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Immunoprecipitation of human adrenal microsomal antigen

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Human adrenal microsomes have been labelled with ^{125}I and immunoprecipitated with sera from patients with Addison's disease. The immunoprecipitates were then analysed by SDS-PAGE and autoradiography. 13 of the 23 sera from the Addison patients studied contained antibodies which reacted with a 55 kDa adrenal microsomal protein. The same 13 sera were also positive for adrenal antibodies as judged by immunofluorescence. The 55 kDa protein was not immunoprecipitated from placenta or thyroid microsomes by Addison sera. Furthermore, patients with Graves' disease or rheumatoid arthritis did not immunoprecipitate the 55 kDa protein from adrenal microsomes. Our studies suggest therefore that Addison sera contain antibodies to a 55 kDa adrenal specific protein which may well be the antigen observed on immunofluorescence.

Adrenal antigen; Addison's disease; Autoimmunity; Autoantibody

1. INTRODUCTION

The sera from patients with idiopathic Addison's disease contain autoantibodies to adrenal tissue, and immunofluorescence studies have shown that the antibodies are of IgG class, bind to the microsomal fraction of adrenal cortex, and are usually present in low titres [1-4]. More recent studies have suggested that the antibodies can be detected by radioimmunoassay or ELISA techniques based on preparations of adrenal cell microsomes [5,6] but no biochemical characterisation of the antigen(s) involved has been reported.

Consequently, we have used the non-denaturing detergent sodium deoxycholate (DOC) to solubilize human adrenal microsomes and then characterised the material after labelling with ^{125}I by immunoprecipitation with Addison sera and gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE).

Our studies indicate that autoantibodies in the serum of patients with Addison's disease interact with a 55 kDa adrenal specific protein.

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2. METHODS

2.1. *Tissue preparations*

Adrenal tissue was obtained from kidney donors, thyroid tissue at partial thyroidectomy for Graves' disease and human placenta at delivery. The tissue specimens were cut into small pieces (200 mg) and stored at −70°C. Microsomal fractions were prepared from tissue homogenates by differential centrifugation and solubilized using 1% DOC in 50 mM NaCl; 10 mM Tris-HCl, pH 8.3, as described [7,8]. After centrifugation (100 000 × *g*; 4°C 1 h) the supernatants were stored in aliquots at −70°C. The protein content of the solubilized preparations was determined by the method of Bradford [9].

2.2. *Serum samples and autoantibody measurements*

Sera were obtained from healthy normal donors and patients with idiopathic Addison's disease (diagnosed on the basis of clinical and biochemical adrenal insufficiency with no history of tuberculosis; see also table 1), Graves' disease or rheumatoid arthritis, and stored in aliquots below −20°C. Autoantibodies reacting with thyroid, gastric parietal cells, smooth muscle, pancreas or adrenal tissue were assessed by immunofluorescence in 2 separate laboratories using unfixed tissue sections.

2.3. *Partial purification of adrenal microsomal antigen*

DOC solubilized adrenal or control (placenta or thyroid)

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microsomes were run (30 ml · h^{-1}) on a column (2.6 × 35 cm) of Sephacryl S-300 at 4°C in 1% DOC, pH 8.3. Aliquots of the column fractions were diluted in 0.1 M $NaHCO_3$, pH 9.2, and 1 ml aliquots (10 μg of protein) used to coat (2 h at 37°C) plastic tubes (3 ml). The tubes were washed and normal sera or serum containing adrenal autoantibodies (diluted in 200 mM NaCl, 20 mM Tris-HCl, pH 7.5, containing 0.05% Tween and 0.5% bovine serum albumin (diluent) added in duplicate. After 2 h at 37°C the tubes were aspirated, washed, ^{125}I-labelled protein A (10^5 cpm) (labelled to a specific activity of 10 μCi per μg using the Iodogen method [10] added and the incubation continued for 2 h at 37°C. The tubes were finally aspirated, washed, and counted for ^{125}I. Fractions showing specific binding to adrenal antibodies were pooled, concentrated by ultrafiltration (Amicon YM-10 membrane) and stored at −70°C. Fractions from placenta or thyroid preparations eluting in the same positions as the adrenal microsome fractions were treated similarly.

2.4. *Labelling, immunoprecipitation and gel electrophoresis of solubilized adrenal microsomes*

DOC solubilized and partially purified (Sephacryl S-300 gel filtration), adrenal or control (placenta or thyroid) microsomes were labelled with ^{125}I to a specific activity of 10 μCi per μg of protein using the Iodogen method [10] as described [11]. Aliquots (250 μl, 20 μCi) of the labelled material were pre-adsorbed (15 h, 4°C) with 100 μl of a 10% suspension of *Staphylococcus aureus* cells (Pansorbin; Cambridge Bioscience, England) and then (after centrifugation at 15 000 × *g* for 4 min to sediment the Pansorbin) added to test sera diluted (4 times) in 1% DOC, pH 8.3. After 8 h at 4°C, fresh Pansorbin (100 μl) was added and incubation continued overnight at 4°C. The mixtures were then centrifuged (15 000 × *g*, 4 min) the pellets washed, and finally suspended in 50 μl of 4% SDS in 100 mM Tris-HCl, pH 6.8, with or without dithiothreitol at a final concentration of 10 mM. After heating (100°C for 3 min) and centrifugation (15 000 × *g*, 4 min) the supernatants were analysed by SDS-PAGE followed by autoradiography.

2.5. *Immunoblotting*

In some experiments adrenal and placenta microsomes (5 μg of protein) were run on SDS-PAGE as described above and the separated proteins transferred to sheets of nitrocellulose electrophoretically (Trans-Blotcell Biorad, 70 V; 3 h; 4°C) [12]. The sheets were then treated with 5% BSA in 50 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.9 (TBST buffer) followed by: (i) incubation (1 h; room temp.) with 20 ml of Addison sera or normal sera diluted 1:400 in TBST buffer; (ii) incubation (1 h; room temp.) with ^{125}I-labelled protein A (20 000 cpm per ml in

Table 1

Results of immunofluorescence and immunoprecipitation studies on 23 Addison's sera

Addision patient	Immunofluorescence for adrenal antibody	Immunoprecipitation of 55 kDa protein from			Immunofluorescence for other antibodies	Immunoprecipitation of additional protein
		Adrenal	Placenta[a]	Thyroid[a]		
1	+	+	−	nt	a,c	70 kDa
2	−	−	nt	nt	−	−
3	−	−	nt	nt	−	−
4	−	−	nt	nt	−	−
5	−	−	nt	nt	a,b,f	−
6	+	+	−	nt	−	−
7	+	+	−	nt	a,b,c,e	−
8	−	−	nt	nt	−	−
9	+	+	−	nt	a,b,c,h	70 kDa
10	+	+	−	nt	c,d,g	−
11	−	−	nt	nt	c,f	−
12	−	−	nt	nt	a	−
13	−	+/−	−	nt	b	−
14	−	−	nt	nt	b,c,f	−
15	−	−	nt	nt	−	−
16	+	+	−	−	c	−
17	+	+	−	−	a,c,g	−
18	+	+	−	nt	a,c,f	−
19	+	+	−	−	a,b	−
20	+	+	−	−	a,b	−
21	+	+	−	−	a,c	70 kDa
22	+	+	+	+	a,c,f,g	−
23	+	+	−	nt	−	−

[a] Not all sera were tested with placenta and thyroid microsomes

Autoantibodies positive by immunofluorescence: a, thyroid microsome; b, thyroglobulin; c, parietal cells; d, intrinsic factor; e, steroid-producing cells; f, anti-nuclear; g, anti-mitochondria. Immunofluorescence and immunoprecipitation results: +, positive result; −, negative result; nt, not tested

TBST buffer containing 5% BSA) and (iii) washing, drying and autoradiography.

3. RESULTS

Immunoprecipitation of labelled adrenal microsomes and analysis by SDS-PAGE and autoradiography were carried out with 23 Addison sera (table 1). A representative autoradiogram is shown in fig.1. A labelled protein band with molecular mass of 55 ± 3 kDa (mean ± SD under reducing conditions; $n = 35$) was specifically enriched (relative to normal pool human serum) by immunoprecipitation with 13 of the 23 sera studied. All 13 sera which immunoprecipitated the 55 kDa band were positive for adrenal antibodies by immunofluorescence and the 10 antibody negative sera did not immunoprecipitate the 55 kDa band (table 1).

The tissue specificity of the 55 kDa protein was investigated by immunoprecipitating labelled placenta and thyroid microsome preparations with some of the sera. The 55 kDa protein was not precipitated from placenta or thyroid (table 1) in 12 of the 13 positive sera. One serum (no. 22, table 1), however, precipitated a protein with a molecular mass close to 55 kDa from thyroid, placenta (table 1) and also skeletal muscle (not shown).

Three of the 23 Addison's sera (nos. 1, 9 and 21) specifically immunoprecipitated a 70 kDa protein from adrenal thyroid and placenta microsome preparations (table 1). All 3 of these sera also immunoprecipitated the 55 kDa adrenal protein (table 1).

Serum specificity was investigated by carrying out immunoprecipitation of adrenal microsomes

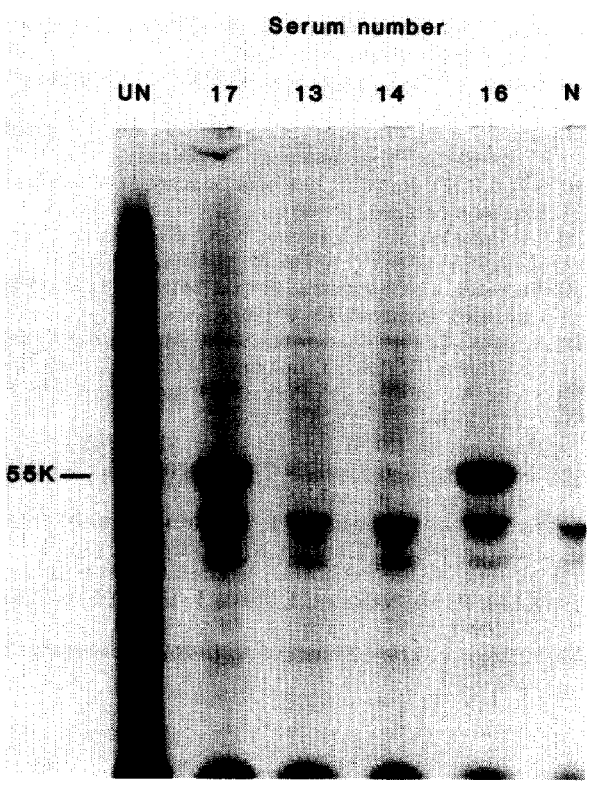

Fig.1. Immunoprecipitation of labelled adrenal microsomes by Addison sera and analysis by SDS-PAGE (under reducing conditions).

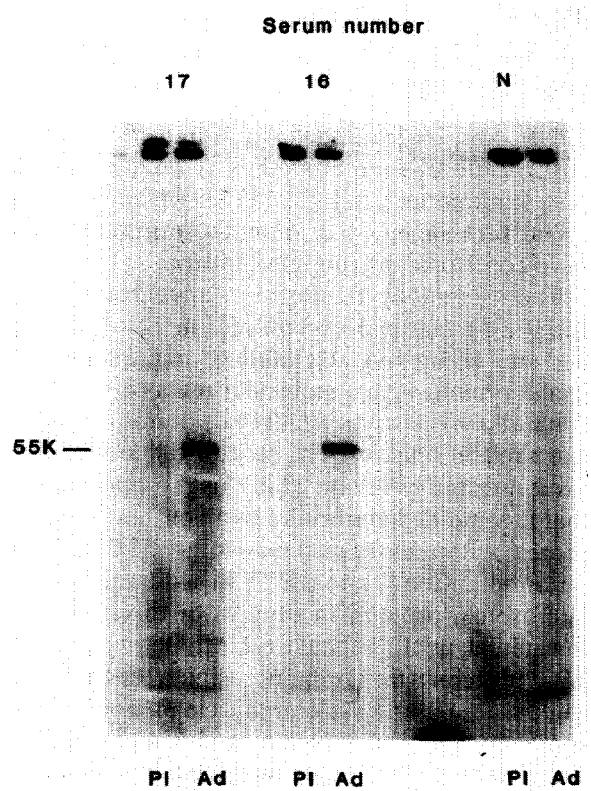

Fig.2. Detection of adrenal 55 kDa protein by immunoblotting of SDS gels. Ad and Pl, adrenal and placental microsomes, respectively, run on SDS-PAGE under reducing conditions followed by blotting and reaction with Addison sera nos. 16 and 17 (table 1) and normal human serum (N) followed by ^{125}I-labelled protein A. See text for experimental details. Data shown are typical of 3 separate experiments. Similar results were obtained under non-reducing conditions.

with sera from patients with Graves' disease (n = 4), rheumatoid arthritis (n = 6) and individual normal subjects (n = 3). None of these samples specifically (relative to normal pool serum) immunoprecipitated the 55 kDa band from adrenal microsomes (not shown). However, the 70 kDa band was immunoprecipitated from adrenal (and placenta and thyroid) microsomes by one of the Graves' and 3 of the rheumatoid arthritis serum (not shown).

In addition to the immunoprecipitation studies analysis of adrenal and placenta microsomes on SDS-PAGE followed by blotting onto nitrocellulose and incubation with Addison sera indicated that the sera reacted specifically with a 55 kDa adrenal microsomal protein under reducing conditions and 58 kDa under non-reducing conditions (fig.2).

4. DISCUSSION

Our data indicate that Addison sera which are positive for adrenal antibodies by immunofluorescence immunoprecipitate a 55 kDa adrenal microsomal protein and the immunoprecipitation data were confirmed by immunoblotting.

The immunoprecipitation appeared to be specific for adrenal microsomes in 12 of 13 cases but one serum (no. 22, table 1) immunoprecipitated a protein with a molecular mass of ~ 55 kDa from all tissues studied (adrenal, thyroid, placenta and skeletal muscle). The lack of tissue specificity demonstrated by serum 22 in this respect suggested that it contained antibodies to a non-tissue specific protein with a molecular mass of ~ 55 kDa in addition to antibodies to the 55 kDa adrenal specific microsomal protein. In support of this suggestion Furmaniak et al. [11] have recently shown that a 54 kDa protein was immunoprecipitated from thyroid, placenta and skeletal muscle preparations by some Graves', Hashimoto and rheumatoid arthritis serum. This tissue non-specific protein was tentatively identified as the cytoskeleton protein desmin [11,13]. Some of the Addison, Graves' and rheumatoid arthritis sera immunoprecipitated a tissue non-specific 70 kDa protein. Previous studies with experimentally produced anti-tubulin antibodies suggested that the 70 kDa band was tubulin and the reported molecular mass of tubulin monomer complexed with DOC is 68 kDa [14].

In conclusion, therefore, our studies suggest that sera from patients with Addison's disease contain antibodies to a 55 kDa adrenal specific protein which may well be the antigen observed on immunofluorescence. The role of this protein in autoimmune destruction of the adrenal requires further investigation.

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