of these bands was not influenced by absorption with thyroglobulin (fig.1). Similar results were obtained

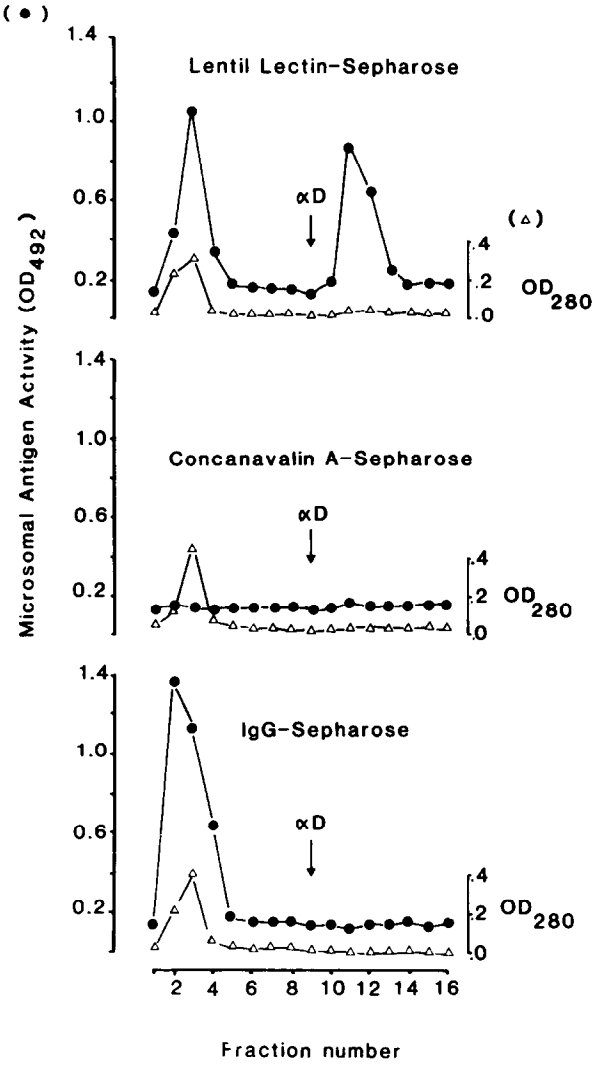

Fig.3. Fractionation of unlabelled DOC solubilized microsomal antigen by lectin affinity chromatography. αD above a vertical arrow indicates point at which elution with 0.5 M α-methyl D-glucoside was started. Microsomal antigen activity is shown as absorbance at 492 nm (OD_{492}) generated in a microsomal antibody ELISA based on horseradish peroxidase-labelled anti-IgG and o-phenylenediamine [2].

with UK serum which had been absorbed by Sepharose-thyroglobulin (fig.2).

Analysis of the 108 and 118 kDa bands under non-reducing conditions (fig.2) gave less precise data but similar values were obtained (115 and 124 kDa, respectively, in one experiment and 112 and 121 kDa in a second experiment).

Repeated analysis of the 108 and 118 kDa bands under reducing conditions gave mean ± SD values of 108.4 ± 4.1 and 118.4 ± 4.0 kDa, respectively (n = 6).

Fractionation of unlabelled DOC solubilised microsomes on lectin columns is shown in fig.3. In the case of lentil lectin columns most of the protein (as judged by absorbance at 280 nm) and about half of the microsomal antigen activity (as judged by ELISA) were eluted unretarded from the column. However, the antigen absorbed by the column under starting conditions was eluted with buffer containing 0.5 M α-methyl D-glucoside.

Concanavalin A columns absorbed all detectable microsomal antigen but this could not be eluted with α-methyl D-glucoside. The Sepharose-IgG (control) column did not absorb detectable amounts of microsomal antigen.

Gel electrophoresis of ^{125}I-labelled microsomal antigen fractionated by lectin affinity chromatography is shown in fig.4. This indicated that the labelled microsomal antigen (108 and 118 kDa bands) was principally located in the fraction eluted with 0.5 M α-methyl D-glucoside from the lentil lectin column.

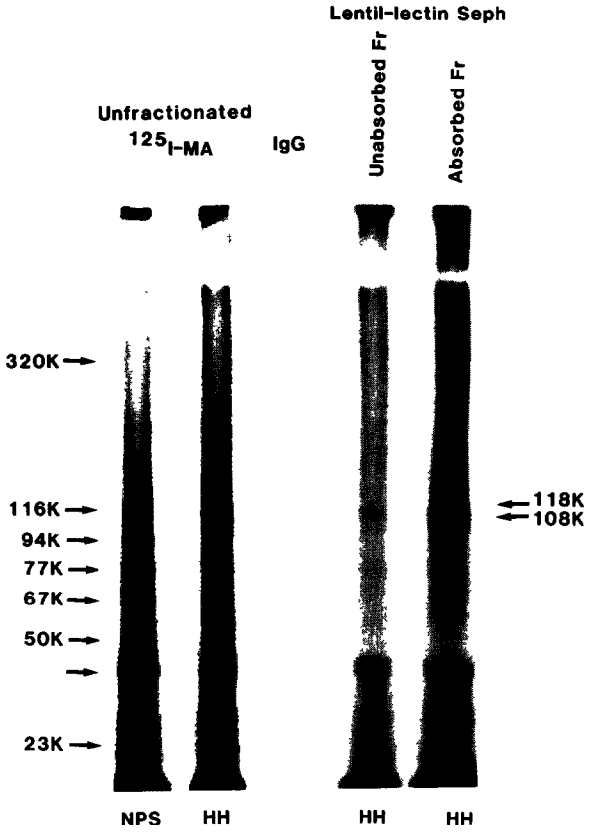

Fig.4. Fractionation of ^{125}I-labelled microsomal antigen by lentil lectin affinity chromatography. Labelled antigen was absorbed onto lentil lectin Sepharose, eluted with α-methyl D-glucoside, precipitated using Hashimoto sera (HH) or normal pool serum (NPS) and analysed by gel electrophoresis and autoradiography.

4. DISCUSSION

Immunoprecipitation of ^{125}I-labelled microsomes with Hashimoto sera, free of thyroglobulin antibody but containing high titers of microsomal antibody, showed that the labelled material contained 2 peptides of 108 and 118 kDa which interacted with autoantibodies other than thyroglobulin antibody. It seemed likely therefore that these two peptides were related to the microsomal antigen.

Similar M_r values were obtained under reducing and non-reducing conditions suggesting that the 2 peptides were not linked to each other or other proteins by disulphide bridges. The slightly higher M_r values observed in the absence of reducing agent probably reflected the difficulties in making accurate M_r determinations in non-reducing gels rather than a true difference in M_r [8].

Microsomal antigen activity measured by ELISA was completely absorbed by Sepharose-concanavalin A columns suggesting that the antigen was glycosylated. Binding to Sepharose-concanavalin A was not readily reversible at least using α-methyl D-glucoside and a similar type of irreversible binding has been observed with solubilised insulin receptor preparations [9]. In experiments with lentil lectin Sepharose about 50% of the antigen was not retained by the column and a similar amount absorbed and eluted with α-methyl D-glucoside. The two fractions from the lentil lectin column could have represented dif-

ferent forms of microsomal antigen or column overload. However, fractionation of labelled microsomes which were derived from much smaller amounts of material showed that the 108 and 118 kDa bands characteristic of microsomal antigen were almost completely absorbed by the lentil lectin column and eluted with α-methyl D-glucoside. Consequently the fraction of unlabelled microsomes not retarded by the lentil lectin column was likely to have been due to column overload rather than antigen heterogeneity. Similar observations have been made with insulin receptor preparations in which a relatively large proportion of the receptor was not retarded by lentil lectin columns [9].

The relationship between the 108 and 118 kDa peptides present in microsomal antigen is not clear at present but it is possible that they represent 2 distinct autoantigens. Alternatively, the 2 peptides may interact through non-covalent bonds to form a single protein of 230 kDa.

REFERENCES

[1] Doniach, D. and Roitt, I.M. (1976) in: Text Book of Immunopathology (Miescher, P.A. and Muller-Eberhad, H.J. eds) pp.715–731, Grune and Stratton, New York.
[2] Schardt, C.W., McLachlan, S.M., Matherson, J. and Rees Smith, B. (1982) J. Immunol. Methods 55, 155–168.
[3] Snary, D., Goodfellow, P., Hayman, M.J., Bodmer, W.F. and Crumpton, M.J. (1974) Nature 247, 457–461.
[4] McLachlan, S.M., Clark, S., Stimson, W.H., Clark, F. and Rees Smith, B. (1982) Immunol. Lett. 4, 27–33.
[5] Fraker, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857.
[6] Buckland, P.R., Rickards, C.R., Howells, R.D., Jones, E.D. and Rees Smith, B. (1982) FEBS Lett. 145, 245–249.
[7] Lissitzky, S. (1984) J. Endocrinol. Invest. 7, 65–76.
[8] Griffiths, I.P. (1972) Biochem. J. 126, 553–560.
[9] Hedo, J.A., Harrison, L.C. and Roth, J. (1981) Biochemistry 20, 3385–3393.