Detection of anti-pituitary autoantibodies by immunoblotting

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A new approach to the detection of anti-pituitary autoantibodies by immunoblotting is presented. This method distinguishes pituitary membrane fraction from cytosolic fraction autoantigens and characterizes them by their molecular weight. A 45 kDa pituitary specific membrane protein was identified as an autoantigen in one of 19 patients with idiopathic growth hormone deficiency and the empty sella syndrome. A 43 kDa membrane protein in pituitary and brain was identified as an autoantigen in one other patient with idiopathic growth hormone deficiency and in one of 14 patients with secondary growth hormone deficiency. These autoantibodies were not seen in any of 27 control subjects. Anti-pituitary autoantibodies can be demonstrated by immunoblotting at titres of up to 1/1000. We conclude that immunoblotting is a useful method for the detection of anti-pituitary autoantibodies.

Key words: Anti-pituitary autoantibody; Immunoblotting; Growth hormone deficiency; Empty sella syndrome

Introduction

Auto-immune pituitary disease is perceived to be rare, in part due to the paucity of clinically recognizable pituitary auto-immune syndromes, such as lymphocytic hypophysitis (Goudie, 1962; Cosman, 1989) and the empty sella syndrome (Komatsu, 1988). In addition, methodological problems inherent to pituitary immunofluorescence studies (Bottazzo, 1975; Pouplard, 1982) have made it very difficult to rely on a specific immunological test for auto-immune hypophysitis.

This paper offers a new approach to the detection of anti-pituitary autoantibodies, namely by immunoblotting, using membrane and cytosolic fractions from post-mortem human pituitary tissue. The search for anti-pituitary autoantibodies was prompted by a patient with idiopathic acquired growth hormone deficiency (IGHD) who had an empty sella on CT scan. We hypothesized that an auto-immune process in the pituitary may have resulted in gland atrophy (hence the empty sella) and subsequent growth hormone deficiency (GHD). Using the immunoblotting technique, autoantibodies to a 45 kDa pituitary specific membrane protein were demonstrated in this patient (Crock et al., 1990). Two other patients with GHD were shown to have autoantibodies to a 43 kDa pituitary membrane protein.

This method seems to successfully overcome the problems of availability of fresh pituitary tissue needed for immunofluorescence and its application to cases of acquired growth hormone
deficiency suggests that at least some of these patients may have an auto-immune basis to their disease.

Materials and methods

Clinical subjects

Sera were collected from the following groups of subjects:

(1) Growth hormone deficiency. (a) 19 children with idiopathic GH deficiency (IGHD), comprising six with congenital hypopituitarism and 13 with acquired IGHD (six girls and 13 boys; age range 6 months–19 years, mean age 12 years 3 months, standard deviation 5 years 2 months). (b) 14 children (seven girls and seven boys; age range 7 years 3 months–18 years 8 months, mean age 12 years 10 months, standard deviation 3 years 8 months) with GH deficiency secondary to intracranial cysts, tumours and/or cranial irradiation.

(2) Normal controls. 27 normal children and young adults (15 males and 12 females; age range 2 years 8 months–30 years, mean age 12 years 3 months, standard deviation 6 years 8 months) with no family history of auto-immune disease.

(3) Positive controls. (a) A female patient whose serum had previously tested positive for anti-prolactin cell antibodies by immunofluorescence (the kind gift of Professor G.-F. Bottazzo, Middlesex Hospital, London). (b) A male patient with idiopathic GH deficiency, treated in the past with growth hormone extracted from human autopsy pituitaries.

The children were attending the Pediatric Endocrine Clinics at the Montreal Children's Hospital, Montreal, Quebec (n = 26) and the Izaak Walton Killam Hospital, Halifax, Nova Scotia, Canada (n = 7).

Ethical approval for the study was given by the Montreal Children's Hospital-McGill University Research Institute Ethics Committee and informed consent given for the collection of blood samples.

Preparation of pituitary tissue antigens

Normal human pituitary tissue was obtained at autopsy 4–8 h post mortem and frozen at −70°C (Dr. M. Chrétien of the Institut de Recherche Clinique de Montréal). 25 glands from persons aged 18–50 years (mean age 25 years) who died from trauma, were used to prepare pituitary membrane and cytosolic fractions. The pituitaries were placed on ice in phosphate-buffered saline (PBS, pH 7.4) with a mixture of protease inhibitors (e-aminocaproic acid, 1,10-phenanthroline, aprotinin, EDTA and benzamidine), cleaned of fibrous tissue, minced with scissors and homogenized using a Polytron mechanical blender. The homogenate was centrifuged at 400 × g (4°C, 20 min) to remove cell debris and nuclei. The supernatant was then centrifuged at 100,000 × g (4°C, 60 min) to give a cytosolic (supernatant/soluble antigen) fraction and a membrane (pellet) fraction. The pellet was further washed in PBS and recentrifuged three times to obtain more purified membrane proteins. The final membrane pellet was resuspended in PBS to give a protein concentration of 10.6 mg/ml (Bio-Rad protein assay, Richmond, CA). The protein concentration of the cytosolic (soluble) fraction was 23.6 mg/ml. The cytosolic fraction was then depleted of IgG (presumably present because of contamination by blood) using protein A coupled to Sepharose (Pharmacia), to give a final protein concentration of 16.0 mg/ml. These preparations were stored in aliquots at −70°C until use.

Other tissue antigens

Membrane and cytosolic fractions were prepared in the same way from other human tissues (brain, thyroid, liver, spleen, gut and skeletal muscle) obtained at autopsy less than 4 h after death, for use in tissue specificity studies.

Fresh, frozen rhesus monkey pituitary glands were obtained from the California Primate Research Center, University of California, Davis, USA, separated into membrane and cytosolic antigens, as outlined above for human tissue, and used in species specificity studies.

Monoclonal and polyclonal antibodies to human GH

Three mouse monoclonal antibodies (2A1, D35 and 3B1) and two rabbit polyclonal antibodies (BR-3-10 and H-6) to human growth hormone were the kind gift of Dr. H. Friesen, Winnipeg, Manitoba, Canada. Their optimal working dilutions were 1/100,000 and 1/10,000 respectively.
in the immunoblotting method. The specificity of binding was assessed by prior absorption of these primary antibodies with excess human growth hormone (50 mg/ml, NIADDK, Bethesda MD). Second antibodies were anti-mouse IgG + M (Kallsted) and anti-rabbit IgG (Kallsted) respectively, conjugated to alkaline phosphatase and used at a dilution of 1/1000.

**SDS-PAGE and immunoblotting**

The method was based on previous experience of immunoblotting, optimized for the detection of anti-eye muscle autoantibodies in patients with thyroid-associated ophthalmopathy (Salvi et al., 1988).

Human pituitary membrane and cytosol preparations were fractionated on sodium dodecylsulphate (SDS)-polyacrylamide gels (10% running gel, 4% stacking gel) by electrophoresis using a mini-apparatus (Mini-Gel, Bio-Rad). Pituitary samples were boiled for 2 min in the presence of 0.72 M β-mercaptoethanol (Sigma, St. Louis, MO) before electrophoresis. Pituitary membrane was loaded at concentrations of 25, 50 and 100

Fig. 1. A: immunoblotting of pituitary membrane homogenate (25 μg protein/lane) fractionated on a 10% SDS polyacrylamide gel, with sera from ten normal control subjects (lanes 1–10) and from a positive control patient (lane 11). Lane 12 = TBS/conjugate control. B: immunoblotting of pituitary cytosol (50 μg protein/lane) fractionated on a 10% SDS polyacrylamide gel. Lanes are as above. H and L = high and low molecular weight markers (kDAs) respectively. P = total protein stain.
\( \mu g \) protein/well in 30 \( \mu l \) aliquots and pituitary cytosol at concentrations of 50 and 100 \( \mu g \) protein/well in 30 \( \mu l \) aliquots. Molecular weight standards (Bio-Rad) were included in each experiment. Monkey pituitary tissue was handled in the same manner as human pituitary tissue (see above). Other human tissue membrane preparations were used at a concentration of 50 \( \mu g \) protein in 30 \( \mu l \)/well, and also analysed under reducing conditions.

Electrophoresis was at 100 V for 20 min and then 110 V for 70 min. Separated proteins were then transferred by wet blotting (Trans Blot, Bio-Rad) to Immobilon PVDF transfer membranes (Millipore) at 95 V for 75 min in Transfer buffer (0.02 mol/l Trizma base and 0.2 mol/l glycine in 20% methanol-80% distilled water, pH 8.3). Molecular weight standard strips were developed in Coomassie Blue and destained with 50% methanol-50% distilled water.

After blocking for 1 h in 3% gelatin in Trizma-buffered saline (TBS) (0.2 mmol/l Tris-HCl pH 7.5 and 0.9% NaCl) at 37°C on a shaker, and washing in TBS 0.05% Tween 20 for 40 min and in TBS for 15 min, Immobilon strips were incubated with experimental or control serum diluted 1/25 in 1% gelatin/TBS for 2.5 h at room temperature on a shaker. Control strips were incubated with 1% gelatin/TBS alone. Following incubation, the Immobilon strips were again washed with TBS-0.05% Tween 20 and then TBS, and incubated with alkaline phosphatase-conjugated goat anti-human IgG anti-serum (\( \gamma \) chain specific; Sigma) diluted 1/1500 or 1/2000 in 1% gelatin/TBS for 1 h at 37°C on a shaker. After a final washing with TBS-0.05% Tween 20 and then TBS, strips were incubated with 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Bio-Rad) for 5–15 min. Finally, strips were rinsed in distilled water for 10 min and dried.

Results

Optimization of immunoblotting

Preliminary experiments indicated that detection of autoantibodies was optimal at pituitary membrane protein concentrations of 25 \( \mu g \) protein/well and a cytosolic protein concentration of 50 \( \mu g \) protein/well. Nonspecific binding was found with a protein of 50 kDa in the pituitary membrane fraction and 54 and 65 kDa in the pituitary cytosolic fraction (see Fig. 1, lane 12). Depletion of IgG in pituitary cytosolic preparations (which was presumably due to unavoidable contamination by blood) by protein A greatly reduced such nonspecific binding. Diffuse background activity was also reduced by using sera that had never been frozen, and by incubating sera at room temperature for 2.5 h instead of 37°C for 1 h. Further modifications to the original method have included the use of 5% Blotto (skim milk powder in PBS) as a blocking agent and overnight incubations at 4°C with sera (primary antibody) in 1% Blotto. There was no difference in immunoreactivity of fresh samples of homogenized tissues and those frozen in aliquots at −70°C for up to 6 months. While lyophilization and multiple freeze-thawing of sera (up to five times) increased diffuse background staining, they did not affect the clarity or intensity of positive bands.

Anti-pituitary autoantibodies detected in normal subjects

No specific reactivity was seen when human pituitary membrane fractions were incubated with the sera from the 27 control subjects (Fig. 1 and Table 1). Incubation with TBS and second antibody conjugate alone, consistently gave a nonspecific band at 50 kDa (Fig. 1, panel A, lane 12). By testing sera at 1/25 dilution, overall diffuse background activity was increased but lower

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<th>Table 1</th>
<th>Reactivity of Patients’ Sera to Pituitary Membrane Autoantigens</th>
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<tr>
<td></td>
<td>43 kDa</td>
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<tr>
<td>Idiopathic GHD</td>
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<tr>
<td>( n = 19 )</td>
<td>1</td>
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<tr>
<td>Secondary GHD</td>
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<td>( n = 14 )</td>
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<td>Normal controls</td>
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serum concentrations were not used for screening as anti-pituitary autoantibodies are traditionally believed to be in low titre. When human pituitary cytosolic fractions were incubated with TBS and second antibody conjugates, non-specific binding was seen at 54 and 65 kDa. (Fig. 1, panel B). A positive band of reactivity to a ~ 30 kDa cytosolic protein was seen in two subjects (one subject shown in Fig. 1, panel B, lane 1).

**Anti-pituitary autoantibodies detected in positive controls**

The serum from a child with idiopathic GH deficiency, who had been treated for many years with growth hormone extracted from human autopsy pituitaries, showed reactivity with pituitary membrane and cytosolic proteins of 43 and 54 kDa. This serum was subsequently used as a positive control (Fig. 1, panels A and B, lane 11). The serum from a patient, previously shown to have anti-prolactin cell antibodies by immunofluorescence (Mirakian et al., 1982) also showed reactivity with a protein of 43 kDa in pituitary membrane (data not shown).

**Anti-pituitary autoantibodies detected in children with idiopathic acquired growth hormone deficiency (IGHD)**

The serum from two of 13 children with idiopathic GHD showed reactivity with pituitary membrane proteins. One patient, who had an empty sella on CT scan, showed reactivity with a 45 kDa protein (Fig. 2, lane 3; see also Table 1). The other, used as a positive control, had reactivity at 43 kDa and 54 kDa (Fig. 1, lane 11 and Fig. 2, lane 6). The 43 and 45 kDa antigens bound by the serum of these two patients were still detected at a serum dilution of 1/1000. The 45 kDa antigen was pituitary membrane specific but serum from the latter child also reacted to pituitary cytosolic antigens of 43 and 58 kDa (Fig. 3, lane 5, PitC).

**Anti-pituitary autoantibodies detected in children with congenital GH deficiency**

The serum of one out of six children with congenital GH deficiency reacted with proteins of 78, 60 and 36 kDa in pituitary membrane fractions and 125, 116, 48 and 37 kDa in cytosolic

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**GH DEFICIENCY**

![GH Deficiency Diagram](image)

Fig. 2. Immunoblotting of pituitary membrane homogenate (25 μg protein/lane). Lanes 1–3: sera from three patients with idiopathic GH deficiency (IGHD) on recombinant GH therapy. Lane 3 shows positive reactivity at 45 kDa with serum from the patient with an empty sella on CT scan. Lanes 4–6: sera from three patients with IGHD who had previously been treated with pituitary extracted GH. Lane 6 shows positive reactivity at 43 and 55 kDa. Lanes 7–9: sera from three patients with secondary GHD. Lane 7 shows weak positive reactivity at 95 kDa. Lane 10: serum from a normal control subject. Lane 11: TBS/conjugate control, shows nonspecific reactivity at 50 kDa. MW = molecular weight markers (kDa).
Fractions. These reactivities were less intense than those to the ~30 kDa protein seen with sera from control subjects (Fig. 1, panel B, lane 1) and the 43 and 45 kDa proteins shown in Fig. 2, lanes 3 and 6. Weak bands of reactivity at 58 kDa (membrane) and at 110 and 30 kDa (cytosol) were seen in one patient each.

Anti-pituitary autoantibodies detected in children with secondary GH deficiency

Serum from one of 14 children with secondary GHD reacted with a protein of 43 kDa in pituitary membrane fractions. This patient was on recombinant GH therapy. Serum from three children, all of whom had received radiotherapy for
cranial tumours, showed weak reactivity to a 95 kDa pituitary membrane antigen (Fig. 2, lane 7) and the same reactivity to a 95–100 kDa cytosolic antigen.

Species specificity

The membrane and cytosol reactivities described above, particularly those with the 45 and 43 kDa antigens, were also found when monkey pituitary membrane and cytosol preparations were tested with the relevant sera (data not shown).

Tissue specificity

The positive sera identified above were tested in immunoblotting against membrane preparations of human brain, thyroid, liver, spleen, small bowel and skeletal muscle tissue. The 45 kDa antigen appeared to be pituitary membrane specific although in one experiment it was seen weakly in thyroid membrane. The 43 kDa membrane antigen was shown in brain membrane preparations and the 54 kDa antigen in thyroid membrane (Fig. 3, lanes 2 and 6, marked THY and BR respectively).

Immunoblotting of human pituitary fractions using monoclonal and polyclonal anti-GH antibodies

The results of immunoblotting with monoclonal antibodies to GH are shown in Fig. 4. There was considerable nonspecific binding to human pituitary membrane fractions with both mouse monoclonal antibodies and rabbit polyclonal antisera. As expected, specific binding at 20–22 kDa was completely blocked by excess GH. Strong reactivity with a 40 kDa protein was shown in all groups, including those incubated with control sera or GH alone but did diminish in intensity with the addition of excess GH (Fig. 4, lanes 2 and 3). However, no binding was shown with proteins of 43 or 45 kDa, the molecular weights which we attribute to the pituitary autoantigens bound by the positive sera.

Discussion

This study describes the application of immunoblotting techniques, using human autopsy pituitary tissue, to detect anti-pituitary membrane and cytosolic reactive autoantibodies.

When Bottazzo (1975) established immunofluorescence as the 'gold standard' test for anti-pituitary autoantibody detection, the observations were made using fresh, frozen human pituitary glands obtained at hypophysectomy for advanced breast cancer. The need for a new approach to anti-pituitary autoantibody testing has arisen because fresh pituitary tissue is now virtually unobtainable and because the results of immunofluorescence on post mortem glands have generally been disappointing (Bottazzo et al., 1975; Pouplard et al., 1980; Pouplard, 1982). Human foetal pituitary cells have been used as an alternative substrate (Scherbaum et al., 1987) but for ethical reasons are not universally available. Human autopsy and fresh monkey tissues were therefore chosen for the immunoblotting technique to avoid
potential problems of species specificity (other than primate) which have led to conflicting reports of the prevalence of anti-pituitary autoantibodies (Pouplard, 1982; Mirakian et al., 1982; Hansen et al., 1983, 1989; Mauerkoff et al., 1987; Suguiru et al., 1987). The choice of these tissues rather than rat GH3 or mouse AtT20 pituitary cell lines (Sugui et al., 1987) also eliminated the potential for cross reactivity between patient sera and fetal calf serum present on the surface of these cells and in their culture media.

In this study the autopsy glands were collected within a short period post mortem (only 4–8 h) and homogenized with a mixture of protease inhibitors to minimize proteolysis. Both these factors are important as tissue collected after 18–24 h or prepared without protease inhibitors has not given reproducible results (Crock et al., unpublished observations). This may explain in part why our method has been successful.

Anti-pituitary autoantibodies have always been thought of as low titre antibodies (Bottazzo et al., 1975; Mirakian et al., 1982). In our study, reactivity was seen with positive sera up to dilutions of 1/1000, whereas earlier immunofluorescence studies reported positive results using undiluted sera for screening or rarely at dilutions up to 1/8 (Bottazzo et al., 1975, 1980; Mirakian et al., 1982). More recent IF studies have used serum dilutions of 1/4 up to 1/25 (Suguiru et al., 1987; Komatsu et al., 1988). Dilutions of 1/100 have been used routinely with immunocytochemistry on rat and porcine pituitary tissue (Hansen et al., 1989; Sauter et al., 1990). The positivity of some of the sera in our study at dilutions of up to 1/1000 tends to argue against nonspecific reactivity of natural antibodies, which are usually present in low titre.

The major problem in setting up this assay was the lack of established positive control sera. For this reason, sera from patients with growth hormone deficiency who had received growth hormone (GH) extracted from human pituitaries obtained at autopsy were initially screened on the assumption that this relatively impure preparation may have 'sensitized' some of them to pituitary antigens. The first positive serum to be identified by immunoblotting came from such a patient whose serum reacted to 43 kDa and 54 kDa proteins in pituitary membrane and 43 and 58 kDa proteins in cytosolic fractions. Although none of the other patients treated with GH derived from autopsy pituitaries had this reactivity in our assay, in the absence of pre-treatment sera in this patient, it is not possible to confirm that the reactivity represents 'true' auto-immune pituitary disease rather than an effect of 'impure' GH treatment. The tissue specificity studies with this serum demonstrated reactivity to a 43 kDa protein in brain membrane and a 54 kDa protein in thyroid membrane. These bands could represent reactivity to impurities in the original pituitary growth hormone extracts. In contrast, the patient whose serum reacted with the 45 kDa protein had never received GH extracted from human pituitaries and the band was confined to pituitary membrane fractions. Furthermore he had an empty sella on CT scan, which has been associated with pituitary auto-immune disease (Komatsu et al., 1988).

Immunoblotting has the advantage over immunofluorescence in that it enables identification of target autoantigen(s) by molecular weight and their localization to membrane and cytosolic fractions. We have focussed on the 45 kDa and 43 kDa bands of reactivity because they appear to be of the greatest intensity and the corresponding antibodies have positive titres of up to 1/1000. Although the other bands detected were not of the same intensity as the 43 and 45 kDa bands, it is possible that they may also prove to be of significance, although some may represent 'natural autoantibodies' which are seen when sera are screened at low titre. Also, we do not dismiss the possible importance of the 95 kDa band seen following radiotherapy in three children with growth hormone deficiency, as pituitary irradiation has been associated with the development of anti-pituitary autoantibodies (Etzrod et al., 1984).

The problem of detecting a multiplicity of bands is well known from immunoblotting studies in organ specific diseases such as type I diabetes mellitus (Karounos et al., 1990). Some of these bands, such as the 64 kDa autoantigen (glutamic acid decarboxylase), have been well characterised (Bakekeskov et al., 1990), although the significance of the other bands is not yet known. In addition, Karounos et al. (1990), found that im-
munoblotting detected a different set of reactivities than those previously identified by immunoprecipitation.

The possibility was raised that the 45 and 43 kDa reactivities could represent binding to a 'big' variant of pituitary growth hormone (40–44 kDa instead of the normal 20–22 kDa), although several studies have shown that anti-pituitary autoantibodies are directed to cellular components and not to the pituitary hormones themselves (Bottazzo et al., 1975). 'Big' GH variants consist of two 20 kDa or two 22 kDa forms of GH, bound by a disulphide bond. Gels run under reducing conditions should break this bond and thus dissociate any 40 or 44 kDa variant into its two component forms. Studies using monoclonal and polyclonal anti-GH antibodies showed considerable binding, although mainly nonspecific, to a 40 kDa pituitary membrane protein. There was no binding, specific or nonspecific, at 43 or at 45 kDa. As expected, reactivity at 20–22 kDa was abolished by the addition of excess GH. In addition, any antibody to GH (whether to the 20 or 22 kDa forms or to a higher molecular weight pituitary variant) would be expected to show greater reactivity to the cytosolic fraction, and in the patient with autoantibodies to the 45 kDa membrane antigen no equivalent cytosolic reactivity was seen. These data suggest that the pituitary antigens identified by these patients' sera are not growth hormone. We postulate that the reactivities at 45 and 43 kDa represent anti-pituitary autoantibody binding to uncharacterized membrane antigens that may be involved in GH processing or transport, or that have been in some way upregulated by GH therapy.

In conclusion, using an immunoblotting method, anti-pituitary autoantibodies to pituitary antigens with molecular weights of 45 kDa and 43 kDa, have been identified in three patients with growth hormone deficiency, one of whom has an empty sella on CT scan. These autoantibodies have been demonstrated at titres of up to 1/1000 by immunoblotting, whereas traditionally antipituitary autoantibodies have been described as being of low titre. Thus, immunoblotting may prove to be a valuable complementary technique to immunocytochemistry in the study of pituitary auto-immune disease. Finally, although not addressed by this technique, the role of cell mediated immunity in pituitary auto-immune disease also needs to be elucidated.

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