Autoimmune Hypophysitis: Identifying the Molecular Mechanisms

Patricia Anne Crock

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I dedicate this thesis to my parents, Gerard and Jacqueline Crock,
for their loving support and encouragement

and

to Dr. Dieter Konrad Hans Lüdecke, my wonderful husband, pituitary neurosurgeon and poet,
who shares my passion for pituitary research and compassion for our patients

Hoar frost in front of Uppsala Castle on the way to the laboratory at Akademiska sjukhuset – Uppsala University Hospital.

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SYNOPSIS

As part of my research to improve the diagnosis and management of pituitary diseases, I chose the important field of pituitary autoimmunity. Little was known at the time. My aim has been to develop a specific and sensitive diagnostic assay for pituitary autoantibodies, to identify the relevant target autoantigens and to understand the molecular mechanisms. This specific body of work spans 25 years and includes 21 publications of which six are reviews.

I developed and validated the first immunoblotting assay for the detection of pituitary autoantibodies using autopsy pituitary tissue. One of the first target autoantigens we identified, a 49 kDa pituitary cytosolic protein, was purified and found to be enolase. Serum from a patient with peripartum lymphocytic hypophysitis reacted to both alpha-enolase and gamma-enolase (neuron specific enolase) in the pituitary and placenta, thus explaining, for the first time, the link between pregnancy and hypophysitis. I identified the first pituitary membrane autoantigens in paediatric patients and we described the first convincing case of hypophysitis in a child with APECED (Autoimmune PolyEndocrinopathy Candidiasis and Ectodermal Dystrophy).

Together with many national and international collaborators, we applied the immunoblotting assay across the clinical spectrum from acute to chronic autoimmune pituitary disease and other autoimmune conditions. I found several novel clinical associations in the largest series of 32 Australian patients with hypophysitis which was a single author publication. A number of other pituitary autoantigens were also identified by molecular weight. I showed that pituitary autoantibodies are found in patients with other endocrine autoimmune diseases and in up to 20% of pituitary tumour cases. This latter group has secondary tumoural hypophysitis, which makes it impossible to differentiate immunologically from primary autoimmune hypophysitis. We found evidence for, and proposed that, the slow progression of post-partum hypopituitarism in Sheehan's syndrome may have an autoimmune basis. We showed that some cases of idiopathic hypopituitarism are likely autoimmune and concluded that most patients with empty sella syndrome and normal pituitary function are unlikely to have chronic autoimmune hypophysitis. We demonstrated seroconversion time points for pituitary autoantibodies and their persistence in patients with APECED/APS1 from Finland. In a cohort of Polish patients with isolated ACTH deficiency we found 49 kDa autoantibodies in 20% and a novel 36 kDa pituitary autoantigen that correlated with co-existent Hashimoto's disease, strongly supporting a role for autoimmunity in this syndrome.

My focus then changed from immunoblotting to immunoscreening of a pituitary cDNA library to identify further autoantigens and develop *in-vitro transcription translation* (ITT) assays. We found a number of novel target autoantigens; CHD8, piccolo and CADPS, the latter two involved in dense core vesicle processing, the mechanism for endocrine peptide hormone secretion. Although none was specific in isolation, 8 of 86 patients with hypophysitis, but none of 90 controls reacted to two or more of these proteins ($p=0.0093$). We were the first to show

the corticotroph-specific transcription factor, TPIT was a target autoantigen in 10% of hypophysitis patients.
APS1 (Autoimmune Polyendocrine Syndrome type 1 – also known as APECED) is a rare, monogenic autoimmune disease and hypopituitarism has been reported in up to 7% of patients. In general, APS1 patients have very high autoantibody titres. Sera from GH-deficient APS1 patients identified three major, novel target autoantigens: a tudor domain containing protein 6 (TDRD6), a testis specific protein TSGA10 and endothelin converting enzyme-2 (ECE-2). The latter was abundantly expressed in endocrine pancreas as well as in the pituitary and brain. Immunostaining of the pituitary showed that it was localized to GH producing cells.

In summary, my work in pituitary autoimmunity started with the development of a new diagnostic assay by immunoblotting and extended to the identification of the first target autoantigens. These autoantigens can be enzymes, transcription factors or proteins involved in dense-core vesicle transport. However, at present pituitary autoantibodies specific for primary autoimmune lymphocytic hypophysitis cannot be differentiated from those secondary to peri-tumoural hypophysitis found in a significant percentage of pituitary tumours and hypothalamic tumours (such as germinomas).

Therefore, my focus has now shifted to the new paradigm of diagnosis with epigenetics and biomarkers including miRNA profiling. The ultimate aim is to develop minimally invasive diagnostic testing in paediatric endocrinology.

DECLARATIONS

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been given in the text.

I give consent for this copy of my thesis, when deposited in the University of Newcastle Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

STATEMENT OF AUTHORSHIP

I hereby certify that this thesis is a collection of publications of which I am first author, senior author or co-author.

I hereby certify that the publications where I am first author were initiated by me and I was the major contributor. The laboratory work in my first author publications was done by myself with due reference to the many outstanding scientists and colleagues who taught me these skills and techniques.

STATEMENT OF COLLABORATION

I hereby certify that part of the work embodied in this thesis has been done in collaboration with other researchers.

I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

Signed in Newcastle, 25th January, 2016

Karolinska Institutet

2/27/2016

To whom it may concern,

This letter is to verify that Patricia Crock has been a long-term collaborator with us.

Our collaboration started in 1996 when Patricia Crock kindly invited Sophie Bensing (née Strömberg) to her laboratory in Newcastle, Australia to detect pituitary autoantibodies with the western blotting technique that Patricia had developed. Over the following years we have together identified several target autoantigens by immunoscreening a pituitary cDNA library. Patricia Crock's PhD student Casey Smith spent several months at Olle Kämpe's laboratory in Uppsala working with the immunoscreening and characterization of autoantigens.
Patricia Crock has visited our laboratories in Sweden on several occasions and among many things conducted cDNA library immunoscreenings by herself.
Our collaboration with Patricia Crock has been highly rewarding and resulted in several publications.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophin
ANA	Antinuclear antbodies
AIIMS	All India Institute of Medical Sciences
AIRE	**A**uto**I**mmune **RE**gulator
APECED	**A**utoimmune **P**olyendocrinopathy **C**andidiasis **E**ctodermal **D**ystrophy
APS1	**A**utoimmune **P**olyglandular **S**yndrome Type **1**
CADPS	**CA**lcium-**D**ependent activator **P**rotein for **S**ecretion
cDNA	complementary deoxyribonucleotide
CHD8	Chromodomain helicase DNA-binding protein 8
CHARGE	**C**oloboma of the eye, **H**eart defects, **A**tresia of the choanae, **R**etardation of growth and/or development, **G**enital and/or urinary abnormalities, and **E**ar abnormalities (including deafness)
CJD	Creutzfeld-Jakob Disease
CMRI	Children's Medical Research Institute (Westmead, Sydney)
CRH	Corticotrophin releasing hormone
CSF	cerebrospinal fluid
CT	computerized tomography
CTLA4 or CTLA-4	cytotoxic T-lymphocyte-associated protein 4
D4Z4	an array of repeated sequences in the terminal region of chromosome 4 (4q35 locus)
DUX4	Double homeobox 4
ECE-1	Endothelin converting enzyme - 1
ECE-2	Endothelin converting enzyme - 2
ESS	Empty Sella Syndrome
FRG1	FSHD candidate region gene 1
FSH	Follicle stimulating hormone
FSHD	Facio-scapulo-humeral dystrophy
GAD65	Glutamic acid decarboxylase 65
GH	Growth hormone
GHD	Growth hormone deficiency
IB	Immunoblotting
IF	Immunofluorescence
IRCM	Institut de Recherches Clinicques de Montréal
ITT assay	*in vitro* transcription and translation assay
KI	Karolinska Institutet
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
MSH	Melanocyte stimulating hormone
NALP5	NACHT leucine-rich-repeat protein 5
NSE	Neuron specific enolase
PC1/3 and PC2	Prohormone-convertases 1/3 and 2
PGSF1a	Pituitary gland specific factor 1a
PGSF2	Pituitary gland specific factor 2
PitAb	Pituitary autoantibodies

PIT1	Pituitary transcription factor 1 (also known as POU1F1a, Pit-1, CPHD1)
PNA	Pituitary Network Association
POMC	Pro-opiomelanocortin
PPH	Post-partum haemorrhage
SCC	Side-chain cleavage enzyme
SLE	Systemic Lupus Erythematosus
TDRD6	Tudor domain containing protein 6
TPH	Tryptophan hydroxylase
TPIT	T-box transcription factor specific to corticotrophs
TSGA10	Testis-specific protein
TSH	Thyrotrophin / Thyroid Stimulating Hormone

INTRODUCTION

Pituitary autoimmune disease has been one of the "last frontiers" of organ-specific autoimmunity. The case reported by Goudie and Pinkerton in 1962 (Goudie and Pinkerton 1962) is regarded as the first description linking lymphocytic infiltration of the pituitary with Hashimoto's lymphocytic thyroiditis and coining of the term anterior hypophysitis. The current terminology is lymphocytic hypophysitis (Crock 1997) or autoimmune hypophysitis (Caturegli, Newschaffer et al. 2005) (Falorni, Minarelli et al. 2014), with some variants such as infundibulo-neurohypophysitis (Takao, Asaba et al. 2000, Weetman 2013) that encompass involvement of the pituitary stalk and hypothalamus.

In organ-specific endocrinopathies, of which Hashimoto's disease is regarded as the classic example (Weetman 2013), there is lymphocytic infiltration of the gland and autoantibodies in serum that target tissue-specific proteins (autoantigens). These target autoantigens can be hormones but are often tissue-specific enzymes involved in hormone production (Winqvist, Karlsson et al. 1992). In high titre, these autoantibodies are diagnostic because they are specific, for example thyroid peroxidase autoantibodies in Hashimoto's thyroiditis, and can be predictive of gland failure, for example 21-hydroxylase autoantibodies in Addison's disease (Reato G 2011) or multiple islet specific autoantibodies in type 1 diabetes. When I started my research in the pituitary autoimmune field, there was no reliable diagnostic test for pituitary autoantibodies and no autoantigens were known.

My aim was to develop a specific and sensitive diagnostic assay for the detection of pituitary autoantibodies in autoimmune lymphocytic hypophysitis and to identify the target autoantigens involved in the process. In some cases presenting with an unidentified pituitary mass, a specific autoantibody result could obviate the need for pituitary biopsy. In others with high titre autoantibodies, it may be possible to predict the onset of hypopituitarism. More importantly, if I could elucidate the underlying pathophysiology, then we could

develop new, targeted therapies, such as immune-modulatory drugs. Perhaps, in turn, these could be applicable to other autoimmune diseases.

The pituitary gland is more complex than other endocrine glands as it comprises six different cell types that interact at autocrine, paracrine and endocrine levels. It is under control from the hypothalamus by numerous releasing and inhibitory factors as well as neural inputs. Hormonal output from the pituitary is also directing multiple glands in the periphery. If each pituitary cell type could develop autoimmunity, either in isolation or in combination with others, then the spectrum of autoantibodies is potentially great. A further layer of complexity could exist, where the process could target both the pituitary cell type and its corresponding peripheral counterpart, for example the corticotroph and the steroid-producing cells of the adrenal cortex – lymphocytic hypophysitis and Addison's disease.

The first challenge was to develop a new autoantibody assay that did not rely on immunofluorescence. The reasons for this are outlined in my review articles. Immunofluorescence can identify cell type and can localize autoantibody reactivity within a cell, but it cannot identify the actual target protein. The other significant issue is that of species specificity when choosing the pituitary tissue substrate (Gluck and Scherbaum 1990).

The six review publications constitute **Chapter 1** of my thesis (Crock 1996, Crock 1997), (Crock 2006) (Crock 2008) (Crock 2011), (Maltby, Crock et al. 2014).

Chapter 2 outlines the development of a new assay for the detection of pituitary autoantibodies by western or immunoblotting (Crock, Salvi et al. 1993). This work was part of my Clinical and Research Fellowship at McGill University, Montreal. I successfully developed and validated the first immunoblotting assays for the detection of human pituitary membrane and pituitary cytosolic autoantibodies. I identified a positive control serum amongst children previously treated with human cadaver-derived growth hormone. In the cohort of

paediatric patients that we studied, I also identified pituitary-specific autoantibodies to a 45 kDa membrane protein in a child with idiopathic GH deficiency and empty sella syndrome. I took this assay back with me to Australia and established it in Melbourne and subsequently in my own research laboratory in Newcastle, NSW.

Using the pituitary membrane assay, I was the first to identify a 45 kDa membrane protein as the target autoantigen in a paediatric patient with APECED (Autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy), also known as APS1 (Autoimmune polyglandular syndrome type 1) (Ward, Paquette et al. 1999). This was also the first convincing report of hypophysitis with characteristic MRI findings in a paediatric patient with APS1. It is unclear if the autoantigen is the same 45 kDa protein that I had identified in our original IB paper.

Chapter 3 is a collection of six publications looking at a wide range of clinical situations that are thought to represent the clinical spectrum of autoimmune hypophysitis, from acute to chronic.

My first report, a single author publication, applied the immunoblotting assay to screen a large cohort of adult patients with biopsy-proven hypophysitis and suspected hypophysitis (Crock 1998). At the time, this was the largest published series of hypophysitis patients and I found novel clinical associations. It covered the whole clinical spectrum of hypophysitis from acute presentation with pituitary apoplexy to end stage disease of empty sella syndrome (ESS) with isolated pituitary hormone deficiencies. I prefer the term CSF sella syndrome, as the sella is filled with CSF (cerebrospinal fluid) and often a rim of pituitary tissue and so is not truly "empty". However, here we still refer to the empty sella syndrome as this is more widely used in the literature. Autoantibodies to 49 kDa and 40 kDa pituitary cytosolic proteins were found in a significant number of hypophysitis patients, but were also found in other clinical situations. <u>This was the first unequivocal demonstration of target autoantigens in hypophysitis</u>.

I had asked Professor James Goding (in whose Immunology laboratory I did the

work), to be my co-author, but he felt the results were too preliminary, so he requested to be in the Acknowledgements only.

The rest of the publications in this chapter came out of close collaborations with Endocrinologists in Sweden, India, Poland and Finland.

The second report with Dr. Sophie Bensing (née Strömberg) showed that some cases of idiopathic hypopituitarism are likely to have an autoimmune basis (Stromberg, Crock et al. 1998). Patients with selective pituitary hormone deficiencies, rather than panhypopituitarism, were more likely to have another autoimmune condition.

The third publication was in collaboration with Dr. (now Professor) Ravinder Goswami from New Delhi (Goswami, Kochupillai et al. 2002). He was the first to propose the concept that the slow progression of hypopituitarism in Sheehan's syndrome may have an autoimmune basis. Using my immunoblotting assay, we were the first to provide convincing evidence for this phenomenon of pituitary autoimmunity secondary to pituitary damage from severe post-partum haemorrhage.

The fourth publication, also a Swedish collaboration, screened a series of patients with empty sella syndrome (ESS) to look for evidence of pituitary or other autoimmunity (Bensing, Rorsman et al. 2004). We found that patients with ESS who had no evidence of hypopituitarism, are unlikely to have had autoimmune hypophysitis. The clinical features of the cohort of ESS patients we studied were more indicative of pituitary atrophy secondary to a hypothalamic process. There may have been a referral bias in the selection of these patients.

The fifth publication examined serum samples from a large and unique cohort of APECED (APS1) patients collected at multiple time points over 25 years by one Finnish paediatric endocrinologist, Prof. Jaakko Perheentupa (O'Dwyer, McElduff et al. 2007). In addition to identifying multiple target autoantigens by molecular weight, we were also able to demonstrate, for the first time, seroconversion time points. Once seroconversion occurred, autoantibodies persisted for years. In

contrast, autoantibodies detected by immunofluorescence in many other conditions tend to disappear over time. Our novel finding highlights at least one fundamental difference between these two assay techniques.

In the sixth publication with Swedish (Dr Sophie Bensing and Dr Anna Lena Hulting) and Polish collaborators (led by Dr Anna Kasperlik-Załuska), we investigated another large and unique cohort of patients, this time with isolated ACTH deficiency (Kasperlik-Zaluska 2008). 49 kDa autoantibodies were found in 20% of patients. We also identified a novel 36 kDa cytosolic pituitary protein as a target autoantigen. Many of these patients had co-existent Hashimoto's thyroiditis and the presence of thyroid autoantibodies correlated with 36 kDa positivity. Our results strongly supported a role for autoimmunity in the syndrome of isolated ACTH deficiency.

Chapter 4 focuses on the further characterization of the 49 kDa target autoantigen by protein purification and sequencing. The publications formed the major part of the PhD thesis of Damien O'Dwyer, my first PhD student.
The first report identified this protein as alpha-enolase, the first time a pituitary target autoantigen had been characterized (O'Dwyer, Smith et al. 2002).
The second report showed that autoantibodies from a patient with hypophysitis also reacted with gamma-enolase, the isoform present in the pituitary and other neuroendocrine tissues (O'Dwyer, Clifton et al. 2002). We used a number of different technical approaches including 2D – gel electrophoresis and tissue immunohistochemistry. More importantly, we demonstrated reactivity against gamma-enolase in placenta, thereby making, for the first time, a direct link between hypophysitis and pregnancy. Hypophysitis is six times more common in women and very often associated with pregnancy, so our data explaining this potential link was a significant breakthrough in the field.

Chapter 5 represents a change in my focus from immunoblotting and protein chemistry to the immunoscreening of a pituitary cDNA library to identify further target autoantigens and the development of novel *in-vitro transcription translation* (ITT) assays.

The first publication in this chapter from Dr. Sophie Bensing was part of her PhD thesis (Bensing, Fetissov et al. 2007). The laboratory work was done in Uppsala and Stockholm, and I supplied the hypophysitis sera from my laboratory. We identified a tudor domain containing protein 6 (TDRD6) as a major autoantigen in 42 of 86 (49%) APS1 patients and showed that sera from GH-deficient patients (with APS1) stain specific cell populations and nerves in the pituitary gland.

The second and third publications were part of the PhD thesis of Casey Jo Anne Smith (now Smith-Anttila). The library screening was also done in Uppsala, partly during my sabbatical visit in 2001 and then by Casey Smith. The ITT assays were further developed in my laboratory and in the laboratory of Prof. Phillip Robinson at CMRI (Children's Medical Research Institute), Sydney.

The initial library screening by Casey Smith identified a number of novel target autoantigens (CHD8, piccolo and CADPS). Many of these proteins are involved in dense core vesicle processing, the mechanism for peptide hormone release from endocrine glands including the pituitary. Although reactivity was not specific for each in isolation, eight of 86 patients with lymphocytic hypophysitis but none of 90 controls, had autoantibodies to two autoantigens ($p= 0.0093$). Therefore, a panel of autoantigens, such as is routinely used in the type 1 diabetes field, may be more appropriate as a screen. The potential significance of these observations is discussed at length in this chapter.

I also chose the corticotroph-specific transcription factor TPIT, as a potential candidate autoantigen and we found it to be a target in 10% of patients with hypophysitis. Again, this was a completely novel finding (Smith, Bensing et al. 2012).

The second report by Casey Smith, identified another novel autoantigen in APS1 and in systemic lupus erythematosus; this time, TSGA10, a novel testis specific protein that has recently been found to have other functions (Smith, Oscarson et al. 2011).

The third manuscript (submitted) by Casey Smith-Anttila focused on further screening of a pituitary cDNA library with sera from APS1 patients. Another novel autoantigen was found; endothelin converting enzyme-2 (ECE-2). This enzyme is an isoform of ECE that is most abundantly expressed in the endocrine pancreas, but also at high levels in the pituitary and brain. ECE-2 reactivity was highly specific to APS1 patients (48 of 104, 46%) and was not seen in any hypophysitis patients, nor in any normal controls. This data potentially opens up whole new fields of study as diverse as Alzheimer's disease and amyloidosis.

Chapter 6

After working on my immunoblotting assay for many years, I wanted to re-visit the immunofluorescence technique. In collaboration with Dr Sophie Bensing at the Karolinska Institute, Stockholm, we approached Dr. Tomas Hökfelt, a renowned neuroscientist with extensive experience in the field of immunostaining. The ensuing publication formed part of the PhD thesis of Casey Smith (Smith, Bensing et al. 2014).

Chapter 7

Two publications of case reports (Tran HA 2012) (Maltby, Crock et al. 2014).

Chapter 8

In this final chapter, I have put the information and my contributions in the previous chapters into the context of the field of pituitary autoimmunity as it stood when I first started and now, many years later, with the advent of new technologies. Over the years, I have developed a strong and vibrant network of collaborators worldwide. We have adopted many new technologies and adapted these to the study of pituitary autoimmunity with fascinating results. Finally, I discuss the future direction of our research.

Chapter 1. Review articles

This chapter contains six review articles, five of which were by invitation.

Publication 1. Pituitary Autoantibodies and Hypopituitarism

Crock PA.

Clin Pediatr Endocrinol 1996; 5 (Supl 8): 1-8. (Crock 1996)

Publication 2. Lymphocytic Hypophysitis

Crock PA.

Curr Opin Endocrinol Diabet 1997;4: 115-123. (Crock 1997)

Professor Ashley Grossman, then at St. Bartholomew's Hospital, London, now at Oxford University, approached me personally to write this review article.

Publication 3. Pituitary autoantibodies

Crock PA, Bensing S, Smith CJA, Burns C, Robinson PJ.

Curr Opin Endocrinol Diabet 2006; 13:344-350. (Crock 2006)

Publication 4. Autoimmune Hypophysitis

Crock PA, Bensing S, Smith CJA, Burns C, Robinson PJ.

Chapter 15; pp 357-392.

In Contemporary Endocrinology: Autoimmune Diseases in Endocrinology.

Ed. A.P. Weetman. Humana Press, Totowa, NJ. 2008. (Crock 2008)

The Editor, Dr Anthony Weetman invited me to contribute a chapter for this book.

Publication 5. Paediatric Pituitary Disorders

Crock PA and Lüdecke DK.

Australian Doctor How to Treat – February 2011. (Crock 2011)

I was invited to write this publication for Australian General Practitioners. In the paediatric context, primary hypophysitis is rare but secondary hypophysitis is seen with cystic lesions (Rathke's cyst and craniopharyngiomas) and peritumourally around germinomas. Dr Dieter Lüdecke, pituitary neurosurgeon

and my co-author, was the first to describe and publish cases of secondary hypophysitis with cystic lesions (Puchner, Ludecke et al. 1994).

Publication 6. **A rare case of pituitary infarction leading to spontaneous tumour resolution and CSF-sella syndrome in an 11-year-old girl and a review of the paediatric literature.**

Maltby VE, Crock PA, Lüdecke DK.

J Pediatr Endocrinol Metab. 2014 Sep; 27(9-10): 939-46. (Maltby, Crock et al. 2014)

This publication is a case report of a rare event in a peri-pubertal girl, with a detailed review of the paediatric literature on both pituitary infarction and hypophysitis.

Chapter 2. Development of the immunoblotting (IB) assay

Publication 7. Detection of anti-pituitary autoantibodies by immunoblotting

Crock P, Salvi M, Miller A, Wall J, Guyda H.
J Immunol Methods. 1993 Jun 4;162(1):31-40.

Since the original pituitary immunofluorescence studies by Goudie (Goudie 1968) and Nerup (Nerup, Lindholm et al. 1969) and then by Bottazzo's group in London (Bottazzo, Pouplard et al. 1975, Bottazzo, McIntosh et al. 1980), it has been clear that this technique has a number of problems including significant issues with species specificity. At that time, total hypophysectomy for advanced breast cancer was common and so the investigators had access to a large amount of fresh, human pituitary tissue. However the pituitary tissue was not "normal" as many of these women were on stilbœstrol and prednisone leading to prolactin and GH cell hyperplasia and reduced number of basophils, respectively. Ready availability of normal human tissue remains an issue.

As a Research Fellow at McGill University, I had access to excellent immunofluorescence laboratories and obtained human cadaveric pituitary tissue from Dr Chrétien and fresh, frozen monkey pituitaries from the Primate Colony in Portland, Oregon. I encountered the technical challenges of high background staining due to cross-species reactivity, a problem that continues to dog the field, as well as the lack of a known positive control sample.

It was while in this predicament that I sought to develop a new method that could overcome the issues of immunofluorescence and also identify the target autoantigens in autoimmune pituitary disease. I was fortunate to meet Dr. Nicole Bernard, an immunologist and Dr. Mario Salvi, an endocrinologist who were both working on the immunopathology of Graves' ophthalmopathy with Prof. Jack Wall at the Montreal General Hospital. I looked at their immunoblotting assay method for eye muscle autoantigens and adapted it for use in pituitary autoimmunity. Pituitary tissue in particular can degrade rapidly, so the addition

of multiple protease inhibitors was vital, as was working with the tissue at 4 degrees Celcius.

These and other issues are outlined in my first paper in the field, published in the Journal of Immunological Methods (Crock, Salvi et al. 1993). This publication was the first description of an immunoblotting assay for pituitary autoantibodies.

Since my Research Fellowship was in Paediatric Endocrinology with Dr Harvey Guyda at the Montreal Children's Hospital, our focus was on finding evidence of pituitary autoimmunity in children. An important issue for the development of a novel assay was the lack of known positive control sera. I reasoned that children in the Growth Clinic at the Montreal Children's Hospital who were old enough to have received human growth hormone (purified from autopsy pituitaries and withdrawn from the market in 1985 after the discovery of CJD - Creutzfeld-Jakob Disease - transmission) may have been "immunized". I found one such patient with high titre antibodies to pituitary membrane proteins, so we had our positive control. The second issue was, and still is, that autoimmune pituitary disease is exceedingly rare in children. Therefore, more studies were needed in clinically relevant adult populations and these are outlined in the next Chapter.

Nevertheless, in this original cohort of patients, we did identify pituitary autoantibodies to a 45 kDa membrane protein in a child with growth failure who was the index case that prompted the study. This was the first description of pituitary autoantibodies by immunoblotting and the first in a paediatric patient.

Following my publication of the immunoblotting method in 1993, the Japanese laboratory of Kobayashi published data using rat pituitary tissue in a western blotting assay (Yabe, Murakami et al. 1995) and subsequently identified the 22 kDa autoantigen as GH (Kikuchi, Yabe et al. 2000). Reactivity to pituitary hormones themselves, GH, TSH and ACTH (by IB) was found in five of 11 patients with pituitary tumors or empty sella syndrome but was neither specific nor sensitive (Mau, Phillips et al. 1993).

Dr. Kozo Hashimoto from Kochi, Japan, also adopted my assay methodology and published widely in the field. He invited me to Japan to present a plenary lecture at the Japanese Endocrine Society meeting in 1995.

In 2001, Nishiki et al. described human pituitary membrane autoantibodies by IB to 68, 49 and 43 kDa proteins in five of 13 patients with hypophysitis and one of 12 with infundibulohypophysitis (Nishiki, Murakami et al. 2001). To my knowledge, these proteins have not been further characterized.

Publication 8. Severe autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy in an adolescent girl with a novel AIRE mutation: response to immunosuppressive therapy.

Ward L, Paquette J, Seidman E, Huot C, Alvarez F, **Crock P**, Delvin E, Kämpe O, Deal C.

J Clin Endocrinol Metab. 1999 Mar;84(3):844-52.

Subsequently, I used the IB assay to find pituitary membrane autoantibodies to a 43-45 kDa protein in a young Québecoise girl with APECED or APS1, a rare monogenic form of polyglandular endocrine autoimmunity due to recessive mutations in the AIRE (AutoImmune REgulator) gene. She had presented at the age of 4 years to the Montreal Children's Hospital with hypocalcemic tetany and a seizure when I was a Fellow, and in fact I diagnosed her condition. Her disease phenotype was severe. She subsequently developed biochemical GH deficiency with growth failure that responded well to GH therapy and her pituitary MRI scan was suggestive of autoimmune hypophysitis with a "halo-effect" of peripheral ring enhancement with gadolinium.

It is not known if the 43-45 kDa pituitary membrane protein was the same as that described in a child in our original IB paper. In addition to her pituitary autoantibodies, she was also found to have antibodies to a 45 kDa membrane protein in liver. It is unclear whether the liver protein is, in any way, related to that targeted in the pituitary. This was the first convincing description of hypophysitis in a patient with APECED, paediatric or adult, and the first to

describe pituitary autoantibodies by immunoblotting in this disease. The data highlighted the importance of screening these patients for evidence of pituitary hormone insufficiency.

Since this publication, a number of papers have explored pituitary autoimmunity in APECED or APS1 patients. The majority has been published by my research group and our collaborators and will be discussed in the following chapters.

Chapter 3. Application of the IB assay to autoimmune hypophysitis, other pituitary diseases and other autoimmune diseases

After completing my Clinical and Research Fellowship at McGill University, I returned to Melbourne and continued my research in the Department of Pathology and Immunology, Monash University under the supervision of Professor James Goding.

Immunopathology of the index case of autoimmune hypophysitis in 1962

I contacted Professor Robert Goudie in Glasgow, the pathologist who described the original case of anterior (lymphocytic) hypophysitis in the autopsy of a young woman with Hashimoto's thyroiditis who died of undiagnosed central adrenal insufficiency following acute appendicitis (Goudie and Pinkerton 1962). She was 14 months post-partum and had a 12 month history of goitre and lassitude. He kindly sent me sections from the case.

These sections were immunostained in Prof Wayne Hancock's laboratory in the Dept. of Pathology and Immunology, Monash University by Ms. Julie Maguire and are shown in Figures 1a-d. The results demonstrated that the majority of lymphocytes are OPD4 positive indicating the presence of T helper cell subsets.

Prof. Goudie coined the term "anterior hypophysitis" as it resembled the lymphocytic infiltrate in her thyroid due to Hashimoto's thyroiditis.

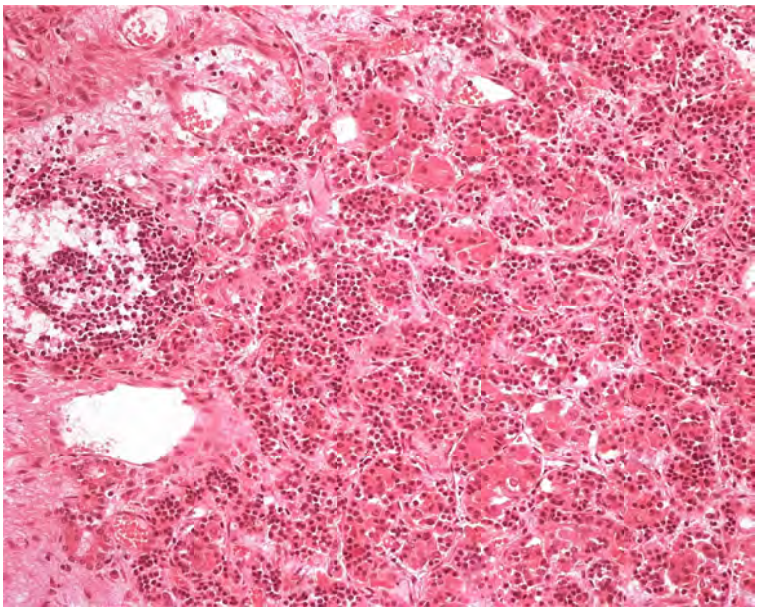

Figure 1a. Haematoxylin and eosin (H & E) stain of the anterior pituitary gland (adenohypophysis) at autopsy of a 22 year old woman who died of central adrenal insufficiency. There are islands of residual acini, which are heavily infiltrated with lymphocytes.

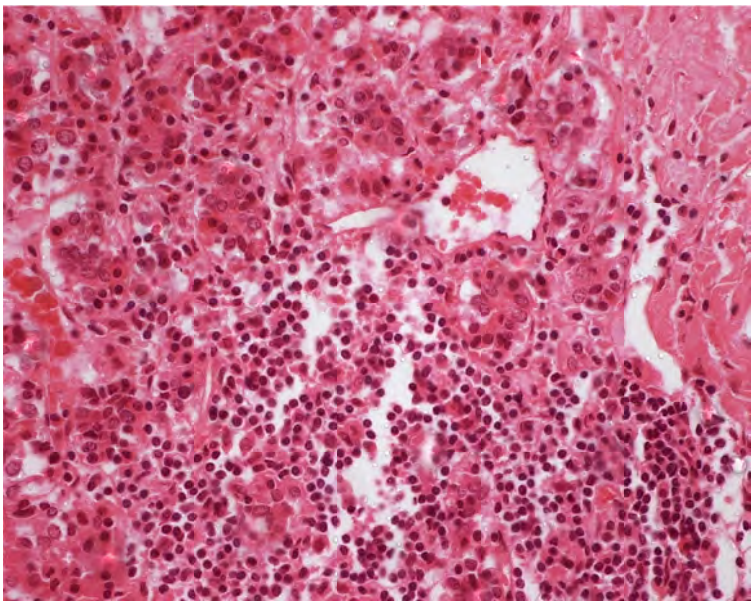

Figure 1b. High power of the anterior pituitary section shown in Figure 1a. demonstrating the lymphocytic infiltrate with occasional plasma cells and some residual pituitary acini.

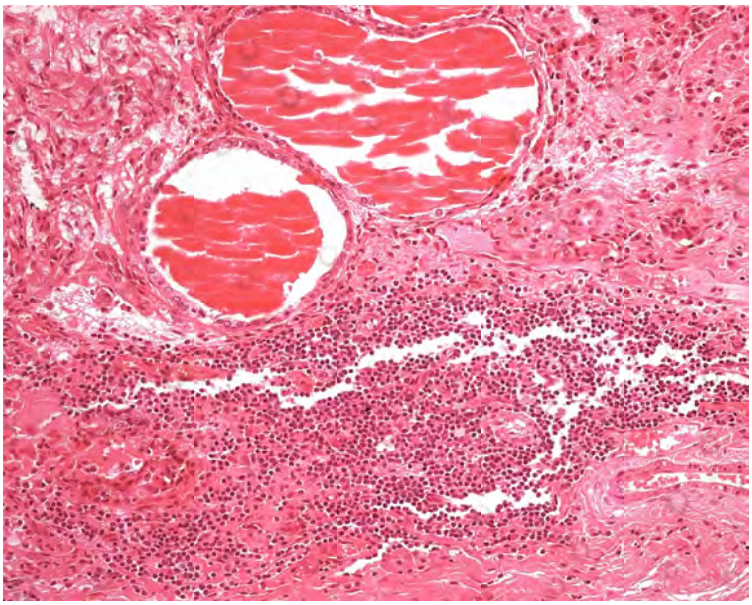

Figure 1c. Haematoxylin and eosin stain of the pars intermedia of the pituitary, showing two small intermediate lobe cysts. The posterior pituitary is on the upper left. The lower half is heavily infiltrated with lymphocytes.

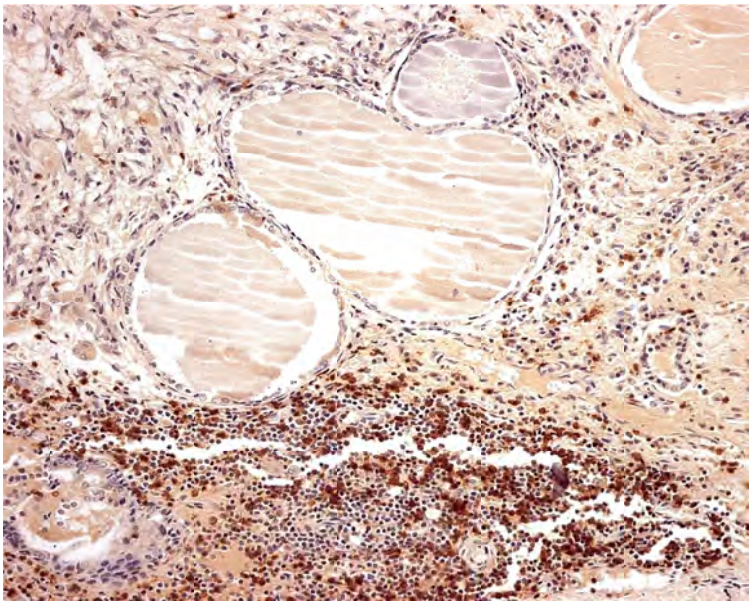

Figure 1d. Immunostaining of the section shown in Figure 1c. with an antibody to OPD4. The majority of lymphocytes are positive indicating the presence of T helper cell subsets.

The monoclonal antibody OPD4 reacts with a helper/inducer subset of T cells in formalin-fixed, paraffin-embedded tissue sections. It is an IgG1 antibody that recognizes the leukocyte common antigen. It does not recognize non-hematopoietic cells, suppressor/cytotoxic T cells, B cells or monocytes (Poppema, Lai et al. 1991).

Publication 9. Cytosolic Autoantigens in Lymphocytic Hypophysitis.

Crock PA.

J Clin Endocrinol Metab **83:** 609 – 618, 1998.

This publication focused on the detection of pituitary autoantibodies in adult patients using the immunoblotting assay that I had developed in Canada, for pituitary cytosolic, not membrane, proteins. The ten patients with biopsy proven hypophysitis represented one of the largest series of such patients internationally at that time. Patient sera from another 22 cases of suspected hypophysitis were included. Sera were referred from endocrinologists throughout Australia and New Zealand as well as from Halifax, Nova Scotia and Boston, Massachusetts. The cases covered a wide spectrum of acute to chronic endocrine presentations ranging from pituitary apoplexy to peri-partum hypopituitarism with a pituitary mass to empty sella syndrome (or CSF sella syndrome) associated with isolated or complete pituitary hormone deficiencies and other autoimmune conditions.

In addition to collecting serum samples, I was also fortunate to be sent histopathological slides from the patients with biopsy-proven hypophysitis. I reviewed these with neuropathologist, Dr Ross McD. Anderson. Dr Wayne Hancock and his technician, Ms Julie Maguire, in the Department of Immunology and Pathology at Monash University did the immunostaining of the sections shown here to define the lymphocyte subsets in the inflammatory infiltrates.

The two tables summarizing the clinical data (Tables 1 and 2) contain a significant amount of information and some surprising coincidences and novel observations.

1. Pituitary apoplexy was described for the first time in the literature in cases of hypophysitis; in one biopsy proven patient and three others (two peri-partum cases). The histopathology from the biopsy proven patient is shown below in Figure 2a-d.

2. The most surprising "coincidence" was that there were two female patients with the constellation of type 1 diabetes mellitus, facio-scapulo-humeral dystrophy and hypophysitis (one biopsy proven). Both had high titre autoantibodies to the 49 kDa protein. My interpretation of this data is that this may not be a "coincidence" at all and that this represents a new syndrome, with the muscular dystrophy potentially being caused by an autoimmune phenomenon. The histopathology from one of these patients is shown below in Figure 3a-d. Although much of the pituitary was infiltrated with lymphocytes ("lymphocytic hypophysitis"), there were some areas with granuloma formation ("granulomatous hypophysitis"). This shows that there are areas of overlap, sometimes termed "lympho-granulomatous hypophysitis".

3. Type 1 diabetes was present in a surprising 25% overall; two of ten biopsy proven patients and five of 15 female and one of seven male suspected patients. This may represent a referral bias by Australian endocrinologists, but is a higher percentage than that reported in the literature.

4. The number of normal CT/ MRI scans in 16 of 22 suspected patients is difficult to comment on. Some scans were taken post-partum and the process may have spontaneously resolved. Also, I could not assess the scans personally and had to rely on the referring endocrinologist and their radiologists for these results. This sub-group of patients fall into the

subacute to chronic part of the disease spectrum. It is possible that some patients had other aetiologies for their symptoms and hypopituitarism, hence the importance of putting more weight on the data from biopsy proven patients.

5. In one patient, I was able to obtain the electron microscopy findings of lymphocytic hypophysitis, courtesy of Neuropathologist Dr Ross McD. Anderson. Shown in Figure 4. below.

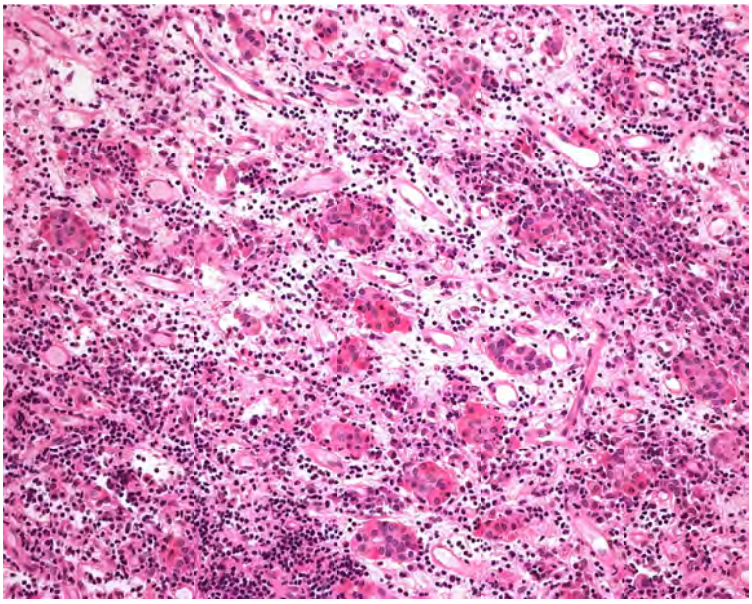

Figure 2a. Haematoxylin and eosin staining of anterior pituitary tissue taken at hypophysectomy. The 57 year old woman with a past history of rheumatic heart disease presented with pituitary apoplexy and a suprasellar mass (Case 1, Table 1) (Crock 1998). Note the islands of residual pituitary acini and the dense lymphocytic infiltrate. Higher power of the same field is shown below.

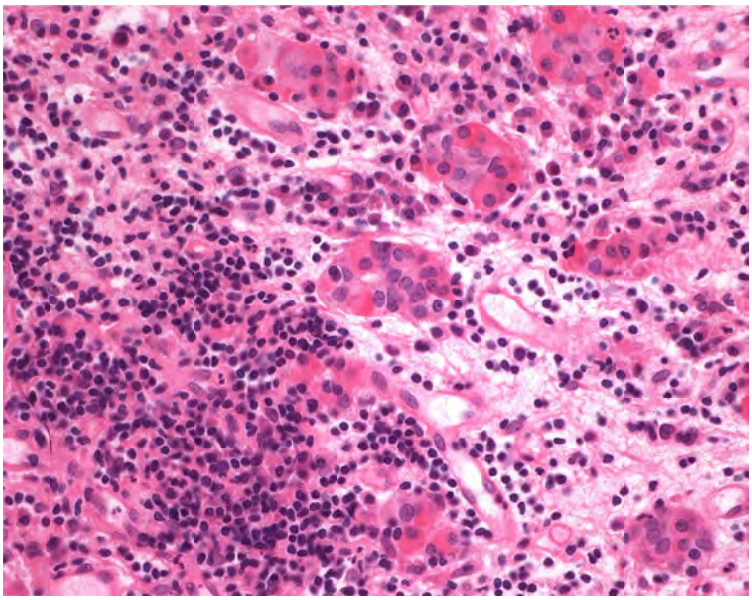

Figure 2b. Higher power section of Figure 2a.

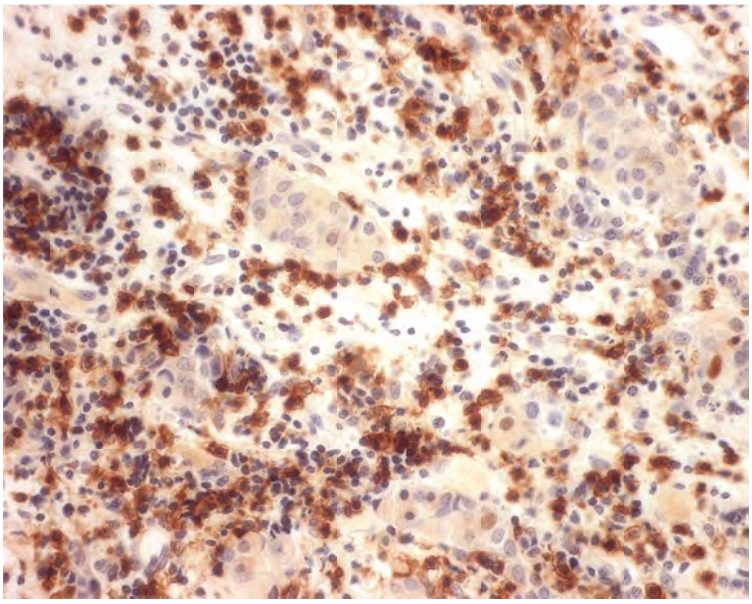

Figure 2c. Immunostaining of the pituitary section shown in Figure 2b. with a monoclonal antibody to OPD4, indicating that the infiltrate is predominantly of T helper cells.

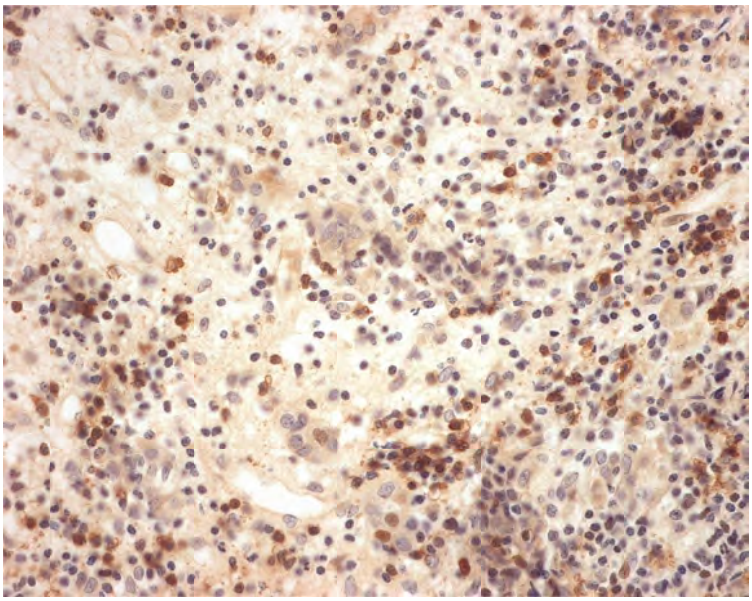

Figure 2d. Immunostaining of the pituitary section shown in Figure 2a. with a monoclonal antibody to L26, indicating B cells. B cell infiltration is less marked than that of T cells. The monoclonal antibody (L26) has excellent specificity and sensitivity for B lymphocytes, and tumours derived from them, in formalin- and B5-fixed, paraffin-embedded tissue (Cartun, Coles et al. 1987).

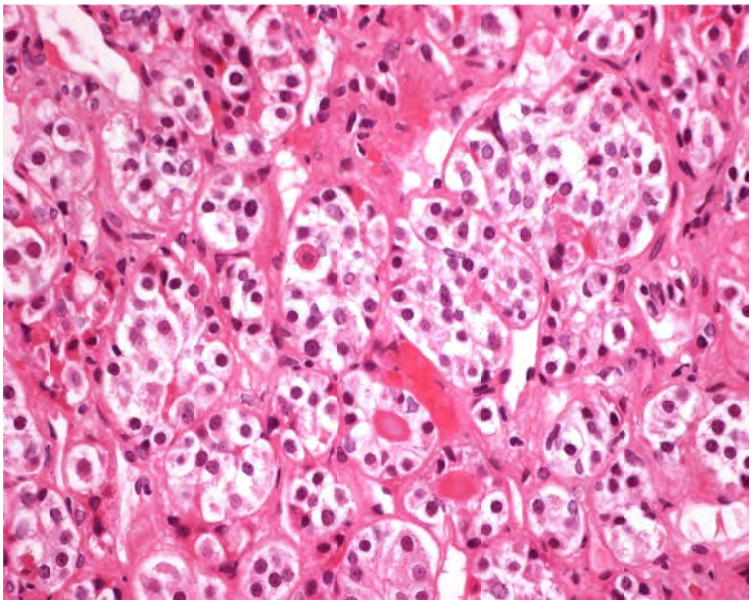

Figure 3 a. Case 8 Table 1 (Crock 1998). A 52 year old woman with insulin dependent diabetes, pernicious anaemia and facio-scapulo-humeral dystrophy.

She underwent hypophysectomy for a pituitary mass causing visual disturbances.

H&E staining of an area of normal pituitary adenohypophyseal tissue with chromophobe cells. Normal acinar pattern with no lymphocytic infiltration.

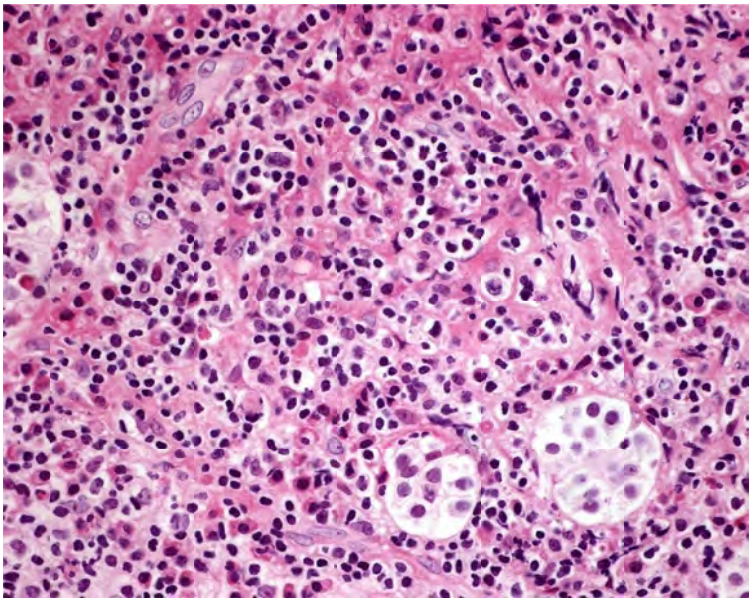

Figure 3b. H&E staining of an area with widespread lymphocytic infiltration with occasional plasma cells and eosinophils. In the acinus at the far right hand bottom corner, several adenohypophyseal cells with pyknotic nuclei can be seen.

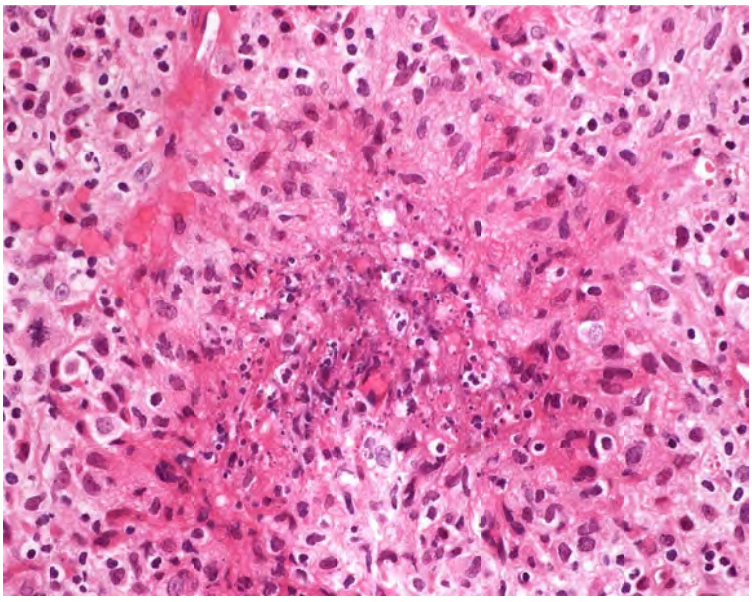

Figure 3 c. H&E staining of a pituitary section showing an area of granulomatous reaction with multinucleated giant cells and an area of necrosis.

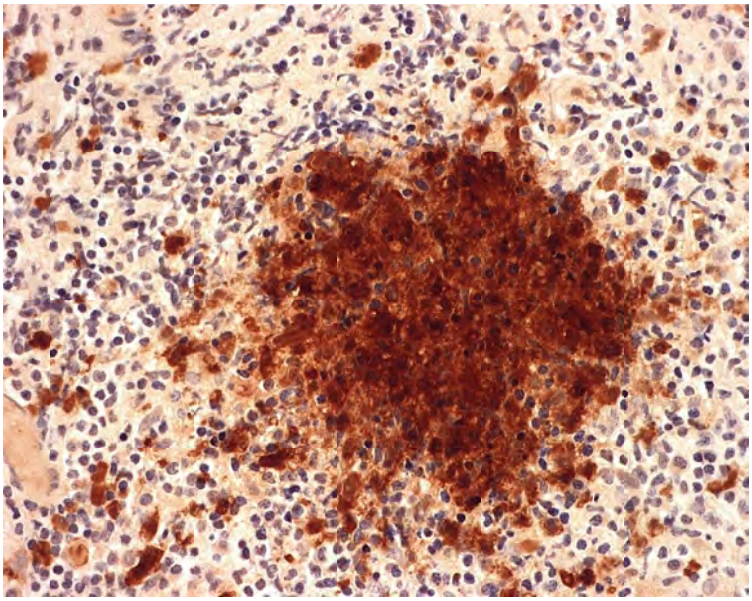

Figure 3d. Immunostaining of a pituitary section adjacent to that in Figure 3c. with a monoclonal antibody to CD68 indicating the presence of macrophages (Kunisch, Fuhrmann et al. 2004).

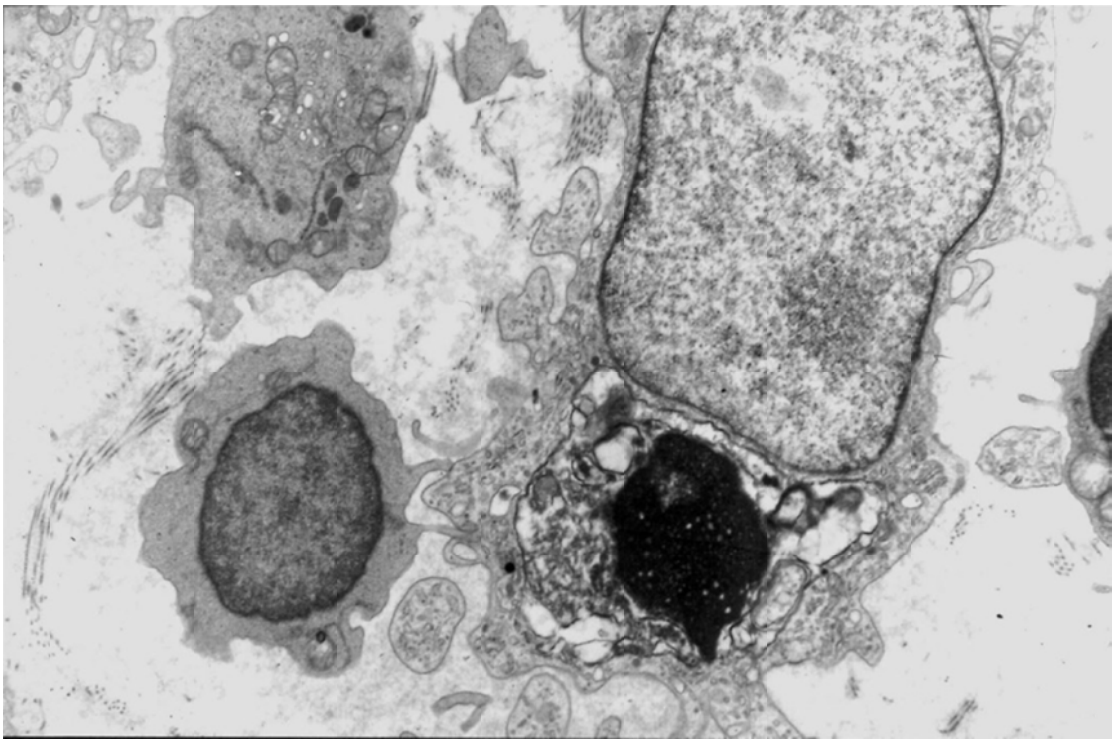

Figure 4.

Electron microscopy of the pituitary gland from a classic peripartum case of lymphocytic hypophysitis. A degenerating adenohypophyseal cell with a pyknotic nucleus is "embraced" by a lymphocyte (* to the left). A normal adenohypophyseal cell is adjacent and above.

The image was taken by the late Dr Ross McD. Anderson, neuropathologist, University of Melbourne.

Photograph reproduced with the kind permission of *Current Opinion in Endocrinology and Diabetes (Crock 1997).*

The laboratory results

Autoantibodies to 49 kDa and 40 kDa pituitary cytosolic proteins were found. This was the first demonstration of target autoantigens in hypophysitis. It also showed that the autoantibodies were at much higher titres than those found by immunofluorescence; 1:50 to >1:1,000 versus undiluted to 1:32 (Bottazzo, Pouplard et al. 1975). The paper also showed that 49 kDa autoantibodies were seen in 9.8% of control sera, but at lower titres. We subsequently identified the

49 kDa protein as enolase (see Chapter 4) and it has been shown in a number of studies that 10% of normal controls have anti-enolase antibodies. Therefore my original data is completely consistent with this more recent data.

Autoantibodies were seen in 13 to 42 % of patients with other autoimmune diseases. Although clinically this makes them non-specific for hypophysitis, there have been isolated reports of pituitary autoantibodies (by IF) in Graves' disease (Hansen, Hegedus et al. 1989), Hashimoto's disease (Kobayashi, Inukai et al. 1988) and Addison's disease (Nerup, Lindholm et al. 1969). A resurgence of interest in this phenomenon has lead to data confirming that pituitary antibodies are more common in newly diagnosed patients with Hashimoto's (Guaraldi, Landek-Salgado et al. 2012). As these studies were done with IF, the target autoantigens are unknown.

A Japanese group postulated and demonstrated that pituitary antibodies in patients with autoimmune thyroid disease recognized D2 (deiodinase 2), present in pituitary (Nakahara, Tsunekawa et al. 2005). Another Japanese group have demonstrated autoantibodies to 49 kDa and 68 kDa pituitary membrane proteins using an IB assay, in a patient with both hypophysitis and Graves' disease (Yamamoto, Murakami et al. 2002).
Therefore, my original data concerning pituitary autoimmunity in patients with autoimmune thyroid disease has been borne out by further studies.

Importantly, 49 kDa autoantibodies were also seen in 20% of pituitary adenoma patients, the one group of patients where a specific assay would be of most clinical benefit to differentiate a pituitary mass as an adenoma or inflammatory. At the time of publication there was only one report in the literature of concurrent lymphocytic hypophysitis with a GH-producing pituitary adenoma. There are now a number of such papers, including one showing 10.5% of patients with acromegaly have antibodies by IF (Guaraldi, Caturegli et al. 2012). The same group has also shown that tumor-infiltrating lymphocytes, not pituitary antibodies, are associated with poor clinical outcome post-surgically in

patients with pituitary adenomas (Lupi, Manetti et al. 2010). The reason for this is unclear.

So, in my opinion, it is very unlikely that a diagnostic test for pituitary autoantibodies will be able to discriminate primary lymphocytic hypophysitis from secondary (peri-tumoural) hypophysitis.

Caturegli et al. published a review of Autoimmune Hypophysitis in 2005 in Endocrine Reviews (Caturegli, Newschaffer et al. 2005). Unfortunately, they misquoted my publication by writing that the autoantibodies were "low titre" (only the controls had low titre antibodies), whereas it was clear that the titres of > 1:1,000 were high compared to those reported in the literature using immunofluorescence (undiluted serum up to a 1:16 dilution only). They also did not reference our cases with apoplexy, nor those with type 1 diabetes, all of which are clearly outlined in Tables 1 and 2 and in the Discussion.

Publication 10. Pituitary autoantibodies in patients with hypopituitarism and their relatives.

Strömberg S, **Crock P**, Lernmark A, Hulting AL.

J Endocrinol. 1998 Jun;157 (3):475-80.

This study was conducted in Sweden at the Karolinska Hospital, Stockholm and the laboratory work was done by Dr. Sophie Strömberg (later Bensing) in my laboratory in Newcastle. It formed part of her PhD thesis.

The premise was that some patients with idiopathic hypopituitarism could have an underlying autoimmune aetiology. The first studies of pituitary autoantibodies in idiopathic hypopituitarism using immunofluorescence by Goudie (Goudie 1968) Nerup (Nerup, Lindholm et al. 1969) and Bottazzo (Bottazzo, Pouplard et al. 1975) were unsuccessful. Therefore, in this study we applied my new immunoblotting assay.

Twenty-one patients were studied, eight of whom had another autoimmune

condition, including three with type 1 diabetes. Six patients with panhypopituitarism, two since childhood, had no evidence of other autoimmune diseases. First-degree relatives or spouses were included (on the assumption that autoimmune disease clusters in families) and 44 healthy Swedish controls.

"Reactivity to an M_r 49 000 cytosolic protein was significantly more frequent in patients (6/21 (28%) P<0·05) as well as in relatives (10/35 (28%) P<0·02) compared with controls (3/44 (6·8%)). Many patients also had reactivity to a range of other proteins, but these have not been further characterized. It is well recognized that immunoblotting identifies multiple reactivities."

In summary, this report showed that some cases of idiopathic hypopituitarism are likely to have an autoimmune basis. Patients with selective pituitary hormone deficiencies were more likely to have another autoimmune condition, however the type of deficiency did not correlate with autoantibodies to the M_r 49 000 cytosolic protein.

More recently, immunofluorescence assays have been revisited by two research groups; one in Naples, Italy (Bellastella, Rotondi et al. 2010) and the second in Baltimore, USA (Lupi, Manetti et al. 2011) to diagnose pituitary autoimmunity in idiopathic hypopituitarism.

Publication 11. Pituitary Autoimmunity in Patients with Sheehan's Syndrome

Goswami R, Kochupillai N, **Crock PA**, Jaleel A, Gupta N.
J Clin Endocrinol Metab 87: 4137– 4141, 2002

The clinical aspects of this study were conducted at AIIMS (All India Institute of Medical Sciences) in New Delhi and the immunoblotting assays for pituitary autoantibodies were performed by Dr. Ravinder Goswami in my laboratory in Newcastle.

Dr. Goswami hypothesized that pituitary necrosis secondary to severe post-partum haemorrhage (PPH) could trigger an autoimmune process and partly

explain the progressive hypopituitarism and delayed presentation in many more than five years after the event. In India, there is still a relatively high maternal mortality rate and many cases of severe, life-threatening PPH, hence Dr. Goswami's cohort of patients is very large by Western standards.

Twenty-six consecutive patients with postpartum hypopituitarism were studied, 19 with Sheehan's syndrome based on a history of PPH and hormone profile suggesting pituitary failure and seven patients with no history of PPH. Controls included 28 healthy women with no prior pregnancy and 28 women with normal pregnancies.

"Twelve of 19 (63.1%) patients with Sheehan's syndrome and one of seven (hypopituitary women) had PitAb against the 49-kDa autoantigen; neuron-specific enolase. Four of 28 (14.2%) controls without prior conception and 5 of 28 (17.8%) controls with prior conception had PitAb positivity ($P < 0.001$ and <0.01 *vs.* Sheehan's syndrome, respectively)."

The prevalence of autoantibodies to the 49 kDa protein was relatively high in the normal controls in this study, but interestingly, so was their percentage of thyroid autoantibodies; 21.4% with no prior conception and 7.4% in those with pregnancies. In comparison, the Australian prevalence study of post-partum thyroid dysfunction showed that 11.5% of normal women had abnormal results at 6 months (Kent et al. 1999).

In summary, Dr. Goswami's paper was the first to propose the concept and the first to provide convincing evidence of a role for autoimmunity in the slow progression of hypopituitarism in Sheehan's syndrome. Since our study, Dr. Kelestimur, a Turkish endocrinologist, has published data using the immunofluorescence assay of de Bellis et al. from Italy with similar findings to our own (De Bellis, Kelestimur et al. 2008). A recent French paper from Dr Thierry Brue, Marseilles has reported the delayed diagnosis and development of hypopituitarism in Sheehan's syndrome (Ramiandrasoa, Castinetti et al. 2013), but they did not attempt to look for pituitary autoantibodies by any method.

Publication 12. No evidence for autoimmunity as a major cause of the empty sella syndrome.

Bensing S, Rorsman F, **Crock P**, Sanjeevi C, Ericson K, Kämpe O, Brismar K, Hulting AL.

Exp Clin Endocrinol Diabetes 2004 May;112(5):231-5.

This study was also conducted partly in Sweden at the Karolinska Hospital, Stockholm and was also part of Dr. Bensing's PhD thesis.

Dr Kerstin Brismar, a co-author, has a particular interest in empty sella syndrome (ESS) and published her PhD thesis on the subject. Many of the patients came from her clinic. The rationale for this study was that empty sella syndrome could represent the end-stage of lymphocytic hypophysitis, when the pituitary gland is atrophic and fibrotic. A similar process is seen in the thyroid gland in Hashimoto's thyroiditis. I would prefer the term CSF-sella syndrome to ESS, as the sella is not really "empty" and there is often a small rim of anterior pituitary tissue – especially in these patients who did not have hypopituitarism

The study included 30 patients with ESS and 50 healthy controls. In addition to pituitary autoantibodies measured by my immunoblotting assay, ten other autoantibodies were assayed to screen for any evidence of autoimmunity.

"The majority of the ESS patients (18/30) exhibited no immunoreactivity at all. None of the remaining 12 ESS patients reacted against more than one autoantigen. Immunoreactivity was found to be similar among ESS patients compared to healthy blood donors."

My main comments regarding the absence of any positive association are as follows:

1. The majority of patients with ESS had no clinical or laboratory evidence of hypopituitarism. As discussed in the manuscript, it seems unlikely that ESS without pituitary hormone deficiency is due to hypophysitis.

2. Nearly 50% of the ESS patients had type 2 diabetes and obesity. My opinion is that these patients are a specific sub-group that has a "hypothalamic" phenotype of diabetes with secondary atrophy of the pituitary.
3. The healthy, Swedish blood bank controls had an unusually high prevalence (7%) of pancreatic autoantibodies, even for Sweden! In Australia and other countries, less than 0.5% of normal controls have islet cell autoantibodies. This clearly affected the statistics.

In summary, patients with ESS and no evidence of hypopituitarism are unlikely to have had autoimmune hypophysitis. ESS is clearly not a homogenous condition and the patients in our study did not fit the "profile" of ESS as an end-stage of hypophysitis.

An earlier publication showed the presence of anti-pituitary hormone antibodies by IB in 45% of 11 patients with ESS or pituitary adenomas (Mau, Phillips et al. 1993), but found them neither specific for, nor predictive of, endocrine deficiencies. A recent publication by Lupi I et al. using an IF assay has demonstrated pituitary autoimmunity in patients with ESS and hypopituitarism (Lupi, Manetti et al. 2011).

Publication 13. Autoantibodies against pituitary proteins in patients with adrenocorticotropin-deficiency.

Bensing S, Kasperlik-Załuska AA, Czarnocka B, **Crock PA**, Hulting A.
Eur J Clin Invest. 2005 Feb;35(2):126-32.

This study was also conducted partly in Sweden at the Karolinska Hospital, Stockholm and was likewise part of Dr. Bensing's PhD thesis. The laboratory work was performed in my laboratory in Newcastle.
It was a collaborative project with Dr Anna Kasperlik-Załuska, Endocrinologist from Warsaw, Poland. She has extensive experience with a unique and large cohort of patients with isolated ACTH deficiency (n=151), in whom she has demonstrated a 73% incidence of endocrine autoimmunity, either thyroid or

ovarian. We wanted to study this group to see if we could detect pituitary autoantibodies using my IB assay and thus confirm the autoimmune pathophysiology underlying the ACTH deficiency. Most reports of isolated ACTH in the literature to this date had been single cases only (Jensen, Handwerger et al. 1986) (Sauter, Toni et al. 1990).

"Autoantibodies to a novel 36-kDa pituitary (cytosolic) autoantigen were seen in sera from 18.5% (12/65) patients and only 3.5% (2/57) of control subjects (P = 0.0214) ... Immunoreactivity to a 49-kDa pituitary autoantigen was observed in 21.5% (14/65) of ACTH-deficient patients compared with 8.8% (5/57) of control subjects (P = 0.0910). Autoantibodies to thyroglobulin were positively correlated to immunoreactivity against the 36-kDa pituitary autoantigen (P = 0.014)."

We have not further identified the novel 36 kDa target autoantigen.

Our results support a direct role for pituitary autoimmunity in about 20% of a large cohort of well-characterized patients with isolated ACTH deficiency.

Publication 14. Pituitary autoantibodies in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).
O'Dwyer DT, McElduff P, Peterson P, Perheentupa J, **Crock PA.**
Acta Biomed. 2007;78 Suppl 1:248-54.

I approached Dr Jaakko Perheetupa, a Paediatric Endocrinologist from Helsinki, Finland to collaborate with us on this project. He made a career-long study of the largest cohort of patients with APECED or APS1 (Autoimmune polyglandular syndrome type 1). This rare, monogenic form of polyendocrinopathy is more frequent in Finnish and Sardinian populations.

Dr Perheentupa sent me sera from his cohort, many of whom had had sequential serum samples over many years. My PhD student Damien O'Dwyer tested these for pituitary autoantibodies with my IB assay.

"We determined the prevalence of pituitary autoantibodies in a cohort of 67

Finnish patients with APECED from 217 serum samples collected over 26 years by one investigator. Autoantibodies to the 49 kDa cytosolic autoantigen, human pituitary enolase were detected in 39 of the 67 patients (58%)."

The most interesting aspect of this study was that we were able to identify time to seroconversion in 14 patients and that autoantibody reactivity persisted once it had developed. In contrast, for example, it is known that autoantibodies in type 1 diabetes detected by immunofluorescence are more likely to be seen at diagnosis and disappear over time. It is unclear why there is this dichotomy of results depending on antibody detection method.

Chapter 4. Identification and purification of the first pituitary target autoantigen using immune serum.

Publication 15. Identification of the 49-kDa autoantigen associated with lymphocytic hypophysitis as alpha-enolase.

O'Dwyer DT, Smith AI, Matthew ML, Andronicos NM, Ranson M, Robinson PJ, **Crock PA.**

J Clin Endocrinol Metab. 2002 Feb;87(2):752-7.

This publication was part of the PhD thesis of Damien O'Dwyer and involved close collaboration with protein biochemists Prof. Phil Robinson (currently at CMRI, Westmead) and Dr. Ian Smith (Baker Institute, Melbourne).

In the final analysis, the protein sequence obtained was 100% identical to alpha-enolase, an ubiquitous glycolytic enzyme. I had shown in 1998 that the 49 kDa protein was not present in skeletal muscle (Crock 1998). This observation was now explained by the fact that the enzyme isoform in muscle, beta-enolase, was not recognized by our patient sera. Of greater importance, the sera also recognized gamma-enolase, the isoform found in neuroendocrine tissues.

<u>These results represented the first time that an autoantigen had been identified in lymphocytic hypophysitis.</u> Unfortunately, the cross-reactivity between alpha- and gamma-enolase meant that these autoantibodies were not specific to pituitary autoimmunity, nor to neuroendocrine autoimmunity. The data also confirmed my original observation that 10% of normal controls had these antibodies, as a number of other investigators have found since that 10% of normal controls have autoantibodies to enolase (see references in the publication).

Publication 16. Pituitary autoantibodies in lymphocytic hypophysitis target both gamma- and alpha-Enolase - a link with pregnancy?

O'Dwyer DT, Clifton V, Hall A, Smith R, Robinson PJ, **Crock PA.**

Arch Physiol Biochem. 2002 Apr;110(1-2):94-8.

This work was part of the PhD thesis of Damien O'Dwyer. We collaborated with Dr Vicki Clifton and Prof. Roger Smith, Mothers and Babies Institute, John Hunter Hospital, who provided the placental tissue and contributed their expertise in placental immunohistochemistry and physiology.

In this study, we wanted to explore the known association between lymphocytic hypophysitis and pregnancy and to see if we could explain the pathogenesis. We used two-dimensional gel electrophoresis and immunoblotting to identify proteins targeted in the pituitary and the placenta by autoantibodies from patients with lymphocytic hypophysitis.

The most important finding was that we were able to demonstrate that the ubiquitous alpha-enolase and the neuroendocrine specific isoform, gamma-enolase were present in both pituitary and placental tissue and were recognized by hypophysitis sera. For the first time, this was a concrete link between the pituitary and pregnancy and could explain the predilection for hypophysitis in female patients in and around the time of pregnancy. Our work has been cited numerous times in this regard (Caturegli, Lupi et al. 2008) (Landek-Salgado, Gutenberg et al. 2010).

Chapter 5. Immunoscreening of a pituitary cDNA library to detect autoantigens and the development of new ITT assays

During my time in Professor Jim Goding's laboratory, Dept of Pathology and Immunology, Monash University, Melbourne, I started to immunoscreen a pituitary cDNA library that I had purchased from Clontech. Using a serum with high titre 49 kDa autoantibodies, I detected four different clones. The first clone isolated and manually sequenced by myself was a 29 kDa protein identified as *FRG1*- FSHD candidate region gene 1. At that time, the function of this gene was not known. Shortly after this, I moved to Newcastle and molecular work at that time was difficult to pursue.

In a recent review in June 2014, the complexity of this chromosomal region has begun to be appreciated (Tawil, van der Maarel et al. 2014). *FRG1* lies at 120 kb distance from the D4Z4 repeat array at the telomeric end of chromosome 4q that is involved in the pathogenesis of Facio-scapulo-humeral dystrophy (FSHD) (Ferri, Huichalaf et al. 2014). Each D4Z4 repeat contains the homeobox sequence of the DUX4 retrogene. My hypothesis is that the two patients with hypophysitis, diabetes and FSHD in my 1998 publication on cytosolic autoantigens are likely to have an autoimmune cause for their FSHD and that the FRG1 protein is the target. Unfortunately both these women are now deceased and their samples in the serum bank were lost in my 2007 "freezer catastrophe".

After a number of years concentrating on protein purification and the application of the immunoblotting assay to a variety of clinical scenarios, I revisited immunoscreening. It was important to pursue the identification of specific pituitary autoantigens in order to develop a more specific and sensitive diagnostic assay.

In the immunoblotting assay, pituitary proteins are separated electrophoretically by molecular weight and transferred to a PVDF membrane. Patient autoantibodies are exposed to the membrane proteins and recognize linear epitopes of the antigens. Similarly with immunoscreening, the cDNA clones from

a phage library are expressed by E.coli and the expressed proteins in plaques are transferred to filters for autoantibody probing. In contrast, immunoprecipitation assays involve three different steps; firstly, the in-vitro transcription translation (ITT) of proteins, secondly, S^{35} labeling of methionine residues in these proteins and thirdly autoantibody recognition of the three-dimensional structure of the target antigen with immunoprecipitation of this Ag-Ab complex. The last step can be crucial, as some proteins undergo post-translational modification (e.g. glycosylation of the TSH receptor) that is needed for normal folding of the protein. Hence, not all target proteins will be recognized in ITT assays and different methods may have very different results.

I had already established a close collaboration with Drs. Sophie Bensing and Anna Lena Hulting from the Karolinska Institute, Stockholm. Dr. Bensing started using the commercially available Clontech pituitary library but the cDNA clones were found to have relatively short sequences. Therefore, she constructed an "in-house" human pituitary cDNA library with expert help from the laboratory of Prof. Olle Kämpe at Uppsala University. This library was then used for the following publications.

Publication 17. Pituitary autoantibodies in autoimmune polyglandular syndrome type 1

Bensing S, Fetissov SO, Mulder J, Perheentupa J, Gustafsson J, Husebye ES, Oscarson M, Ekwall O, **Crock PA**, Hökfelt T, Hulting AL, Kämpe O.
Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):949-54. Epub 2007 Jan 10.

This study was conducted in Sweden at the Karolinska Institute, Stockholm and at Uppsala University in Prof. Olle Kämpe's laboratory. It was part of Dr. Bensing's PhD thesis. I provided control sera from patients with biopsy proven and suspected hypophysitis.

The aim of this study was to identify novel pituitary target autoantigens in patients with APS1 (Autoimmune Polyglandular Syndrome Type 1) or APECED (Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy), and to

correlate the autoantibodies with the clinical features of hypopituitarism. APS1 is a monogenic form of autoimmunity due to mutations in the AIRE (AutoImmune REgulator) gene on chromosome 21 (Finnish-German 1997) (Nagamine, Peterson et al. 1997). APS1 is characterized by very high titre autoantibodies, so patient sera can be used at high dilution for immunoscreening, which reduces background staining.

"A pituitary cDNA expression library was screened with APS1 sera (from two patients with GH deficiency) and a tudor domain containing protein 6 (TDRD6) cDNA clone was isolated. Positive immunoreactivity against *in vitro* translated TDRD6 fragments was shown in 42/86 (49%) APS1 patients but not in patients with other autoimmune diseases (including hypophysitis) or in healthy controls."

In APS1 patients, there was no clinical correlation between autoantibodies to TDRD6 and pituitary dysfunction or to other clinical manifestations. Two of six patients with GHD had these autoantibodies, but patient numbers were too small for meaningful statistical interpretation. In spite of these limitations, it is clear that immunoscreening can be a powerful tool to identify novel proteins and potentially inform disease mechanisms.

The function of TDRD6 is now better understood (Vasileva, Tiedau et al. 2009). TDRD6 is specifically expressed in germ cells in the testis and is essential for spermiogenesis, for chromatid body structure, and for proper mature and precursor miRNA expression. Whether autoantibodies to TDRD6 could impair fertility in male patients with APS1 is not clear to date. It is also possible that other roles will emerge, just as they have for TSGA10 and ECE-2, other target autoantigens that we have identified (see below).

Publication 18. Identification of TPIT and other novel autoantigens in lymphocytic hypophysitis: immunoscreening of a pituitary cDNA library and development of immunoprecipitation assays.

Smith CJ, Bensing S, Burns C, Robinson PJ, Kasperlik-Załuska AA, Scott RJ, Kämpe

O, **Crock PA**.
Eur J Endocrinol. 2012 Mar;166(3):391-8. Epub 2011 Dec 22.

This publication was part of the PhD thesis of Casey Jo Anne Smith (now Smith-Anttila). The laboratory work was done partly in Australia; in my laboratory in Newcastle and in Prof. Phillip Robinson's laboratory at CMRI, Westmead, and partly in Sweden; in Prof. Olle Kämpe's laboratory, Uppsala.

In addition to library screening, I chose to study a potential candidate target autoantigen, the corticotroph-specific transcription factor, TPIT, because corticotroph cells appear to be preferentially targeted in lymphocytic hypophysitis. Dr. Jacques Drouin, IRCM, Montreal was the first to identify this transcription factor (Lamolet, Pulichino et al. 2001) and he kindly donated a full-length cDNA TPIT clone to me and Casey Smith.

Two other previously reported candidate autoantigens, PGSF1a and PGSF2 were kindly donated by Dr Ke-ita Tatsumi, Japan (Tanaka, Tatsumi et al. 2002) at my request.

Potential candidate autoantigen

Our results showed that TPIT was indeed a significant target autoantigen in nine of 86 (10.5%) patients with lymphocytic hypophysitis. The publication was the first description of this transcription factor as a pituitary autoantigen. It was also the first description of an ITT assay for the detection of these autoantibodies.

The results were expressed as a TPIT autoantibody index. The upper limit of the normal range was calculated by the average index of the negative healthy Blood Bank controls plus 3 standard deviations. Analysis of the data in Figure 1. shows that only one or two patients in each category of other autoimmune diseases were positive for TPIT autoantibodies. Except for one patient with APS 1, the autoantibody index was less that 2, but higher in hypophysitis patients. It is also possible that the patients with Addison's disease and ACTH deficiency with TPIT autoantibodies were true positives. Lymphocytic infiltrates in the pituitary have

been described at autopsy in patients with Addison's disease and ACTH deficiency is believed to be part of the spectrum of hypophysitis (Crock 1998). Otherwise, it was unexpected and counter-intuitive that this latter group did not have a higher incidence of TPIT autoantibodies.

Autoantigens identified by cDNA library screening

The initial library screening by Casey Smith was done with sera from four patients; one patient with biopsy-proven hypophysitis and three with suspected hypophysitis. A total of 58 individual cDNA clones were isolated and sequenced.

I will focus on the clones that we chose as being most significant.

CHD8 was identified independently by two of the four screening sera, which I felt was a very significant finding. Given the rarity of hypophysitis, it seemed unlikely that this was coincidental. Another five patients, including two biopsy proven cases, reacted to this protein but so did three normal controls. The chromodomain helicase DNA-binding (CHD) family is a member of the SNF2 family of chromatin remodeling ATPases and acts as an anti-apoptotic factor. Enzymes are frequently autoimmune targets and are usually those involved in hormone synthesis. Yet this enzyme is involved in complex neurological patterning. Insight into its function has been widened by recent genetic, proteomic and biochemical studies. It is involved reciprocally with BAF in WNT/β catenin signalling in neural development and in some cancer types (eg. gastric cancer). Clinically, it has been linked to autism, with mutations associated with a phenotype including macrocephaly. It forms a multi-protein complex (see Figure 1.) that links to, amongst others, CHD7, the gene associated with CHARGE syndrome. CHARGE is an acronym for Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities (including deafness). It may include hypopituitarism as one of the clinical features. It is unclear why patients with hypophysitis should develop such autoantibodies.

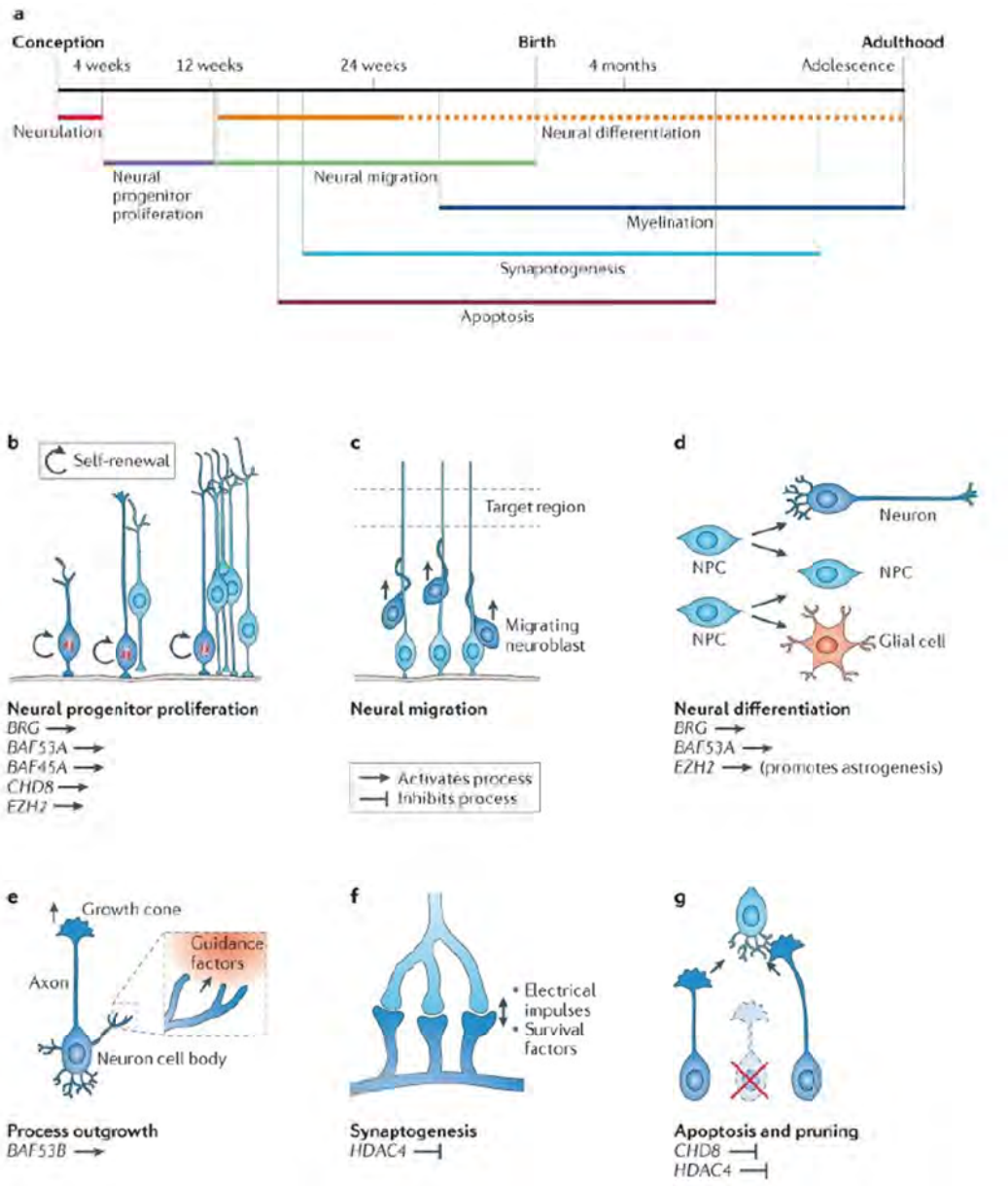

Figure 1. Chromatin regulators have essential roles throughout neural development

The fundamental processes of neural development are illustrated. Chromatin regulators discussed in this Review are noted under the processes in which they have important roles. The key indicates whether a particular regulator promotes or inhibits each neurodevelopmental process. **a** | A timeline of human neural development. **b** | The development of the vertebrate nervous system begins during gastrulation. In the early embryo, neural progenitor cells undergo symmetrical proliferative division. **c** | With the expansion of the number of cell types and the size of the nervous system, the cell bodies of both neural progenitors and resulting postmitotic neurons migrate away from their birthplace to appropriate regions in response to environmental cues. **d** | Neural progenitors asymmetrically divide to give rise to neurons, glial cells or intermediate progenitors. Neural differentiation generates enormous numbers of diverse cell types in the nervous system. **e** | After migrating neurons have reached their destinations, they extend axonal and dendritic processes, which are guided by intricate cellular interactions and guidance molecules to appropriate

Figure taken from Ronan JL, Wu W and Crabtree GR. Nat Rev Genet. 2013; 14 (5): 347-359.

The other two important clones were piccolo and CADPS. These two proteins are involved in dense core vesicle processing, the mechanism required for peptide hormone release from the pituitary. If this functional role were to be adversely affected by antibodies, then hypopituitarism would ensue.

Piccolo was originally isolated with a biopsy proven patient's serum and auto-reactivity found in two other patients and two normal control sera. One of the normal control sera had extremely high titre autoantibodies and could be considered an outlier. Nevertheless, the percentage of patients with positive results was low overall (3.5%).

The **CADPS** clone isolated from the human pituitary cDNA library proved difficult to express in the ITT assay. On the advice of Professor Phillip Robinson, I approached Dr. Thomas Martin, Wisconsin, USA for a full-length rat clone. Although this worked in the ITT assay, the high number of positive results in normal control sera (12% versus 14% in patients) begs the question as to whether there was a problem with species cross-reactivity and/or heterophile antibodies.

Although reactivity to these three target autoantigens and two other previously described autoantigens (NSE and PGSF2) was not specific for each in isolation, there was a significant difference when we looked at combining results for multiple autoantibodies. Eight of the 86 patients with lymphocytic hypophysitis but none of the 90 controls, had autoantibodies to two autoantigens, p= 0.0093 (X^2 test with Yates' correction). Therefore, a panel of autoantigens may be more appropriate as a screen. This approach has been used successfully in type 1 diabetes (Sosenko, Skyler et al. 2013).

Publication 19. TSGA10 - A target for autoantibodies in autoimmune polyendocrine syndrome type 1 and systemic lupus erythematosus.

Smith CJ, Oscarson M, Rönnblom L, Alimohammadi M, Perheentupa J, Husebye ES, Gustafsson J, Nordmark G, Meloni A, **Crock PA**, Kämpe O, Bensing S.
Scand J Immunol. 2011 Feb;73(2):147-53.

This publication was part of the PhD thesis of Casey Jo Anne Smith (now Smith-Anttila). The laboratory work was done in Uppsala, Sweden under the guidance of Dr. Sophie Bensing. I visited the laboratory a number of times whilst Casey was working in Sweden.

The target autoantigen, TSGA10, a testis-specific protein, was identified by immunoscreening the pituitary cDNA library with serum from a patient with APS1 and GH deficiency, but no signs of hypogonadism. No correlation could be found between serum reactivity to TSGA10 and any pituitary dysfunction, hypogonadism or infertility. It is considered a minor autoantigen, seen in only 5% of APS1 patients.

Interestingly, this protein was also found to be a novel target autoantigen in five of 135 (3.7%) patients with SLE, albeit in only one patient at high titre.

Recent studies have shown that TSGA10 not only codes for a protein localized in sperm-tail and conserved in ciliary structures, but that it is involved in signalling and expression during embryogenesis and brain development (Behnam, Modarressi et al. 2006). The protein is also upregulated in some malignancies including brain tumors, such as glioblastoma (Behnam, Chahlavi et al. 2009). The most intriguing data so far linking TSGA10 with immune function, is the finding that Filament-associated TSGA10 protein is expressed in professional antigen presenting cells and interacts with the cytoskeletal protein, vimentin (Roghanian, Jones et al. 2010). Thus, proteins may have multiple functions that seem unrelated and the fact that they can be target autoantigens is intriguing.

Manuscript submitted: Identification of Endothelin Converting Enzyme-2 as an Autoantigen in Autoimmune Polyendocrine Syndrome Type 1

Casey JA Smith-Anttila, Sophie Bensing, Mohammad Alimohammadi, Frida Dalin, Mikael Oscarson, Ming-Dong Zhang, Jaakko Perheentupa, Eystein S Husebye, Jan Gustafsson, Peyman Björklund, Anette Fransson, Gunnel Nordmark, Lars Rönnblom, Antonella Meloni, Rodney J Scott, Tomas Hökfelt, Patricia A Crock, Olle Kämpe.

This publication was part of the PhD thesis of Casey Jo Anne Smith (now Smith-Anttila). The laboratory work was done in Uppsala, Sweden under the guidance of Dr. Sophie Bensing and Prof. Olle Kämpe and at the Karolinska Institute under the guidance of Dr. Tomas Hökfelt. I provided all the hypophysitis sera and Australian Blood Bank control sera as well as intellectual input.

The pituitary cDNA library was screened with sera from two APS-1 patients with GH deficiency. A total of 46 positive cDNA clones were isolated, revealing seven different clones in addition to tryptophan hydroxylase (TPH) isoform 1 (a well known APS1 autoantigen). The clone encoding ECE-2 was chosen from these seven for further study as it was recognized by more than 50% of patients.

Immunostaining studies showed that ECE-2 was highly expressed in the pancreas (particularly the endocrine pancreas) and in pituitary and brain. In pituitary tissue, ECE-2 was widely expressed in GH secreting cells.

Chapter 6. Immunofluorescence studies

Publication 20. Intermediate lobe immunoreactivity in a patient with suspected lymphocytic hypophysitis.

Smith CJ, Bensing S, Maltby VE, Zhang M, Scott RJ, Smith R, Kämpe O, Hökfelt T, **Crock PA.**

Pituitary. 2014 Feb;17(1):22-9.

In this manuscript, which was included in Casey Smith's PhD, we looked for pituitary autoantibodies by immunofluorescence in 16 patients with hypophysitis (13 biopsy proven) and 13 matched Australian Blood Bank controls.

The immunofluorescence studies in Dr Tomas Hökfelt's laboratory were unable to detect pituitary autoantibodies in any of 13 biopsy proven patients nor in any of 13 controls. In one of three patients with suspected hypophysitis and diabetes insipidus, we were able to show immunoreactivity to a cell population located within the intermediate lobe of the guinea pig pituitary and weak reticular staining in the posterior lobe. The patient had marked skin pallor, as can be seen in pan-hypopituitarism, however, the staining was not affected by pre-adsorbing the serum with ACTH, alpha-MSH or beta-endorphin. We hypothesized that the autoantibodies were directed at melanotrophs and perhaps proteins involved in POMC signaling.

In unpublished experiments in my laboratory in Newcastle, I had already looked for reactivity to PC1/3 and PC2 (prohormone-convertases 1/3 and 2) by immunoblotting in this patient – with negative results. The enzymes were kindly given to me by Dr. Llloyd Fricker from the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York (Feng, Reznik et al. 2002). He is an expert in PC1/3 and PC2 and is a friend and collaborator of Dr. Ian Smith at the Baker Institute, Melbourne (with whom we worked for the sequencing of enolase). My reasoning was that most organ-specific autoimmune endocrinopathies were known to target enzymes and hence

I thought that PC1/3 and PC2 were good candidates. Later, in 2003, Tatsumi et al. from Japan published the presence of PC1/3, but not PC2, autoantibodies in patients with nonfunctioning pituitary adenomas (Tatsumi, Tanaka et al. 2003). Autoantibodies were more frequent than in patients with suspected lymphocytic hypophysitis. Therefore, these autoantibodies are not specific for hypophysitis.

Interestingly, this patient was one of the four patients whose sera were used to immunoscreen the pituitary cDNA library (Chapter 5). She was included in my original immunoblotting paper in JCEM, 1998 and was found to have very high titre antibodies to the pituitary 49 kDa cytosolic protein.
Her case illustrates many points. It highlights the difficulties in diagnosis of hypophysitis when there is no tissue available for histopathology and adds to the literature on the long-term follow-up of patients presenting with hypopituitarism associated with diabetes insipidus. It is rare for patients with pituitary adenomas to develop diabetes insipidus pre-operatively, so this narrows the diagnostic possibilities and increases the likelihood of conditions such as hypophysitis or infundibulo-neurohypophysitis. However the hypophysitis could be either primary or secondary to a Rathke's cyst or other cystic lesion.

Finally, our study again questions the utility of immunofluorescence to detect pituitary autoantibodies. It can be argued that guinea pig pituitary tissue is an unsuitable substrate when it comes to species specificity, but this would work in the direction of false positive results. It is very unlikely that this tissue would lead to false negative results, especially as most clinically significant target autoantigens are conserved during evolution and across species.

The original description of hypophysitis in 1962 was by Professor Goudie and he tried later, unsuccessfully, to demonstrate pituitary autoantibodies by immunofluorescence (Goudie and Pinkerton 1962). Prof Jørn Nerup, an expert in Addison's disease, also tried unsuccessfully (Nerup, Lindholm et al. 1969). The first successful results came from Bottazzo's laboratory in London in the late 1970's, using human pituitary tissue from women with breast cancer undergoing

hypophysectomy. Later they used baboon pituitary glands. They reported pituitary autoantibodies in newly diagnosed patients with diabetes and in patients with polyglandular autoimmune diseases (Bottazzo, Pouplard et al. 1975). They never tested any sera from patients with hypophysitis. Pouplard et al. from France also had some success with guinea-pig pituitary tissue but again did not investigate patients with hypophysitis (Pouplard, Bottazzo et al. 1976, Pouplard, Job et al. 1985). This is not so surprising given that the first cases of hypophysitis diagnosed ante-mortem were in 1980 (Quencer 1980) (Mayfield, Levine et al. 1980).

Recently, immunofluorescence studies have been revived by two main groups. The first group is in Naples with senior authors, Dr A. de Bellis and Dr A. Bellastella. The second group is led by Dr Patrizio Caturegli, pathologist from Johns Hopkins, Baltimore. Both groups have used monkey or baboon pituitary as the substrate. The results from Naples are sometimes difficult to assess as they do not publish many images and some of those that they do publish appear to show high, non-specific background IF (De Bellis, Kelestimur et al. 2008). They have focused on pituitary autoantibodies in association with other autoimmune diseases (De Bellis, Bizzarro et al. 2003) (De Bellis, Salerno et al. 2006) (Bellastella, Rotondi et al. 2010) or on different forms of hypopituitarism (De Bellis, Pane et al. 2011) (De Bellis, Dello Iacovo et al. 2014) or diabetes insipidus. The paper on the detection of autoantibodies to AVP-producing cells in the hypothalamus in patients with evidence of clinical or subclinical diabetes insipidus was interesting (De Bellis, Sinisi et al. 2012). They have not published data on a significant number of patients with biopsy proven hypophysitis.

Chapter 7. Case Reports

Publication 6*. A rare case of pituitary infarction leading to spontaneous tumour resolution and CSF-sella syndrome in an 11-year-old girl and a review of the paediatric literature.

Maltby VE, **Crock PA**, Lüdecke DK.

J Pediatr Endocrinol Metab. 2014 Sep;27(9-10):939-46.

* This publication is also included in Chapter 1 because of its comprehensive review of the paediatric literature.

The case is of a child with a rare clinical presentation and highlights, yet again, the difficulties in diagnosis when there is no tissue histopathology. I followed her clinical course over 10 years to the present and we demonstrated pituitary autoantibodies to the target autoantigen piccolo. In the final analysis, it is likely that she had pituitary apoplexy in an underlying growth hormone secreting tumour with peritumoral hypophysitis. The publication synthesizes the clinical and research approaches that I took over this time.

Publication 21. Pituitary Disease in Chronic Hepatitis C Infection and Interferon-alpha Related Therapy: Two Case Reports

Tran HA, Crock PA, Reeves GEM *J Endocrinol Metab* 2012;2(4-5):190-194.

This paper was a collaboration with Dr Huy Tran, Director of Biochemistry, Hunter Area Pathology Service. It highlights the unexpected side-effects of immunological therapy, in this instance interferon-alpha, on the development of pituitary autoimmunity leading to pituitary insufficiency.

Another immunomodulator, anti-CTLA4 monoclonal antibodies, have been used to treat advanced melanoma. Interestingly, some patients develop hypophysitis, particularly isolated ACTH deficiency. CTLA4 is a surface receptor on helper T cells and sends inhibitory signals. There is now a significant literature on this subject (Caturegli 2012) (Iwama, De Remigis et al. 2014).

Chapter 8. Final Summary

The aim of my original studies was to develop a new diagnostic test for the detection of pituitary autoantibodies that was more sensitive and specific than immunofluorescence. I developed the first immunoblotting assays for the detection of human pituitary membrane and pituitary cytosol autoantibodies. These assays enabled us to identify a number of target antigens by molecular weight in a range of clinical situations consistent with autoimmune hypophysitis.

Other groups in the field, principally from Japan (Dr Kozo Hashimoto and colleagues), confirmed our findings. The first autoantigen that we purified and characterized was enolase, both the ubiquitous alpha-enolase and the neuroendocrine specific gamma-enolase forms. Unfortunately, they were not specific for pituitary autoimmunity, although can indicate an underlying autoimmune condition. Nevertheless, our identification of gamma-enolase autoantibodies in a patient with suspected hypophysitis that recognized both pituitary and placental tissue was a significant breakthrough. It showed, for the first time, concrete evidence that could explain the well-known link between hypophysitis and pregnancy.

My immunoblotting assay was applied in a wide range of clinical situations and thus helped to better delineate the clinical spectrum of autoimmune hypophysitis. As can be appreciated by our publications, I collaborated at an international level with other experts in their specific fields of interest. Many had unique patient populations that were a privilege to study, such as the APS1 patients from Prof. Jaakko Perheentupa in Finland; the empty sella syndrome cohort from Dr Kerstin Brismar, Dr Anna Lena Hulting and Dr Sophie Bensing at the Karolinksa Institute, Sweden; the isolated ACTH deficiency patients from Prof. Anna Kasperlik-Załuska in Poland and the Sheehan's syndrome patients from Prof. Ravinder Goswami, AIIMS, in New Delhi.

In APS1, we published the first paediatric case of hypophysitis from Québec, identifying pituitary membrane autoantibodies and describing the classical "ring

enhancement" sign of hypophysitis. Also for the first time, we demonstrated seroconversion time-points and that autoantibodies detected by immunoblotting persist for many years. In contrast, autoantibodies detected by immunofluorescence (IF) are known to disappear over time. We showed the complexity of hypothalamic and pituitary involvement in APS1 with IF staining of a fiber-plexus in the pituitary intermediate lobe recognizing enzymes of monoamine and GABA synthesis. Except for our French Canadian paediatric index case, the correlation between pituitary autoantibodies and clinical phenotypes in APS1 was difficult to establish.

Empty sella syndrome (ESS), better termed CSF-sella syndrome, has long been thought to represent the chronic and fibrotic end-stage of autoimmune pituitary disease. We found that patients with no clinical pituitary hormone deficiencies, no pituitary autoantibodies and no other endocrine autoantibodies were unlikely to have had hypophysitis. In conclusion, the cohort of ESS patients from the Karolinska Hospital, half of whom had type 2 diabetes, did not fit the picture of underlying hypophysitis. Therefore, patients with CSF sella syndrome secondary to pituitary autoimmunity are likely to have another autoimmune condition with isolated or multiple pituitary hormone deficiencies that develop in adulthood.

Prof. Anna Kasperlik-Załuska has a large series of patients with isolated ACTH deficiency, many of whom also have Hashimoto's thyroiditis. We were able to further characterize the endocrine autoimmunity in this group and identify a novel 36 kDa pituitary cytosolic autoantigen.

Finally, with Prof. Ravinder Goswami, we were able to confirm his theory of progressive autoimmunity secondary to vascular injury in the puerperium by demonstrating pituitary autoantibodies in women with Sheehan's syndrome. The prolonged delay in diagnosis has been found by other groups, most recently by obstetricians working with Dr. Thierry Brue, an endocrinologist in Marseille, France (Ramiandrasoa, Castinetti et al. 2013). Unfortunately they did not attempt to measure pituitary autoantibodies by any method, IF or IB.

Following on from my immunoblotting work, I wanted to identify more target autoantigens by immunoscreening a pituitary cDNA library. As outlined earlier, immunoscreening recognizes positive clones by western blotting (linear epitopes), whereas the subsequent ITT assays depend on three-dimensional presentation of the autoantigen and immunoprecipitation of the antigen-antibody complex. Hence, immunoblotting assay results and/or positive clones on immunoscreening may not translate into an ITT assay, and we saw that with enolase as an autoantigen. In addition, partial length clones may produce proteins that do not fold normally in the true native state or that are not normally glycosylated. Finally, species specificity and heterophile antibodies may be an issue as with the use of the full-length rat CADPS protein we obtained from Dr Tom Martin, Wisconsin. Notwithstanding these issues, we did go on to identify a number of target proteins of interest.

The target autoantigen CHD8 was a surprising discovery. It is an ATP-dependent chromatin remodelling enzyme that regulates gene transcription (Ronan, Wu et al. 2013). It does not appear to be involved directly in hormone synthesis or secretion, but it is involved in a large complex of interacting factors, including the protein CHD7. CHD7 mutations are associated with CHARGE syndrome of multiple congenital anomalies that can include hypopituitarism. This may explain the rather complex, convoluted link with pituitary autoimmunity.

The identification of the proteins CADPS and piccolo was intriguing as they are both involved in dense-core vesicle transport. Pituitary protein hormones use this pathway for secretion and thus disruption would cause pituitary hormone deficiency. In contrast, most other organ specific autoimmune endocrinopathies involve enzyme pathways for hormone synthesis, such as thyroid peroxidase in Hashimoto's thyroiditis, 21-hydroxylase in Addison's disease and GAD65 (glutamic acid decarboxylase) in type 1 diabetes. Although we did not identify any specific pituitary enzyme targets, I did choose the corticotroph-cell specific T-box transcription factor, TPIT, as a potential autoantigen. We showed for the first time that TPIT is a target autoantigen and that 10% of patients with hypophysitis had TPIT autoantibodies. After we had already started our work

with TPIT, Japanese colleagues Yamamoto et al. (Yamamoto, Iguchi et al. 2011) published a case series of three adult patients with GH and TSH deficiency who were found to have autoantibodies to the pituitary transcription factor Pit1, that is involved in the differentiation of somatotrophs and thyrotrophs. One of the three patients subsequently died and at autopsy was shown to have multiple autoimmune endocrinopathies, including hypophysitis with loss of somatotrophs and thyrotrophs in the pituitary, that secrete GH and TSH respectively. Thus, our description of TPIT as a target autoantigen, is the second pituitary transcription factor to be so identified.

One of the most important findings to come out of our ITT assays, was that only patients with hypophysitis had autoantibodies to more than one target autoantigen. It would therefore seem that a panel of autoantigens would be a more sensitive and specific test for pituitary autoantibodies than any one autoantigen in isolation. The best autoantigens for such a panel are yet to be determined.

Finally, especially in young patients, the most pressing need is for a sensitive and specific autoantibody test that can differentiate between pituitary tumours and a mass due to hypophysitis. After many years in the field, my belief is that this will not be possible with the current immunological tools alone, as many patients have peri-tumoural hypophysitis.

Contribution to the Paediatric Literature

In the paediatric age-group, the diagnosis of lymphocytic hypophysitis should be made with great caution. When a condition is rare, there is a great temptation to publish “The first case of lymphocytic hypophysitis in a child”. The literature is replete with cases of intrasellar, suprasellar or infundibular masses that were called hypophysitis and subsequently found to be germinomas with peri-tumoural hypophysitis or cases of Langerhans cell histiocytosis. In the case of cystic pituitary lesions, the presence of diabetes insipidus makes the diagnosis of an adenoma very unlikely. It also suggests the presence of secondary

autoimmune inflammation (hypophysitis), as first described by Puchner et al. (Puchner, Ludecke et al. 1994). When this inflammation extends into the anterior lobe, it can lead to hypopituitarism, independent of, but additive to, the mass effect of the cyst. Therefore, immune-modulatory therapy may have a role in minimizing the inflammatory component to preserve pituitary function. To my knowledge, there are no published manuscripts discussing such a possibility.

In my publications, we have described one convincing case of hypophysitis in a child with APS1, who had both clinical and radiological features. We also identified a 45 kDa pituitary membrane target auto-antigen in that case. In my original immunoblotting methods paper, I described the presence of pituitary membrane autoantibodies in children treated with cadaveric-derived growth hormone (a form of inadvertent "pituitary immunization") and similar autoantibodies in a boy with growth failure and CSF sella syndrome. I found no evidence that they were against GH itself. However, using my immunoblotting method, Prof. Kozo Hashimoto's group found autoantibodies to pituitary growth hormone (a 22 kDa protein) in some patients with suspected hypophysitis (Takao, Nanamiya et al. 2001).

Finally, in my paediatric case report of a probable GH secreting tumour, we found autoantibodies to piccolo that may have developed in response to the infarction (as I published in Sheehan's syndrome with Dr. Goswami) or have been part of a peri-tumoural hypophysitis (Maltby, Crock et al. 2014). The case highlights the uncertainty that exists when there is no tissue diagnosis and when the autoantibody testing is non-specific. This scenario also applies to adult patients. The review that we included in the paper made it clear that many cases in the paediatric literature of "lymphocytic hypophysitis" have been mis-called.

Future directions

In the future, the emphasis will move towards identifying epigenetic and other biological markers in serum or plasma, such as miRNAs or exosomes (Choi, Kim et al. 2014). I think that exosome profiling (Yoon, Kim et al. 2014) will give a

more integrated look at the biological processes that are going on in autoimmune conditions. Until recently, the focus has been on cancer biology but articles are now appearing in the autoimmune field (Salama, Fichou et al. 2014). Exosomes and microvesicles are mechanisms by which cell-cell interaction can occur with very complex sets of molecules, rather than the paradigm of a single hormone binding to its specific receptor. There is a component of the tumour response that is targeted at the immune system, specifically cytotoxic T cells. It may be this component which is relevant to the concept of peri-tumoural hypophysitis. The information in this emerging field is now accessible via EVpedia (Kim, Lee et al. 2014), a proteomic database that can link whole sets of protein interactions and discover new networks.

Our laboratory is now developing miRNA profiling across the spectrum of autoimmune diseases and we hope that this will not only have diagnostic but also therapeutic implications. A main aim is the further development of less invasive diagnostic tools, e.g. differentiating the causes of obesity and diabetes in paediatric endocrinology.

Acknowledgements

Firstly, I would like to acknowledge all the patients and normal control subjects who provided sera for our studies and all the endocrinologists and physicians with whom we have collaborated over so many years.

In particular, I want to thank my mentors for their wise advice and support and the many talented, generous and helpful scientists in the laboratories in which I have worked including those of Dr Harvey Guyda, Prof Jack Wall and Dr Mario Salvi, Prof James Goding, Dr Wayne Hancock, Dr Ian Smith, Prof Olle Kämpe, Dr Anna Lena Hulting, Dr Tomas Hökfelt and Professors Roger Smith, Phillip Robinson and Rodney Scott.

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MD THESIS Publications for Patricia Anne Crock

Publication 1. Pituitary Autoantibodies and Hypopituitarism

Crock PA.

Clin Pediatr Endocrinol 1996; 5 (Supl 8): 1-8. (Crock 1996)

Official Journal of the Japanese Soc. of Pediatric Endocrinology - no Impact factor

Publication 2. Lymphocytic Hypophysitis

Crock PA.

Curr Opin Endocrinol Diabet 1997;4: 115-123. (Crock 1997)

Impact Factor 3.37

Publication 3. Pituitary autoantibodies

Crock PA, Bensing S, Smith CJA, Burns C, Robinson PJ.

Curr Opin Endocrinol Diabet 2006; 13:344-350. (Crock 2006)

Impact Factor 3.37

Publication 4. Autoimmune Hypophysitis

Crock PA, Bensing S, Smith CJA, Burns C, Robinson PJ.

Chapter 15; pp 357-392.

In Contemporary Endocrinology: Autoimmune Diseases in Endocrinology.

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Publication 5. Paediatric Pituitary Disorders

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Publication 6. A rare case of pituitary infarction leading to spontaneous tumour resolution and CSF-sella syndrome in an 11-year-old girl and a review of the paediatric literature.

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Publication 7. Detection of anti-pituitary autoantibodies by immunoblotting

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Impact factor – 1.74

Publication 20. Intermediate lobe immunoreactivity in a patient with suspected lymphocytic hypophysitis.

Smith CJ, Bensing S, Maltby VE, Zhang M, Scott RJ, Smith R, Kämpe O, Hökfelt T, **Crock PA.**

Pituitary. 2014 Feb;17(1):22-9. (Smith, Bensing et al. 2014)

Impact factor - 3.2

Publication 21. Pituitary Disease in Chronic Hepatitis C Infection and Interferon-alpha Related Therapy: Two Case Reports

Tran HA, Crock PA, Reeves GEM *J Endocrinol Metab* 2012;2(4-5):190-194. (Tran HA 2012)

No current Impact factor

Clin Pediatr Endocrinol 1996; 5(Suppl 8): 9-16

Pituitary Autoantibodies and Hypopituitarism

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Abstract The clinical spectrum of autoimmune pituitary disease is still being defined. In the pediatric age group, autoimmune mechanisms have been postulated for some cases of growth hormone (GH) deficiency and the empty sella syndrome. The classical presentation in adulthood is of peripartum hypopituitarism associated with a pituitary mass and visual failure. Biopsy reveals lymphocytic infiltration of the pituitary, mainly with CD4+ T helper cells, and is termed lymphocytic hypophysitis. Secondary adrenal insufficiency is a prominent feature of the hypopituitarism and isolated adrenocorticotropin (ACTH) deficiency and the empty sella syndrome are thought to be endstages of the disease spectrum. There has been no specific pre-operative serological test for this condition.

We have developed a new assay based on immunoblotting, for evaluating autoantibodies in pituitary disease. The assay uses autopsy pituitaries which are homogenized and ultracentrifuged to give membrance and cytosolic fractions. Proteins are immunoblotted and reactivity of patient sera to autoantigens is detected using an alkaline-phosphatase conjugated second antibody.

A pituitary specific 45 kDa membrane protein was identified by serum from a child with isolated GH dificiency and an empty sella. The target autoantigen in some cases of isolated ACTH deficiency appears to be a 49 kDa cytosolic protein.

Key words: anti-pituitary autoantibodies, pituitary autoimmunity, empty sella syndrome, growth hormone deficiency, isolated adrenocorticotropin deficiency, immunoblotting

Introduction

This presentation is an overview of the clinicopathological spectrum of pituitary autoimmunity and of the field of antipituitary autoantibody testing. The development of a new assay for the detection of antipituitary autoantibodies is described using an immunoblotting method with discussion of its clinical applications.

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Pituitary Auto immune Disease

Histopathological Description

The first recognised histological description of autoimmune pituitary disease was made in 1962 by Goudie and Pinkerton (1).

They reported the case of a 22 year old woman with Hashimoto's thyroiditis who died post-appendicectomy from secondary adrenal failure. The onset of symptoms of hypopituitarism had been temporally related to her second pregnancy, fourteen months previously. At autopsy, the lymphocytic infiltrate in her pituitary gland resembled that in her thyroid and the authors suggested that these two conditions shared an autoimmune basis related to "the reaction to antigens released during the puerperal involution of these glands". Review of Professor Goudie's original sections showed the diffuse lymphocytic infiltrate of the anterior pituitary with some extension into the adjacent dura mater. Immunohistochemical studies in our laboratory in collaboration with Dr. Wayne Hancock (Sandoz Center for Immunobiology, Deaconess Hospital, Harvard Medical School, Boston) showed that the infiltrate was predominantly composed of T cells. A CD4+ marker UCHL-1, showed that these lymphocytes were mainly CD4+ T helper cells, and a B cell marker, L26 showed that only a few of the cells were of this lineage. Other studies have also shown a significant CD4+ infiltrate (2-5) and one study that CD8+ cells were more common (6). However, it is possible that the nature of the infiltrate changes with time.

There appears to be a progression of histological changes in lymphocytic hypophysitis. In the early stages the gland is enlarged and infiltrated by lymphocytes, some of which may form follicles with germinal centres (2). Lymphocytic infiltration may extend into the dura as far as the cavernous sinuses, even causing internal carotid artery occlusion (7). The adenohypophysial cells atrophy and are destroyed (8), often showing oncocytic change. Electron microscopy studies have shown lymphocytes interdigitating with pituitary cells (8, Crock *et al.* unpublished data). Later, there is fibrosis which gives the gland a characteristic yellowish colour and firm consistency at neurosurgical exploration, quite unlike pituitary adenomata (3). Although there may still be a pituitary mass at this stage, which may last many years, ultimately the gland shrinks in size and in some cases may leave an empty sella (9). Thus, end-stage lymphocytic hypophysitis probably accounts for some cases of the empty sella syndrome.

Clinical Spectrum

There are now nearly 100 cases of biopsy-proven lymphocytic hypophysitis in the world literature. The advent of computed tomography (CT) and magnetic resonance imaging (MRI) scans has been associated with an increase in the number of reported cases. Lymphocytic hypophysitis cannot be distinguished from a pituitary adenoma by radiological imaging as both lesions cause a mass which will enhance with contrast (10-12). However, there are some clinical features which make the diagnosis more likely and these will be discussed within the spectrum of disease presentation.

The most acute presentation recorded is a fatal adrenal crisis during labour (13), but patients may also present with sudden headache. Classically the presentation is as a pituitary mass associated with headache, visual symptoms and hypopituitarism in the last trimester of pregnancy or in the post-partum period (8, 11, 15). Approximately fifty percent of cases have an association with preg-

nancy and only twelve cases have been recorded in men, the first case being described in 1987 (16). The striking feature of the hypopituitarism is the presence of adrenocorticotropin (ACTH) deficiency, which can be an isolated deficit (2, 17). By contrast, ACTH is usually the last hormone to be lost with pituitary tumours. There are some cases which appear to resolve spontaneously (18-22). Other, non-tumourous causes of hypopituitarism should be included in the differential diagnosis, the most important of these being Sheehan's syndrome (post-partum pituitary necrosis). It is likely that some cases of Sheehan's syndrome, in which there has not been major peri-partum blood loss, are due to unrecognized lymphocytic hypophysitis.

Another feature of lymphocytic hypophysitis which should arouse suspicion that the lesion is not a pituitary adenoma is its association with diabetes insipidus, which is rarely seen pre-operatively with pituitary tumours (23). The term infundibuloneurohypophysitis has been coined by Imura *et al.* (24) and it is unclear if this is a new entity or part of the spectrum. Other unusual presentations have included infertility (4), hyperprolactinemia (25), transient cranial nerve palsies (7), cavernous sinus involvement (7, 27) and internal carotid artery occlusion (20).

The histological and immunohistochemical studies described above and the incidence of coexistent autoimmune disease in up to 40 % of patients (28, 29), all support the concept of lymphocytic hypophysitis as one of the endocrine organ-specific autoimmune diseases. However until now, pituitary biopsy or hypophysectomy have been the only means of making this diagnosis definitively in a living patient. The need for a pre-operative serological diagnostic test is evident.

Antipituitary Autoantibody Testing

The detection of circulating autoantibodies is one of the criteria of autoimmunity and has proved more problematic in pituitary autoimmune disease than in other endocrine organ-specific autoimmune diseases. The ability to make a serological diagnosis with positive antipituitary autoantibodies would help to define the clinical spectrum of the disease and perhaps avoid neurosurgery in selected cases.

Complement Fixation and Immunofluorescence Assays

The first paper detailing antipituitary autoantibody activity was by Engelberth and Jezkova in 1965 who used a complement-fixation test but there was no objective endocrine data on the clinical state of these patients (30). Testing by immunofluorescence was developed by Bottazzo *et al.* in 1975 using fresh, frozen human pituitary tissue from hypophysectomies performed for breast cancer (31). Anti-pituitary prolactin cell autoantibodies were detected in 19 of 287 patients with polyglandular autoimmune disease, but again none of these patients had evidence of pituitary insufficiency (31). These autoantibodies were also said to be primate specific and of low titre.

Dr. Sugiura *et al.* from Sohgo Biomedical Laboratories have developed an immunofluorescence assay using AtT20 cells (a mouse pituitary ACTH-secreting cell line) and GH3 cells (a rat pituitary GH-and prolactin-producing cell line) (32). Reactivity to AtT20 cells

was seen in five of five patients with isolated ACTH deficiency and 75 % of patients with the primary empty sella syndrome (33). Professor Kobayashi *et al.* have demonstrated autoantibodies to AtT20 cells in 11 of 14 pregnant women just prior to delivery but this phenomenon decreased to 4 out of 14, (14 %) by day 6 postpartum (34).

Immunoblotting Method

The original case which prompted this study was a boy aged 11 years, who presented growth failure over 2 years due to isolated GH deficiency. Apart from an empty sella turcica on CT scan of his head, no cause could be found. We postulated that atrophy of his pituitary gland could have been due to the end stage of an autoimmune process and set out to determine if antipituitary autoantibodies could be identified in his serum. The unavailability of fresh, frozen human pituitary tissue for immunofluorescence studies led to the search for an alternative approach.

Immunoblotting Method

This method for the detection of antipituitary autoantibodies has been published previously (35). In brief, human autopsy pituitary glands were obtained within 4-8 hours of death and frozen at –70 °C. The glands were homogenised with a mixture of protease inhibitors, and centrifuged at 100,000 × g to give cytosolic and membrane fractions. The pituitary preparations were fractionated on sodium dodecyl sulfate (SDS) -polyacrylamide gel by electrophoresis and separated proteins transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membranes. Immobilon strips were incubated with experimental or control serum diluted 1:50 in 1 % skim milk powder in phosphate buffered saline (PBS) overnight at 4 °C. Positive immunoreactivity was detected using goat anti-human IgG conjugated to alkaline phosphatase (BioRad) as the second antibody step. Strips were developed with 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Bio Rad). Non-specific binding by second antibody alone was seen at approximately 50 kDa and 25 kDa, the sizes of the heavy and light chains respectively of immunoglobulins from blood present in the pituitary tissue.

Clinical Subjects

We studied the prevalence of antipituitary autoantibodies as detected by immunoblotting, in 20 children with idiopathic GH deficiency, 15 children with acquired GH deficiency secondary to irradiation or surgery and 29 normal paediatric control subjects (35).

At this time we were also sent sera from three men (aged 44, 45 and 49 years) with isolated ACTH deficiency, one of whom had a partial empty sella on CT scan. One of these patients had been reported by Sauter *et al.* from Tufts University, Boston, to have autoantibodies directed against 200 nm secretory granules in rat corticotrophs using immunohistochemistry (36). Ethical approval for this study was given by the Human Ethics Committee of the Alfred Hospital, Prahran.

Pituitary Membrane Autoantibodies

None of the sera from normal pediatric subjects had significant reactivity to pituitary membrane antigens (35). The child with isolated GH deficiency and the empty sella

syndrome had autoantibody reactivity to a 45 kDa pituitary-specific membrane protein. This reactivity could not be demonstrated in serum which had been taken prior to recombinant GH treatment and which had been stored for many years at –20 °C. Two children with idiopathic GH deficiency, previously treated with pituitary-extracted GH, had autoantibodies to a 43 kDa membrane protein that was also present in brain membrane fractions. Two children with radiation-induced hypopituitarism had autoantibodies to a 95 kDa pituitary membrane protein. The clinicopathological significance of these autoantibodies is unclear. The induction of autoantibodies by irradiation has been reported (37) and it is possible that impurities in the pituitary-extracted GH used for therapy, "immunized" the two patients with reactivity to the 43 kDa protein (35), although it was not seen in any of the other patients' sera. The empty sella syndrome has been associated with antipituitary autoantibodies detected by other methods (32, 33) and so our original case with reactivity to a 45 kDa pituitary membrane protein may represent autoimmune-mediated GH deficiency.

Pituitary Cytosolic Autoantibodies

Antipituitary autoantibodies of high titre (>1:1,000) were found in the sera of two of the three patients with isolated ACTH deficiency (38). Immunoreactivity was to a pituitary cytosolic protein of molecular weight 49 kDa and was not found in the sera of any of the pediatric control patients (38). Isolated ACTH deficiency is a rare condition that has been reported in association with lymphocytic hypophysitis (2, 17) and the prediliction for corticotroph destruction in the latter has already been discussed above. It has also been reported with the empty sella syndrome as was seen in one of our cases (39, 40).

The high titre autoantibodies detected by immunoblotting are supporting evidence that isolated ACTH deficiency and the empty sella syndrome both have an autoimmune basis. Further characterization of the 49 kDa autoantigen targetted by these autoantibodies is currently in progress.

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Lymphocytic hypophysitis

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The clinical spectrum of lymphocytic hypophysitis is reviewed in 113 cases (96 female and 17 male) of biopsy-proven or suspected disease. Predominantly a disease of young women, the classic acute-phase presentation mimics an enlarging pituitary tumor with partial hypopituitarism or panhypopituitarism in late pregnancy. Distinctive clinical features include isolated corticotropin (ACTH) deficiency and diabetes insipidus, both unusual with pituitary adenoma, and coexistent autoimmune disease. CT and magnetic resonance imaging show a homogeneous, intensely enhancing mass, sometimes involving the pituitary stalk and hypothalamus, termed *infundibuloneurohypophysitis*. Subacute disease is increasingly being recognized in the postpartum period, when both spontaneous regression of the mass and hypopituitarism may occur. Neurosurgical intervention often results in permanent hypopituitarism. Steroid therapy is discussed. Chronic disease can mimic Sheehan's syndrome and lead to isolated ACTH deficiency and the empty-sella syndrome. Antipituitary autoantibody testing is reviewed, including an immunoblotting assay that has identified autoantibodies to a 49-kD cytosolic protein in some patients with isolated ACTH deficiency.

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Abbreviations

ACTH corticotropin
LyH lymphocytic hypophysitis

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Lymphocytic hypophysitis (LyH) was the last of the autoimmune endocrinopathies to be described [1]. The classic presentation is that of a young woman in late pregnancy or in the postpartum period with symptoms of a pituitary tumor—headaches and visual disturbances with hypopituitarism. Although certain clinical features may help distinguish this condition from pituitary adenoma and Sheehan's syndrome, diagnosis still rests on biopsy. Some new antipituitary autoantibody tests have been developed [2–4], but these do not yet provide definitive preoperative diagnosis.

This review concentrates on the advances in clinical diagnosis and treatment of the condition since the major reviews by Cosman *et al.* in 1987 [5] and Parent in 1990 [6] and provides discussion of the current status of antipituitary autoantibody testing.

Clinical spectrum

The identification of LyH as a clinical entity is relatively recent. In 1980 the first premortem case was recognized by surgical biopsy [7]. There are now 93 biopsy-proven cases in the English literature [5,6,8••,9–33,34••,35•,36,37•,38,39•,40–44]; the largest is a series by Thodou *et al.* [8••] from Toronto, Canada. Another 20 cases of suspected LyH [13,45,46•,47–59] lack biopsy proof but represent the subacute spectrum of the disease, in which spontaneous regression of the pituitary mass and recovery of pituitary function are more likely. Chronic forms of the condition, which may include some cases of isolated corticotropin (ACTH) deficiency [3,51,60] and the empty-sella syndrome [50], will become better classified as antipituitary autoantibody assays are refined (Table 1).

As in some other autoimmune conditions, LyH occurs predominantly in women ranging in age from 15 to 74 years [34••,61]; two thirds of the cases related to pregnancy. Bettendorf *et al.* [62] reported the case of a 7-year-old prepubertal girl. Seventeen cases have been reported in men between the ages of 27 and 74 years [5,8••,9,10,14,19,23,24,27,34••,36,39•,40,63].

Mass effect

The symptoms and signs of pituitary enlargement secondary to autoimmune LyH are indistinguishable from those of any expanding intrasellar mass with or without suprasellar extension. Extension into the cavernous sinuses with cranial nerve palsies [13,14,34••], internal carotid artery occlusion [11,19], meningoencephalitis [64], and sixth nerve palsy with

raised intracranial pressure and papilledema [7] have been reported.

Endocrine effects

The two distinctive features of the hypopituitarism associated with LyH are the predilection for corticotroph destruction and extension of the disease into the pituitary stalk and neurohypophysis, causing diabetes insipidus (Table 1).

Corticotropin deficiency

Unrecognized secondary adrenal insufficiency has been the major cause of mortality in LyH [1,5,8••]. Isolated ACTH deficiency is a rare condition, but it is the most common form of isolated anterior pituitary hormone deficiency seen in these patients [65,66], whereas ACTH is often the last hormone to be affected by pituitary tumors. Hypoglycemia in late pregnancy is an indicator of ACTH deficiency [67], as are falling insulin requirements in the pregnant diabetic patient and prolonged postpartum malaise [40]. The detection of antipituitary autoantibodies in patients with isolated ACTH deficiency [3,60,68•] and its association with autoimmune diseases [69] further support a more central role for the corticotroph in the autoimmune process.

Diabetes insipidus

It is surprising that the association between LyH and diabetes insipidus was not reported until 1987 [64], but there are now 22 reported cases [8••,9,11,14,20, 22–28,38,40,43,45,64]. If present, diabetes insipidus is a major distinguishing feature from pituitary tumors. Even patients with very large primary pituitary tumors rarely present with diabetes insipidus.

The new concept of infundibuloneurohypophysitis [9,23], in which inflammation is confined to the pituitary stalk and neurohypophysis, as a distinct entity is controversial. There are two case reports of LyH being confined to the suprasellar area [8••,10]. Similarly, necrotizing infundibulohypophysitis [24] may be part of the disease spectrum.

Granulomatous diseases such as sarcoidosis, tuberculosis, and syphilis have a predilection for the pituitary stalk, causing diabetes insipidus. Although it is unusual, these diseases have been reported to cause a simultaneous pituitary mass [70]. Langerhans cell histiocytosis and germinoma also occur in the pituitary stalk.

Other hormonal effects

Prolactin levels can vary from normal to elevated or deficient in approximately 30% of cases apiece [5,8••]. Hyperprolactinemia is physiologically normal in pregnancy and lactation but otherwise may be caused by stalk pressure. Prolactin levels are not extremely elevated [8••]. Failure to lactate post partum is common with hypophysitis but may occur with any cause of hypopituitarism, including Sheehan's syndrome.

Table 1

Disease spectrum: clinical features suggestive of lymphocytic hypophysitis

Acute disease
Pituitary mass and/or hypopituitarism associated with
Pregnancy
Autoimmune disease
Diabetes insipidus
Subacute disease
Postpartum hypopituitarism (may be transient)
Differential diagnosis of Sheehan's syndrome
Spontaneous regression of a pituitary mass
Chronic disease
Isolated ACTH deficiency
Empty-sella syndrome

ACTH–corticotropin.

Thyroid function may be abnormal as a result of coexistent autoimmune thyroid disease, including transient postpartum thyrotoxicosis [8••,49] with hypercalcemia [46•,52] or secondary hypothyroidism [5,8••].

Growth hormone levels may be normal or low [5,8••]. Elevated levels occur with an associated adenoma (*see* "Association with tumors").

Gonadotroph function is often preserved except with panhypopituitarism. Successful pregnancy has followed a diagnosis of suspected [46•,47,48,59] and proven LyH [33,66].

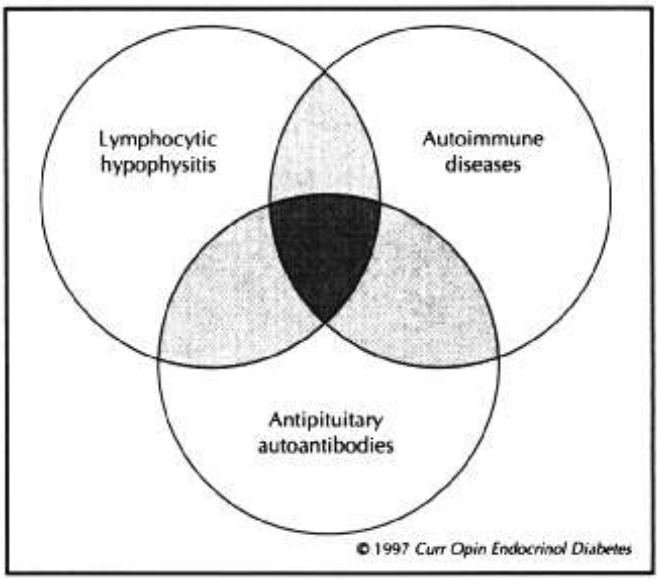

Fig. 1. Proposed schema for pituitary autoimmune disease.

Association with autoimmune disease

Lymphocytic hypophysitis has been associated with other autoimmune conditions in 20% to 25% cases [5,8••], characteristically with Hashimoto's thyroiditis and transient postpartum thyroiditis (Fig. 1).

Association with tumors

Lymphocytic infiltration to a degree considered typical of LyH has been reported in association with tumors (somatotroph adenoma [16,33,40] and craniopharyngioma [39•]). Sautner *et al.* [39•] have suggested that this phenomenon be called "secondary hypophysitis." The biologic significance of this inflammation is unknown but may be akin to the "tumor lymphocytes" seen in breast cancer and other malignancies.

Imaging investigations

The first CT scan report of LyH in 1980 could not distinguish it from a pituitary adenoma [7]. There are features emerging on CT and magnetic resonance imaging that are suggestive of hypophysitis, although they are not diagnostic (Table 2).

Computed tomography

In the early phases of the disease, the pituitary gland is enlarged owing to inflammation. The mass usually enhances intensely with contrast and is homogeneous [34••], neither of which distinguishes it from an adenoma. In pregnancy it can mimic lactotroph cell hyperplasia [71]. The gland may vary in size from slightly bulky with a convex upper border to a mass with suprasellar extension [10]. In some cases it may appear normal. Unusual features that should alert the clinician to the diagnosis of LyH include stalk enlargement and extension of contrast enhancement up into the infundibulum and hypothalamus or into the cavernous sinus laterally.

Magnetic resonance imaging

On T_1-weighted precontrast images, LyH appears as an isointense mass (relative to gray matter), which may extend into the suprasellar region causing chiasmal compression [8••,34]. Extension of the lesion into the pituitary stalk, with enlargement of the stalk or obliteration of the recesses of the anterior third ventricle, is highly suggestive of infundibuloneurohypophysitis [34••]. Diabetes insipidus may be associated with the absence of the high-intensity signal of the neurohypophysis [23], but not in all cases.

Table 2

CT and magnetic resonance imaging: features suggestive of lymphocytic hypophysitis

Pituitary mass with stalk thickening and diabetes insipidus
Intense contrast enhancement–usually homogeneous
"Ring enhancement"
Enhancement extends into
The stalk, infundibulum, and/or hypothalamus
The dura ("dural tail")
The cavernous sinus with carotid artery involvement
No calcification

Table 3

Terms used in the literature to describe inflammatory conditions of the pituitary

Terminology	First used by	Year
Anterior hypophysitis	Goudie and Pinkerton [1]	1962
Hypophysitis	Hume and Roberts [see 5]	1967
Lymphoid hypophysitis	Lack [see 5]	1975
Lymphocytic adenohypophysitis	Quencer [see 5]	1979
Lymphoid adenohypophysitis	Mayfield *et al.* [7]	1980
Lymphocytic hypophysitis	Topliss and Volpe [see 5]	1981
Lymphocytic infundibuloneurohypophysitis	Kojima *et al.* [9]	1989
Lymphoplasmacytic hypophysitis	Blisard *et al.* [22]	1992
Necrotizing Infundibulohypophysitis	Ahmed *et al.* [24]	1993
Granulomatous and lymphocytic hypophysitis	McKeel [76] Miyamoto *et al.* [75]	1983 1988
Giant cell granuloma of the anterior pituitary	Oelbaum and Wainwright [see 39•]	1950
Granulomatous hypophysitis	Kiaer and Norgaard [74]	1969
Secondary hypophysitis (tumor related)	Sautner *et al.* [39•]	1995
Pituitary abscess	Whalley [see 39•]	1952

Contrast (gadolinium, gadopentetic acid, or Magnevist; Berlex Imaging, Wayne, NJ) produces intense enhancement, which is usually homogeneous and sometimes appears more intense than is seen with pituitary tumors [34••,44]. "Ring enhancement," in which enhancement is confined to the periphery of the lesion [27,34••], and extrapituitary enhancement along the dura mater ("dural tail") and into the cavernous sinus or up into the hypothalamus are suggestive of hypophysitis [23]. Enhancement of the cavernous sinus [19] with occlusion of one or both internal carotid arteries [11,31] has been seen and has correlated with extensive fibrosis sometimes found during surgery [28].

These features may also be seen with complicated or invasive pituitary adenomata [34••] and with granulomatous hypophysitis or pituitary abscess.

Evolution of disease

A pituitary mass caused by LyH may persist for at least 8 years [30]. Resolution of the mass in the postpartum period, to either a normal gland or progression to an empty sella [40,48,50,55], has been shown by sequential CT and magnetic resonance imaging scans [18,72].

Pathologic findings

Gross appearance

The appearance of the pituitary gland at autopsy usually reveals atrophy [1,5,8••]. Neurosurgical descriptions are of an enlarged pituitary gland that does not look like a

pituitary adenoma, with a consistency varying from soft to densely fibrotic [28]. The dura is often markedly adherent [27].

Histopathology

The different terms used to describe autoimmune pituitary disease and other inflammatory conditions are summarized in Table 3. The inflammatory infiltrate consists of lymphocytes, plasma cells, and scattered eosinophils [5,8••,73]. Foci of necrosis have been reported in classic cases of LyH [8••] and in turn have been associated with isolated giant cells [5]. Thus, necrotizing infundibulohypophysitis [24] may prove to be part of the spectrum, depending on whether one is a "lumper" or "splitter."

There are cases in the literature in which granulomatous hypophysitis is associated with otherwise classic lymphocytic thyroiditis and adrenalitis [74] or coexistent extensive lymphocytic infiltration of the pituitary [75,76]. Whether giant cells alone are enough to rule out the diagnosis of LyH is controversial.

Granulomatous hypophysitis due to sarcoidosis, tuberculosis, syphilis, actinomycosis, or Wegener's granulomatosis is obviously outside the scope of this review, although pulmonary sarcoidosis has been associated with LyH [42].

Immunohistochemistry

The lymphocytic infiltrate in LyH is consistent with an organ-specific autoimmune disease and has been studied primarily in patients undergoing transsphenoidal biopsy in the earlier phases of the disease. The infiltrate contains T lymphocytes that are predominantly CD4+ cells with a CD4:CD8 ratio of 2:1 [12,26,31,66,68•]. In one postpartum case, CD8+ cells were more common [32]. Plasma cells, B cells, and eosinophils were also present in the infiltrate.

Electron microscopy

Ultrastructural studies have shown lymphocytes [8••,26,77] and plasma cells [66,78] interdigitating with adenohypophyseal cells, some of which are being phagocytosed [66]. The electron micrograph in Figure 2 shows not only the close proximity of a lymphocyte to an adenohypophyseal cell but also that the lymphocyte is actively communicating with it. Taken together, these findings suggest that the hypopituitarism of hypophysitis is caused by T-cell–mediated cytotoxicity within the pituitary.

Antipituitary autoantibodies

The other arm of the immune system is the B-cell–mediated or humoral response, which produces autoantibodies in the sera of patients with autoimmune disease. Proteins targeted by these autoantibodies are called *autoantigens.* Autoantibodies to anterior pituitary tissue have been detected using indirect immunofluorescence on a variety of substrates, including human pituitary (fresh-frozen surgical tissue [79], autopsy glands, or fetal pituitaries [2]), primate glands (baboon, rhesus, and cynomolgus monkey [79]), nonprimate pituitaries (rat, guinea pig [80], and pig [81]), and finally certain cell lines (murine AtT20 cells and rat GH_3 cells [3,82]).

Apart from the issues of species specificity and the presence of cell surface Fc receptors on corticotrophs, the main problem has been that antipituitary autoantibodies have been detected by immunofluorescence in only three

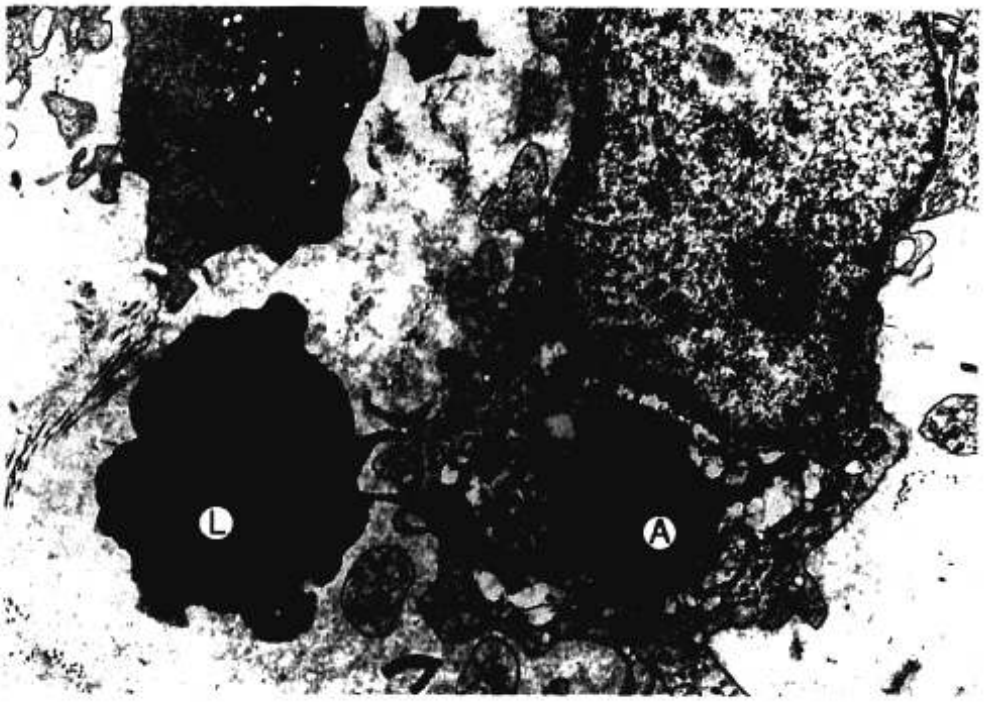

Fig. 2. The ultrastructural appearance of lymphocytic hypophysitis. Electron microscopy demonstrates a lymphocyte *(L)* extending out to embrace a degenerating adenohypophyseal cell *(A).*

patients with histologically proven LyH [7,83,84]. Perhaps there is a window when autoantibodies appear, akin to rubella-induced experimental hypophysitis [85], although no data in the human literature address this question.

Biopsy proof has been lacking in patients with isolated ACTH deficiency and the empty-sella syndrome, both of which are believed to be end-stage manifestations of the disease. There are reports of autoantibodies in these conditions using rat and mouse substrates [3,60,86], the validity of which has been challenged on the basis of species specificity [87]. With an alternative approach of immunoblotting human autopsy glands, antipituitary autoantibodies to a 49-kD cytosolic protein have been detected in two of three patients with isolated ACTH deficiency [68], one of whom had been shown previously to have antibodies to 200-nm secretory granules in rat corticotrophs [60]. Immunoblotting and immunofluorescence tend to detect different epitopes and there is no proof as yet that the autoantibodies detected by these two methods are targeting the same protein.

According to Glück and Scherbaum [87], heterophile antibodies due to cross-species reactivity accounted for 5% to 14% of false-positive antipituitary autoantibodies. Porcine and fetal cynomolgus monkey tissue had the lowest cross-

Table 4

Steroid therapy in lymphocytic hypophysitis

Study	Clinical case	Steroid therapy	Progress	Comments
Mayfield *et al.* [7]	23-y-old woman, 7 mo post partum, pseudotumor cerebri, headache, 6th nerve palsy, papilledema, enlarged sella on skull radiography	Prednisolone, 60 mg/d Acetazolamide, 250 mg twice daily	Headache and abducens palsy resolved quickly; symptoms recurred 2 mo later, with hypopituitarism	Gritty, nonencapsulated pituitary mass; antipituitary antibody positive
Meichner [see 5]	24-y-old woman at 7 mo gestation, headache, diplopia on CT scan post partum, 2-cm intrasellar mass with suprasellar extension	Dexamethasone, 4 mg four times daily	Fewer headaches; superotemporal field defect, no change with therapy	Pituitary fibrosis; smooth muscle antibody positive; subnormal cortisol and growth hormone levels postoperatively
Pestell *et al.* [10] and Stelmach and O'Day [15]	22-y-old woman, bitemporal hemianopia at 30 wk gestation	Prednisolone, 15 mg twice daily Betamethasone, 0.5 mg twice daily	Improved over 5 wk, then relapsed	Hypophysitis in an ectopic suprasellar pituitary
Feigenbaum *et al.* [13]	30-y-old woman, multipara, at 36 wk gestation, bitemporal hemianopia	Prednisolone, 2.5 mg three times daily Bromocriptine, 2.5 mg twice daily	Vision improved 1 wk post partum; mass with suprasellar extension decreased	Subnormal cortisol and growth hormone levels
Nussbaum *et al.* [14]	40-y-old man with bilateral 6th nerve palsies and diabetes insipidus with cavernous sinus and stalk involvement	Dexamethasone, no dose published	Steroids stopped for *Staphylococcus aureus* septicemia; resumed for 3rd nerve palsy postoperatively	Developed avascular necrosis of both femoral heads as a complication of steroid therapy
Bitton *et al.* [18]	27-y-old woman at 24 wk gestation, headaches, bitemporal hemianopia	Postoperative hydrocortisone, 60 mg/d	Full recovery of vision by 1 mo and hypopituitarism by 2 mo	Pituitary mass, yellow, swollen, and soft
Reusch *et al.* [21]	29-y-old woman, primigravida, at 20 wk gestation, headache; at 27 wk gestation, bitemporal hemianopia	Dexamethasone, 4 mg/d for 5 d Bromocriptine, 2.5 mg/d	No response	Partial debulking of tumor
Ahmed *et al.* [24]	33-y-old man with frontal headaches, hypothyroidism, hypogonadism, and diabetes insipidus	Prednisone, 60 mg/d for 3 wk	Responded, but relapsed twice on tapering steroids; spontaneous regression	Necrotizing infundibulohypophysitis on biopsy
Nishioka *et al.* [28]	33-y-old woman, bitemporal hemianopia, diabetes insipidus, no lactation 5 d post partum	Prednisolone, 20 mg/d for 2 wk	No response	Massive fibrosis at operation; antipituitary antibody negative
Beressi *et al.* [29]	27-y-old woman 13 mo post partum	Prednisone, 60 mg/d	Relapsed 5 mo later	Biopsy confirmed lymphocytic hypophysitis
Thodou *et al.* [8]	41-y-old woman, postpartum headaches, visual loss	Decadron for 2 mo	No response, but progressive improvement off therapy	Biopsy only
	29-y-old woman, diabetes insipidus, visual loss, hyperprolactinemia	Therapeutic dose of steroids 1 y preoperatively	No response	–
Bettendorf *et al.* [62]	14-y-old girl with a 7-y history of progressive panhypopituitarism and diabetes insipidus	Dexamethasone	No response, went on to cranial irradiation	Biopsy confirmation of disease; cushingoid side effects

reactivity, and beef the highest. Their study consisted mainly of patients with pituitary tumors and none with LyH. These authors have previously reported a high percentage of pituitary autoantibodies, using human fetal pituitaries, in patients with Cushing's disease [2,88]. Antipituitary *hormone* antibodies have been associated with other pituitary tumors [86]. The significance of all these autoantibodies as well as their relation to LyH is not yet understood.

Reversible hypopituitarism after interferon-α therapy

Pituitary autoantibodies against GH_3 cells have been reported in a patient on interferon therapy for chronic hepatitis C associated with hypopituitarism [89•]. ACTH and thyroid-stimulating hormone deficiency resolved when therapy was ceased. Interferon is known to induce thyroid autoimmunity [90].

Virus-induced autoimmunity

Viruses have been implicated as triggers for autoimmune disease in susceptible individuals. Two animal models of virally induced pituitary autoimmunity have been reported.

In the most recent study by Yoon *et al.* [85], male golden Syrian hamsters developed LyH when injected with rubella virus E1 and E2 glycoproteins. Within 3 weeks of injection, 95% of the animals had autoantibodies against pituitary cells, as detected by immunofluorescence, declining to 20% by 8 weeks. All animals had diffuse inflammatory cell infiltration in the pituitary. The disease was almost completely prevented by neonatal thymectomy, but it could not be transferred passively by autoantibodies. This implies that LyH is a T-cell–mediated disease and that the autoantibodies may be merely transient epiphenomena. T-cell–transfer experiments have yet to be conducted.

In a second model, mice infected with reovirus type 1 developed an autoimmune polyendocrinopathy with autoantibodies directed against anterior pituitary, islets of Langerhans, and gastric mucosa [91]. Some of the pituitary autoantibodies cross-reacted with rat, pig, and human pituitary tissue, and some were directed against hormones, such as insulin and growth hormone.

Treatment

The majority of patients who present with a rapidly enlarging pituitary mass and visual impairment require surgical decompression [5,8••]. A conservative approach, including frozen section and minimal biopsy, has been advocated by many authors [8••,13,17] in a bid to preserve pituitary function. Spontaneous regression of the mass [45,47,57,58] and partial or total recovery of pituitary function are well documented, whereas surgical intervention has led to permanent hypopituitarism in all but a few cases [8••,72]. Relapse with progression to panhypopituitarism has been described. Bromocriptine may lower prolactin levels and improve visual field loss but does not produce a concomitant reduction in the mass [8••].

Therapy with steroids

Lymphocytic hypophysitis has a predilection for corticotroph destruction, so that the majority of patients with hypopituitarism have secondary adrenal insufficiency and require cortisol replacement [5,8••]. The rationale for using steroids as anti-inflammatory therapy is to reduce the size of the pituitary mass and avoid surgery.

Nine patients received anti-inflammatory doses of prednisolone, 60 mg/d, or dexamethasone, up to 4 mg/d, as summarized in Table 4. All but one responded with improvement of symptoms and signs. However, symptoms recurred when the dose was tapered or stopped. One patient went on to have spontaneous regression of the mass after two relapses off steroids [24]. Complications of dexamethasone therapy included avascular necrosis of both femoral heads [14] and marked cushingoid features [14,62]. No patient received lymphocytotoxic doses (up to 1 g) of prednisolone or methylprednisolone [92].

Four patients received hydrocortisone or prednisolone, equivalent to maintenance or stress replacement doses. Two of these women improved post partum, which was probably related to natural disease evolution rather than steroid use [13,18], and two showed no response [8••].

Conclusions

Lymphocytic hypophysitis is being recognized with increasing frequency and should be considered when a pituitary mass is associated with pregnancy, autoimmune disease, or diabetes insipidus. The development of a definitive preoperative serologic test is keenly awaited.

Acknowledgments

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Pituitary autoantibodies

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Purpose of review

The aim of this article is to review recent advancements in pituitary autoantibody assays.

Recent findings

The newest assay is based on the in-vitro transcription and translation of pituitary specific proteins followed by immunoprecipitation with patient sera. The two proteins, PGSF1a and PGSF2, were identified as pituitary specific from a human pituitary gland cDNA library. Autoantibodies were found in one patient with biopsy proven lymphocytic hypophysitis and seven with suspected hypophysitis, including idiopathic hypopituitarism. Patients with rheumatoid arthritis, especially if rheumatoid factor negative, also had autoantibodies to PGSF1a. An immunoblotting method identified the autoantigen enolase (both α and neuron-specific), as a marker of neuroendocrine autoimmunity but an in-vitro transcription and translation assay has shown that enolase autoantibodies are nonspecific. Enolase autoantibodies have also been found in Sheehan's syndrome. Immunoblotting identified a novel 36 kDa pituitary cytosolic autoantigen in adrenocorticotropin (ACTH) deficiency and pituitary membrane proteins of 68, 49 and 43 kDa in patients with lymphocytic hypophysitis. Indirect immunofluorescence using baboon pituitary has been revisited and somatotroph autoantibodies found in patients with idiopathic growth hormone (GH) deficiency. High titre antibodies were thought to be clinically significant. Enyme-linked immunosorbent assays using human pituitary adenoma cells or rat tissue have identified antibodies in patients with type 1 diabetes, Hashimoto's thyroiditis and various pituitary disorders but not hypophysitis.

Summary

The search for reliable and specific pituitary autoantibody markers continues.

Keywords

lymphocytic hypophysitis, pituitary autoantibodies, pituitary autoimmunity

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Abbreviations

ELISA	enzyme linked immunoabsorbent assay
GAD	glutamic acid decarboxylase
ICA	islet cell antibody
ITT	in-vitro transcription translation

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Introduction

A sensitive and specific diagnostic assay for pituitary autoantibodies is keenly awaited. Such an assay (or assays) would be particularly useful for clinicians managing patients with atypical pituitary masses, peripartum hypopituitarism or idiopathic hypopituitarism with or without an empty sella [1]. These case scenarios represent the spectrum of autoimmune pituitary disease or lymphocytic hypophysitis, from its acute presentation mimicking a pituitary adenoma, through to sub-acute and chronic forms. The classical sub-acute presentation is a woman in the peripartum period presenting with a pituitary mass and hypopituitarism that spontaneously resolve. Chronic manifestations (e.g. empty sella syndrome) are more difficult to define because such patients rarely, if ever, undergo pituitary biopsy. Until there is a suitable assay, the gold standard for the diagnosis of lymphocytic hypophysitis will remain pituitary biopsy.

The recent advancements in the field have centred on the development of new assay techniques incorporating molecular technology and immunoprecipitation. Some new autoantigens have been identified but none as yet are confirmed as completely pituitary specific [2]. Immunoblotting has also identified some novel autoantigens in ACTH deficiency and hypophysitis [3••]. Enzyme linked immunoabsorbent assay (ELISAs) have been developed in Japan [4,5] and indirect immunofluorescence has been revisited [6] with interesting results in patients with idiopathic growth hormone deficiency. A summary of the techniques and substrates used for their detection is given in Table 1 [2,3••,4–7,8•,9–23].

The concept of 'organ-specific autoimmune endocrinopathies'

The autoimmune endocrinopathies are regarded as organ-specific diseases from 'the traditional' viewpoint and lymphocytic hypophysitis is considered part of this group. Classic examples are Hashimoto's thyroiditis, Addison's disease, type 1 diabetes mellitus and Graves'

Table 1 Studies on pituitary autoantibodies published in the last 10 years

Technique	Tissue substrate	References
Indirect immunofluorescence	Rat pituitary	Fetissov *et al.* 2002 [7]
	Baboon pituitary	De Bellis *et al.* 2003 [6]
		De Bellis *et al.* 2005 [8•]
Immunoblotting	Human autopsy cytosolic or membrane tissue preparations	Crock 1998 [9]
		Strömberg *et al.* 1998 [10]
		Nishiki *et al.* 2001 [11]
		Takao *et al.* 2001 [12]
		Goswami *et al.* 2002 [13]
		O'Dwyer *et al.* 2002 [14]
		O'Dwyer *et al.* 2002 [15]
		Bensing *et al.* 2004 [16]
		Bensing *et al.* 2005 [3••]
	Rat tissue preparation	Yabe *et al.* 1998 [5]
		Kikuchi *et al.* 2000 [17]
		Nishino *et al.* 2001 [18]
	Porcine tissue preparation	Kobayashi *et al.* 1997 [19]
		Kobayashi *et al.* 1998 [20]
ELISA	Human adenoma cell line	Keda *et al.* 2002 [4]
	Rat pituitary cells	Yabe *et al.* 1998 [5]
		Kikuchi *et al.* 2000 [17]
		Nishino *et al.* 2001 [18]
		Keda *et al.* 2002 [4]
	Porcine pituitary cells	Kobayashi *et al.* 1998 [20]
ITT and immunoprecipitation of pituitary proteins	Human cDNA library	Tanaka *et al.* 2002 [2]
		Tanaka *et al.* 2003 [21]
		Tanaka *et al.* 2003 [22]
		Tatsumi *et al.* 2003 [23]

ELISA, enzyme linked immunoabsorbent assay; ITT, in-vitro transcription translation.

disease. The corresponding target autoantigens are tissue-specific or cell-specific enzymes: thyroid peroxidase [24], 21-hydroxylase [25], glutamic acid decarboxylase 65 (GAD65) [26], hormones: (insulin) [27] or receptors: thyrotropin receptor (TSHR) [28] respectively. Conversely, ubiquitous antigens may be the target of autoantibodies that are 'organ-specific', such as transglutaminase in coeliac disease [29] and pyruvate dehydrogenase in primary biliary cirrhosis [30].

Yet on closer inspection, this concept of 'specificity' does not hold completely true. Islet cell antibodies (ICAs) detected by immunofluorescence in patients with type 1 diabetes recognize not only insulin-secreting β cells but react with islet α cells, δ cells, those making pancreatic polypeptide [31•] and multiple pituitary cells [32]. Preabsorption of these sera with GAD and islet cell antigen (IA2) does not abolish ICAs suggesting that there are other relevant islet cell autoantigens that are of sialoglycolipid nature [33•]. In Hashimoto's thyroiditis, patients can rarely develop an encephalopathy picture that appears to correlate with their thyroid autoantibody status and not some concurrent neurological condition [34,35]. Patients with Graves' disease have been shown to have antibodies that cross-react with pituitary cells [36].

Translating 'traditional' logic to pituitary autoimmunity or lymphocytic hypophysitis, the target autoantigens should be cell-specific enzymes, hormones or receptors. The enzymes in the pituitary are present in hypothalamic tissue and neuroendocrine tissues throughout the body, for example neuron-specific enolase [14], prohormone convertase 1/3, neuroendocrine protein 7B2, [23] and the family of carboxypeptidases [37]. Although we identified enolase as a target autoantigen in lymphocytic hypophysitis, reactivity is found in the sera of many other patients, including 20–46% of those with pituitary adenoma [9,21] and at low titres in 5–10% of normal controls. Proteins found to be pituitary-specific (PGSF1a and PGSF2) from a human pituitary cDNA library [2] were targeted by sera from some Japanese patients with hypophysitis and hypopituitarism [38]. Subsequent experiments showed that PGSF1a was also recognized by sera from patients with rheumatoid arthritis [22]. There is past and recent evidence that growth hormone can be a target autoantigen [12,17] but there is no body of work on any receptors as potential targets in hypophysitis.

Finally, different arms of the immune system interact with target antigens in different ways. Epitopes are the components of antigen that are recognized by the immune system. Epitopes are recognized as linear epitopes by T cells, whilst conformational ('three-dimensional') structures are the typical target of B cell or autoantibody recognition. Critically, the major 'endocrine' autoantigens, thyroid peroxidase [24], adrenal 21-hydroxylase [25] and GAD65 [26], all have such complex three-dimensional binding sites. In addition, the sites recognized by patient

sera are different from those recognized by normal control sera at low titres [39]. The significance of this differential epitope mapping is unclear.

The latest assays in development will be discussed in the context of the above comments, including a brief section on technical issues in the laboratory.

In-vitro transcription translation and immunoprecipitation of pituitary proteins

The newest assay for pituitary autoantibodies involves the production of recombinant pituitary proteins *in vitro* using rabbit reticulocyte lysate. Methionine residues in the proteins are labelled with ^{35}S and the proteins then used in an immunoprecipitation step with patient sera and protein-A sepharose [40]. This approach implies that the target autoantigen has already been identified in some way. Tanaka *et al.* [2] chose two novel pituitary gland specific factors, PGSF1a and PGSF2, as potential candidates because of their pituitary specific tissue expression. They also tested enolase [21] and growth hormone in this system on the basis of our original immunoblotting results [9] and on previous work from Japan, identifying growth hormone as the 22 kDa protein autoantigen on immunoblots [12]. Subsequently, other potential candidates, the pro hormone-processing enzymes prohormone convertases 1/3 and 2, carboxypeptidase E (CPE) and prohormone convertase 2 regulatory protein 7B2 were studied [23]. Again, these are not pituitary specific.

One of three patients with biopsy proven hypophysitis and two patients with isolated ACTH deficiency had positive antibody indices to PGSF1a. Further studies have shown that PGSF1a can also be a target autoantigen in rheumatoid arthritis [22], particularly in patients with more erosive disease and in four of eight patients who were rheumatoid factor negative. This assay appears promising but studies of pituitary function in rheumatoid patients are needed to confirm the specificity of the assay. Two of 14 patients with suspected hypophysitis or infundibulohypophysitis and three of 14 patients with hypopituitarism had reactivity to PGSF2. Anti-growth hormone antibodies were detected in four patients with suspected hypophysitis or hypopituitarism and in two patients with other autoimmune diseases, but the antibody indices were relatively low with none above two. No patients with a pituitary adenoma had positive indices to any of these autoantigens [2]. Two of 14 patients with lymphocytic hypophysitis had a PC1/3 autoantibody index over 1.5 but so did five of 11 patients with nonfunctioning pituitary macroadenoma [23].

A separate publication by Tanaka *et al.* [21] looked at enolase in the in-vitro transcription translation (ITT) assay. They confirmed similar results to our immunoblotting study, demonstrating positive autoantibodies in 41% of patients with lymphocytic hypophysitis, 20% with other autoimmune diseases and 4.3% of healthy controls but found even higher levels (46% versus 20% with immunoblotting) of autoantibodies in patients with pituitary adenoma. Reactivity to enolase cannot be used as a specific marker in pituitary autoimmunity but such a high level in tumour patients also raises the question of its use as an indicator of an autoimmune diathesis.

Theoretically, the ITT assay could be used to express any number of possible target autoantigens. Some autoantigens, such as GAD in type 1 diabetes, can best be detected by immunoprecipitation [26], as they are not recognized by patient sera in their denatured form. ITT has, however, been problematic with certain proteins. In the case of the thyrotropin receptor (TSH), it was possible to obtain adequate amounts of protein but not the normal highly glycosylated form which is required for conformational binding of Graves' sera [41]. The addition of canine pancreatic microsomes had been used by Li *et al.* [42] to improve production, and presumably folding, of the autoantibody reactive calcium-sensing receptor but was ineffective for TSHR. Expression of adequate amounts of bioactive TSH receptor could only be obtained after transfection into a leukaemia cell line [43]. Future studies with potential pituitary target autoantigens will need to consider these technical issues.

Immunoblotting

The immunoblotting assay was developed to provide an alternative approach to immunofluorescence [44]. Immunoblotting identifies target autoantigens based on linear epitopes and characterizes them by molecular weight. The proteins are in a denatured form and there may be hundreds of proteins represented at any particular molecular mass on a gel. Nevertheless, this technique enables the purification of relevant target proteins using column chromatography and immunoblotting of each protein fraction as was described with enolase [14]. It can also be used to characterize isoforms on two-dimensional gels [15]. In this latter paper, serum from a peripartum woman with lymphocytic hypophysitis recognized neuron-specific enolase in both the placenta and pituitary. This observation provides an intriguing link to the frequent presentation of lymphocytic hypophysitis in pregnancy and to a recent study in Sheehan's syndrome [13]. In a large series of Indian women with true Sheehan's syndrome Goswami *et al.* [13] found 12 of 19 or 63.1% had developed antienolase antibodies compared with 17.8% (five of 28) of women with normal pregnancies and 14.2% (four out of 28) of women who had never conceived. The evolution of their hypopituitarism was often over many years rather than immediately postpartum, which supported the theory that the insult

at the time of pregnancy triggered a subsequent autoimmune process.

In 2001, Takao *et al.* [12] identified autoantibodies to a 22 kDa human pituitary cytosolic protein in 73% or 11 of 15 patients with lymphocytic hypophysitis and 77.8% or seven of nine patients with isolated ACTH deficiency. Sequencing of this protein showed it to be growth hormone. Interestingly nine of the 11 patients with positive results had growth hormone deficiency on formal testing, supporting a pathogenic role for these antibodies. Kobayashi *et al.* [20] made the interesting observation that preabsorption with pancreatic antigens, but not liver, spleen or kidney extracts, abolished pituitary autoantibody reactivity to the 22 kDa protein. This data correlates well with Bottazzo's observations [32] in patients with diabetes and positive ICA, whose sera also cross-reacted with pituitary proteins.

Recent studies have identified a novel 36 kDa pituitary cytosolic autoantigen in patients with ACTH deficiency (12 of 65 or 18.5% versus two of 57 or 3.5% in healthy controls, statistically significant $P < 0.021$) [3••]. ACTH deficiency is a prominent feature of lymphocytic hypophysitis and isolated ACTH deficiency is known to co-exist with several other autoimmune disorders [45]. In this large series of patients collected over many years in Poland, 61 of 65 had isolated ACTH deficiency, 51% had another autoimmune disease and 85% (55 of 65) had positive thyroid autoantibodies [3••]. Patients with autoantibodies to the 36 kDa protein had a higher frequency of thyroglobulin autoantibodies than the patients who were not immunoreactive to the 36 kDa protein. Studies in other pituitary diseases and identification of the 36 kDa autoantigen are necessary before further conclusions can be drawn from these results.

The empty sella syndrome is not a homogenous entity and in some cases it may represent the fibrotic end-stage of lymphocytic hypophysitis. Bensing *et al.* [16] described a group of patients with empty sella syndrome who did not have evidence of high titre pituitary autoantibodies. The fascinating observation in this group was that 15 of 30 patients had Type 2 diabetes or impaired glucose tolerance and a body phenotype of central obesity. One could speculate that these patients represent a phenotype of 'a hypothalamic syndrome with centrally mediated diabetes' and secondary pituitary atrophy, but not underlying autoimmunity.

Studies looking at human pituitary membrane antigens are limited. Nishiki *et al.* [11] identified specific antibodies to 68, 49 or 43 kDa proteins in five of 13 patients with lymphocytic hypophysitis, one of 12 patients with infundibuloneurohypophysitis and none of four patients with isolated ACTH deficiency. These proteins are of interest but have not yet been further characterized.

There are several technical considerations with immunoblotting. The quality of the pituitary tissue preparation is critical. Autopsy tissue taken more than 24 h *post mortem* is likely to be markedly autolysed and to give poor results. Dissection and homogenization of tissues needs to be done at 4°C and in the presence of a cocktail of antiproteolytic enzymes [44]. In the centrifugation process, the initial low speed pellet containing nuclear debris and mitochondria is discarded, so any autoantigen in this fraction would be lost. The pituitary contains at least five different hormone secreting cell types and some such as thyrotrophs and corticotrophs, make up less than 5–10% of the gland volume. Conceivably, a relevant target autoantigen from one of these cell types may be present in such small quantities in pituitary tissue preparations as to be undetectable.

Finally, the quality of both primary and secondary antibodies is important. Primary antibodies are those in patient sera and they tend to give very high background activity if the sera have not been stored optimally (personal observation).

Indirect immunofluorescence

In 1975 Bottazzo *et al.* [46] first described autoantibodies to pituitary prolactin-secreting cells using indirect immunofluorescence in 19 of 287 patients with endocrine autoimmunity but no hypopituitarism. In general, the titre of pituitary autoantibodies found by immunofluorescence was low. The immunofluorescence assay has been the most widely used technique but very few patients with biopsy-proven and suspected lymphocytic hypophysitis have been studied and the results have been particularly disappointing and unilluminating. Immunofluorescence recognizes the conformational structure of antigens and has the advantage of identifying the pituitary cell type and subcellular structures that are targeted by pituitary autoantibodies, but it cannot identify the target autoantigen proteins themselves.

The choice of pituitary substrate is critically important but problematic. Although fresh human tissue would be ideal, the ethical issues of using fetal glands and the limited supply of surgical tissue make this untenable. Bottazzo's original publications concluded that baboon pituitary was the most suitable alternative [46]. A detailed species specificity study outlining the problems of heterophile antibodies was published by Gluck and Scherbaum in 1990 [47]. Human sera positive for pituitary autoantibodies on human fetal substrate were only recovered 4% with adult baboon, 0% with fetal cymologous monkey, 20% with porcine, 11% with bovine,

11% with ovine and 7% with rat tissue, suggesting the use of animal tissue produced results with no clinical significance. Heterophilic antibodies to the animal substrates were also detected at a rate of 4–15%. The extent of this nonspecific species cross-reactivity can also be seen in immunoblotting experiments [9]. Important autoantigens, however, usually are conserved across species.

Recently De Bellis and colleagues [6] have revisited indirect immunofluorescence using cryostat sections from young baboon pituitary glands. They found high titre pituitary autoantibodies in 33% of patients with idiopathic GH deficiency and low titres in six of 20 patients with adenoma. Twenty-two percent of patients with autoimmune endocrine diseases had antibodies (40/180) of whom five had high titres. High titres were universally associated with severe isolated GH deficiency and the target cells were the somatotrophs, whereas low titres appeared to have no effect on pituitary function.

Table 2 Pituitary autoantigens identified to date and the methodology employed for their detection

Autoantigen	Technique	References
49 kDa protein	Immunoblotting	Crock 1998 [9] Nishiki *et al.* 2001 [11]
α-enolase	Immunoblotting ITT	O'Dwyer *et al.* 2002 [14] Tanaka *et al.* 2003 [22]
γ-enolase	Immunoblotting	O'Dwyer *et al.* 2002 [14]
43 kDa protein	Immunoblotting	Nishiki *et al.* 2001 [11]
68 kDa protein	Immunoblotting	Nishiki *et al.* 2001 [11]
22 kDa	Immunoblotting	Kikuchi *et al.* 2000 [17]
Growth hormone	Immunoblotting ITT	Takao *et al.* 2001 [12] Tanaka *et al.* 2002 [2]
PGSF1a	ITT	Tanaka *et al.* 2002 [2] Tanaka *et al.* 2003 [21]
PGSF2	ITT	Tanaka *et al.* 2002 [2]
Prohormone convertase 1/3	ITT	Tatsumi *et al.* 2003 [23]
Prohormone convertase 2 regulatory protein, 7B2	ITT	Tatsumi *et al.* 2003 [23]
36 kDa	Immunoblotting	Bensing *et al.* 2005 [3••]

ITT, in-vitro transcription translation.

Enzyme linked immunoabsorbent assay

An ELISA was first developed in Japan for pituitary autoantibody detection [5]. Using rat pituitary homogenate as an antigen source, pituitary autoantibodies were detected in patients with type 1 diabetes [5], autoimmune thyroiditis [18] as well as various pituitary disorders [17]. This research group has also found the prevalence of pituitary autoantibodies to be significantly higher in type 2 diabetes patients than in control subjects using porcine instead of rat pituitary as antigen [20].

Keda *et al.* [4] measured autoantibodies to cell surface antigens of human pituitary adenoma cells and rat pituitary cells with a cellular variant of an ELISA. In this study, patients with idiopathic hyperprolactinemia or idiopathic isolated GH deficiency had autoantibodies more frequently to prolactin-secreting cells and GH-secreting cells respectively, than patients with other forms of pituitary diseases.

No sera from biopsy-proven lymphocytic hypophysitis patients have been tested using ELISA methodology.

Autoantibodies to pituitary hormones

In the original immunoblotting method paper [44], preabsorption studies showed that in children with GH deficiency, pituitary membrane and cytosolic autoantibodies were not targeting growth hormone itself. De Bellis *et al.* [6] showed that 33% of patients with isolated GH deficiency of childhood onset, had somatotroph cell, but not GH, autoantibodies.

Nevertheless, there are a number of studies showing that pituitary hormones themselves can be targets, just as insulin is a recognized autoantigen in type 1 diabetes. Mau *et al.* 1993 [48], demonstrated anti-ACTH and anti-growth hormone antibodies in two of six patients with empty sella syndrome and anti-ACTH and anti-TSH antibodies in three of five patients with pituitary tumours. No antibodies were found in six controls and there was no correlation with hormonal status. Autoantibodies reacting with ACTH have also been reported in patients with eating disorders as well as in some healthy controls [7].

Immunoblotting studies from Kikuchi *et al.* [17] and Takao *et al.* [12] identified a 22 kDa protein as a target autoantigen and they subsequently showed this to be growth hormone. To our knowledge there are no similar studies looking at prolactin.

The autoantigens that have been characterized so far are summarized in Table 2.

Conclusion

That a single diagnostic assay will cover the broad clinical spectrum of lymphocytic hypophysitis is unlikely. There is strong evidence that there are multiple autoantigens in lymphocytic hypophysitis. The pituitary autoantibodies in a patient with lymphocytic hypophysitis and isolated ACTH deficiency are almost certainly going to be different to those from a patient with isolated TSH deficiency or panhypopituitarism and the empty sella syndrome. The challenge is to match the target autoantigens with each scenario.

Acknowledgements

We thank Professor Rodney Scott and Dr Glenn Reeves for constructive comments on the manuscript.

References and recommended reading

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- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 401).

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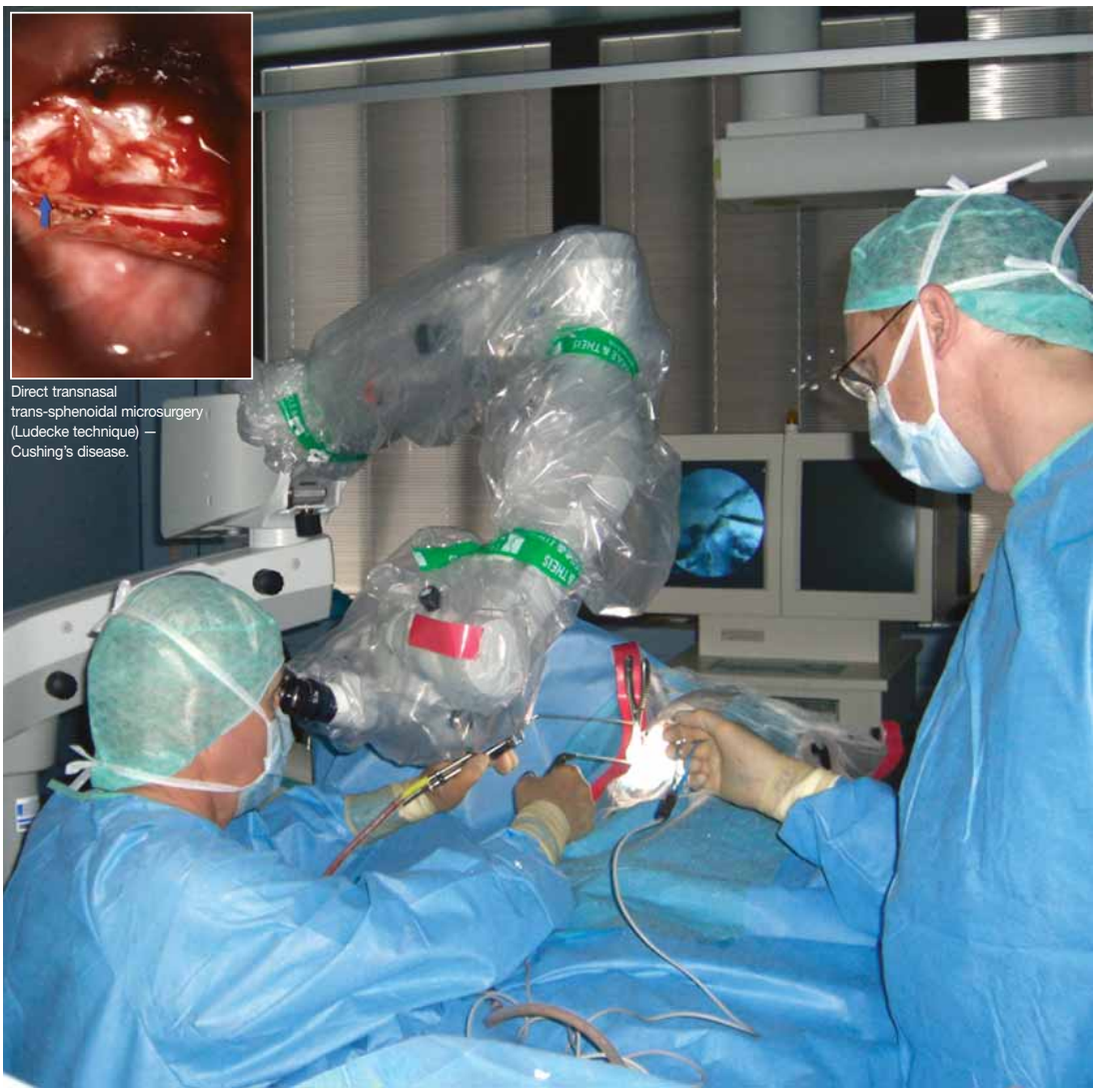

Direct transnasal trans-sphenoidal microsurgery (Ludecke technique) — Cushing's disease.

Paediatric pituitary disorders

Background

IT is now 30 years since Drs Wettenhall and Vines formed the Australasian Paediatric Endocrine Group. In the past three decades the diagnosis of pituitary conditions has improved significantly, with the availability of new, sensitive hormonal assays and MRI. The molecular basis for congenital hypopituitarism and pituitary tumourigenesis is being unravelled and therapeutic growth hormone is no longer derived from autopsy pituitaries. Trans-sphenoidal pituitary neurosurgery (also referred to as transnasal trans-sphenoidal neurosurgery) performed in specialist centres achieves high rates of remission if the tumours are diagnosed as microadenomas (<1cm) or at a resectable stage. There are new medications to control some of these tumours.

Pituitary problems in paediatrics are rare in general practice, but if diagnosed in time, significant morbidity and life-threatening situations can be avoided. Accurate height and weight measurements taken regularly over time are critical to decision making. An orchidometer for pubertal staging in boys is also helpful. We outline the important clinical signs and symptoms, including laboratory tests and imaging and how to interpret them.

Imaging of the pituitary gland and hypothalamus

Today, MRI provides pictures of the brain and pituitary in exquisite detail, and CT, with its lower resolution, is often unnecessary radiation.

The range of scan abnormalities in a child with hypopituitarism includes a normal or hypoplastic pituitary gland. Small glands are particularly seen in children with panhypopituitarism or with GH deficiency, as GH-producing cells make up almost two-thirds of the gland. A mere rim of tissue is called CSF sella (CSF occupies the empty sella) or empty sella syndrome, which paradoxically may not be associated with glandular hypofunction.

Pituitary stalk interruption syndrome is also known as ectopic posterior pituitary syndrome and is a disorder of neuronal migration. Common midline anomalies include:

- Absence of the corpus callosum.
- Absence of the septum pellucidum.
- Optic nerve hypoplasia.
- Arnold–Chiari malformation.

Incidental pituitary lesions on MRI are found in about 16% of adults without clinical symptoms. The incidence in children is unknown and follow-up is as for adults. The range of pathologies is similar to that for adults, but some are found more frequently in children.

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inside

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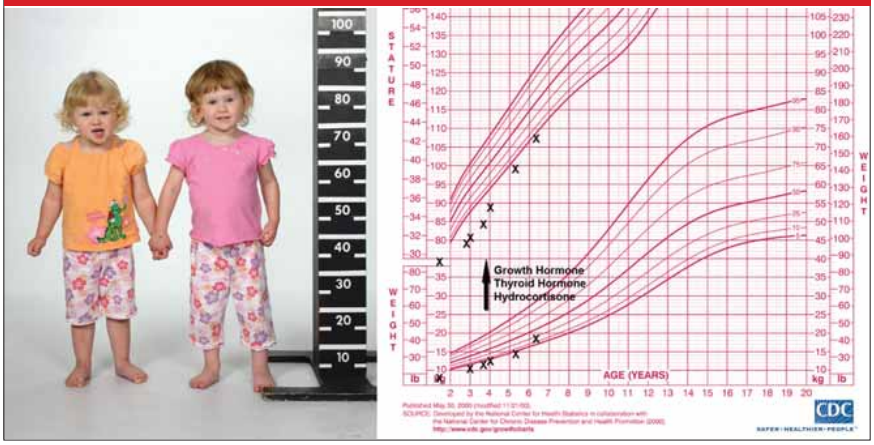
Congenital hypopituitarism

THE incidence of congenital hypopituitarism has been difficult to estimate. The incidence of growth hormone deficiency is about 3.5 per 100,000 children and can be seen in isolation or in combination with other hormone deficiencies. Optic nerve hypoplasia has an incidence of 6.3 per 100,000 and is frequently associated with hypopituitarism.[1]

Figure 1: Growth hormone deficiency: A five-year-old girl (R) with progressive growth failure and her 2.5-year-old sister. Presentation was a hypoglycaemic episode with gastroenteritis. Free T4 low. MRI showed ectopic posterior pituitary syndrome, absent pituitary stalk and anterior pituitary hypoplasia. Photo used with permission from Stephen McInally, John Hunter Hospital .



If hypopituitarism is not diagnosed neonatally, the most common reason to suspect it is poor postnatal growth.

Symptoms and signs in the newborn period

Babies with hypopituitarism usually have a normal birthweight and length because their own growth hormone is not essential for growth in utero. Growth hormone (GH), thyroid hormone and cortisol are all necessary for the maturation of hepatic enzymes. Therefore, these babies tend to develop hypoglycaemia and jaundice. Male babies may have small genitalia, as both testosterone and growth hormone contribute to phallic growth. Hypogonadism also causes cryptorchidism. Female babies do not have these extra clinical clues. The triad of hypoglycaemia, jaundice and micropenis is pathognomonic of congenital hypopituitarism.

Central adrenal insufficiency due to ACTH deficiency

Babies with cortisol deficiency are particularly prone to hypoglycaemia; this is the most potentially life-threatening of the hormone deficiencies. The babies may appear pale and jittery and then become lethargic, mottled and feed poorly. Vomiting and excessive drowsiness with high fevers (above 39°C) can also be signs of low cortisol levels. It may lead to loss of consciousness and seizures.

The diagnosis is confirmed by finding low ACTH and cortisol levels at the time of hypoglycaemia. The hypoglycaemia responds quickly to relatively low doses of IV glucose and frequent feeds, but ultimately appropriate hormone replacement is essential — of cortisol, and of growth hormone and/or thyroxine if these are also deficient. Jaundice may be prolonged but usually resolves, even without hormone replacement. Occasionally liver function is quite disturbed and biopsy shows giant cell hepatitis.

Treatment is oral hydrocortisone given three times daily with dose increases for intercurrent illness. Intramuscular hydrocortisone is given in emergency situations, and parents instructed in its use. A medic-alert should be worn.

Central hypothyroidism due to TSH deficiency

In Australia, the Newborn Screening Program for congenital hypothyroidism is based on TSH measurements alone. High levels indicate a primary thyroid problem and are promptly reported. However, TSH levels in central hypothyroidism may be low or in the normal range with a matching free T4 level that is also low or low normal. As low TSH levels are not reported and free T4 levels not measured, congenital hypopituitarism will be missed in our system and has to be diagnosed by aware GPs or paediatricians.

Symptoms of central hypothyroidism include neonatal jaundice, lethargy, hypoglycaemia and hypothermia, but are often more subtle than in primary hypothyroidism. Fontanelles may be large and slow to close.

Free T4 must be specifically requested if pituitary disease or hypothalamic disease are suspected. Mildly elevated TSH (eg, 5-6 mIU/L) can be seen in hypothalamic disease. TSH is glycosylated, and in hypothalamic disease there is a different pattern of glycosylation that makes the TSH less biologically active and increases its half-life, thus increasing TSH levels somewhat. It is common in this clinical context yet tends to be dismissed, which means that these children are missed.

Mildly elevated TSH can also indicate coexistent central hypoadrenalism, as cortisol suppresses TSH. Adrenal insufficiency should be excluded before thyroxine therapy is started, as thyroxine can precipitate an adrenal crisis.

Thyroxine replacement is extremely important for normal brain development and the aim is free T4 levels in the upper normal range. The dose cannot be adjusted based on TSH levels, as the problem is TSH insufficiency.

Hypogonadism caused by deficiency of LH or FSH (hypogonadotrophic hypogonadism)

Male babies present with micropenis with or without cryptorchidism. Testosterone therapy is most effective in the first three months of life, as this corresponds to the physiological period of 'mini-puberty' in males. Children with Kallmann's syndrome often have anosmia (loss of smell), which occurs due to abnormalities in neural migration that affect both the olfactory bulb and the GnRH neurons in the hypothalamus.

Evidence of a midline syndrome — 'the face predicts the brain'

The developmental problems associated with congenital hypopituitarism are called midline syndromes. The greater the severity of the anomaly, the greater is the likelihood of pituitary involvement. Common associations are:

- Cleft palate (not cleft lip alone).
- Single central incisor syndrome.
- Optic nerve hypoplasia (septo-optic dysplasia).
- Absent septum pellucidum (the midline brain structure below the corpus callosum).
- Agenesis of the corpus callosum.

Optic nerve hypoplasia presents in the first few weeks after birth with failure to fix and follow, and with nystagmus (often roving). Referral to a paediatric ophthalmologist is important.

Diabetes insipidus

Excessive thirst and urination may indicate antidiuretic hormone (ADH) deficiency. Many children with septo-optic dysplasia have partial or complete diabetes insipidus. This may be masked by co-incident cortisol and thyroid hormone deficiencies because both these deficiencies impair free water clearance in the kidney. Hypernatraemia and dehydration will occur if the baby cannot drink enough.

Diagnosis is confirmed with high plasma osmolality in the face of inappropriately low urine osmolality. Neonatal diabetes insipidus is difficult to manage, and referral to a paediatric endocrinologist is important. Infants may be more safely treated with a low renal solute load formula (that reduces obligatory urinary water losses) and hydrochlorthiazide (which concentrates the urine) than with oral desmopressin solution.

Symptoms and signs in infancy and childhood

If hypopituitarism is not diagnosed neonatally, the most common reason to suspect it is poor postnatal growth.

Growth hormone deficiency

Children with growth hormone (GH) deficiency progressively lose height relative to their peers (figure 1). At the time of diagnosis their height is usually under the third centile, unless they have tall parents, when the child's height centile is well below the centile for their mid-parental height.

Mid-parental height = (mother's height + father's height)/2 ± 6.5cm (+ for a boy, – for a girl). It is the genetic adult height target for the offspring.

The second feature is a relative excess of weight for height, carried as abdominal fat that may look like cellulite (called fat dimpling). Central hypothyroidism exacerbates this central adiposity. In contrast, in children who also have cortisol deficiency, excess abdominal fat may not be a feature. Some children may continue to be at risk of hypoglycaemia beyond the neonatal period, especially if they are fasting or vomiting. This was the presentation of the child shown in figure 1.

As skeletal growth is retarded, the child's body proportions resemble those of a younger child. Their facial growth is also slowed and so their facial appearance is 'cherubic'. The forehead is prominent, the bridge of the nose depressed, the voice high-pitched and anterior fontanelle slow to close. Baby teeth may be slow to appear, termed delayed dental eruption. There may be irregular development and setting of permanent teeth. Bone age is delayed.

The diagnosis is made if GH levels are low at the time of hypoglycaemia and insulin-like growth factor 1 (IGF-1) levels are low or low normal. (IGF-1 is synthesised in the liver in response to GH and is the effector molecule for GH in the tissues.) If the child is not hypoglycaemic, formal growth hormone stimulation testing may be needed. Random GH levels are not diagnostic, as normal children can have undetectable levels. Treatment is with daily growth hormone injections. Initiation of GH therapy may unmask central hypothyroidism and even central hypoadrenalism.

Midline syndromes

Any child with a cleft palate, significant hypotelorism or hypertelorism (facial features too close or too far apart, respectively), and short stature should be assessed for GH deficiency. More subtle forms of cleft palate are only obvious when the child starts to speak. Children with a submucous cleft of the soft palate have symptoms such as 'nasal' speech and regurgitation of liquids out of the nose.

Symptoms and signs in adolescence

The two issues in this age group are the delayed onset of puberty and the development of further pituitary hormone deficiencies.

Delayed puberty and hypogonadotrophic hypogonadism

Delayed puberty is defined as no signs of puberty by 13 years in girls or 14 years in boys. Bone age is delayed and bone mineral density may be already subnormal.

Spontaneous onset of puberty is less likely in a child who had signs of sex hormone deficiency in the neonatal period or has multiple pituitary hormone deficiencies. Patients may also have anosmia (Kallmann's syndrome).

Induction of puberty with sex hormones at an appropriate age is important for psychological as well as physical health and to optimise bone mineral density.

Progressive hypopituitarism

In children with certain genetic causes for hypopituitarism such as *PROP1* defects, there is progressive loss of pituitary cells, the last to fail being the ACTH-secreting cell (corticotrophs).

If patients who have previously coped well with intercurrent illness start vomiting, have high fevers and are more lethargic than expected, one must consider that these could be symptoms of cortisol deficiency. Cortisol deficiency can mimic gastroenteritis. Respiratory distress and 'air hunger' can also be a feature of cortisol deficiency and be incorrectly diagnosed as an asthma crisis.

Progressive hypopituitarism, including diabetes insipidus, occurs in patients with optic nerve hypoplasia.

Hypopituitarism and late effects in oncology patients

Hypopituitarism may develop after cranial or total body irradiation and/or high-dose chemotherapy. This is an expanding field and space restrictions do not allow us to do it justice.An excellent mongraph from Dr M Zacharin is available from the Royal Children's Hospital, Melbourne.

cont'd page 28

Precocious puberty

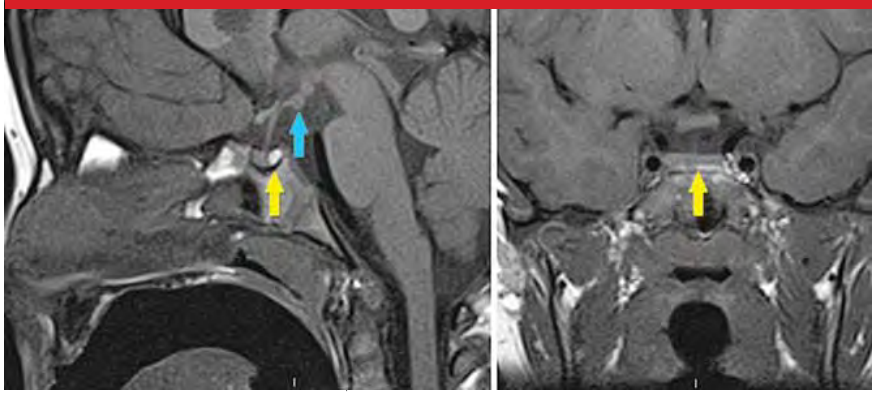
PUBERTY occurs when an area of the hypothalamus called the gonadostat begins to secrete gonadotrophin-releasing hormone (GnRH) in pulses of increasing frequency and magnitude. In turn, increasing LH and FSH pulses from the pituitary stimulate the gonads. A genetic or structural problem at any point in this pathway can affect puberty. Precocious puberty is defined as signs of sexual maturation before the age of eight years in girls or nine years in boys.

Hypothalamic hamartomas

Hypothalamic hamartomas cause central, gonadotrophin-dependent precocious puberty. They are a congenital malformation that consists of heterotopic neural tissue attached to the floor of the third ventricle or that is pedunculated (figure 2). They contain GnRH neurons. Intrahypothalamic lesions cause gelastic seisures (seizures characterised by laughing or crying).

Precocious puberty is far more common in girls. It may begin as early as the first or second year of life and is successfully treated by long-acting GnRH analogues. (Sustained high levels of GnRH analogues inhibit LH and FSH secretion from the pituitary, whereas pulsatile GnRH is stimulatory.)

Figure 2: Hypothalamic hamartoma (blue arrow) in a four-year-old girl with central precocious puberty and normal anterior and posterior pituitary (yellow arrow) and stalk on sagittal MRI. Coronal MRI at level of normal pituitary. Treatment with gonadotrophin-releasing hormone analogue. Images used with permisison from Stephen McInally, John Hunter Hospital.



Pituitary adenomas

PITUITARY adenomas can arise from any of the six pituitary cell types (secreting prolactin, ACTH, GH, LH, FSH or TSH). They are nearly always benign. They are classified according to their diameter as micro-and macroadenomas (>10mm). If diagnosed late as large invasive tumours, they are not completely resectable, even with the best surgical techniques.

In adolescents, particularly girls, prolactinomas predominate, but are nearly always managed with medical therapy. In surgical series, corticotroph adenomas causing Cushing's disease are the most common in children, followed by somatotroph (GH) adenomas causing gigantism and acromegaly. Non-functioning adenomas are rare, in contrast to the situation in adults, where they make up nearly 50% of tumours. TSH-omas are exceedingly rare.

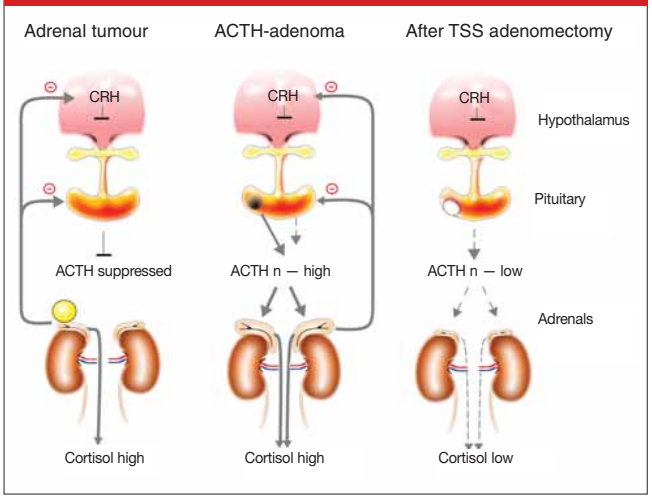
A family history of pituitary tumours may indicate there is an underlying genetic condition, such as multiple endocrine neoplasia type 1 (MEN1).

Figure 3: Girl with Cushing's disease three days after transnasal trans-sphenoidal microsurgery, showing the classic 'moon face' and short stature next to her normal younger sister.

Diagnostic path of Cushing's disease

Night salivary cortisol elevated
↓
Referral to paediatric endocrinologist
↓
Plasma-ACTH normal or high
↓
CRH stimulation test positive
↓
MRI of pituitary
↓
Consultation with specialised pituitary neurosurgeon
↓
ACTH catheter study of central venous drainage

Figure 4: Pathophysiology of (i) adrenal adenoma with low ACTH due to autonomous cortisol hypersecretion from a unilateral lesion (left); (ii) an ACTH-secreting pituitary adenoma inducing bilateral adrenal hyperplasia (middle); (iii) after selective removal of the ACTH-adenoma (right), with suppressed ACTH-secretion from the anterior lobe. Schematic drawing by Dr Mark Read.



Cushing's disease

Cushing's disease is a severe metabolic disease with cortisol excess caused by an ACTH-secreting (corticotroph) pituitary adenoma. Cushing's syndrome, by contrast applies to any cause of hypercortisolaemia that is not ACTH-dependent (figure 4). It is 100 years since Harvey Cushing described the first case — a young woman with secondary amenorrhoea at age 16, with severe headache, facial plethora and hirsutism, central obesity and severe hypertension. Her disease spontaneously remitted, presumably from bleeding into the tumour. All Cushing's descriptions of pituitary cases were autopsy based, not surgical.

In children ACTH-secreting pituitary adenomas account for 90% of operated adenomas, and more often occur in pre-pubertal boys than in girls. In contrast, in adults they represent only 10% of all diagnosed pituitary tumours and occur predominantly in women.

The first cases described by Cushing already showed the main difficulty in diagnosis and management, namely, the minute size of pituitary adenomas, often <4mm. Despite modern MRI, these lesions may not be detected in up to 50% of cases. Advanced MRI techniques with 3 Tesla, available in some centres in Australia, could improve the detection rate.

The main clinical signs and problems in children include:

- So called 'moon face' with plethora (figure 3).
- Weight gain, which, unlike in adults, may be more generalised than central.
- Proximal myopathy, which is less evident than in adults.
- Growth failure (crossing the centiles downward).
- Signs of androgenisation occuring at the same time as delayed puberty and amenorrhoea.
- Hypertension, headaches, impaired glucose tolerance or diabetes and osteoporosis possibly also developing.

The average time from symptoms to diagnosis is two and a half years, compared with about five years in adults.

Endocrine investigations

With hypercortisolism there is a loss of diurnal rhythm, which can be easily confirmed by the measurement of free cortisol in the saliva after 10pm. Elevated night salivary cortisol differentiates children with Cushing's syndrome from children with obesity. Obesity is almost reaching epidemic proportions yet Cushing's disease is rare. Beware the short, fat child! Most obese children are tall for their age and may have an advanced bone age. Importantly, their growth is normal. The obese child who is not growing should have a night salivary cortisol level done and see a paediatric endocrinologist. Morning cortisol levels alone are not diagnostic.

Measurement of 24-hour urinary free cortisol and low-dose dexamethasone suppression testing (1mg or 30µg/kg/day in children <40kg) are still used. Urinary free cortisol levels may be falsely low if collection is incomplete. The high sensitivity, non-invasiveness and ease of use in outpatients support the use of the salivary cortisol technique, especially in children. If the result is pathological, referral to a paediatric endocrinologist is advised.

Polycystic ovary syndrome often presents with obesity, oligo-amenorrhoea and hirsutism, so it is reasonable to screen for Cushing's disease in this clinical setting.

Differential diagnosis is the next step to be done by the specialist.

Normal or slightly elevated ACTH levels exclude an adrenal pathology and exogenous steroid use (oral, inhaled or topical). In these latter causes ACTH is undetectable.

Significant suppression of cortisol in the low-dose dexamethasone test, and ACTH or cortisol stimulated by corticotrophin-releasing hormone are the generally accepted ways to differentiate pituitary-dependent from ectopic Cushing's syndrome (neither of adrenal nor pituitary origin). If both tests are positive, an ectopic source is ruled out. Ectopic disease is exceedingly rare in paediatric Cushing's syndrome (thymoma, carcinoids).

For a summary of the investigation algorithm see box, left.

MRI of the pituitary

MRI is the next diagnostic step, after endocrine results indicate pituitary-dependent Cushing's disease. T1WI coronal and sagittal sequences, with and without gadolinium-DTPA enhancement and thin slices, have to be performed.

Invasive catheter techniques with venous blood sampling

The role of these techniques is unclear at present and not widely available. Blood sampling from the inferior petrosal sinus has been developed to distinguish between pituitary and ectopic Cushing's syndrome. Cavernous sinus sampling, as initiated

Figure 5: Boy, 15, with gigantism two days after selective transnasal trans-sphenoidal microsurgery of a 12mm GH-secreting adenoma. Complete remission.

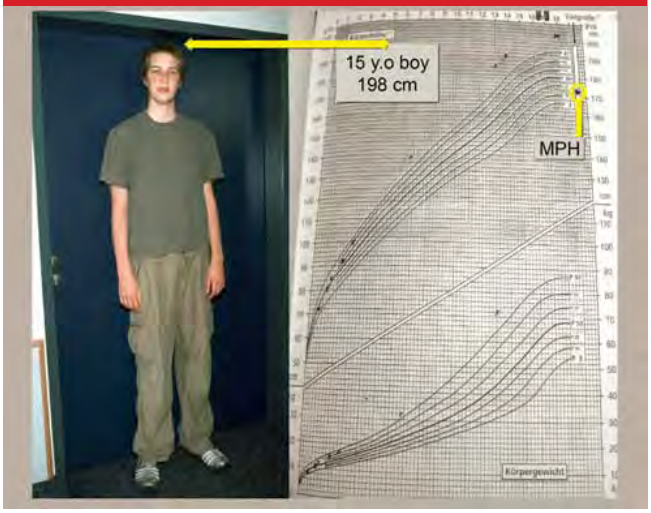


Figure 6: Nineteen-year-old woman with gigantism and acromegaly, with her mother, after transnasal trans-sphenoidal microsurgery of a large, invasive GH-secreting pituitary adenoma (blue arrow 45mm) — partially resectable. Inset: preoperative MRI.

by one of the authors (Dieter Ludecke) in 1989 , is gaining wider acceptance as a more precise localisation aid for minute adenomas within the pituitary, according to Teramoto.[2]

Therapy

Primary therapy of Cushing's disease in children is trans-sphenoidal surgery (figure 3). Selective removal of microadenomas by experienced microsurgeons achieves remission in more than 90% of cases. A postoperative decline of ACTH and cortisol levels in the subnormal range persists for about a year (shorter than in adults) and needs adequate replacement and retesting by the endocrinologist. In 10% of cases, some pituitary deficits occur.

In case of failure of first surgery or recurrence of the adenoma, pituitary re-operation is an option but with a higher risk of pituitary deficits. Radiotherapy or medical therapies have also been used. Bilateral adrenalectomy is another option but this may stimulate growth of residual pituitary adenoma tissue (Nelson's syndrome).

Pituitary gigantism and acromegaly

Gigantism is usually due to excessive production of growth hormone from a somatotroph adenoma before the closure of epiphyseal growth plates at puberty. These tumours tend to be macroadenomas. Familial acromegaly occurs. Somatotroph hyperplasia can occur in McCune–Albright syndrome or Carney complex.

The acceleration in growth in these children crosses growth centiles upwards and predicted final height is well above their mid-parental height (figure 5). As children grow proportionately, they do not have the classic signs of acromegaly until after puberty, when, if untreated, they will develop the features seen in adults of large hands and feet and coarsening of facial features (figure 6). Sweating is often a prominent symptom, as is headache. Visual symptoms due to optic chiasm compression may be relatively late to appear.

Differential diagnosis of tall stature and rapid growth

Rapid growth in children may be due to sex steroids, either from precocious puberty or androgen excess such as in late-onset congenital adrenal hyperplasia. Signs of sexual development will be present and bone age significantly advanced. Thyrotoxicosis also accelerates growth. Genetic causes of tall stature include Marfan syndrome and Sotos' syndrome.

Endocrine investigations

IGF-1 is synthesised under GH stimulation, mainly in the liver, and levels are stable, unlike GH, which is pulsatile. A single high serum IGF-1 level may be diagnostic but levels need to be interpreted using age- and puberty-adjusted normal ranges.

For evaluation of GH levels, an oral glucose tolerance test usually needs to be performed. If GH levels do not fall in response to the glucose load, this indicates gigantism. Unfortunately, studies in normal tall adolescents have shown that up to 30% will not show complete suppression of GH. It is also important to measure prolactin, as tumours may be mixed somato-lactotroph and may respond to dopamine-agonist therapy.

Imaging

MRI shows a pituitary adenoma in nearly every case.

Therapy for gigantism

Trans-sphenoidal surgery is first-line therapy and cure rates are high for microadenomas with an experienced pituitary neurosurgeon. Increasingly, preoperative treatment with long-acting somatostatin analogue (SSA) injections every 4-6 weeks is being used to shrink the larger tumours.

If IGF-1 levels do not normalise after surgery, postoperative SSA is started. Somatostatin suppresses insulin secretion, so it may cause glucose intolerance or precipitate diabetes. Other side effects include gastrointestinal symptoms and gallstone formation requiring cholecystectomy.

Large and locally invasive tumours may require a multipronged approach including preoperative SSA, surgical reduction of the accessible tumour and postoperative SSA or the new GH-receptor antagonist, pegvisomant. Pegvisomant has to be given daily and reduces IGF-1 levels by blocking hepatic production. It does not block tumour growth. Radiation may be needed in some cases but often leads to pan-hypopituitarism.

Prolactinoma

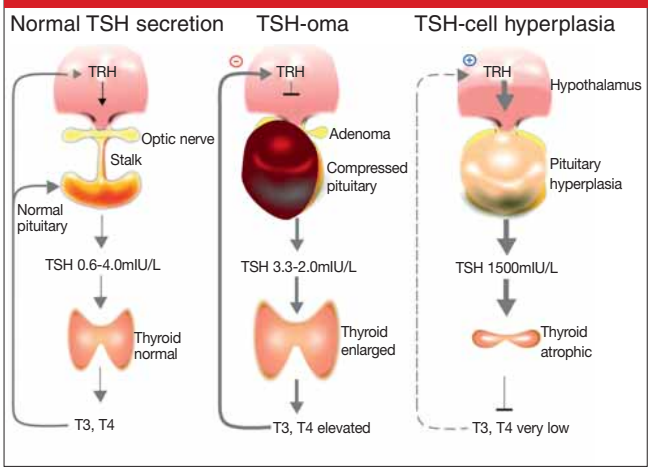
Lactotroph adenomas or prolactinomas are the most common tumour in adolescents. They are mainly seen in girls, who tend to present with microadenomas causing primary or secondary amenorrhoea and galactorrhoea (spontaneous or provoked in up to 75% of cases). Boys nearly always present with a macroadenoma and the symptoms of mass effect, namely headache, optic chiasm compression, visual loss and hypopituitarism. Gynaecomastia is not obligatory. The size of prolactinomas correlates well with the baseline level of prolactin.

Family history is important. Prolactinomas may be part of an inherited syndrome such as MEN1, familial isolated pituitary adenomas or Carney complex.

Differential diagnosis of prolactinoma

The differential diagnosis of hyperprolactinaemia is extensive (table 1). It includes all causes of loss of dopaminergic suppression. High levels of prolactin are pathognomonic of a prolactinoma and even in children on antipsychotic therapy, the cause should be clarified by an MRI.

Figure 7: Physiological TSH secretion (left). Pathophysiology of autonomous TSH secretion from a TSH-oma without suppression by high thyroid hormone levels (middle). Primary thyroid insufficiency leading to TSH-cell hyperplasia with compression of the chiasm (right).
Schematic drawing by Dr Mark Read.



Treatment

First-line management in prolactinomas is medical, with dopamine agonists — bromocriptine, carbergoline or quinagolide. The aim of therapy is to normalise prolactin levels and other pituitary function (LH and FSH are suppressed by high prolactin), and to decrease the tumour size.

In older children and adolescents, restoring or maintaining gonadal function will mean resumption of normal pubertal development, attainment of peak bone mass and potential fertility. In older adolescents and young women with amenorrhoea, it is important to consider the need for contraception once dopamine agonist therapy is started. Medical therapy is successful in 70-90% of patients.

Carbergoline and quinagolide (selective dopamine receptor 2 [D2] agonists), are better tolerated and more effective than bromocriptine. Gentle dose titration is important to minimise side effects. The most common are GI symptoms such as nausea, vomiting and abdominal pain. Non-specific symptoms include headache, tiredness, drowsiness and weakness. Behavioural and mood changes may also occur with exacerbation of underlying psychiatric disease.

Dizziness due to orthostatic hypotension has been reported. Valvular cardiac problems have been reported with the much higher doses (>10) used to treat Parkinson's disease. Since the cardiac risk of long-term low-dose therapy in a young person is unknown, an echocardiogram is probably advisable.

Indications for surgery in prolactinomas include acute threat to vision and intolerance and/or resistance to dopamine agonist therapy

cont'd next page

Table 1: Causes of hyperprolactinaemia

Hypothalamic	Tumours, irradiation, histiocytosis
Pituitary	Adenomas, pituitary stalk lesions
Endocrine	Hypothyroidism, polycystic ovary syndrome, breast stimulation, pregnancy, lactation
Drug induced	Drugs with antidopaminergic action (eg, antipsychotics, metoclopramide)
Neurological/neurogenic	Seizures, raised intracranial pressure, chest trauma
Systemic	Renal and liver failure
Macro-prolactinaemia	Immune complex with IgG (assay artefact, not a true high prolactin)

from previous page

with persistent hyperprolactinaemia and/or increasing tumour growth. Complications of dopamine agonist treatment that may precipitate surgery include rapid tumour shrinkage, leading to CSF rhinorrhoea, or bleeding into an adenoma, causing visual disturbance, headache and pituitary deficits. Patient preference to avoid long-term medical therapy, especially when dopamine agonist therapy is not well tolerated, is also a valid reason.

TSH-secreting pituitary adenomas

'TSH-omas' are extremely rare and nearly always overlooked in both adults and children. Even when the tumour is relatively large, the TSH level is only 3.3-20.0mIU/L with free T4 levels above the upper limit of normal (figure 7, page 27). The differential diagnosis is thyroid hormone resistance syndrome, also a rare entity. Very high TSH levels with low free T4 levels from longstanding primary hypothyroidism are due to TSH cell hyperplasia and can mimic an adenoma on MRI scan (figure 7). Careful interpretation of thyroid hormone test results is the key to early diagnosis and treatment.

MRI scan often shows a TSH macroadenoma, and first-line therapy in children and adolescents is neurosurgical. In adults, somatostatin analogues may be used first line.

Non-functioning (non-secreting) pituitary adenomas

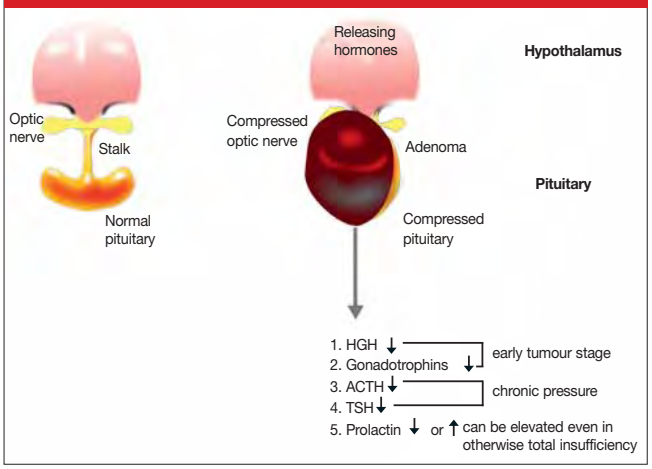
Hormonally silent pituitary tumours represent only 5% of cases in children, whereas they represent up to 50% of pituitary lesions in adults. Most present with symptoms of mass effect, including headache and visual disturbances with temporal field defects. Hypopituitarism due to compression of the normal pituitary may result in GH deficiency, delayed puberty or central adrenal insufficiency (figure 8). Prolactin levels may be elevated due to stalk compression, but initiating dopamine agonist therapy will have no effect on the adenoma.

Trans-sphenoidal microsurgical removal with preservation of the compressed pituitary is the first treatment option. Rarely, in cases of excessive supra- and parasellar growth, transcranial surgery and radiotherapy become necessary.

Since there is no serum marker and residual tumour may grow slowly, long-term follow-up with MRI has to be performed for more than five years, even if MRI is negative postoperatively.

Incidental small tumours, without neurological or hormonal impairment, may be monitored by endocrine observation and MRI.

Figure 8: Non-functioning pituitary macro-adenoma with asymmetrical upward pressure on the right optic nerve and compression of the anterior lobe of the pituitary to the left, resulting in pituitary deficits. Schematic drawing by Dr Mark Read.



Craniopharyngiomas

CRANIOPHARYNGIOMAS are the most common cause (80-90%) of a mass in the pituitary region in children. They arise from embryonic remnants of Rathke's pouch, the invagination of oral ectoderm from which the anterior pituitary develops. The incidence is 0.5-2.0 cases per million persons per year, with a bimodal peak at 5-15 years and at about 60 years. Half of all cases present in childhood. There is an equal sex distribution.

There are two different histological types. Predominant in the paediatric age is the adamantinomatous variant, which tends to be cystic.

Craniopharyngiomas often impair pituitary function. Intrasellar tumours develop between the adeno- and neuro-hypophyseal parts of the pituitary, so as they enlarge they displace the pituitary anteriorly. This has implications for surgery via the transnasal route (figure 9). Craniopharyngiomas often contain calcification that is best seen on CT scan.

As they expand above the sella they first impinge on the optic chiasm, then go into the hypothalamus and to the third ventricle, causing hydrocephalus (figure 10). Extension laterally into the cavernous sinus may affect ocular movements. Strabismus due to a sixth nerve palsy can also be a false localising sign of raised intracranial pressure.

The typical clinical presentation is headache and visual loss, but symptoms of hypopituitarism such as growth failure, fatigue and delayed puberty are common. Visual loss in a child may be profound before it is diagnosed, as the loss is gradual and may affect one eye more than the other. However, optic nerves that are already atrophic may decompensate acutely, with complete loss of vision within hours. In this sight-threatening situation surgical decompression is an emergency procedure.

GH deficiency is seen in 75% of patients, gonadotrophin deficiency in 40%, and hypothyroidism and hypoadrenalism in one-quarter. Diabetes insipidus is found in about 15% of patients. Any given patient will have their own particular 'cocktail' of pituitary hormone problems that can interact in a complex fashion, as discussed under 'Congenital hypopituitarism' (page 24).

Figure 9: Intrasellar craniopharyngioma. Cystic and partially calcified craniopharyngioma impinging on the optic nerve. Arrow indicates transnasal surgical approach. Schematic drawing by Dr Mark Read.

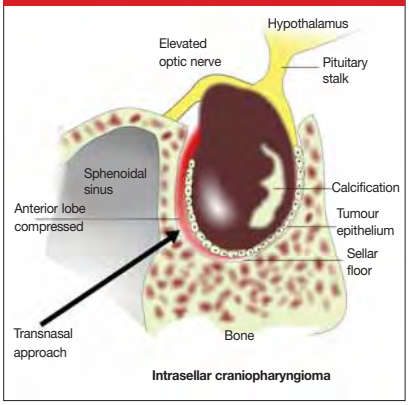
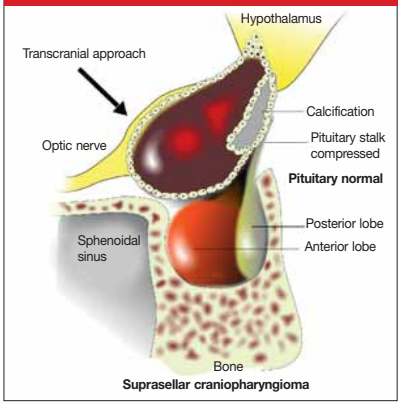


Figure 10: Suprasellar craniopharyngioma (aubergine) with partial invasion into the hypothalamus and compression of the pituitary stalk. Arrow indicates transcranial surgical approach. Schematic drawing by Dr Mark Read.



Treatment

Observation with close follow-up by MRI may be the choice in a subgroup of paediatric patients with small cystic tumours. Some may not grow or even spontaneously regress, such as cysts due to *PROP1* mutations.

Surgery

In most cases of craniopharyngioma, microsurgical resection is the treatment of choice. Tumours with predominant suprasellar extension (about 70%) have to be operated on by the transcranial route, mainly sub-fronto-temporal (see figure 10). There are risks of damage to the chiasm, hypothalamus and pituitary stalk. This can be avoided in cases of sellar enlargement, when transnasal trans-sphenoidal surgery with its low complication rate can be chosen. Effectiveness and morbidity of surgical treatment depends on the extension of the tumour, the equipment and the skill of the surgeon. Endoscopic approaches can achieve more radical tumor removal, eventually resulting in more function loss.

CSF fistulas are a major problem and have to be differentiated from rhinitis by beta-transferrin measurement. Frequently, reoperation is needed. Additional pituitary deficits are quite common. Postoperative adiposity occurs in about 40%, especially with hypothalamic extension, but rarely after transnasal surgery.

Radiotherapy

Since craniopharyngiomas are radiosensitive, postoperative radiotherapy has to be considered when a tumour rest (residual tissue) is present or a small rest increases during the mandatory MRI follow-ups. We suggest MRI scans at six months then yearly postoperatively unless there are other postoperative indications. Localised recurrences with distance from the optic system may be treated with stereotactic radiation. The most modern form (CyberKnife) is not yet available in Australia. This is also applicable to adenomas if medical treatment is not effective. Most centres use gamma-knife or fractionated radiation by linear accelerator.

Hypothalamic syndrome

Tumour infiltration of the hypothalamus and damage to the hypothalamus after surgery or radiotherapy may lead to a number of problems. The most distressing is hyperphagia, which may be extreme and lead to morbid obesity. Emotional lability and rage attacks often result from attempts by parents to restrict food. Obesity may lead to sleep apnoea and daytime somnolence. There is no generally accepted pharmacological therapy. Gastric banding surgery and laparoscopic truncal vagotomy have shown immediate normalisation of the food craving.

Abnormal thirst (excessive or absent) may cause major electrolyte problems, with either hypo- or hypernatraemia. Abnormal temperature control may lead to problems of hyperthermia in summer and hypothermia in winter. Memory and intellectual impairment may compound the problems outlined above.

Hypothalamic tumours

THESE include hamartomas (see 'Precocious puberty' section, page 26), germinomas (see below), Langerhans' cell histiocytosis, and hypothalamic and optic nerve gliomas.

Germinomas

Germinomas are relatively slow-growing germ cell tumours occurring between three and 21 years of age. In one-third of patients with intracranial lesions they are localised in the pituitary stalk with the early clinical sign of diabetes insipidus. The main localisation is at the pineal gland. Serum markers such as beta-HCG are often negative. The specific marker human placental alkaline phosphatase in CSF or serum may be negative for a long time. Germinomas are highly radiosensitive and have a good prognosis with irradiation alone. Even in advanced stages, chemotherapy alone is also successful in the long term. Monitoring by experienced neuro-oncologists is essential.

Further reading, references, online resources and relevant PubMed search strategies

Available on request from julian.mcallan@reedbusiness.com.au

cont'd page 32

GP's contribution

DR ROSS WILSON
Bathurst, NSW

Case study

MISS JR presented at age six with premature adrenarche. Her parents had noted the appearance of pubic hair three months prior, with no axillary hair, no vaginal discharge and no facial acne. Increased sweating and body odour were also noted. From birth her growth had always been on the 90th centile for height and weight. No headache or visual disturbance were present.

Past medical history include tonsillectomy for obstructive tonsillitis at age 4½. Immunisation was complete. No allergies were noted and childhood milestones were unremarkable.

There was no family history of early puberty or tall stature, and academic performance was normal. Pubertal Tanner stage was B1 P3 on presentation (B1: prepubertal breasts; no glandular tissue, areola follows the skin contours of the chest with only the nipple raised. P3: pubic hair becomes more coarse and curly, and begins to extend laterally). JR was normotensive. Neurological and ocular exams were normal. All tests (EUC, FSH, LH, oestradiol, 17-OH progesterone, testosterone, androstenedione and DHEAS) as well as bone age (wrist) were normal for age. Short synacthen test was also normal.

On discussion with the parents I raised the strong possibility of this being precocious puberty.

Questions for the author

Is it reasonable to continue to observe developments clinically?

Although "true" or gonadotrophin-dependent puberty may start with pubic hair alone, the history here is more suggestive of adrenarche as stated. Androgens may be adrenal or ovarian. Breast budding, ovarian and uterine enlargement on ultrasound and vaginal discharge are all signs of oestrogen action from "true" puberty. In early adrenarche, there is often a history of prematurity or

family history of polycystic ovarian disease. Bone age reports are dependent on the paediatric experience of the reporter, so re-read it, if clinically you would expect bone age to be accelerated.

Should I arrange CT brain with or without contrast?

There is really no role for brain imaging in a girl with adrenarche, but imaging of the adrenals and ovaries may be considered. If the child had gonadotrophin-dependent precocious puberty, it would be reasonable to do an MRI of the hypothalamic–pituitary area. There is a diminishing role for CT brain scans due to the considerable radiation in a child and the lack of high definition images.

What should the management involve if further Tanner development occurs medically and psychologically?

Tanner 3 pubic hair is usually associated with axillary hair, so I wonder if this child's Tanner staging is closer to 2. To reach stage 3 in three months is quite dramatic and exclusion of an adrenal tumour would be warranted. If signs of true puberty emerge and progress quickly, then consideration would be given to suppression of puberty with GnRH analogue therapy. Early adrenarche does not necessarily imply puberty will be early too.

Given JR's parents are of discrepant heights (mother >185cm; father about 168cm), could this be purely a genetic issue?

Yes. Her tall stature is probably from her mother, but this does not explain her early adrenarche.

General question for the author

At what age with precocious puberty does one discuss eventual height of the patient and the outlook for sensitive issues such as contraception?

Estimated mature height predictions are only available for bone ages above six years. Puberty may start even in the first one or two years of life! The youngest pregnancy recorded was at age five years in the Andes.[1] A study in Sweden showed early menarche (<10 years) was associated with shorter stature, excess weight as an adult, earlier sexual experiences and disruption to academic pursuits.[2] Parents need to be informed, but the child should be given age-appropriate explanations.

1. *La Presse Medicale* 1939; 47:744.
2. Johansson T, Ritzèn EM. Very long-term follow-up of girls with early and late menarche, In: Delemarre-van de Waal HA (editor). *Abnormalities in Puberty. Scientific and Clinical Advances*. Karger, Basel, 2005.

How to Treat Quiz

Paediatric pituitary disorders — 18 February 2011

INSTRUCTIONS

Complete this quiz online and fill in the GP evaluation form to earn 2 CPD or PDP points. We no longer accept quizzes by post or fax.

The mark required to obtain points is 80%. Please note that some questions have more than one correct answer.

ONLINE ONLY
www.australiandoctor.com.au/cpd/ for immediate feedback

1. Which TWO statements are correct?
a) Regular and accurate height and weight measurements are critical for the detection and management of pituitary disease in children
b) Babies with hypopituitarism with growth hormone deficiency are usually small and underweight at birth
c) Hypoglycaemia and jaundice in infants may be due to deficiency of growth hormone, thyroid hormone or cortisol
d) Cortisol deficiency in infants is usually a mild sub-clinical condition

2. Which TWO statements are correct?
a) Central adrenal insufficiency is confirmed by finding high ACTH and low cortisol levels at the time of hypoglycaemia
b) The Australian Newborn TSH Screening Program reliably detects primary hypothyroidism
c) The Australian Newborn TSH Screening Program reliably detects central (pituitary) hypothyroidism
d) Symptoms of central hypothyroidism include neonatal jaundice, lethargy, hypoglycaemia and hypothermia

3. Which TWO statements are correct?
a) Both TSH and free T4 are needed to diagnose pituitary or hypothalamic hypothyroidism
b) Mildly elevated TSH excludes hypothalamic hypothyroidism.
c) In an infant with hypothyroidism, thyroxine replacement is essential for normal brain development
d) In central hypothyroidism, thyroxine replacement dosage is adjusted according to TSH levels

4. Which TWO statements are correct?
a) Male babies with a deficiency of LH or FSH usually have normal genitalia
b) In male babies with a deficiency of LH or FSH, testosterone therapy is most effective if started in the pre-pubertal period
c) 'Midline syndromes' associated with congenital hypopituitarism include cleft palate, single central incisor syndrome, or optic nerve hypoplasia
d) Optic nerve hypoplasia presents in the first few weeks after birth with failure to fix and follow, and with nystagmus

5. Which TWO statements are correct regarding GH deficiency in children?
a) For a child of tall parents, the height centile is well below the centile for their mid-parental height
b) There is a relative excess of weight for height, carried as abdominal fat
c) The child's body is small and the proportions resemble those of an adult
d) Low random GH levels are diagnostic

6. Which THREE statements are correct?
a) Delayed puberty is defined as no signs of puberty by 13 years in girls or 14 years in boys
b) Induction of puberty with sex hormones is important for psychological and physical health and to optimise bone mineral density
c) Precocious puberty is defined as signs of sexual maturation before the age of eight years in girls or nine years in boys
d) Cushing's disease in children can mimic both gastroenteritis and asthma crises

7. Which TWO statements are correct regarding Cushing's disease in children?
a) In Cushing's disease the cortisol excess is caused by an ACTH-secreting pituitary adenoma
b) Macroadenomas (>10mm) are the most common pituitary lesions in Cushing's disease and are easily detectable with imaging
c) Weight gain in children may be more generalised than central
d) Height is unaffected

8. Which TWO statements are correct regarding Cushing's disease in children?
a) Androgenisation occurs as well as delayed puberty and amenorrhoea
b) Night salivary free cortisol level is low
c) Low ACTH levels exclude an adrenal pathology and exogenous steroid use
d) Elevated night salivary cortisol level differentiates children with Cushing's syndrome from those with obesity

9. Which TWO statements are correct regarding gigantism and acromegaly?
a) Sweating and headache are common symptoms
b) Differential diagnosis of rapid growth in children includes sex steroid excess and thyrotoxicosis
c) Random elevated GH levels are always diagnostic
d) Medical therapy for GH-secreting adenomas involves somatostatin inhibitors

10. Which TWO statements are correct?
a) First-line management in prolactinomas is surgery
b) Indications for surgery in prolactinomas include acute threat to vision and intolerance and/or resistance to dopamine agonist therapy
c) Non-secreting pituitary adenomas are never associated with endocrine dysfunction
d) Craniopharyngiomas may present with headache, visual loss and symptoms of hypopituitarism

CPD QUIZ UPDATE

The RACGP requires that a brief GP evaluation form be completed with every quiz to obtain category 2 CPD or PDP points for the 2011-13 triennium. You can complete this online along with the quiz at **www.australiandoctor.com.au.** Because this is a requirement, we are no longer able to accept the quiz by post or fax. However, we have included the quiz questions here for those who like to prepare the answers before completing the quiz online.

Australian Doctor Education

HOW TO TREAT Editor: **Dr Giovanna Zingarelli**
Co-ordinator: **Julian McAllan**
Quiz: **Dr Giovanna Zingarelli**

NEXT WEEK The next How to Treat looks at blistering skin disorders. The authors are **Dr Adrian Lim**, dermatologist and phlebologist, director of training, NSW Faculty of the Australasian College of Dermatologists, and in private practice, Bondi Junction and Sydney CBD, NSW; and **Dr Patricia Lowe**, staff specialist, Royal Prince Alfred Hospital, Camperdown, and dermatologist in private practice, Drummoyne and Sydney CBD, NSW.

Patient report

Vicki E. Maltby[a], Patricia A. Crock[a,*] and Dieter K. Lüdecke

A rare case of pituitary infarction leading to spontaneous tumour resolution and CSF-sella syndrome in an 11-year-old girl and a review of the paediatric literature

Abstract: Pituitary infarction or apoplexy with spontaneous cure of the underlying pituitary adenoma is rare. In the paediatric population, we found only a few reported cases. We report a rare case of pituitary infarction progressing to CSF-sella syndrome (or empty sella) in an 11-year-old girl. She presented with sudden onset vomiting, moderate headaches, lethargy, weight loss, and tall stature above her mid-parental height. She did not have any severe symptoms of apoplexy. Her clinical and radiological findings suggested infarction of a pituitary lesion, such as a pituitary adenoma or infarction of a cystic lesion, such as a Rathke's cleft cyst. In this report, we discuss her case of probable infarction of a growth hormone secreting adenoma with a phase of accelerated growth ending up with total anterior pituitary insufficiency. The differential diagnosis and review of the rare cases of paediatric pituitary infarction in the literature will be discussed.

Keywords: apoplexy; CSF-sella syndrome; gigantism; lymphocytic hypophysitis; pituitary; pituitary infarction.

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Introduction

Pituitary infarction or apoplexy in the paediatric population is rare, with only a few cases reported in association with pituitary adenomas. Pituitary infarction, with or without haemorrhage, leading to spontaneous cure of the underlying adenoma, is even rarer (1–4). Haemorrhage can occur into other lesions, such as a Rathke's cleft cyst (RCC) or less commonly, other cystic lesions (5, 6). Symptoms include sudden headache, visual field defects, low blood pressure, nausea and vomiting, and hypopituitarism (7, 8). In the most severe form, there is rupture of the haemorrhagic lesion into the hypothalamus (7) or into the subarachnoid space with signs of fever, meningismus, loss of consciousness, and death. Conversely, haemorrhage can be asymptomatic and only detected during surgery or by the pathologist on tissue sections. This spectrum from asymptomatic to catastrophic explains why the term "pituitary apoplexy" is often used imprecisely. In adults, precipitating factors for pituitary haemorrhage may include arterial hypertension, head trauma, anticoagulant therapy, radiotherapy, and hormone stimulation testing (8–10).

Another condition that can present acutely is lymphocytic hypophysitis, although apoplexy is rare. It is thought to be caused by self-reactive T-lymphocytes targeting pituitary cells, resulting in hypopituitarism (11). Symptoms include headache, vision loss, fatigue, nausea and vomiting, and loss of anterior pituitary hormones, particularly ACTH (12). In children, hypophysitis is usually peritumoral associated with craniopharyngioma or RCC (13) or germinoma (14), and is often associated with diabetes insipidus. There are no reported cases of paediatric hypophysitis presenting with apoplexy. Ongoing autoimmune inflammation can result in pituitary gland atrophy and empty sella syndrome (15). Empty sella or CSF-sella syndrome (as the sella is not truly "empty") also can occur when a pituitary adenoma or other lesion has been surgically removed, treated with radiotherapy, or undergone haemorrhagic infarction.

Here, we report an unusual case of sudden onset hypopituitarism resulting from pituitary infarction and progression to CSF-sella syndrome in an 11-year-old girl. We report on her investigations, treatment, and follow-up over the course of 10 years and an overview of the literature on pituitary infarction in the paediatric population.

Case report

An 11-year 4-month-old girl presented with symptoms of a urinary tract infection (UTI) and sudden onset vomiting. She was significantly dehydrated, with low blood pressure (90/60), and symptoms out of proportion to a UTI. Treatment with antiemetic and 2.5 L of intravenous fluids (IVF) improved her symptoms. She was discharged from the Emergency Department after rehydration, but symptoms returned 5 days later. Prior to her first admission, she had experienced moderate frontal headaches with no visual disturbances, but with vomiting, fever, lethargy, and 6 kg weight loss over 3 weeks. Laboratory tests revealed normal electrolytes, low TSH of 0.09 mU/L (normal 0.4–4.0) and free thyroxine (FT4) 11.1 pmol/L (normal 12–26) but normal free triiodothyronine (FT3) 4.6 pmol/L (normal 3.7–5.7). Random cortisol was 44 nmol/L at the time of the emergency visit but was undetectable in subsequent tests, prompting referral.

On examination, the patient was centrally obese, with height 159.5 cm (90th percentile), weight 59.05 kg (>97th percentile), BMI 23.2 (+1.44 SDS) with pale complexion, and no skin or buccal pigmentation. Pubertal development was normal for her age (Tanner 3 breast and pubic hair development). Past medical, surgical, and family histories were unremarkable. A Synacthen® test (Novartis, Austria) showed very poor response (cortisol <30 nmol/L, 86 nmol/L, and 100 nmol/L at 0, 30, and 60 min, and undetectable ACTH). Baseline assessment of pituitary hormones revealed the following: TSH 0.12 mU/L (normal 0.4–4), IGF1 0.7 U/mL (0.5–2), LH 0.2 IU/L (normal 0.0–2.3), FSH 2.3 IU/L (1.1–7.9), Prolactin 60 mIU/L (normal 0–42), and Oestradiol <10 pmol/L (Table 1). Tumour markers (AFP, HCG, CEA, CA125), viral serology, tuberculosis screen, and adrenal antibodies were negative (Table 1). Hormone replacement was started, and brain MRI was performed.

With the MRI precontrast, the pituitary showed some decreased signal within its centre but was normal in size (approximately 7 mm in height). The stalk was midline and hypothalamus was normal. There was loss of the posterior pituitary bright spot, although she had no symptoms of diabetes insipidus. After gadolinium, the pituitary stalk enhanced normally; however, there was no enhancement on the delayed images suggesting either a cystic lesion or no vascular supply and nonhaemorrhagic infarction of the pituitary (Figure 1 A–D). A repeat MRI 1 month later showed the pituitary gland had decreased in size and was now smaller and flatter. Only some peripheral enhancement persisted, leading to initial diagnosis of atrophy following nonhaemorrhagic infarction of the pituitary gland (Figure 1 E–H).

Pituitary autoantibody testing was performed using both immunofluorescence (IF) against monkey pituitary sections (EuroImmun; Lübeck, Germany) and ITT (in vitro transcription translation) for the corticotroph-specific transcription factor Tpit, pituitary gland specific factor 1a and 2 (PGSF1a PGSF2), Ca^{2+}-dependent secretion activator (CADPS), chromodomain-helicase-DNA binding protein 8 (CHD8), presynaptic cytomatrix protein (Piccolo) and

Table 1 Laboratory findings on initial evaluation.

	Presentation	Normal values
Evaluation of tumour markers		
β-HCG, mU/mL	<2	0–5
Alpha-fetoprotein, IU/L	2	0–9
CEA	1	0–5
CA125	16	<21
Hormonal evaluation		
Cortisol, nmol/L	44	180–720
Synacthen test (250 μg, IV) cortisol levels (0, 30, 90 min)	<30, 86, 100	
Free T4, pmol/L	11.1	8.4–29.6
Free T3, pmol/L	4.6	3.7–5.7
TSH, mU/L	0.09	0.4–4.0
FSH, IU/L	2.3	0.0–7.9
LH, IU/L	0.2	0.0–2.3
Oestradiol, pmol/L	<10	
DHEAS, μmol/L	<0.8	<5.5
Prolactin, mIU/L	60	0–42
IGF-1, U/mL[a] 0, 15, 45, 90, 150, 225 min	1.25, 0.67, 0.66, 0.70, 0.70, 0.69	1.00–2.9
GH, U/mL[a] 0, 15, 45, 90, 150, 225 min	<0.1	
Renin, ng/mL/h	1.8	1.2–2.8
Infection evaluation		
CMV	Negative	
Toxo	Negative	
EBV	Negative	
Rubella	Negative	
Echo-neuro	Negative	
Coxsackie	Negative	
Autoimmune evaluation		
Adrenal Ab	Negative	
Pituitary Ab	Piccolo	

[a]1 year after presentation.

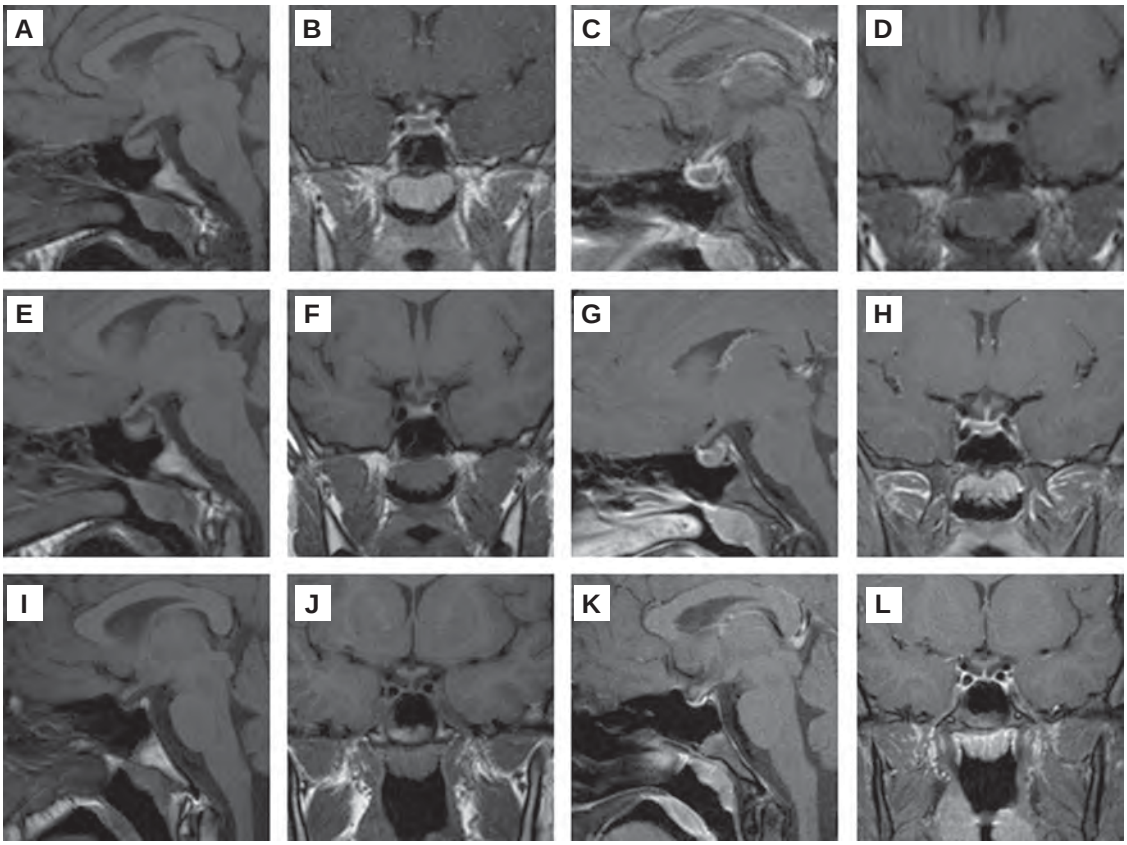

Figure 1 MRI scans.
Saggital unenhanced T1-weighted MRI at (A) initial visit, (E) on month after initial visit, and (I) 4 years postinfarction.
Coronal unenhanced T1-weighted MRI at (B) initial visit, (F) on month after initial visit, and (J) 4 years postinfarction.
Saggital contrast-enhanced T1-weighted MRI at (C) initial visit, (G) on month after initial visit, and (K) 4 years postinfarction.
Coronal unenhanced T1-weighted MRI at (D) initial visit, (H) on month after initial visit, and (L) 4 years postinfarction.

neuron specific enolase (NSE) (16). No autoantibodies were detected using the IF method with serum titre of 1:10 (data not shown), but autoantibodies were detected with specificity for Piccolo, a protein involved in dense-core vesicle transport (16).

By 12 years of age, 1 year after initial referral, the patient showed significant weight gain (69.85 kg=10 kg weight gain) and growth arrest (161.3 cm) (Figure 2), although her symptoms of vomiting and headaches had ceased. Bone age X-ray examination corresponded with her chronological age. Her appearance had become Cushingoid, her skin was dry, and she still complained of lethargy. Growth hormone stimulation testing with arginine and clonidine revealed growth hormone deficiency (GHD) (undetectable GH and low IGF-1 (0.66–1.25 U/mL, normal 1.00–2.90) (Table 1). Recombinant growth hormone therapy was commenced at 4.66 mg/m^2/week. Oestradiol (<100 pmol/L) was low, and breast and axillary hair development remained at Tanner 3, indicating pubertal arrest. Pubertal induction through oestrogen (E2) replacement therapy was started.

By 13 years 6 months, the patient remained centrally obese but had grown 10 cm in height (170.6 cm tall and 79.3 kg). Pubertal development progressed on treatment and menarche was induced. A repeat brain MRI showed the pituitary gland was flatter and reduced to 3 mm in height, half the original size (Figure 1 I–L).

The patient was seen for follow-up over 7 years and continues to struggle with central obesity. While the patient was taking growth hormone therapy she achieved some weight loss; unfortunately, at age 16.5 years, funding for recombinant growth hormone therapy was no longer available, and therapy had to be discontinued, resulting in rapid weight gain (Figure 3). It is well documented that growth hormone deficiency in adults has detrimental effects on body composition and metabolic processes, most of which can be reversed with recombinant growth hormone therapy (17–19).

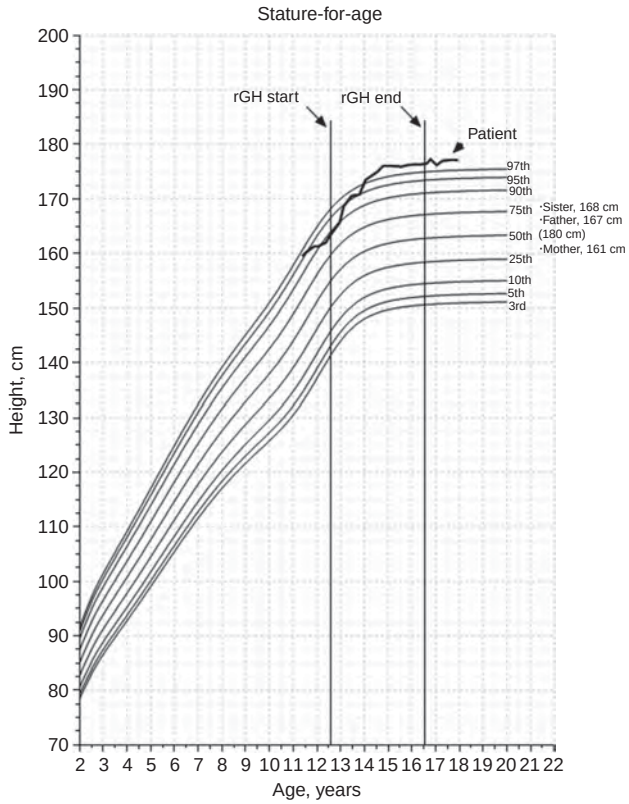

Figure 2 Growth chart.
Patient's 7-year stature-for-age (dark black line) with father's height (square) and mother's height (black circle) indicated. Source for percentiles: Developed by the National Centre for Health Statistics in collaboration with the National Centre for Chronic Disease Prevention and Health Promotion (2000) http://www.cdc.gov/growthcharts.

Our patient's most recent MRI, at age 15, showed a flattened pituitary with only a thin rim of tissue remaining and a CSF-sella (Figure 1 M–P). Now, 10 years after her initial episode, she remains on full hormone replacement therapy with the exception of growth hormone. No definite cause was found for pituitary infarction in this 11-year-old girl, but in retrospect, we suspect she had a pituitary tumour, possibly GH secreting, that was spontaneously cured by the event and lead to hypopituitarism and subsequent CSF-sella syndrome.

Discussion

This report presents a novel case of sudden onset hypopituitarism due to pituitary infarction in an 11-year-old girl in early puberty. As she had no visual field defects and her headaches were not debilitating, a conservative management strategy was chosen over surgical intervention. The lack of any diagnostic tissue sample limits our ability to determine the exact cause. On the basis of her clinical and radiological findings, the differential diagnosis includes infarction of a pituitary adenoma, infarction of a cystic lesion, such as a Rathke's cleft cyst, or craniopharyngioma. Intrasellar germinoma with secondary hypophysitis seems very unlikely.

Pituitary adenomas account for <3% of childhood brain tumours (20). They present with similar symptoms to other sellar lesions except that the majority (80%–97%) are hormonally active. ACTH adenomas are more common in younger male children, followed by GH tumours, whereas in older adolescents, particularly females, prolactin secreting adenomas predominate (21–27). Prolactinomas presenting with apoplexy are uncommon (28, 29). Nonsecretory tumours are rare.

Our patient had no evidence of long standing hypopituitarism as her growth had been excellent, although not obviously excessive, and she had started puberty. A tumour secreting GH could have contributed to her tall stature at presentation (on the 97th percentile) as her midparental height is just above the 50th percentile. In children, growth is proportionate, so there are often no signs of acromegaly, but our patient did have a prominent lower jaw. Spontaneous remission of gigantism was described as early as 1905, and more recent case reports include (1–4, 30). Growth hormone secreting tumours in children are more likely to cosecrete prolactin as they are derived from mammosomatotrophs (31) and are more likely to become haemorrhagic than in adults (32). However, our patient was in early puberty, and this is inconsistent with a prolactin-secreting component.

Other possible differential diagnoses include infarction of a cystic lesion, such as a Rathke's cleft cyst (RCC). Craniopharyngioma, xanthogranuloma, and dermoid or epidermoid cysts (33) are less likely given the MRI findings. RCCs are rare in the paediatric population, with 94 cases reported in the literature by 2011, [reviewed by Jahangiri et al. (34)] and an additional 14 cases published by the KRANIOPHARYNGEOM 2000 Study (5). They are thought to arise from remnants of Rathke's pouch (33, 35) and are generally asymptomatic. RCCs have been reported to undergo intracystic haemorrhage, which present with symptoms of pituitary apoplexy (6). A retrospective study identified 21 cases of RCC apoplexy in the literature, and two additional cases have been subsequently reported (6, 36). At least three cases occurred in patients under the age of 18; however, one study did not provide patient age, so it is possible that there are additional cases (6, 37, 38). None of the paediatric cases reported empty sella as a final outcome, as is seen in our patient. Diabetes insipidus has

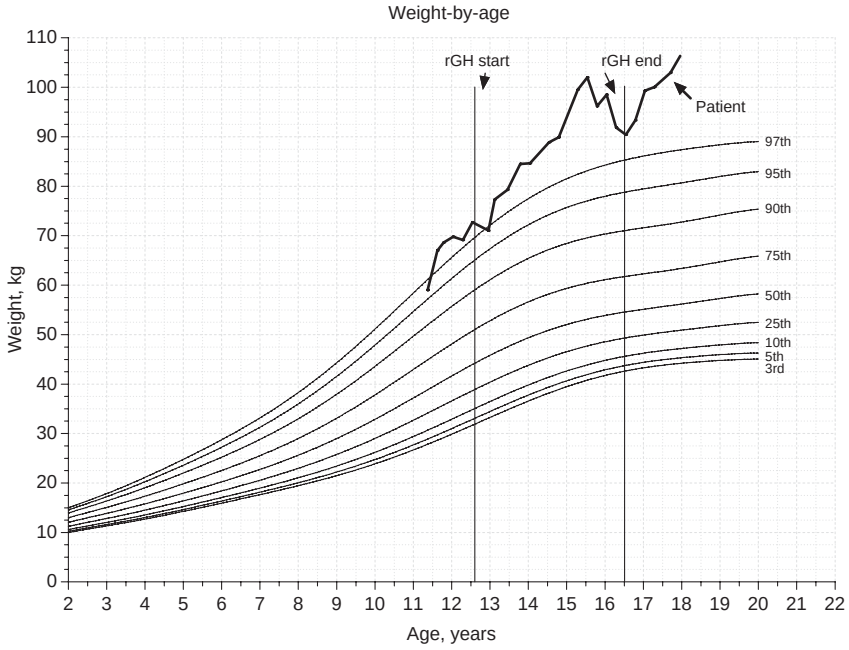

Figure 3 Weight chart.
Patient's 7-year weight-by-age (dark black line). Source for percentiles: Developed by the National Centre for Health Statistics in collaboration with the National Centre for Chronic Disease Prevention and Health Promotion (2000) http://www.cdc.gov/growthcharts.

been reported in up to 19% of cases (35). Thin peripheral rim enhancement, as seen on the MRI of our patient, has been attributed to inflammation or squamous metaplasia of a cyst wall (39). The secondary inflammation, pathologically identified as secondary hypophysitis, appears to be associated with diabetes insipidus and more loss of anterior pituitary function (13, 40). Additionally, most cysts display homogenous signal intensity as seen on our patient's MRI (41). In our patient, there was no evidence of diabetes insipidus at any stage. Finally, cystic lesions associated with hypopituitarism have been reported in association with PROP-1 mutations. These patients usually present with short stature due to growth hormone deficiency, so our patient's clinical picture does not fit this scenario (42, 43).

Germinomas affect primarily prepubertal children and often localise to the pineal gland or suprasellar region (44, 45). They can present as a pituitary stalk lesion alone, often with preceding diabetes insipidus (46). Our patient had pituitary involvement alone with no diabetes insipidus, making intrasellar germinoma unlikely. In the literature we found 19 cases of childhood germinoma initially misdiagnosed as lymphocytic hypophysitis (14, 47–55). The issue with germinoma is that the area of peritumoural or "secondary" hypophysitis can be extensive and can mask the underlying tumour, resulting in a false-negative biopsy. The most specific tumour marker in CSF and plasma is PLAP (human placental alkaline phosphatase) (56).

In the paediatric population, autoimmune hypophysitis is rare. In our review of the literature we found only four cases of hypophysitis not accompanied by diabetes insipidus or secondary to another pituitary condition or systemic disease (57–60). In contrast, Kalra and colleagues included 95 children with "hypophysitis", 85% of whom presented with symptoms of diabetes insipidus (61). This review included many children with undiagnosed stalk lesions (53), children with known germinoma (48–52) including one case cited twice (14, 47), children with coeliac disease and slow growth (62), children with isolated or central DI (52, 63–67), and one child with a Rathke's cleft cyst (67), none of whom are likely to have had primary autoimmune hypophysitis.

Pituitary autoantibodies against the dense-core vesicle transport protein, Piccolo, were found in the serum of our patient (16). The significance of a single pituitary autoantibody is unclear. Autoantibodies against a range of pituitary antigens have been described in the sera of patients with lymphocytic hypophysitis (16, 68–73). However, they also are described in other diseases including autoimmune thyroid disease, APS1, and in some cases of germinoma (74–76). Pituitary autoantibodies also are found at low titre in up to 12% of individuals with normal pituitary function, suggesting that these low

titre antibodies are epiphenomenon rather than causative of disease (16).

In summary, our patient presented acutely with no previous symptoms of pituitary dysfunction other than tall stature above her mid-parental height centile. Her MRI suggested infarction of a pituitary lesion that was most likely a GH-secreting adenoma. On reviewing the paediatric literature, we could find only four other cases where there was spontaneous cure of such a tumour (1–4). This case adds to the literature of pituitary infarction in the paediatric literature and highlights the difficulties in diagnosis of such rare events without the ability to obtain tissue confirmation.

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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.

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Abbreviations: CT, computerised tomography; GH, growth hormone; IGHD, idiopathic growth hormone deficiency; IF, immunofluorescence; TBS, Tris-buffered saline.

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 (ϵ-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 $400 \times g$ (4°C, 20 min) to remove cell debris and nuclei. The supernatant was then centrifuged at $100{,}000 \times g$ (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, 3D5 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

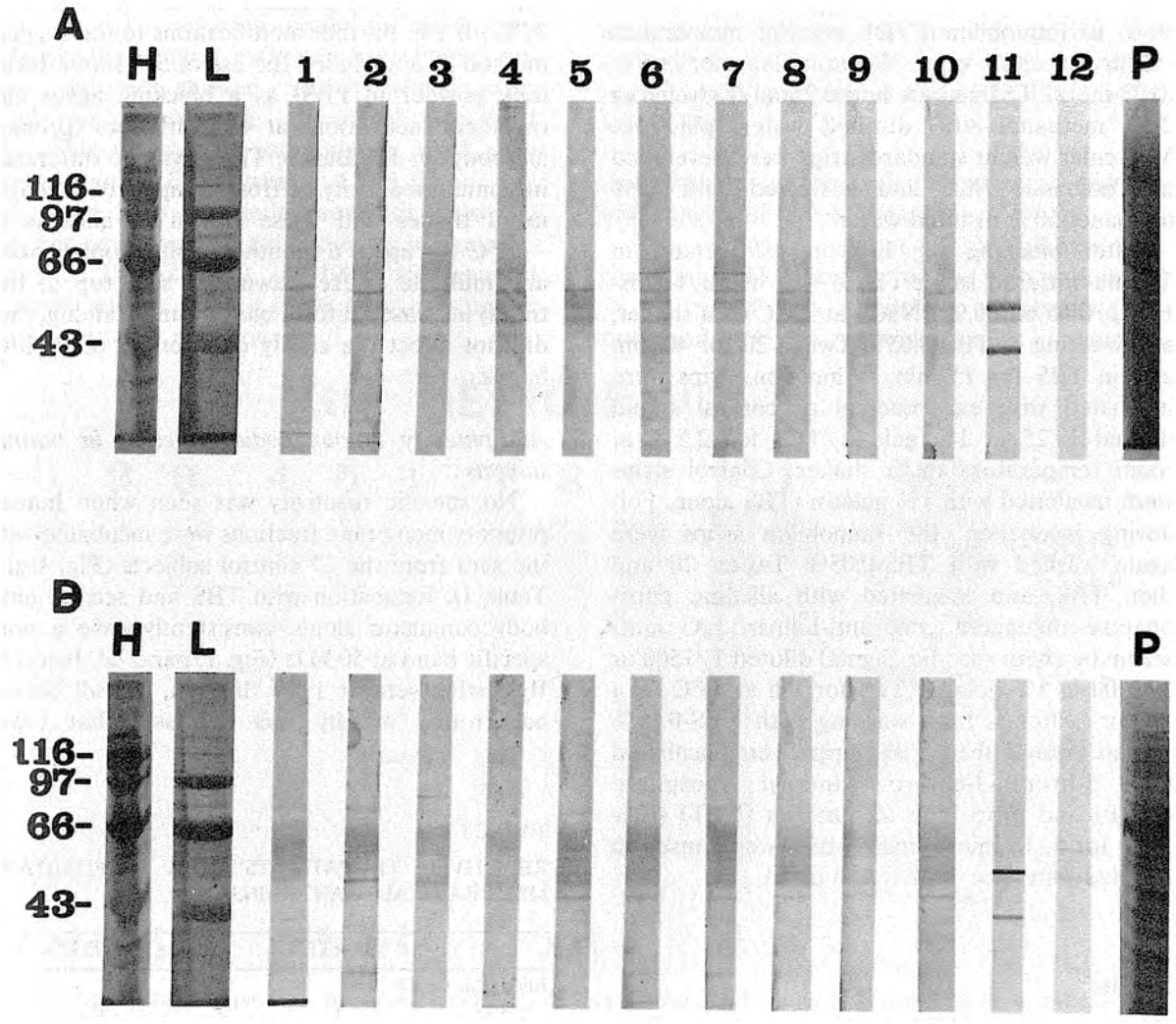

Fig. 1. *A*: immunoblotting of pituitary membrane homogenate (25 μg protein/lane) fractionated on a 10% SDS polyacrylamide gel, with sera from ten normal control subjects (lanes 1–10) and from a positive control patient (lane 11). Lane 12 = TBS/conjugate control. *B*: immunoblotting of pituitary cytosol (50 μg protein/lane) fractionated on a 10% SDS polyacrylamide gel. Lanes are as above. H and L = high and low molecular weight markers (kDa) respectively. P = total protein stain.

μg protein/well in 30 μl aliquots and pituitary cytosol at concentrations of 50 and 100 μg protein/well in 30 μl 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 μg protein in 30 μl/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 antiserum (γ 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 μg protein/well and a cytosolic protein concentration of 50 μg 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 I). 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

TABLE I

REACTIVITY OF PATIENTS' SERA TO PITUITARY MEMBRANE AUTOANTIGENS

	43 kDa	45 kDa	95 kDa
Idiopathic GHD $n = 19$	1	1	0
Secondary GHD $n = 14$	1	0	3
Normal controls $n = 27$	0	0	0

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 I). 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

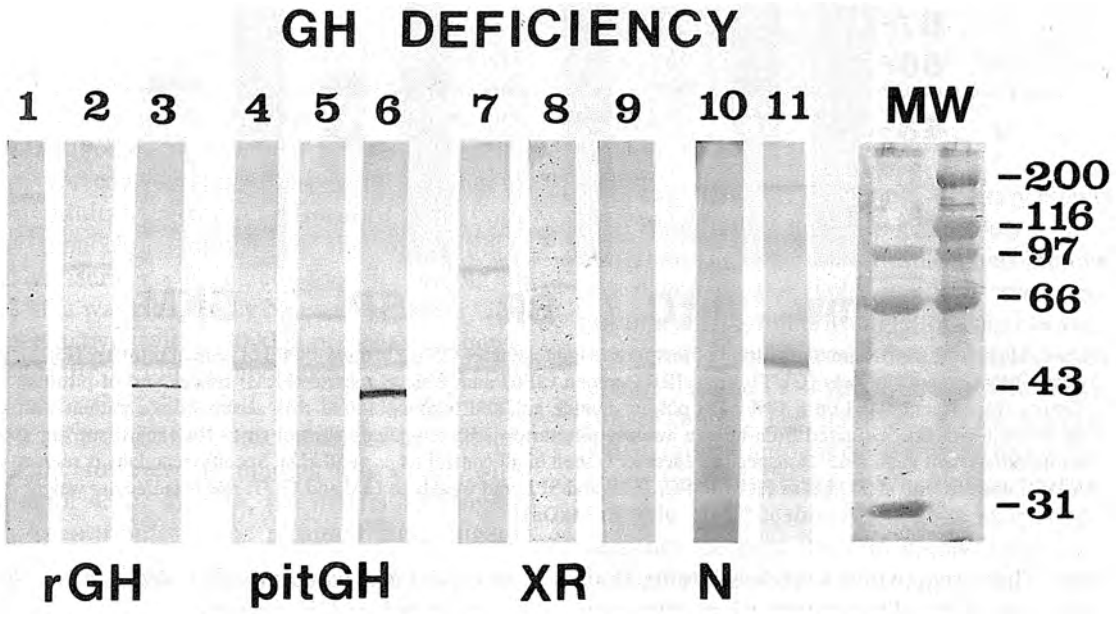

Fig. 2. Immunoblotting of pituitary membrane homogenate (25 μg protein/lane). Lanes 1–3: sera from three patients with idiopathic GH deficiency (IGHD) on recombinant GH therapy. Lane 3 shows positive reactivity at 45 kDa with serum from the patient with an empty sella on CT scan. Lanes 4–6: sera from 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).

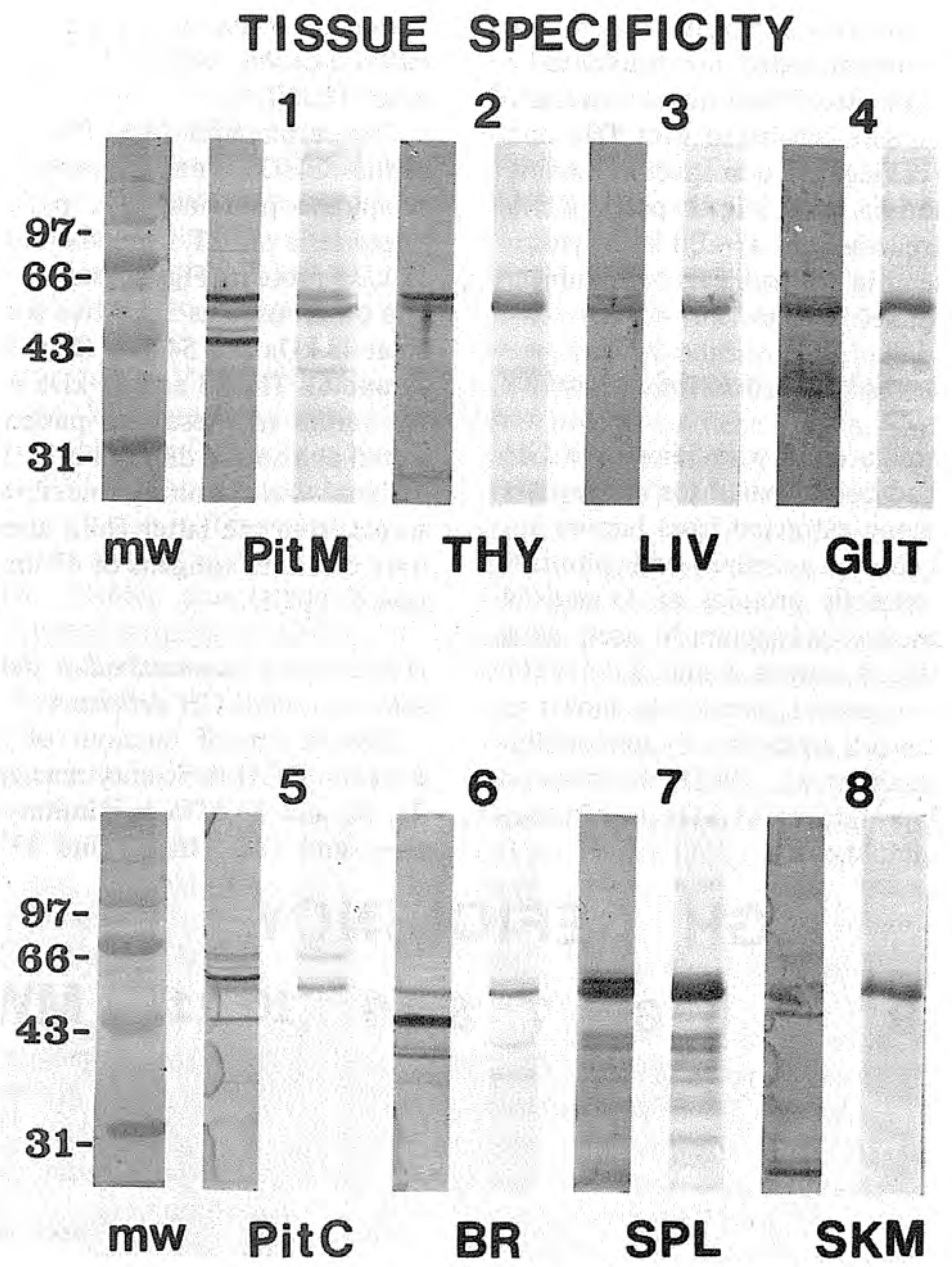

Fig. 3. Tissue specificity study. Immunoblotting of membrane homogenates (25 μg protein/lane) of human pituitary (PitM), thyroid (THY), liver (LIV), smooth muscle (GUT), brain (BR), spleen (SPL) and skeletal muscle (SKM) tissues and of pituitary cytosol (PitC). Tissues were fractionated on a 10% SDS polyacrylamide gel, and immunoblotted with serum from a patient with IGHD, previously treated with GH extracted from human autopsy pituitaries. TBS/conjugate control strips for each tissue are shown on each corresponding right hand lane. Nonspecific reactivity is seen in all control lanes at 50 kDa. Specific reactivity is seen at 43 kDa to Pit M, Pit C and BR and at 55 kDa to Pit M, Pit C, THY and SPL and weakly to LIV and GUT. 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.

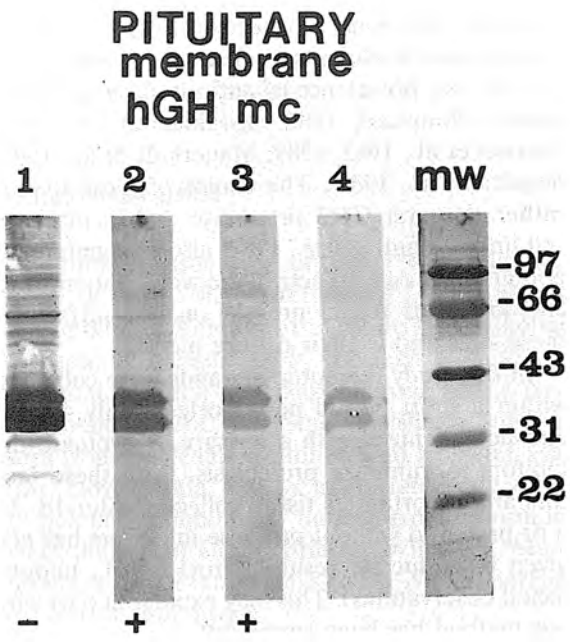

Fig. 4. Immunoblotting of human pituitary membrane fraction analysed on a 15% SDS polyacrylamide gel, with mouse anti-human GH monoclonal antibody 3D5 (hGH mc, dilution 1/100,000) and second antibody, anti-mouse IgG + M (dilution 1/1000). Lane 1: monoclonal antibody 3D5 alone. Lane 2: monoclonal antibody 3D5 with excess GH + (50 mg/ml), showing specific binding competed out at ~ 22 kDa. Lane 3: excess GH + (50 mg/ml) alone. Lane 4: TBS/conjugate control. mw = molecular weight standards (kDa).

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; Suguira et al., 1987). The choice of these tissues rather than rat GH3 or mouse AtT20 pituitary cell lines (Suguira 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 (Suguira 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 (Etzrodt 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 (Baekkeskov 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 anti-pituitary 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.

Acknowledgements

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Severe Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy in an Adolescent Girl with a Novel *AIRE* Mutation: Response to Immunosuppressive Therapy*

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ABSTRACT

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal recessive disorder for which the gene (*AIRE*) has recently been identified on chromosome 21q22.3. We present the mutational analyses of a French-Canadian family with APECED, in which there are two affected siblings, as well as the response to cyclosporine A(CyA) therapy in the index patient, the eldest sibling.

Haplotype analysis suggested compound heterozygozity at the *AIRE* locus. Direct sequencing of exon 8 revealed a previously described mutation, a 13-bp deletion (1085–1097) of maternal origin, found in the index patient, her affected sister, and her unaffected sister. A novel missense mutation characterized by a T→G transversion at nucleotide position 398, resulting in a leu→arg amino acid substitution (L93R), was found in exon 2. The mutation was present in the father, the brother, the index patient, and the affected sister. The presence of the mutation in the propositus was verified by cloning of PCR products from genomic DNA. The mutation destroys a *Pst*I restriction enzyme site, as confirmed in the aforementioned patients. Screening of 50 French-Canadian controls with *Pst*I digestion did not show destruction of the restriction-enzyme site.

The index patient's phenotype was severe, manifested by classic features of the illness (adrenal insufficiency, hypoparathyroidism, candidiasis, and keratoconjunctivitis with alopecia universalis), as well as by severe exocrine pancreatic insufficiency, diabetes mellitus, hepatic inflammation, growth hormone (GH) deficiency due to lymphocytic hypophysitis, and primary ovarian failure. Oral CyA (5 mg/kg/day) was initiated at 13 yr of age. After 8 months of therapy, stimulated pancreatic lipase increased 24-fold with normalization of stool fat (from 31.5 g/day to 2.5 g/day, normal(N) < 5). There was complete resolution of her photophobia, and considerable hair regrowth was diffusely apparent. Minimal side effects were noted. Our experience supports the use of oral CyA for the treatment of severe APECED-associated exocrine pancreatic failure and keratoconjunctivitis. (*J Clin Endocrinol Metab* **84:** 844–852, 1999)

AUTOIMMUNE polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a potentially debilitating, even lethal, autosomal recessive disorder characterized by variable endocrine gland failure, including hypoparathyroidism and adrenal insufficiency, chronic mucocutaneous candidiasis, and dystrophy of the nails and teeth with alopecia and keratitis (1–5). In 1994, the locus for APECED was mapped to chromosome 21q22.3 by linkage analysis in Finnish families, among whom there is a relatively high incidence of the disease (1:25,000) (6). Recently, the gene responsible for APECED was identified and found to code for a nuclear protein characterized by two plant homeodomain-like zinc-finger domains, which are expressed in a variety of tissues including thymus, lymph nodes, and fetal liver (7–9). The discovery of the *AIRE* (autoimmune regulator) gene (7, 8) represents the first single-gene defect resulting in a multisystem autoimmune disease. To date, eight mutations have been reported in various geoethnic groups (7–10). This paper describes the haplotype and *AIRE* analyses of a French-Canadian pedigree and the discovery of a novel missense mutation in addition to the 13-bp deletion previously identified.

The phenotype of APECED is highly variable; however, conclusions regarding genotype-phenotype correlations have not been possible (1–3). Although recent reviews have discussed the natural history of APECED in depth (1, 3), little emphasis has been placed on therapy. With increasing experience in the use of immunosuppressive therapy (11, 12), more aggressive and comprehensive treatment of APECED is feasible.

We investigated a French-Canadian pedigree including the adolescent index case who presented rare features of the disease that posed diagnostic and therapeutic challenges,

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notably severe exocrine pancreatic insufficiency and isolated growth hormone (GH) deficiency secondary to lymphocytic hypophysitis. We further report the clinical investigations in the evaluation of this girl's severe disease and the results of treatment with cyclosporine A (CyA).

Subjects and Methods

Family

The index case, a 13.6-yr-old girl, was the first of four children born to healthy, nonconsanguineous, French-Canadian parents. A 12-yr-old sister has suffered only mild episodes of vaginal candidiasis. An 11-yr-old sister also presented with features of APECED, including mucocutaneous candidiasis, hypoparathyroidism, and alopecia areata. An 8-yr-old brother has been treated for oral candidiasis, but is otherwise unaffected.

Clinical course of index patient (refer to Table 1 for clinical summary)

Candidiasis and ectodermal dystrophy. The patient presented in infancy with recurrent mucocutaneous candidiasis, unresponsive to topical antifungal agents, though controlled with systemic therapy. Immunological investigations showed a cell-mediated immune deficiency with a low thymocyte-helper to thymocyte-suppresser cell ratio. Photophobia and visual impairment occurred at 6 yr of age followed by alopecia universalis at age 9 yr. The keratoconjunctivitis was of such severity that home schooling became necessary. Systemic vitamin A was initially prescribed, with some improvement. This was eventually changed to topical vitamin A ointment (Dulcis, Allergan, Inc., Mougins, France) because of concern for potential hepatoxicity; the topical vitamin A treatment proved remarkably effective in preventing new corneal ulceration and allowing resumption of classroom studies, although photophobia persisted. Dental enamel hypoplasia was present without evidence of nail dystrophy.

Gastrointestinal dysfunction. Mild, intermittent elevations of liver enzymes (maximum aspartate aminotransferase and alanine aminotransferase values between two and three times the upper limit of normal) were apparent from the age of 2 yrs, although liver function tests were otherwise normal. A liver biopsy at 11.8 yrs of age revealed a mild chronic, focal hepatitis with lymphocytic infiltration. Hepatitis viral serology was negative. Liver-kidney-microsomal (LKM_1) antibodies and liver cytosol antibodies were negative. However, using previously described methodology, immunoblotting detected reactivity to a 45–47 kDa protein in the microsomal fraction of human liver (13).

Gastrointestinal symptoms associated with growth failure occurred between the ages of 7.5 and 9 yrs. These included severe abdominal distension (Fig. 1A), cramping pain, diarrhea, and constipation. The subject was normocalcemic at the time. Upper gastrointestinal series and rectal manometry were normal. Upper endoscopy with quantification of intestinal disaccharidases revealed hypolactasia (1.2 μmol substrate/min/g, normal (N = 11–85); sucrase and maltase levels were normal (55 μmol substrate/min/g, N = 27–145 and 174 μmol substrate/min/g, N = 112–618, respectively). In the face of normal serum immunoglobulin A, G, and M levels, anti-enterocyte and anti-gliadin antibodies were negative. Bacterial overgrowth was present: duodenal fluid cultures were positive for two strains of aerobic bacteria (*streptococcus viridans*, 2.0×10^8 CFU/L) and coagulase negative *staphylococcus*, 2.0×10^8 CFU/L). She was treated with a lactose-free diet, lactase supplements, and oral metronidazole, without recuperation of weight or improvement in symptoms. Endoscopy-directed small bowel biopsies at ages 8 and 10 yr confirmed normal villous architecture with mildly increased lymphocytic infiltration of the lamina propria. Silver staining of the duodenal biopsies obtained at 8 and 10 yr demonstrated an absence of serotonin-secreting enterochromaffin cells. Lymphangiecstasia was ruled out on these biopsies and by a normal albumin $^{51}CrCl_3$ test to quantify stool protein loss (0.16%, N <0.7).

However, severe malabsorption of fat was evident (stool fat 14.3 g/day; N = <5). We therefore carried out pancreatic function tests endoscopically, using stimulation with secretin (14). This confirmed the presence of an exocrine pancreatic insufficiency (50 min post-stimulation with secretin: lipase 51 IU/kg/50 min (N = 1,212–1,636). A sweat chloride test was normal (17 mEq/L, N <60). Pancreatic enzyme replacement was initiated at 9 yr of age (pancrelipase, Cotazyme ECS-20, Organon, 8 tablets/day), with a transient improvement in stool fat excretion However, little improvement in weight gain was achieved (Fig. 1B). Nine months after the initiation of pancreatic enzyme therapy, the stool fat was still extremely elevated (31.7 g/day). Despite maximal enzyme replacement (pancrelipase, Creon 25, Solvay Pharma, 22 capsules/day), weight loss was evident. Nocturnal nasogastric gavages with a fat-deficient elemental diet (Tolerex, Novartis Nutrition) were thus added at 10.6 yr of age, resulting in satisfactory weight gain. However, abdominal distension and cramps persisted.

Endocrine dysfunction. At 4.6 yr of age, the proband presented with seizures and tetany secondary to hypocalcemia. Hypoparathyroidism was documented, and parathyroid antibodies were positive by indirect immunofluorescence. She was successfully treated with calcitriol. At 10 yr of age, first-phase insulin release during an intravenous glucose tolerance test was performed as described (15) and was compared to age-matched, female controls. A decrease in the first-phase insulin release from greater than the 95th to less than 10th percentile (140.0 μU/ml to 70.3 μU/ml) (1, 15) was observed over the ensuing year. In addition, very high serum anti-islet cell antibodies (ICAs) were detected (>80 JDF units). After complaints of polyuria and polydipsia at age 11, home blood glucose monitoring revealed an elevated post-prandial glucose (270 mg/dL) with preprandial glucose values between 126 and 162 mg/dL. The HbA_{1C} was normal (0.052, N = 0.043–0.058), and urine ketones were negative. Long-acting subcutaneous insulin was initiated at 11.6 yrs of age (0.15 U/kg/day). At the time of this report, the proband was receiving 0.4 U/kg/day long-acting human insulin before supper as a once daily injection to maintain euglycemia.

Despite significant improvement in the patient's nutritional status (height-to-weight ratios above the 50th percentile) after treatment for pancreatic insufficiency, growth velocity continued to decrease [3.0 cm/yr between 11 and 12 yr of age, < 3rd percentile for bone age (16)].

TABLE 1. Index patient's clinical course

Age (yr)	Problem	Diagnosis
Infancy	Mucocutaneous candidiasis	Cell-mediated immune deficiency
2	Mild elevation of liver enzymes	Hepatic inflammation confirmed on biopsy at 11.8 yr of age
4.6	Seizures secondary to hypocalcemia	Hypoparathyroidism (positive parathyroid antibodies)
6	Photophobia, decreased vision, blepharospasm	Severe keratoconjunctivitis, corneal ulceration
7.5	Steatorrhea, growth failure, abdominal bloating	Lactase deficiency, bacterial overgrowth, and severe exocrine pancreatic insufficiency
9	Alopecia universalis, dental enamel hypoplasia	Ectodermal dystrophy
11	Decrease in first phase-insulin release, post-prandial glucose 270 mg/dL, fasting glucose 126–162 mg/dL	Diabetes mellitus
12	Decrease in height velocity despite improvement in nutritional status	Growth hormone deficiency secondary to lymphocytic hypophysitis
13.6	Hyperpigmentation, nausea, fatigue	Addison's disease
13.6	Delayed puberty	Primary ovarian failure

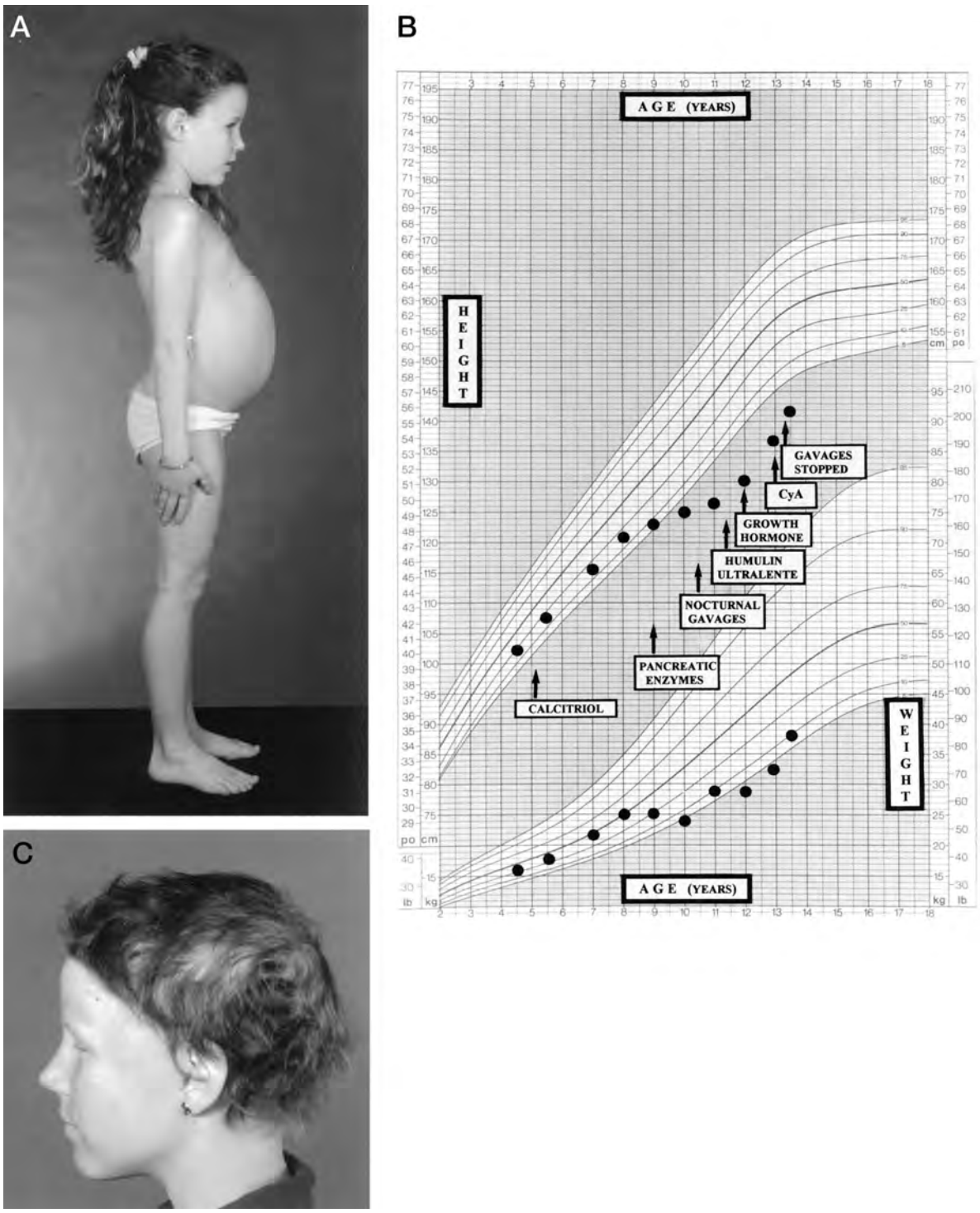

FIG. 1. A, Index patient at 8.0 yr of age. Severe abdominal bloating was accompanied by alternating diarrhea and constipation. B, Index patient's growth curves showing correlation between initiation of various therapies and subsequent growth response. C, Index patient at 13.6 yr of age. Despite 4 yr of alopecia universalis, hair regrowth after 8 months of CyA therapy included scalp hair, eyebrows, and eye lashes. Most notably, scalp hair texture returned to normal.

At 11.9 yr of age her growth parameters were as follows: height 129.6 cm (−2.9 SD), weight 28.1 kg (−1.2 SD), and bone age 8 yr. Plasma insulin-like growth factor (IGF)-I was low before nutritional supplementation (46 ng/mL), but demonstrated a modest rise after the sequential initiation of pancreatic enzyme supplementation, gavage feedings, and insulin (46, 64, and 93 ng/mL, respectively). Provocative GH

testing after confirming euthyroidism revealed inadequate peak responses of 0.8 and 0.3 ng/mL to L-dopa-propranolol and to clonidine, respectively. The maximal nocturnal GH peak was 3.1 ng/mL. The peak GH response to clonidine with estrogen priming (20 μg ethinyl estradiol once daily for three days) was 4.6 ng/mL. Magnetic resonance imaging demonstrated a perihypophyseal "halo effect" with gadolinium enhancement, suggestive of hypophysitis. Immunoblotting of human pituitary membranes with consecutive samples of the patient's sera detected antipituitary autoantibody reactivity to a 43–45 kDa protein (17). These findings were highly suggestive of autoimmune hypophysitis complicated by GH deficiency. GH replacement therapy was provided with recombinant human growth hormone (0.18 mg/kg/week, given as a daily injection sc) at age 12.0 yr, and over a 12 month period there was significant increase in growth velocity (9 cm/yr) and an increase in plasma IGF-I to 483 ng/mL.

At age 12.7 yr, four months before the initiation of immunosuppressive therapy as outlined below, the gonadotropin and corticotropin axes were assessed by dynamic testing. A baseline cortisol was 13.9 μg/dL at 0800 (N = 5–25), with a peak of 15.4 μg/dL (N > 20) after 250 μg ACTH, although 24-h urine cortisol was normal. An LHRH stimulation test (100 μg) demonstrated a prepubertal response (baseline LH 0.8 mIU/mL, FSH 5.47 mIU/mL; peak LH 2.3 mIU/mL, FSH 16 mIU/mL). Anti-ovarian antibodies were positive. To date, posterior pituitary function has been unaffected.

At 13 yr of age, immunosuppressive therapy was initiated with CyA, 5 mg/kg/day, after the informed consent of both the patient and her parents. Indications and treatment goals were: 1), to improve pancreatic function, given that the patient remained dependent upon nocturnal gavages to prevent weight loss despite maximal therapy with pancreatic enzyme supplements; 2), to prevent further progression of the severe keratoconjunctivitis and of the hepatic inflammation; and 3), to prolong residual pancreatic endocrine function.

Methods

Autoantibodies and hormonal assays. Antimicrosomal liver antibodies (13) and antipituitary membrane antibodies (17) were measured by Western immunoblots as previously described. Anti-adrenal antibodies were detected by indirect immunofluorescence using monkey adrenal tissue (Inova Diagnostic Inc., San Diego, California). Methods for antiparathyroid antibodies (18), anti-IA-2, and antiglutamate decarboxylase (GAD) antibodies (19), anti-aromatic-L-amino-acid decarboxylase (AADC) (2, 20), and antitryptophan hydroxylase antibodies (21) have been detailed elsewhere. Anti-ovarian and anti-ICAs were kindly performed by Dr. Noel MacLaren (Harahan, LA).

Plasma insulin was measured by a semi-automated microparticle enzyme immunoassay (Abbott Laboratories, Montréal, Québec). Plasma growth hormone, cortisol, and gonadotropins were measured by time-resolved immunofluorometric assay (AutoDELFIA, EG&G Wallac, Kirkland, Québec). Glycated hemoglobin was performed with an immunoturbidometric inhibition assay (Boehringer Mannheim, Montréal, Québec). Plasma aldosterone was measured by a one-step solid-phase radioimmunoassay (Diagnostic Product Corp., Los Angeles, CA), plasma renin with a solid-phase radioimmunometric assay (Sanofi Diagnostics Pasteur, Montréal, Québec) and ACTH by a two-antibody, equilibrium radioimmunoassay (INCSTAR Corp., Stillwater, MN). Plasma IGF-I was measured after acid-ethanol extraction using an immunoradiometric assay (Diagnostic Systems Laboratories, Webster, TX).

Mutation analysis. Genomic DNA was isolated from peripheral blood samples taken from the propositus, her three siblings, parents, and extended family members after informed consent (Fig. 2). Haplotype analysis with the markers D21S1912 (GDB Accession ID: 665559), D21S171 (GDB ID: 60660) and PFKL (GDB ID: 60455) was performed using Human MapPairs and PCR conditions from Research Genetics, Inc., Genome Services protocol (Research Genetics, Inc., Huntsville, AL). The *AIRE* gene (GenBank accession no. AB006684) is located just centromeric (<10 kb) to the PFKL gene and approximately 150 kb from the other two flanking markers noted above. Exons 2, 6, 8, and 10 of the *AIRE* gene, together with adjacent intronic sequences were amplified using primers and conditions specified by the Finnish German APECED Consortium (*http://chr21.rz-berlin.mpg.de/APECED.html*) and a Gene Amp 9600 PCR system (Perkin Elmer, Cetus Instruments, Norwalk, CT). The

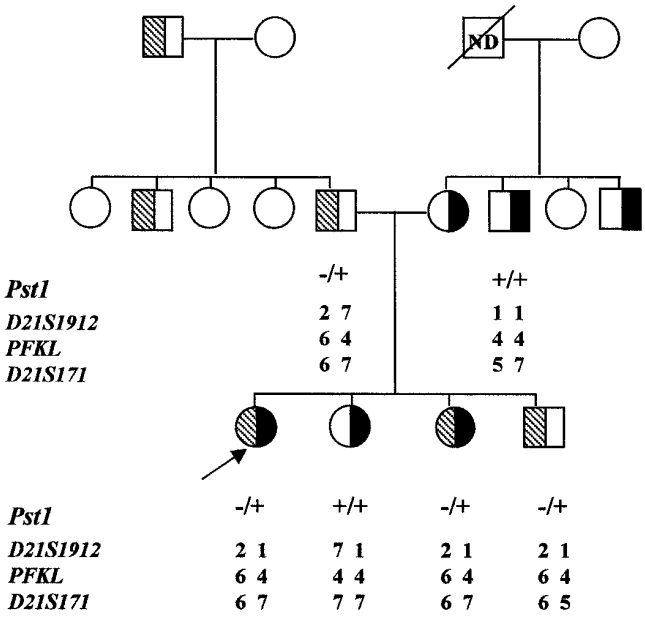

FIG. 2. Familial pedigree showing *Pst*I RFLP analysis of the *AIRE* exon 2 PCR product and haplotype at 21q22.3 (see *Subjects and Methods* section). The index patient and her younger sister are compound heterozygotes as suggested by the haplotype analysis and as confirmed by sequencing of exons 2 and 8. The novel, exon 2 mutation disrupts a *Pst*I site, permitting rapid screening of the maternal relatives. *Hatched symbols*, exon 2 mutation inherited from the paternal grandfather; *Solid symbols*, exon 8, 13-bp deletion presumably inherited from the maternal grandfather (N.D., genotype not determined).

PCR products were then purified with the Qia-quick PCR purification columns kit (Qiagen Inc., Ontario, Canada) according to the manufacturer's instructions, directly sequenced by the dideoxy nucleotide chain termination method (Thermo Sequenase terminator cycle sequencing kit, Amersham Life Science), and reaction products were run on a 6% denaturing polyacrylamide gel. The exon 2 PCR product containing the mutant *Pst*I restriction site in the heterozygous form was subcloned into pBluescript II KS (Stratagene, La Jolla, CA). Plasmids (0.25 pmol) with either the mutant or the normal allele were then directly sequenced in the manner described above.

*Pst*I restriction enzyme analysis of the exon 2 PCR product was performed for the family as well as for 50 French-Canadian controls. After amplification by PCR as described above, 5 μL purified PCR product was digested for 2 h with 20 U *Pst*I (Life Technologies, Grand Island, NY), separated on a 7% polyacrylamide gel, and the products were visualized after ethidium bromide staining.

Results

Mutation analysis

Haplotype analysis suggested compound heterozygosity at the *AIRE* locus (Fig. 2). Direct sequencing of exon 8 revealed a previously described mutation, a deletion of nucleotides 1085–1097 (EMBL Accession no: mRNA Z97990) found in the index patient, her affected sister, and her unaffected sister. This 13-bp deletion mutation was of maternal origin, presumably inherited from the maternal grandfather. It results in a frameshift and produces a truncated 371 amino-acid protein. The defect is predicted to lead to the loss of at least one of the two *AIRE* PHD fingers (7).

Direct sequencing of exons 6 and 10 failed to show any of

the previously described or any novel mutations. However, a T→G transversion at nucleotide position 398, resulting in a leu→arg amino acid substitution (L93R) was detected in exon 2. The mutation was found in the father, the brother, the index patient, and the affected sister. The unaffected sister and mother were not carriers of the mutation. The presence of the mutation in the propositus was confirmed by cloning of PCR product from genomic DNA (Fig. 3). The mutation destroys a *Pst*I restriction-enzyme site. Using *Pst*I digestion, its presence was confirmed in the index patient, the clinically affected sister, the father, the brother, one paternal uncle, and the paternal grandfather. Screening of 50 French-Canadian controls with *Pst*I digestion did not show destruction of the restriction-enzyme site. Thus, none of the controls were carriers of the novel mutation.

Immunosuppressive therapy

An outline of the response to CyA is shown in Table 2. The pancreatic dysfunction and ectodermal dysplasia responded dramatically to therapy. Stimulated pancreatic lipase increased 24-fold, accompanied by a marked decrease in the quantity of stool fat and requirements for pancreatic enzyme supplements. Nocturnal gavage feedings were successfully discontinued 5 months after CyA. The patient also reported marked improvement in her symptoms of abdominal bloating, diarrhea, and cramps. Eight months after initiating CyA, ophthalmological re-evaluation showed no evidence of new corneal ulcerations. The severe photophobia resolved completely, obviating the need for artificial tears and vitamin A ointment. Hair regrowth was apparent diffusely, most notably on the scalp (Fig. 1C).

The endocrine assessment after 8 months of CyA demonstrated progression from a prepubertal gonadotropin profile in the presence of anti-ovarian antibodies to that of primary ovarian failure. Similarly, there was progression of the adrenal insufficiency. The suboptimal stimulation of cortisol with 250 μg of ACTH before the initiation of immune therapy evolved to a complete absence of cortisol response to the supraphysiological dose of ACTH on CyA. Plasma ACTH, measured after 8 months of CyA, was 285.7 pg/mL (N = 0–71), consistent with primary adrenal failure. Anti-adrenal antibodies were positive, and titres remained unchanged before and after 8 months of immunosuppressive treatment. Glucocorticoid replacement therapy was started. Results of the iv glucose tolerance test revealed that insulin secretion increased 6-fold with CyA, and at the same time, the dose of human long-acting insulin increased from 0.14 U/kg/day to 0.4 U/kg/day in the absence of recurrent episodes of hypoglycemia. Thyroid and posterior pituitary function remained unaffected over the 8-month period.

Hypomagnesemia (1.5 mg/dL, N = 1.7–2.4) and hyperuricemia (147 ng/mL, N = 55–110) developed as a result of the CyA therapy. The patient was treated with magnesium supplements and allopurinol (100 mg twice daily) with complete normalization of these parameters. Serum urea and creatinine were within normal limits both before and after CyA. The glomerular filtration rate (GFR) by ^{99}Technetium DTPA scanning before initiation of immunosuppressant therapy was 150 cc/min/1.73 m^2, followed by 100 cc/min/1.73 m^2 after 8 months of treatment (N = 125 ± 13).

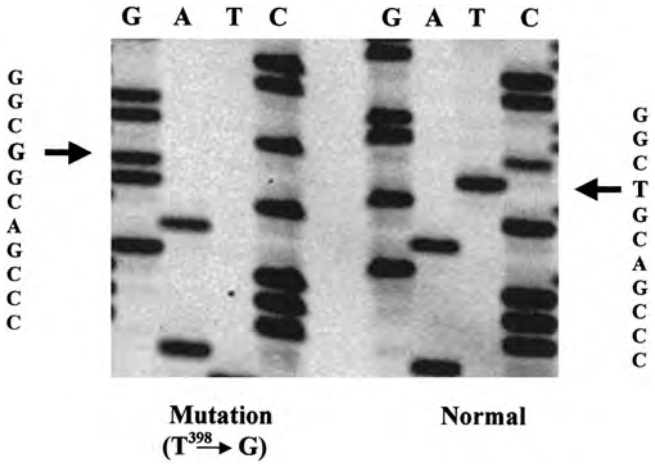

FIG. 3. Novel missense mutation in exon 2 revealed by sequencing of cloned alleles obtained as described in *Subjects and Methods* section. The thymidine at nucleotide position 398 was replaced by a guanine (transversion), resulting in the substitution of an arginine for a leucine at position 93 of the protein (missense mutation).

Discussion

This patient, in whom we discovered a novel *AIRE* mutation, demonstrated a particularly severe APECED phenotype characterized by well-described features of the disease. These included hepatitis and diabetes mellitus, in addition to two unusual diagnoses: severe exocrine pancreatic insufficiency and isolated GH deficiency secondary to autoimmune hypophysitis.

APECED complicated by steatorrhea is well-documented, as it occurs in up to 24% of cases in the Finnish population (1). However, the etiology of fat malabsorption in this setting is poorly defined. Malabsorption was initially attributed to hypoparathyroid-induced hypocalcemia (22); more recently it has been proposed that malabsorption is the primary event that subsequently leads to poor absorption of calcium and vitamin D and exacerbation of diarrhea (23, 24). Steatorrhea in APECED has also been linked to intestinal infection (23). In the present case, bacterial overgrowth of the small bowel was a contributing, though not the only, factor. Bereket *et al.* (24) report a patient with APECED-associated steatorrhea who had histological evidence of dilated lacteals in the presence of normal villous architecture. However, other clinical features of intestinal lymphangiectasia, such as edema, hypoproteinemia, and lymphocytopenia were absent, thus weakening the possibility that lymphangiectasia was the sole source of the patient's malabsorption. Steatorrhea has also been associated with autoimmune enteropathy, recently described in a patient with APECED, intractable diarrhea and hypocalcemia unresponsive to supplementation (25). Villous atrophy and mild subacute inflammation were present on intestinal biopsy, with positive serum autoantibodies to the brush border of normal gut enterocytes. This patient was successfully treated with iv high-dose methylprednisolone and maintenance oral methotrexate (25). Interestingly, pancreatic function tests were consistent with mild pancreatic

TABLE 2. Response to cyclosporine A therapy

	Parameter	Pre-CyA	8-month evaluation on CyA
Gastrointestinal dysfunction	Dose of pancreatic enzymes (Creon 25)	22/day	6/day
	AST (N: 11–43 U/L)	92	27
	ALT (N: 5–25 U/L)	73	23
	Stool fat quantification (N: <5 g/day)	Maximum 31.7	2.5
	Stimulated Lipase (N: 1,212–1,636 IU/kg/50 mins)	51	1244
	Anti-AADC antibodies[20] (N: <20)[a]	109	103
	Antitryptophan hydroxylase antibodies[21] (N: <14)[a]	76	53
	Antiliver microsomal antibody (45–47 kDa)	Positive	Positive
Endocrine dysfunction	First-phase insulin release (1′ + 3′ minus baseline insulin) μU/mL	70.3 (<10thth percentile)	427.1 (>95th percentile)
	Anti-GAD antibodies (N: <5 U)	77	74
	Anti-IA-2 antibodies (N: <3 U)	0.1	0.2
	Anti-ICAs	>80 JDF units	N.D.
	Antipituitary membrane antibodies (43–45 kDa)	Positive	Positive
	Antiadrenal antibodies	Positive 1/40	Positive 1/40
	ACTH stimulation test (μg/dL) (250 μg Cortrosyn) (N baseline cortisol: 5–25; stimulated cortisol >20)	Baseline cortisol 13.9 at 0800; peak 15.4 at 30 min	Baseline cortisol 8.7 at 0800; no rise in response to ACTH
	Plasma renin (N: 5.1–38.7 ng/L)	N.D.	34.2
	Aldosterone (N: 3.6–21.6 ng/dL)	N.D.	3.3
	LHRH test (mIU/mL) (100 μg Leuprolide)	Baseline LH 0.8, FSH 5.5; peak LH 2.3, FSH 16.0	Baseline LH 10.9, FSH 48; peak LH 51.2, FSH 72.2
	TRH Test (μU/mL) (200 μg TRH)	N.D.	Baseline TSH 2.9, peak TSH 6.8 at 30 min
Ectodermal dystrophy	Alopecia	Universalis	Diffuse regrowth
	Keratoconjunctivitis	Corneal ulceration, photophobia, blepharospasm	No new ulcerations of the cornea, no photophobia

N.D., Not determined.
[a] Reported as antibody index[21].

insufficiency, which was unresponsive to pancreatic enzyme replacement. Evidence for autoimmune destruction of the exocrine pancreas has been suggested by others: Scirè *et al.* (23) reported a 15-yr-old girl with APECED and exocrine pancreatic insufficiency who was successfully treated with enzyme replacement and in whom pancreatic, smooth muscle, and mitochondrial auto-antibodies were present.

In the present report, exocrine pancreatic insufficiency and duodenal inflammation heralded the onset of islet-cell failure in the presence of anti-ICAs. This temporal association suggests that the pathogenesis of the exocrine and endocrine failure, as well as the enteritis, may have a common mechanism. Recently, antibodies to the autoantigen tryptophan hydroxylase have been identified in the sera of patients with APECED (21). Tryptophan hydroxylase is a 230 kDa tetramer that is expressed in serotonin-producing cells of the central nervous system and of the intestine. In the gut, tryptophan hydroxylase and serotonin are present in enterochromaffin cells of the mucosa, the neuronal cells of the submucosa, and in the myenteric plexus. Ekwall *et al.* (21) reported that patients with APECED-associated gastrointestinal dysfunction and positive tryptophan hydroxylase antibodies had complete absence of enterochromaffin cells on duodenal biopsy, as was noted in our patient. Antitryptophan hydroxylase antibodies have been found to almost completely inhibit the enzyme (21), which converts L-tryptophan to 5-hydroxy-L-tryptophan, the precursor of serotonin. Intestinal serotonin depletion has been shown in mice to result in diarrhea (26), and serotonin receptors have been found in ganglia and acinar nerves of the pancreas (26). Communication between the gut and the pancreas via serotonin is suggested by the finding that serotoninergic enteropancreatic axons terminate near the pancreatic serotonin receptors (27). The absence of enterochromaffin cells in the gut of patients with APECED-related intestinal dysfunction and the occurrence of pancreatic exocrine as well as endocrine insufficiency in these patients raises the possibility that abnormal regulation of serotonin plays a role in the development of both the gastrointestinal and pancreatic disease.

The risk of developing type 1 diabetes has clearly been associated with the occurrence of high antibody titers, including anti-ICAs, anti-GAD antibodies, anti-IA-2 and anti-insulin antibodies (28). Within the context of APECED, however, their significance is harder to evaluate, as anti-ICA and anti-GAD antibodies can be present for many years without progression to overt diabetes (29). High anti-ICAs were iden-

tified in our patient at the age of 9.6 yr, and progression to diabetes mellitus, as recently defined (30), was evident by the age of 11 yr. Anti-AADC antibodies have also been implicated in the pathogenesis of diabetes mellitus (20) and were positive in our patient. Like GAD, AADC is a pyridoxal phosphate-dependent enzyme that catalyzes the decarboxylation of amino acids in the production of neurotransmitters (the former leads to γ-aminobutyric acid production, while the latter produces serotonin). Thus the AADC antigen, located in the pancreas, liver, and enterochromaffin cells of the intestine, may also be involved in the pathogenesis of the gastrointestinal and the pancreatic dysfunction (20).

It is noteworthy that insulin-dependent diabetes mellitus is seen in up to 18% of APECED patients, in contrast to the higher prevalence of most of the other disease components (3). In contrast to isolated diabetes mellitus type 1 and diabetes in the context of types 2 and 3 autoimmune polyglandular syndromes, no linkage to the histocompatibility leukocyte antigen (HLA)-DR/DQ alleles has been demonstrated (29, 31, 32), although questionable linkage of APECED to the HLA-A region has been described in the Finnish population (33), and linkage to the HLA-B region has been seen among North Americans (31). We have therefore explored the possible contribution of other loci implicated in the genetic susceptibility for development of type 1 diabetes. The genotyping of the insulin-dependent diabetes mellitus type 2 locus as previously described (34, 35) revealed that our index patient was homozygous for the class I susceptibility alleles (814 bp, 828 bp), whereas her siblings were heterozygous for the class I and III alleles (data not shown). Class III alleles are dominantly protective in the development of type 1 diabetes mellitus. While our intention is not to imply causality, it is of interest that none of the siblings manifest type 1 diabetes to date, including the sister with APECED, thus future studies should seek to define the *IDDM2* locus in APECED.

Autoimmune hypophysitis is another rare feature of this patient's disease. Lymphocytic hypophysitis classically presents as a pituitary mass in young women in late pregnancy or early post-partum. It preferentially causes ACTH deficiency, sometimes in isolation or with TSH deficiency, in contrast to this child with isolated GH-deficiency (36). Antipituitary antibodies in the present case to a 43–45 kDa membrane protein were more in keeping with previous findings in GH-deficient children (17) than were autoantibodies to a 49 kDa cytosolic protein in women with hypophysitis (37). The ring-enhancement on magnetic resonance imaging is highly suggestive of autoimmune hypophysitis (38), but the immunological mechanism may be different between patients with APECED and those with polyglandular autoimmune disease type II. It is interesting to note that immunoblotting in the index case showed reactivity to a protein in the microsomal fraction of human liver of 45–47 kDa and to a pituitary membrane protein of 43–45 kDa, suggesting the possibility of a common antigen in both tissues. The migration of this antigen appears to be distinct from that of P450 1A2 (52 kDa), another hepatic autoantigen recently reported in at least two patients with APECED and autoimmune hepatitis (39).

Hepatitis, although a rare feature of APECED, has been associated with fulminant hepatic failure, accounting for 25% of deaths in the Finnish group of patients (1). Our patient's aspartate aminotransferase and alanine aminotransferase levels have returned to normal on CyA. Immunosuppressive therapy has been used to halt the liver disease in APECED, but transplantation has ultimately been necessary in some cases (40). Similarly, while oral CyA has been used for isolated autoimmune enteropathy (12) and hepatitis (41), as well as for a number of other autoimmune diseases (42) including Behcet's syndrome, uveitis, psoriasis, atopic dermatitis, rheumatoid arthritis, Crohn's disease, and nephrotic syndrome, its use for APECED-associated pancreatic exocrine insufficiency has not, to our knowledge, been reported.

Our experience suggests that CyA is useful in the treatment of APECED, although we would only recommend its use for specific components of the illness. The gastrointestinal dysfunction, alopecia universalis, and keratoconjunctivitis responded dramatically to the immunosuppressive therapy: these aspects of the disease are associated with organ systems in which there is a high cell turnover. It is possible that the regenerative capacity of the gut, the hair follicle, and the corneal epithelium is more amenable to therapy for this reason. In this report, it does not appear that CyA altered the course of at least two of the endocrinopathies, as progression to primary ovarian and adrenal failure ensued over the 8 months of therapy.

Although it has been shown that the remission period in patients with non-APECED-associated diabetes mellitus type 1 is prolonged when oral CyA is administered early in the course of the disease, it has been clearly demonstrated that this is effective only temporarily in patients with both preclinical and established diabetes mellitus (43), and clinical trials in this instance have been abandoned. Our patient developed hyperinsulinemia after the initiation of subcutaneous, long-acting insulin and then CyA. This may, in part, be explained by GH-replacement therapy, which was initiated after the pre-CyA intravenous glucose tolerance test presented in Table 2 and was maintained in conjunction with CyA treatment. GH replacement in GH-deficient children is known to increase insulin secretion and hepatic insulin resistance, although peripheral insulin sensitivity appears to be unchanged (44, 45). Anti-insulin receptor and anti-insulin antibodies in the context of multisystem autoimmune disorders are associated with hyperinsulinemia and insulin resistance (46), a possible explanation in this case which remains to be verified. Given that the pathogenesis of diabetes mellitus in APECED may be different than in isolated diabetes mellitus or diabetes associated with types 2 and 3 polyglandular syndromes, it is possible that the effect of CyA may also be different in APECED; that is, the positive effect of immunosuppression may persist longer. However, this remains to be confirmed in our patient and in others with APECED-associated diabetes mellitus and will be difficult to prove given the paucity of patients.

Our patient experienced minimal short-term side effects of the therapy, including hypomagnesemia and hyperuricemia. Both were easily remedied with supplementation of magnesium and allopurinol. Although the GFR fell during the 8 months of treatment, the pre-CyA GFR was supranormal, possibly secondary to renal hyperfiltration seen in the early course of diabetes mellitus. The assessment one year later on

CyA was in the normal range. Renal dysfunction is relatively common during CyA therapy, though serious and irreversible damage is rare (42). No other adverse effects of the therapy were noted during the 8-month period. Common side effects such as neurological and gastrointestinal symptoms, when they do occur, are usually mild to moderate and resolve on dosage reduction. In the present report, trough CyA levels were maintained between 200–300 ng/mL on 5 mg/kg/day for the first 8 months; we now aim for a nadir (pre-dose) level of 100 ng/mL, with a lowering of the CyA dose to 3.5 mg/kg/day. From the patient's and family's perspective, the marked improvement in vision and the ameliorated body image are worth the risks of low dose CyA therapy.

The recent cloning of the *AIRE* gene has permitted investigation of the molecular basis of APECED in a number of patients from various geo-ethnic groups. The *AIRE* gene encodes a nuclear protein containing two zinc-finger (PHD-finger) motifs, suggestive of a transcription factor. Eight mutations have been reported (7–10), and here we report a novel missense mutation in exon 2, the same exon in which another missense mutation (K83E) has been described in the Finnish population (8). The mutation we describe also predicts a change in the protein conformation as a nonpolar amino acid (leucine) is replaced by a polar (arginine) amino acid. We also report the common 13-bp deletion mutation (1085–1097) that results in a frameshift producing a truncated 371 amino-acid protein. This mutation has been previously identified in a number of populations (British, Dutch, German, Northern Italian, Finnish, New Zealand, and American) (8–10). Haplotype analysis suggests that the 13-bp deletion in this French-Canadian family is not due to the founder effect; rather, it appears to represent an independent mutational event (8, 9). It has been proposed that the *AIRE* gene contains hypermutable sites, accounting for the recurrence of the 13-bp deletion mutation and the Finnish major mutation (R257X, producing a truncated protein) in patients from a variety of geographical locations and ethnic groups (9).

Genotype-phenotype correlations have been difficult to ascertain to date, as there is tremendous variability in phenotypes among family members with APECED (1, 47). Furthermore, Scott *et al.* (9) noted that although the X546C mutation, which is expected to produce a functional protein, was found in compound heterozygozity with the R257X mutation in two Finnish patients, the clinical features of patients with this mutation were indistinguishable from the phenotype of the Finnish patients who were homozygous for the R257X mutation, which is expected to lead to complete loss of function of the zinc-finger protein. Phenotype-genotype correlations may not be possible until long-term follow-up establishes the full manifestations of the disease, as it tends to evolve over years. Our index patient was severely affected at a young age, suggesting that compound heterozygozity for the novel missense mutation and the common 13-bp deletion may be associated with a particularly difficult clinical course.

In summary, we describe a French-Canadian adolescent with severe failure to thrive in the context of unique features of APECED, including isolated GH deficiency secondary to autoimmune hypophysitis and exocrine pancreatic insufficiency. It is apparent that immunosuppressive therapy is generally underused in severe APECED, and we recommend a trial of CyA in the treatment of APECED-associated exocrine pancreatic failure and keratoconjuctivitis. The genetic analyses of this French-Canadian pedigree revealed a novel missense mutation in exon 2 as well as the common 13-bp deletion mutation in exon 8 of the *AIRE* gene. Haplotype analysis suggests the 13-bp deletion represents an independent mutational event.

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Cytosolic Autoantigens in Lymphocytic Hypophysitis*

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ABSTRACT

Lymphocytic hypophysitis was first recognized postmortem, then by biopsy, but detection of antipituitary autoantibodies by immunofluorescence has proved unsatisfactory. Immunoblotting has the dual advantages of increased specificity and identification of the mol wt of autoantigens.

Sera from 115 patients and 52 normal subjects were immunoblotted against human autopsy pituitary cytosolic proteins. Among the neurosurgical cohort (30), 10 patients had biopsy-proven lymphocytic hypophysitis, and 20 had hypopituitarism secondary to tumor. There were 22 cases with suspected hypophysitis; 47 with either Hashimoto's, Graves', or Addison's diseases; and 15 with rheumatoid arthritis.

Antipituitary autoantibodies reactive to a 49-kDa pituitary cytosolic protein were found in 70% of biopsy-proven lymphocytic hypophysitis, 55% of suspected hypophysitis, 42% of Addison's disease, 20% of pituitary tumors, 15% of patients with thyroid autoimmunity, 13% of rheumatoid arthritis patients, and 9.8% of normal subjects. Reactivity to a 40-kDa cytosolic protein was also found in 50% of patients with biopsy-proven disease. These 49- and 40-kDa autoantigens are conserved across species and are not exclusive to pituitary tissue.

Immunoblotting has demonstrated antipituitary autoantibodies to 49- and 40-kDa cytosolic proteins in biopsy-proven cases of lymphocytic hypophysitis. (*J Clin Endocrinol Metab* **83:** 609–618, 1998)

LYMPHOCYTIC hypophysitis is considered an autoimmune reaction in the anterior pituitary (1–3). The classical presentation is peripartum hypopituitarism, often with a pituitary mass and visual failure (3–8). Secondary adrenal insufficiency is an almost universal feature, which, when undiagnosed, has proven fatal (9, 10). In the early stage, the pituitary gland is enlarged like a pituitary tumor (11–13), from which it cannot be distinguished on computed tomography or magnetic resonance imaging (MRI) scanning (11, 14). In the later stages, the gland may atrophy, leaving an empty sella (15), as occurs in Sheehan's syndrome. Spontaneous resolution of both the mass (16, 17) and the hypopituitarism (18–21) have been reported. Neurosurgical intervention has led to irreversible pituitary failure in some cases (6, 22).

Although the literature records 101 biopsy-proven cases, the relevant target autoantigens have not been identified previously. A serological test for the detection of antipituitary autoantibodies would confirm the autoimmune nature of this disease and help to determine its prevalence and define the clinical spectrum. The need for neurosurgery, with its attendant risks, may be reduced. An immunoblotting test has been developed for the detection of antipituitary autoantibodies (23). We have now used this to identify at least two target autoantigens in lymphocytic hypophysitis.

Materials and Methods

Patient and normal control sera

Serum samples were obtained from sources in Australia, Canada, and the USA. Sera from 10 patients (8 women and 2 men; mean age, 38.5 yr) with biopsy-proven lymphocytic hypophysitis and 22 patients [15 women (mean age, 34 yr) and 7 men (mean age, 50 yr)] with suspected disease were studied. The criteria for suspecting hypophysitis included the diagnosis of isolated ACTH deficiency or the presence of hypopituitarism in a patient with autoimmune disease or during the peripartum period. Patients with diabetes insipidus were tested for sarcoidosis by angiotensin-converting enzyme levels and were negative. Clinical details are outlined in Tables 1-3. The histology from all the biopsy-proven patients was reviewed by one neuropathologist, Dr. R. McD. Anderson, who confirmed the diagnosis of lymphocytic hypophysitis.

Sera from 20 patients with hypopituitarism secondary to irradiation or pituitary adenomata, from 47 patients with organ-specific autoimmune diseases (Hashimoto's thyroiditis, n = 21; Graves' disease, n = 12; Addison's disease, n = 14), and from 15 patients with rheumatoid arthritis were also assayed for antipituitary autoantibodies. Sera were collected from 52 normal subjects (32 women and 20 men; age range, 19–60 yr; mean, 29.0 yr) who were laboratory and general staff attending the Staff Clinic at the Alfred Hospital (Melbourne, Australia) for routine posthepatitis B vaccine serology. Exclusion criteria included any major illness or an autoimmune disease. Sera from all patients with hypophysitis and from 27 normal subjects (age range, 20–60 yr; mean, 33.2 yr) were also assayed for other autoantibodies.

Ethics approval for the study was obtained from the Alfred Hospital ethics committee, and informed consent was given before the collection of blood samples.

Detection of antipituitary autoantibodies by immunoblotting

Normal human autopsy pituitary tissue was homogenized in phosphate-buffered saline with protease inhibitors (aprotinin, leupeptin, pepstatin, phenylmethylsulfonylfluoride, and ethylenediamine tetraacetate) and centrifuged at 400 × *g* and then at 100,000 × *g* to give cytosolic and membrane fractions. Pituitary cytosol preparations were fractionated on SDS-polyacrylamide gels by electrophoresis under reducing conditions. The total protein loaded was constant at 50 μg/well. Monkey, rat, and ovine pituitary tissues and the mouse ACTH-secreting AtT20 cell line were handled in the same manner. Separated proteins were transferred to Immobilon (Bio-Rad, Hercules, CA) polyvinylidene difluoride (PVDF) membranes and incubated with experimental or control serum diluted 1:50 in 1% BLOTTO-phosphate-buffered saline overnight at 4 C. Reactivity to pituitary proteins was detected using alkaline phosphatase-conjugated goat antihuman IgG antiserum (Bio-Rad, Richmond, CA) and a color reaction with 5-bromo-4-chloro-3 indolyl phosphate and nitro blue tetrazolium. Autoantibody

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TABLE 1. Biopsy-proven lymphocytic hypophysitis

Patient no.	Age (yrs)	Sex	Past history	Presenting symptoms	CT/MRI	Deficits	Progress	Autoimmune disease	49-kDa antibody reactivity	40-kDa antibody reactivity	Time from biopsy to pituitary antibody test
1	57	F	Rheumatic heart disease	Collapse due to pituitary apoplexy	Mass SSE	Panhypopituitarism	Hypophysectomy, permanent hypopituitarism	ANF 1:200	+ >1:1000	+	17 months
2	43	F	G1P1, psoriasis	2^{o} amenorrhoea	Mass 3.5 cm	ACTH, TSH, LH/FSH	Biopsy	IDDM (7 yr) Graves' (11 yr)	+ >1:500	+	4.5 yr
3	51	M		Panhypopituitary, 12–18 months	Mass	Panhypopituitarism	Hypophysectomy	Nil	+	+	16 months
4 (Ref. 8)	29	F	G1P1	Rapid visual failure 25/40, gestation, H/A	Mass, SSE	ACTH, GH, TSH, LH/FSH	Biopsy, menses returned	Nil	−	+	16 months
5 (Ref. 24)	34	F	G4 P4, 10 yr postpartum nephrogenic diabetes insipidus (lithium)	Fatigue, blurred vision, headache, 2^{o} amenorrhoea	Mass, SSE	ACTH, LH/FSH, PRL 4× N	Failed trial of bromocriptine, hypophysectomy, hypopituitarism	ANF 1:400	+	−	3 months
6 (Ref. 7)	21	F	G1P1	Rapid visual failure 28/40 gestation, H/A	Mass, SSE	PRL 7× N	Failed trial of steroids, hypophysectomy, hypopituitarism	Nil	−	−	7 yr
7	25	F	G3P1	Third trimester H/A, bitemporal, hemianopia	Mass, SSE	ACTH, TSH	Hypophysectomy, hypopituitarism	Nil	−	−	15 months
8	52	F	Facioscapulohumeral dystrophy	6 months visual disturbance	Mass, SSE	Panhypopituitarism	Hypophysectomy, hypopituitarism	IDDM, pernicious anemia	+ >1:1000	+	5 yr
9	32	F		Meningoencephalitis	Mass, contrast ring		Hypophysectomy, hypopituitarism	ANF 1:200	+	−	12 months
10	60	M	Ischemic heart disease	Persistent fevers 12 months of diabetes insipidus, hypogonadism, hypothyroidism	6-mm mass in stalk	LH/FSH, TSH, diabetes insipidus	Hypophysectomy, panhypopituitarism	Nil	+	−	18 months

CT, Computed tomography; MRI, magnetic resonance imaging; SSE, Suprasellar extension; G, gravida; P, para; N, normal; HA, headache; ANF, antinuclear factor; IDDM, insulin-dependent diabetes mellitus.

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TABLE 2. Suspected lymphocytic hypophysitis: female patients

Patient no.	Age (yrs)	Sex	Past history	Presenting symptoms	CT/MRI	Hormone deficits
1	24	F	G2P2	Headaches, postpill amenorrhoea, 2^0 infertility	Mass SSE, contrast ring	LH/FSH, normal vision
2	27	F	Pill-induced hepatic adenomata, G2P1	Severe headache, hypoglycemic coma in 3rd trimester	N postpartum	ACTH, GH, transient diabetes insipidus, normal vision
3	31	F	Facio-scapulo-humeral dystrophy, Guillain-Barre syndrome, G2P1	Headache, vomiting, recurrent hypoglycemic coma in 1st and 2nd trimesters	Mass, 5 mm	Panhypopituitarism, normal vision
4	31	F	G2P2	Fatigue, anorexia, hypoglycemic coma in 3rd trimester, 29/40	N postpartum	ACTH, LH/FSH, PRL, TSH
5	20	F	G0	2^0 amenorrhoea, galactorrhoea		PRL 7× N, LH/FSH, TSH
6	29	F	Diabetes insipidus for 9 yr	2^0 amenorrhoea	N	GH, LH/FSH, diabetes insipidus
7	24	F	G2P2, alopecia	Failure to lactate 9 weeks postpartum	N	LH/FSH PRL
8	50	F		Hypoadrenalism, isolated ACTH deficiency	N	ACTH, TSH
9	35	F	G2P1	Headaches in 2nd and 3rd trimesters, failure to lactate postpartum	Mass	Panhypopituitarism
10	32	F	G1P1	Failure to lactate postpartum, transient visual field defect 10 days postpartum	N	ACTH, LH/FSH, PRL
11	24	F	G1P1	Secondary amenorrhoea 2 yr postpartum	N	ACTH, GH, PRL 2 × N
12	42	F	G2P2	Hypoadrenalism	N	Isolated ACTH deficiency
13 (Ref. 44)	30	F	G3P3	3rd trimester fatigue, wt loss, anorexia, amenorrhoea, and failure to lactate postpartum	N	Transient T_4 increase and hypercalcemia, panhypopituitarism
14	43	F	G3P3	Fatigue	Thick stalk	Isolated ACTH deficiency
15	68	F	G2P2	Marked fatigue, weight loss	N	Isolated ACTH deficiency

titers were also performed at serum dilutions of 1:100, 1:200, 1:500 and 1:1,000. For further details, see Crock *et al.* (23).

Species specificity

Positive sera were tested by immunoblotting on pituitary tissue from cynomologous monkeys, sheep, rats, and murine AtT20 cells. Fresh-frozen cynomologous monkey pituitary glands were obtained from the Commonwealth Serum Laboratories (Melbourne, Australia) with ethics and quarantine approvals. Ovine pituitary tissue was obtained from an abbatoir. Rat pituitaries came from laboratory animals killed for other purposes. All tissues were prepared as outlined above for human tissue.

Tissue specificity

Positive sera, as identified above, were tested by immunoblotting against cytosolic preparations from fresh-frozen cynomologous monkey brain, adrenal, thyroid, liver, spleen, serum, and skeletal muscle.

Screening for other autoantibodies

Sera from all patients with hypophysitis and from 27 of the 52 normal subjects were tested for a wide range of autoantibodies, looking for both organ-specific and nonorgan-specific autoimmunity. These included autoantibodies to thyroid, stomach, ovary, adrenal, kidney, liver, smooth muscle, nuclei, and mitochondria. The 25 normal subjects not tested were younger (mean age, 25.8 yr). Sera were screened for antithyroid microsomal and antithyroglobulin autoantibodies, using Thymune*-M and Thymune*-T kits, respectively (Murex Diagnostics, Temple Mill, UK). Antigastric parietal cell, antismooth muscle, and antimitochondrial autoantibodies were detected by indirect immunofluorescence on fresh-frozen sections of mouse stomach, kidney, and liver. Antinuclear antibodies were detected by immunofluorescence on Hep-2 cells, and antiovarian and antiadrenal autoantibodies were detected by indirect immunofluorescence on fresh-frozen sections of cynomologous monkey tissue.

Statistical analysis

Reactivity to the 49- or 40-kDa cytosolic proteins by immunoblotting was compared between groups using Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Antipituitary autoantibodies

Autoantibodies against a 49-kDa pituitary cytosolic protein were demonstrated by immunoblotting in the sera of 7

TABLE 2. *Continued*

Progress	Autoimmune disease (duration)	49-kDa antibody reactivity	40-kDa antibody reactivity	Other autoantibodies
Progressive panhypopituitarism with diabetes insipidus, episode of pituitary apoplexy	Hashimoto's thyroiditis	+ (>1:1000)	−	Thyroid peroxidase (1:409,000), ANF (1:200), antismooth muscle +
Failure to lactate postpartum permanent ACTH deficiency	IDDM (15 yr)	+	−	Antiparietal cell +++, ANF (1:200)
Addisonian crisis 4 months postpartum, permanent hypopituitarism	IDDM (20 yr)	+ (>1:1000)	+	−ve
Failure to lactate postpartum, permanent ACTH and PRL deficiency	IDDM (4 yr)	+	−	−ve
	Hashimoto's thyroiditis	−	−	Thyroid peroxidase (1:1600)
		−	−	−ve
	Addison's (4 yr), gestational diabetes	+	+	Adrenal antibodies +, ANF (1:400)
Persistent hypopituitarism	IDDM (26 yr)	+	+	−ve
Persistent hypopituitarism, mass resolved		+	−	Antimicrosomal (1:25,600)
Amenorrhoea, ACTH deficiency, asymptomatic		+	+	Antireticulin +
Persistent ACTH deficiency, pregnant again		−	−	−ve
Progressive panhypopituitarism over 3 yr		−	−	Antismooth muscle +
ACTH deficiency, amenorrhoea 9 months postpartum		−	−	ANF (1:800)
Isolated ACTH deficiency	Hashimoto's thyroiditis	−	−	Antimicrosomal +
Isolated ACTH deficiency	IDDM (42 yr), Hashimoto's thyroiditis	−	−	Adrenal antibody positive

See Table 1 for abbreviations.

of 10 patients with biopsy-proven lymphocytic hypophysitis (by Fisher's exact test, $P < 0.0001$ *vs.* controls) and in 12 of 22 patients who were suspected of having the disease (by Fisher's exact test, $P < 0.0001$ *vs.* controls) (see Fig. 1). Six of the 19 patients with antipituitary autoantibodies had a titer of more than 1:1000 (see Tables 1–4 and Fig. 2); of whom 3 had pituitary apoplexy and 2 had isolated ACTH deficiency (1 with an empty sella). Five of 10 biopsied patients and 6 suspected patients also had autoantibodies to a 40-kDa pituitary cytosolic protein (see Tables 1–3; by Fisher's exact test, $P < 0.005$ and $P = 0.06$, respectively, *vs.* controls).

Sera from 5 of 52 control subjects were reactive to the 49-kDa cytosolic protein (by Fisher's exact test, $P < 0.0001$ *vs.* both biopsy-proven and suspected patients), but none had a titer of more than 1:200 (see Fig. 1 and Table 4). Four normal control subjects had autoantibodies to the 40-kDa cytosolic protein, of whom one had a titer of more than 1:1000.

Autoantibodies to a 49-kDa protein were detected in 6 of 14 Addison's patients (by Fisher's exact test, $P = 0.18$ *vs.* biopsy-proven hypophysitis), 1 of 12 Graves' disease patients ($P < 0.005$ *vs.* biopsy), 4 of 21 Hashimoto's disease patients ($P < 0.01$ *vs.* biopsy), 2 of 15 rheumatoid arthritis patients ($P < 0.01$ *vs.* biopsy), and 4 of 20 patients with pituitary tumors ($P = 0.01$ *vs.* biopsy; see Table 4). Only 3 of these 62 patients had a titer of more than 1:1000: 1 each with Addison's disease, rheumatoid arthritis, and a postoperative pituitary tumor.

Other polypeptides were detected by individual sera (*e.g.* an 88-kDa band was seen in two patients, one illustrated in Fig. 1, lane 4), but because these were not consistently present in hypophysitis cases, they will not be further considered here. Immunoblotting is known to detect multiple autoantigens (26). Nonspecific binding was seen in all experiments at approximately 25, 50, and 64 kDa with second antibody alone and was reduced by depleting the pituitary cytosolic fraction of IgG. It was not seen when

TABLE 3. Suspected lymphocytic hypophysitis: male patients

Patient no.	Age (yrs)	Sex	Past history	Presenting symptoms	CT/MRI	Hormone deficits	Progress	Autoimmune disease	49-kDa antibody reactive	40-kDa antibody reactive	Other autoantibodies
1	49	M		6-month wt loss, malaise, myalgia, anorexia, nausea, vomiting	Partial empty sella	Isolated ACTH deficiency	Well on replacement therapy		+ >1:1,000	−	
2 (Ref. 25)	44	M		6 months wt loss, fatigue, arthralgia, gynecomastia	N	Isolated ACTH deficiency	Prednisone (7.5 mg), 9-kg wt gain, normotensive		+ >1:1,000	−	Antinuclear antibodies 1:320 (nucleolar)
3	42	M		Hypoglycemic comas, 12-kg wt loss, cramps	N	Isolated ACTH deficiency	Well on cortisone acetate (37.5 mg daily)		−	Weak	
4	72	M	Blind right eye (war injury)	Headaches, hypogonadism	Mass SSE	Panhypopituitarism, diabetes insipidus, normal left eye	10 months later: CT normal diabetes insipidus and hypopituitarism both resolved	Nil	+	−	
5	34	M		Diabetes insipidus	N	Diabetes insipidus	Permanent diabetes insipidus	Iritis	−	+	
6	50	M	Deep venous thrombosis	Idiopathic hypopituitarism	N	ACTH, GH, LH/FSH	Panhypopituitarism		−	−	
7	59	M	Hypertension	Acromegaloid, hypogonadism, bitemporal hemianopia	N	GH, LH/FSH, normal IGF-I	Hypogonadal	Diabetes mellitus	+	−	

SSE, suprasellar extension; CT, computed tomography; MRI, magnetic resonance imaging; N, normal.

TABLE 4. Summary of results of antipituitary autoantibody testing

Subjects with 49-kDa autoantibodies	Serum dilution 1:50	Serum dilution 1:200	Serum dilution 1:1000
Lymphocytic hypophysitis			
1. Biopsy proven	7/10	5/10	2/10
2. Suspected	12/22	7/22	4/22
Normal controls	5/52	0/52	0/52
Hypopituitarism secondary to tumors or radiotherapy	4/20	2/20	1/20
Autoimmune disease			
1. Hashimoto's thyroiditis	4/21	0/21	0/21
2. Graves' disease	1/12	0/12	0/12
4. Addison's disease	6/14	2/14	1/14
5. Rheumatoid arthritis	2/15	1/15	1/15

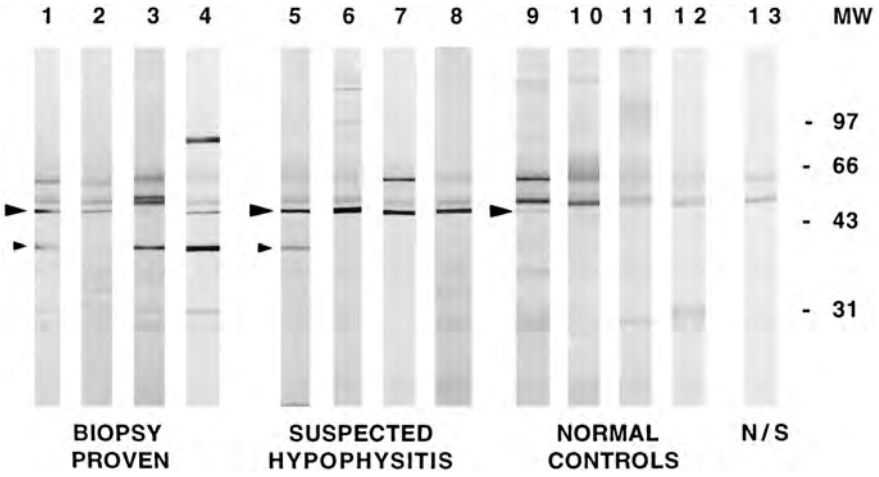

FIG. 1. Immunoblotting assay for antipituitary autoantibodies. Human pituitary cytosolic proteins were separated on 10% SDS-PAGE, transferred electrophoretically to PVDF membranes, and probed with antibodies. Lanes 1–4, Incubated with sera from patients with biopsy-proven lymphocytic hypophysitis. Lanes 5–8, Sera from patients with suspected lymphocytic hypophysitis. Lanes 9–12, Sera from normal control subjects. Lane 13, Nonspecific binding by second antibody (goat antihuman IgG conjugated to alkaline phosphatase) alone. The *large arrow* indicates positive reactivity to a 49-kDa autoantigen, and the *small arrow* indicates positive reactivity to a 40-kDa autoantigen. MW, Molecular mass markers in kilodaltons.

animal tissues were used. Human Igs from blood present in the pituitary glands at homogenization are the most likely explanation.

Other autoantibodies

One or more autoimmune diseases were present in 5 patients with biopsy-proven lymphocytic hypophysitis: type I diabetes, Graves' disease, pernicious anemia, and positive antinuclear factor (Table 1). Sixteen of 22 patients with suspected disease also had 1 or more autoimmune diseases, as outlined in Tables 2 and 3. Seven control sera had positive antinuclear factor antibodies, with titers ranging from 1:200 to 1:1600 (normal range, 0–1:100), but were negative for all other antibodies. Low titer autoantibodies are often found in normal control subjects and are thought to represent natural antibodies.

Species specificity

Patient sera reactive to the 49-kDa cytosolic protein in human pituitary also reacted with a 49-kDa cytosolic protein in pituitary tissue from cynomologous monkey, sheep, and rat and the AtT20 cell line. Positive reactivity is shown in Fig. 3. Many other bands of reactivity are seen, particularly with nonhuman tissues, but these are shared with normal control sera. The 49-kDa band was particularly prominent in AtT20 cytosolic preparations, suggesting that this antigen may be highly expressed in these cells and their normal counterparts, the corticotroph (Fig. 3, lane 10). Thus, the 49-kDa autoantigen is conserved across many species and is not primate specific. The 40-kDa autoantigen was also present in monkey and rat brain (results not shown).

Tissue specificity

Patient sera reactive to the 49-kDa protein in human and monkey pituitary also reacted to a 49-kDa cytosolic protein in some other tissues, including monkey brain, thyroid, liver, and spleen, but not in skeletal muscle or serum (results not shown). Although the 49-kDa autoantigen is not pituitary specific, neither is it ubiquitous.

Discussion

Autoantibodies are the hallmark of autoimmunity, but they have been difficult to detect in pituitary autoimmune disease. Improved imaging of the pituitary by computed tomography scanning and MRI now displays more subtle pituitary masses, but still cannot distinguish a pituitary adenoma from lymphocytic hypophysitis (14). The diagnostic armamentarium needs to be expanded to include a reliable serological test. Our results may represent a first step in this direction.

The original report of antipituitary autoantibody activity described a complement fixation test and found reactivity in the serum of 18% of postpartum women, but gave no objective endocrine data to support their clinical relevance (27). Testing by immunofluorescence was developed in 1975 by Bottazzo *et al.*, using fresh-frozen human pituitary tissue from hypophysectomies (28). The sera of 297 patients with polyglandular autoimmune disease were screened for anti-

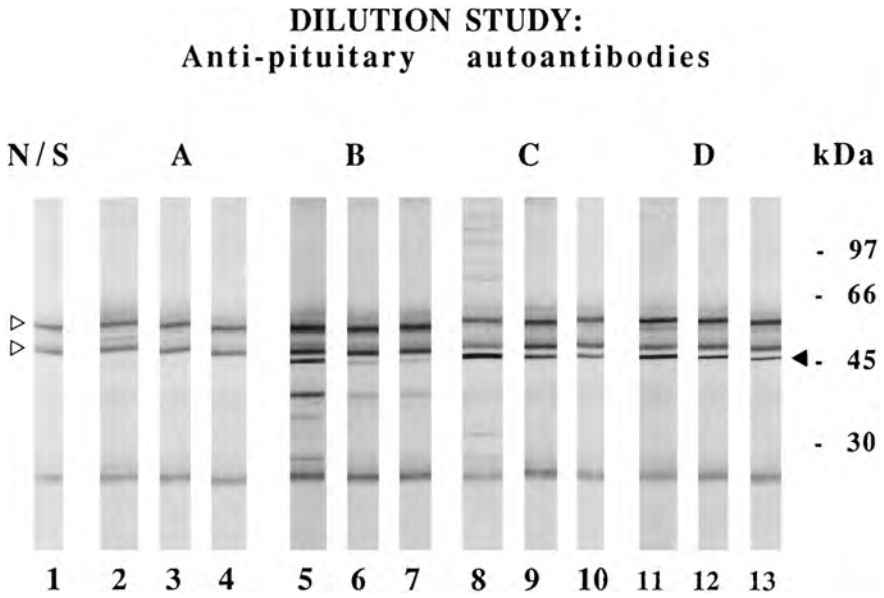

FIG. 2. Dilution study. Human pituitary cytosolic proteins were separated on 10% SDS-PAGE, transferred electrophoretically to PVDF membranes, and probed with antibodies. Lane 1, Nonspecific binding by second antibody (goat antihuman IgG conjugated to alkaline phosphatase) alone (*open arrows*). A, Lanes 2–4, Serum from a normal control subject diluted 1:50, 1:500, and 1:1000, respectively. B, Lanes 5–7, Serum from a patient with biopsy-proven lymphocytic hypophysitis diluted 1:50, 1:500, and 1:1000; C and D, lanes 8–10, and 11–13, serum from two patients with suspected disease diluted 1:50, 1:500, and 1:1000, respectively. Lanes 5–13 show reactivity to a 49-kDa protein, indicated by the *black arrow*. Lanes 5–7 show reactivity to a 40-kDa protein. MW, Molecular mass markers in kilodaltons.

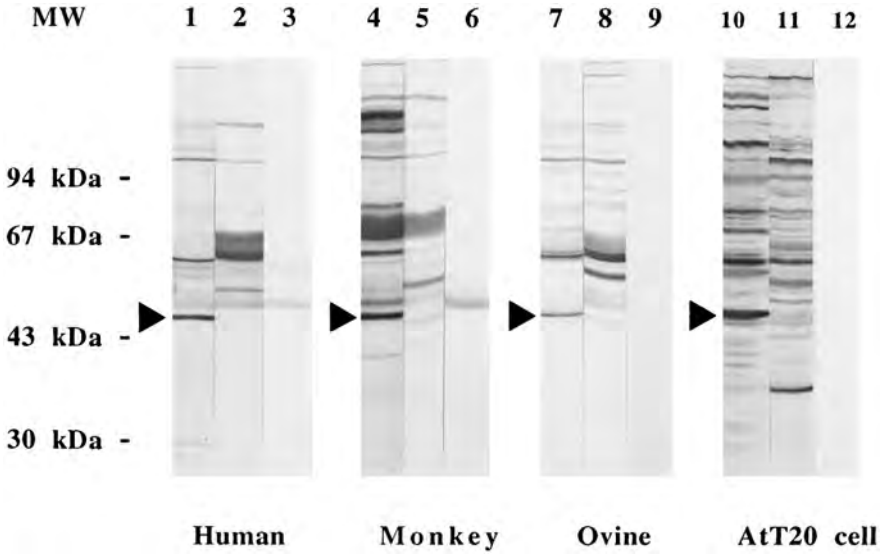

FIG. 3. Species specificity. Pituitary cytosolic proteins from human tissue (lanes 1–3), cynomologous monkey tissue (lanes 4–6), ovine tissue (lanes 7–9), and mouse AtT20 cells (lanes 10–12) were separated on 10% SDS-PAGE, transferred electrophoretically to PVDF membranes, and probed with antibodies. Lanes 1, 4, 7, and 10 were incubated with serum from a patient with suspected lymphocytic hypophysitis and show positive reactivity to a 49-kDa protein (*large arrow*). Lanes 2, 5, 8, and 11 were incubated with serum from a normal subject. Lanes 3, 6, 9, and 12 show nonspecific binding with second antibody (goat antihuman IgG conjugated to alkaline phosphatase) alone. MW, Molecular mass markers in kilodaltons.

pituitary autoantibodies on the premise that autoimmune diseases cluster. Nineteen patients had positive antipituitary autoantibodies to PRL-secreting cells, but again, none of these patients had evidence of pituitary insufficiency (28). Immunofluorescence has detected autoantibodies in only 2 of 7 patients with biopsy-proven lymphocytic hypophysitis (12, 29). These autoantibodies were believed to be of low titer and only specific to fresh-frozen pituitary glands of primate origin (28). Seventy-eight percent of reactions were only detected with undiluted or untitrated sera (28). However, other researchers have used pituitary tissue from guinea pig (30), rat (31), mouse AtT20 cells (31), pigs (32), and fetal tissue (33), with positive results in cryptorchidism (30), empty sella syndrome (34), isolated ACTH deficiency (31), Graves' disease (32), and Cushing's disease (33). A further problem with immunofluorescence has been the nonspecific binding of autoantibodies to corticotroph cells by Fc receptors (33, 35).

An alternative approach to the detection of antipituitary autoantibodies uses immunoblotting to overcome these problems (23). This method has been successful with autopsy pituitary glands as substrate. In the current study, we have detected antipituitary autoantibodies in patients with biopsy-proven and suspected autoimmune pituitary disease at titers of more than 1:1000. At this dilution the assay became more specific for lymphocytic hypophysitis, but did lose some sensitivity. We have identified at least two target autoantigens in pituitary autoimmune disease, which are cytosolic proteins of 49 and 40 kDa. The antigenic determinants recognized by these sera are conserved across species, including monkeys, sheep, rats, and mice. Conservation of autoantigenic epitopes across species is the general rule in autoimmunity.

We speculate that the 49-kDa autoantigen might be related to ACTH deficiency from corticotroph destruction. ACTH deficiency is the most prominent feature of lymphocytic hy-

pophysitis and may be seen in isolation (9, 21). By contrast, ACTH is often the last hormone to be affected in patients with pituitary tumors. Two of three male patients with isolated ACTH deficiency in our series had high titer autoantibodies to the 49-kDa autoantigen. One had an empty sella on MRI scan, suggesting that his condition was the result of pituitary atrophy secondary to lymphocytic hypophysitis. The second patient has been shown by other investigators to have autoantibodies that bound to 200-nm secretory granules in rat corticotrophs (25). Isolated ACTH deficiency is more common in Japan, and an antipituitary autoantibody assay using immunofluoresence on AtT20 cells was reported to be positive in 70% of patients (31). When AtT20 cell cytosolic proteins were immunoblotted against our positive sera, strong reactivity to a 49-kDa protein was detected, and the target autoantigen appeared enriched. This suggests that the 49-kDa pituitary cytosolic protein is both present in corticotrophs and conserved across species. Reactivity to many other proteins in AtT20 cells was detected by sera from patients and normal subjects, highlighting the problems of nonspecific binding with nonprimate tissues and perhaps reflecting natural antibodies to xenoantigens.

Reactivity to the 49-kDa cytosolic protein was found in brain, adrenal, thyroid, liver, and spleen, but not in skeletal muscle or serum. The lack of pituitary specificity of this protein does not necessarily mitigate against its involvement with corticotrophs and ACTH deficiency. The precursor of ACTH, POMC, is processed in many cells in the body, except skeletal muscle (36), and target autoantigens are not always confined to the tissues primarily affected by the autoimmune process. For example, in primary biliary cirrhosis, the major autoantigen detected serologically is a ubiquitous mitochondrial enzyme, pyruvate dehydrogenase, but clinical disease is confined to the liver (37).

Immunoblotting methods often detect multiple autoantigens (26). It is now recognized that there are multiple target autoantigens in type I diabetes (38), Hashimoto's thyroiditis, and Graves' disease (39). Sera from 10 patients in our series reacted with a 40-kDa pituitary cytosolic protein, and this was particularly striking in the biopsy-proven cohort (50% positive). Some patients showed weak reactivity to multiple cytosolic proteins, which disappeared at greater serum dilutions. Some of this reactivity could be explained by the phenomenon of natural antibodies, which are usually in low titer and nonpathogenic. Strong reactivity to an 88-kDa protein, as shown in Fig. 1, lane 4, may represent another target autoantigen, as it was not seen with normal sera. Thus, at least two, if not three, pituitary autoantigens have been identified by our immunoblotting assay.

Unusual clinical presentations of lymphocytic hypophysitis in our series included two women with facio-scapulohumeral muscular dystrophy and diabetes mellitus, both of whom had high titer antipituitary autoantibodies (>1:1000) and one of whom had biopsy-proven disease. There were four cases with pituitary apoplexy, two during pregnancy, which has also not been reported previously in hypophysitis. Apoplexy was associated with high titer autoantibodies and in one case with ring enhancement of the pituitary mass on MRI. Although ring enhancement is seen with pituitary tumors, it has recently been reported as a feature of hypophysitis (14). It is also likely that pituitary apoplexy due to hypophysitis in the peripartum period can be misinterpreted as Sheehan's syndrome (40). One male patient in our series had acromegaloidism, and there are single reports of a coexistent GH-secreting adenoma (41) and elevated GH levels (42) with hypophysitis. One patient in our series presented with meningoencephalitis, and other cases have presented as lymphocytic meningitis (43). One woman presented with hypercalcemia associated with transient hyperthyroidism and secondary adrenal failure (44). Fifteen cases of hypophysitis have been reported in men (8, 45–52), and we add another seven male patients.

Diabetes insipidus occurs with lymphocytic hypophysitis (19, 43, 46, 47, 50), but rarely preoperatively in patients with pituitary adenomata. It was present in four patients in our series, three of whom had antipituitary autoantibodies. The term lymphocytic infundibuloneurohypophysitis has been coined by Imura (50) for patients with diabetes insipidus and lymphocytic infiltration of the pituitary stalk. Necrotizing infundibulo-hypophysitis has also been reported with diabetes insipidus (49). Perhaps these are part of the spectrum of lymphocytic hypophysitis.

A classical feature of lymphocytic hypophysitis is its association with pregnancy (1, 3, 4, 51- 53). PRL levels may be high, simulating a prolactinoma (3, 54, 55), or deficient, causing lactation failure (4). Hyperprolactinemia was seen in four cases in our series. Lactational failure was seen in six patients, five of whom had antipituitary autoantibodies. This latter symptom is commonly seen in Sheehan's syndrome, and we suspect that patients labeled as such but with no history of peripartum hemorrhage have unrecognized hypophysitis.

Four of 20 patients in our series, with known pituitary tumors, had antipituitary autoantibodies. These sera had been collected postoperatively and/or postradiotherapy. Radiation damage and tumors may induce autoantibody production (56), so these results may reflect the release of tissue antigens due to pituitary injury.

A range of other autoimmune diseases was present in 50% of our biopsy-proven patients, which is a little higher than the 30% reported by Cosman (4), but is in keeping with the tendency of autoimmune diseases to cluster. Autoimmune diseases were present in 60% of our suspected hypophysitis patients, but as hypopituitarism with autoimmune disease was often the rationale for requesting antipituitary autoantibodies, our series has this inherent selection bias. The incidence of antipituitary autoantibodies in patients with thyroid autoimmune disease was not significantly different from that in control subjects, suggesting that reactivity to the 49-kDa protein by immunoblotting is not a nonspecific finding in autoimmune disease.

Surprisingly, 42% of Addison's sera had positive reactivity to a 49-kDa cytosolic protein, a protein also present in adrenal tissue. Patients with Addison's disease have been found to have lymphocytes in the pituitary at autopsy (57), and patients with hypophysitis have had adrenal lymphocytic infiltration (58, 59). It is possible that the 49-kDa autoantigen is involved in POMC or ACTH processing (36). Alternatively, Addison's sera may recognize a different autoantigen that coincidentally has the same molecular mass as the 49-kDa protein targeted in hypophysitis. This situation occurred in

type I diabetes, where the 64-kDa autoantigen was found to be both glutamic acid decarboxylase and BSA.

Identification of the 49- and 40-kDa cytosolic autoantigens recognized by immunoblotting should help to elucidate the pathogenesis of lymphocytic hypophysitis.

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Pituitary Autoimmunity in Patients with Sheehan's Syndrome

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Postpartum hemorrhage (PPH) is a frequent complication of pregnancy in India. Sheehan's description of postpartum hypopituitarism promoted the belief that PPH leads to necrosis of the enlarged pituitary gland of pregnancy and hypopituitarism. However, slow clinical progression suggests factors other than ischemia in its pathogenesis. Tissue necrosis could release sequestered antigens, triggering autoimmunity of the pituitary and delayed hypopituitarism in Sheehan's syndrome. Twenty-six consecutive patients with postpartum hypopituitarism were studied, 19 with Sheehan's syndrome based on a history of PPH and hormone profile suggesting pituitary failure [mean (SD) age 32.7 ± 6.4 yr, duration of illness 5.5 ± 3.1 yr], and seven patients with no history of PPH, categorized as "Other." Pituitary imaging and basal T_4, TSH, cortisol, LH, FSH, 17β-estradiol, and autoantibodies against pituitary (PitAb) and thyroid (TMA) were evaluated. Controls included 28 healthy females without prior conception (22 ± 5 yr) and 28 with prior conception (26 ± 5 yr). Twelve of 19 (63.1%) patients with Sheehan's syndrome and one of seven in the Other group had PitAb against the 49-kDa autoantigen; neuron-specific enolase. Four of 28 (14.2%) controls without prior conception and 5 of 28 (17.8%) controls with prior conception had PitAb positivity ($P < 0.001$ and <0.01 *vs.* Sheehan's syndrome, respectively). There was no significant difference in the mean serum hormone values and TMA positivity between patients with Sheehan's syndrome and the Other group as well as patients with or without PitAb positivity. Pituitary autoimmunity may play a role in the cause of hypopituitarism following PPH. (*J Clin Endocrinol Metab* 87: 4137–4141, 2002)

SHEEHAN (1), IN 1937, described the syndrome of postpartum hemorrhage (PPH), pituitary necrosis, lactation failure, and hypopituitarism. With better obstetric care, such complications of pregnancy are rare in the industrialized countries of the West. The National Family Health Survey in India (1998–1999) revealed 66% of childbirths to be home deliveries (2). The prevalence of anemia in pregnancy (<10.9%) in the country is 49.7%, the incidence of PPH 11%, and maternal mortality 428–653/10^5 live births (2). These data make it likely that postpartum pituitary failure is common in India. However, clinically, such patients often present late with severe hypopituitarism and circulatory collapse (3–5). Sheehan's description of the syndrome promoted the belief that PPH leads to necrosis of the hyperplastic pituitary gland of pregnancy with resultant hypopituitarism and failure of lactation (1, 6). Release of sequestered pituitary antigens from necrotic pituitary tissue with the subsequent triggering of pituitary autoimmunity could lead to postpartum hypopituitarism in such cases. Recently autoantibodies against a 49-kDa pituitary cytosolic protein, identified as neuron-specific enolase, have been reported in patients with autoimmune lymphocytic hypophysitis (7–10). Using the above autoantibody as a marker, we report results that demonstrate pituitary autoimmunity among patients with Sheehan's syndrome.

Abbreviations: BMI, Body mass index; NSE, neuron specific enolase ($\gamma\gamma$ isoform); PitAb, pituitary autoantibody; PPH, postpartum hemorrhage; TMA, thyroid microsomal autoantibody.

Materials and Methods

Twenty-six consecutive patients with the onset of postpartum hypopituitarism followed up in the endocrine clinic of All India Institute of Medical Sciences (AIIMS), Delhi, during 1998–2000 were studied. Nineteen patients had Sheehan's syndrome based on a history of PPH along with a clinical picture and hormone profile suggesting pituitary failure. Seven patients without a history of PPH were categorized as "Other" because hypopituitarism in them might have been due to antepartum hemorrhage, lymphocytic hypophysitis, idiopathic empty sella, or other causes (Tables 1 and 2). Clinical features included imaging of the pituitary and pituitary function as assessed by basal serum total T_4, TSH, cortisol (0800 h), LH, FSH, 17β-estradiol measurements using commercial kits for LH, FSH, T_4, and TSH (Medicorp Inc., Montreal, Quebec, Canada), 17β-estradiol (DiaSorin, Inc., Saluggia, Vercelli, Italy), and cortisol (Immunotech, Marseille, France). All hormone assays were batched together. Intraassay coefficient of variation ranged from 4.0% to 7.2% for these hormones. The normal basal ranges in our laboratory are 52–167 nmol/liter for serum T_4, 0.3–4.0 mU/liter for TSH, 260–720 nmol/liter for cortisol, 0.5–15 IU/liter for LH, 0.2–10 IU/liter for FSH, and 110–734 pmol/liter for 17β-estradiol. Informed consent was obtained from all subjects, and the study was approved by the Research Committee of the AIIMS, New Delhi. Clinical features recorded include details of the last childbirth, PPH, blood transfusion, lactation failure, amenorrhea, and neonatal outcome. T1- and T2-weighted magnetic resonance imaging was done in 24 patients and computed tomography in two patients. A basal blood sample was drawn from each patient and sera were preserved in multiple aliquots at −20 C for pituitary (PitAb) and thyroid microsomal autoantibody (TMA) assays. Controls included 28 healthy females with no prior history of conception (mean age 22 ± 5 yr) and 28 healthy females with prior conception and normal obstetric history (mean age of 26 ± 5 yr) who were 4 ± 2 yr postpartum.

PitAb was assayed using Western blot analysis as described previously (7, 11, 12). Briefly, a total of 10 normal human pituitaries, obtained at autopsy, 4–8 h postmortem, and frozen at −70 C were homogenized in PBS with protease inhibitors. The cytosol fraction was depleted of IgG with protein-A Sepharose (Amersham-Pharmacia Biotech, Uppsala,

TABLE 1. Clinical and pituitary autoantibody positivity data in patients with Sheehan's syndrome (n = 19) and "Other" with postpartum hypopituitarism (n = 7)

SN	Age (yr)	Last childbirth	APH	PPH	Childbirth at hospital	Blood transfusion at birth	Neonatal/ infant death (at)	Lactational failure	Year of diagnosis	Presenting complaints
Sheehan's syndrome										
1	45	1988	N	Y	N	Y	N	Y	1998	Amenorrhea, hypotension, hypoglycemia, hyponatremia
2	31	1996	N	Y	Y	N	N	Y	1997	Amenorrhea, hypotension
3	40	1987	N	Y	N	N	N	Y	1996	Facial swelling, amenorrhea
4	26	1993	N	Y (RP)	N	Y	Y (5 mo)	Y	2000	Amenorrhea
5	35	1992	N	Y (RP)	N	Y	Y (3 mo)	Y	2000	Amenorrhea, hypotension
6	35	1987	N	Y	N	N	N	Y	1998	Amenorrhea
7	39	1992	N	Y	N	N	N	Y	1999	Amenorrhea
8	30	1997	N	Y (RP)	N	Y	N	Y	1999	Amenorrhea, hypotension
9	40	1980	N	Y	N	Y	Y (SB)	N	1980	Amenorrhea, hypotension
10	25	1994	N	Y	N	Y	Y (SB)	Y	1999	Amenorrhea, hypotension
11	25	1995	N	Y	N	N	N	Y	2000	Amenorrhea, facial swelling, infertility
12	30	1991	N	Y	N	N	Y (d 1)	Y	1994	Amenorrhea, hypotension, facial swelling
13	28	1992	N	Y	Y	N	Y (d 1)	Y	1998	Amenorrhea, infertility
14	32	1996	N	Y	Y	Y	N	Y	1998	Amenorrhea, psychosis
15	30	1990	N	Y	N	Y	Y (3 mo)	Y	1994	Amenorrhea
16	32	1999	N	Y	N	Y	N	Y	2000	Lactational failure, amenorrhea
17	45	1985	N	Y	Y	Y	Y (d 1)	Y	1993	Hypotension
18	25	1995	N	Y	N	N	Y (SB)	Y	2000	Amenorrhea, hypotension
19	30	1995	N	Y	Y	N	Y (4 mo)	Y	2000	Amenorrhea
Other										
1	30	1990	Y	N	N	N	Y (2 mo)	Y	2000	Infertility, hypotension
2	45	1980	N	N	N	Y	N	Y	1992	Hypotension, amenorrhea
3	30	1992	N	N	Y	N	N	Y	2000	Amenorrhea, hypotension
4	42	1987	N	N	N	Y	N	N	1997	Hypotension
5	30	1996	Y	N	Y	Y	Y (5 mo)	Y	2000	Psychosis, amenorrhea, facial swelling, hypotension
6	30	1995	N	N	Y	Y	N	Y	1997	Hypotension, amenorrhea, psychosis
7	38	1989	N	N	N	N	Y (SB)	Y	1999	Amenorrhea

Sweden) and stored in aliquots at −70 C. Cytosol fractions were boiled for 2 min in the presence of 1,4-dithiothreitol and subjected to 10 × 10.5 cm SDS-PAGE (10% running gel and 4% stacking gel) with 50 μg protein loaded in each well. Molecular weight marker proteins (Bio-Rad Laboratories, Inc., Hercules, CA) were included in each experiment. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.), which were incubated for 1 h in 5% fat-free skim milk (Blotto/PBS) to block nonspecific binding. A Deca-probe incubation manifold (Hoeffer, San Francisco, CA) was used for primary antibody binding with patient sera diluted 1:50 in 1% Blotto/PBS and incubated overnight at 4 C on a rocker. Each blot included a positive control serum and a negative control lane with 1% Blotto/PBS only. Positive control samples included serum from a patient with lymphocytic hyphophysitis, previously shown to have strong 49-kDa pituitary autoantibody positivity (7). The membranes were then washed thrice (15 min each) with PBS-Tween 20 (0.05%), followed with PBS washes (three times, 15 min) and incubated with alkaline phosphatase-conjugated goat antihuman IgG antiserum (DAKO Corp., Glostrup, Denmark) diluted 1:2500 in 1% Blotto/PBS for 1.5 h at room temperature on a shaker. Membranes were washed again as above. Membranes were incubated with 5-bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium until color development was optimal. Two investigators, one of whom was blinded to sample identity (P.A.C.), read the blots (Fig. 1). Serum TMA was assayed by hemagglutination method (Thymune-M, Murex Diagnostics, Dartford, Kent, UK) and a titer of 1:100 or more was considered positive.

Analysis

Chi-square analysis was used to compare the frequency of pituitary antibody positivity between patient and control groups. Chi-square and *t* tests were used to compare the frequency and mean of different variables, respectively, in patients with Sheehan's syndrome and "other" group as well as patients with or without PitAb positivity. A *P* value of less than 0.05 was considered statistically significant.

Results

Clinical details of all patients are given in Tables 1 and 2. There was no significant difference in the mean age, duration of illness, and body mass index (BMI) between the 19 patients with Sheehan's syndrome and the seven categorized as Other. Fourteen of 26 required hospitalization and blood transfusion immediately after delivery (10 of 19 with Sheehan's and 4 of 7 in Other). At the time of this study, the mean postpartum duration was 6.1 ± 3.3 yr. Nine of 19 (47.3%) patients with Sheehan's syndrome and 6 of 7 (85.7%) in Other had hypotension and/or circulatory collapse at presentation ($P = 0.07$). Three of them (two in Sheehan's syndrome and one in Other) had grossly enlarged cardiac silhouettes associated with myocardial dysfunction that were successfully managed conservatively with prednisolone and T_4 replacement (5). History of stillbirth or neonatal/infant mortality in the offspring during the last pregnancy was present in 10 of 19 patients (52.6%) with Sheehan's syndrome and three of seven (42.8%) in the Other group ($P = 0.65$); four children were stillborn, three died on d 1, and six died between 2 and 5 months of age. Imaging revealed an empty sella (pituitary stalk descending to the floor of the cerebrospinal fluid-filled sella) in 14 (73.7%) and a partial empty sella (a thin rim of pituitary tissue along its floor) in 4 of 19 (21.0%) patients with Sheehan's syndrome. Two subjects with partial and one subject with complete empty sella had thickened pituitary stalks. Five of seven patients in the Other group had an empty sella. Pituitary imaging was normal in one patient with Sheehan's

TABLE 1. Continued

BMI (kg/m^2)	TMA titers	Imaging MRI/CT Ant pit	Gona	Thyr	Cort	Pituitary antibody positivity (kDa)
21.9	1:1600	ES	↓	↓	↓	49
28.8	Nil	ES	↓	↓	↓	49
27.4	Nil	PES (Th Inf)	↓	↓	↓	49
17.2	Nil	ES	↓	↓	↓	Nil
13.6	Nil	N	↓	↓	↓	49
24.7	1:1600	PES	↓	↓	↓	49
21.6	Nil	ES	↓	↓	↓	Nil
16.0	Nil	PES Th Inf	↓	↓	↓	Nil
21.1	Nil	ES	↓	↓	↓	Nil
21.1	Nil	ES	↓	↓	↓	Nil
17.4	Nil	ES	↓	↓	↓	49
22.0	Nil	ES	↓	↓	↓	49
25.2	Nil	ES	↓	↓	↓	Nil
18.6	Nil	ES	↓	↓	↓	Nil
21.8	Nil	ES	↓	↓	↓	49
19.0	1:1600	ES Th Inf	↓	↓	↓	49
20.4	Nil	ES	↓	↓	↓	49
20.3	Nil	ES	↓	↓	↓	49
18.1	Nil	PES	↓	↓	→	49, 40
17.0	Nil	ES	↓	↓	↓	Nil
18.0	Nil	N	↓	↓	↓	49
25.3	Nil	ES	↓	↓	↓	Nil
21.5		ES	→	↓	↓	Nil
22.7	Nil	ES	↓	↓	↓	Nil
23.4	Nil	N	↓	↓	↓	40
21.9	1:1600	ES	↓	↓	↓	Nil

Ab, Antibody; Ant pit, anterior pituitary; APH, antepartum hemorrhage; Cort, corticotroph function; CT, computed tomography; ES, empty sella; Gona, gonadotroph function; mo, month; MRI, magnetic resonance imaging; N, no; PES, partial empty sella; RP, retained placenta; SB, stillbirth; Thyr, thyrotroph function; Th Inf, thickened infundibulum; Y, yes; ↓, decreased; →, normal.

TABLE 2. Clinical characteristics, PitAb positivity, hormone profile, and imaging findings among patients with Sheehan's syndrome and "Other" group

Parameters	Sheehan's syndrome (n = 19)	Other (n = 7)	P^a
Mean age (yr)	32.7 ± 6.4	35.0 ± 6.5	0.44
BMI (kg/m^2)	20.9 ± 3.8	21.4 ± 2.9	0.28
Duration between last delivery and diagnosis (yr)	5.5 ± 3.1	8.0 ± 3.6	0.09
49-kDa PitAb positivity	12 (63.1%)	1 (14.2%)	0.07
Hormones[b]			
Total T_4 (nmol/liter)	33.2 ± 23.0	46.0 ± 38.3	0.41
TSH (mU/liter)	2.3 ± 1.3	1.4 ± 1.5	0.23
Cortisol (nmol/liter)	123.6 ± 70.3	88.9 ± 43.7	0.28
LH (IU/liter)	2.1 ± 1.6	2.5 ± 2.0	0.63
FSH (IU/liter)	3.6 ± 1.9	3.8 ± 2.3	0.84
17β-Estradiol (pmol/liter)	245.9 ± 117.8	317 ± 142.4	0.26
Imaging			
Empty sella	14 (73.6%)	5 (71.4%)	0.90
Partial empty sella	4 (21.0%)	Nil	0.55
Thickened stalk	3 (15.7%)	Nil	0.53
Normal pituitary	1 (5.2%)	2 (28.6%)	0.09
Posterior pituitary signal +ve	11/18 (61.1%)	3/6 (50%)	0.99
TMA positivity	3 (15.7%)	1 (14.2%)	0.92

[a] *P* values for patients in the Sheehan's and Other groups.
[b] Hormone data included 11 patients in the Sheehan's and 6 in Other groups (subjects already on T_4, glucocorticoid, and estrogen replacement therapy were not included in the analysis).

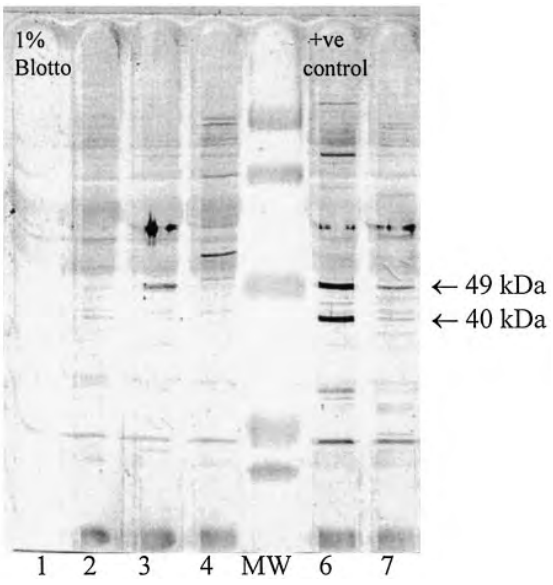

FIG. 1. Immunoblotting of human pituitary cytosolic proteins. A, Ten percent SDS-PAGE Western blot showing specific seropositivity against 49- and 40-kDa pituitary cytosolic autoantigens by positive control serum (lane 6); negative control (1% blotto, lane 1), and healthy controls (lanes 2 and 4) with no seropositivity against 49- and 40-kDa band; patients with Sheehan's syndrome with seropositivity against the 49-kDa autoantigen (lanes 3 and 7) and molecular weight markers (lane 5).

syndrome and in two in the Other group. Gonadotroph, corticotroph, and thyrotroph function was subnormal in all except one each in the Sheehan's and Other groups (Table 1). None of the patients had symptoms of diabetes insipidus, either before or after hormone replacement. A bright posterior pituitary signal on magnetic resonance imaging was present in 11 of 18 patients (61.1%) with Sheehan's syndrome and in 3 of 6 patients (50%) in the Other group.

Western blot analysis revealed PitAb positivity against the 49-kDa autoantigen in 12 of 19 (63.1%) with Sheehan's syndrome and 1 of 7 in the Other group (Fig. 1). In comparison, only 4 of 28 (14.2%) controls without prior conception and 5 of 28 (17.8%) controls with prior conception had PitAb ($P < 0.001$ and < 0.01 *vs.* Sheehan syndrome, respectively). Two subjects each in the Sheehan's syndrome and controls with prior conception, but none in controls without prior conception, had autoantibodies against a 40-kDa autoantigen ($P =$ not significant, Fisher's exact test). When the whole cohort of 26 patients were segregated in relation to PitAb status (13 PitAb +ve and 13 PitAb −ve), no significant difference was observed in the mean age, duration of symptoms, BMI, hormone profile, imaging abnormality, and TMA positivity between the two groups. None of the nine controls with PitAb positivity had symptoms or subnormal hormone values.

Frequency and titer of TMA positivity in the Sheehan's group (3 of 19, 15.7%) was not significantly different (Table 2), compared with the Other group (one of seven, 14.2%, $P = 0.92$), controls without prior conception (21.4%, $P = 0.82$), or to controls with prior conception (7.4%, $P = 0.62$).

Discussion

Most of the present group of patients with Sheehan's syndrome had slow progression of their hypopituitarism with the majority presenting more than 5 yr after delivery, in a state of circulatory collapse. Other clinical features included normal BMI and very high perinatal/infant mortality in the offspring during last pregnancy (52.6%).

The pathogenesis of pituitary failure following PPH is not clear. Sheehan and Davies (6) proposed vasospasm of the pituitary vessels and consequent ischemic necrosis of the pituitary. However, experimental evidence suggests efficient adaptive autoregulation of adenohypophyseal blood flow during hypotension caused by acute blood loss (13, 14). Massive or submassive ischemic necrosis of the pituitary should result in acute pituitary failure in Sheehan's syndrome akin to that observed in pituitary apoplexy. Maccagnan *et al.* (15) assessed pituitary function among patients with pituitary apoplexy managed conservatively . Two weeks following apoplexy, gonadotropin, thyrotropin ,and corticotropin deficiency were observed in 80%, 50%, and 33%, respectively (15). However, in the present study, patients survived acute blood loss and related pituitary failure for several years without any hormone replacement. Despite lactational failure and amenorrhea, indicating pituitary insufficiency dating from the postpartum period, they did not seek medical attention until life-threatening symptoms of circulatory collapse because of adrenal insufficiency supervened (5, 16). This sequence of loss of pituitary trophic hormone function suggests that destruction of the pituitary gland was partial from the inception with progressive loss over time involving thyrotroph and corticotroph function. Such delayed presentation in patients with Sheehan's syndrome could be due to the subtlety of symptomatology and thus the associated failure to recognize hypopituitarism (17). Alternatively, a mechanism, analogous to that of ischemic injury and delayed loss of gonadal function of the type seen among men with unilateral torsion of the testis, may be invoked. Many of them present later with infertility and histological abnormalities of the contralateral testis and antisperm antibodies, presumably caused by antigen release at the time of ischemic infarction (18).

There are two isolated case reports of pituitary autoimmunity in Sheehan's syndrome (19–20). In 1965 Engelberth and Jezkova (19), using a complement consumption test, reported PitAb in a patient with Sheehan's syndrome. In 1982 Pouplard (20) observed PitAb by indirect immunofluorescence in three of four patients with Sheehan's syndrome. However, in 1969 Nerup *et al.* (21) could not demonstrate PitAb in any of six patients with Sheehan's syndrome. The variable reports on PitAb positivity in Sheehan's syndrome could be due to the small numbers of patients studied or technical issues related to the immunofluorescence assays used. In the present study, 63.1% of patients with Sheehan's syndrome had PitAb against a 49-kDa pituitary cytosolic antigen, even 5 yr after the onset of illness. However, only 14.2% of patients in the Other group had PitAb positivity. This trend of higher prevalence of pituitary autoantibody in the Sheehan's syndrome patients with PPH suggests a relationship between the PPH and PitAb positivity. However,

the difference observed in PitAb positivity between the two groups did not attain statistical significance. The comparable prevalence of TMA positivity among the study groups and controls showed no increase in the prevalence of thyroid autoimmunity in the group of Sheehan's syndrome patients with PitAb positivity.

Recently the 49-kDa pituitary cytosolic autoantigen has been identified as the enzyme enolase (2-phospho-D-glycerate hydrolase; EC 4.2.1.11) by one of us (8, 9). The neuron-specific, $\gamma\gamma$ isoform, enolase (NSE) is present in high concentration in cells of the Amine Precursor Uptake and Decarboxylation system including anterior pituitary (22–24). Its distribution correlates with the metabolic activity of the hormone-producing cells (24). Enolase catalyzes the conversion of 2 phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. It is released into serum and cerebrospinal fluid following tissue injuries including hemorrhage and shock (25).

We have demonstrated by two-dimensional gel electrophoresis and immunoblotting that sera from patients with peripartum lymphocytic hypophysitis recognize both $\alpha\alpha$-enolase (the ubiquitous form) and $\gamma\gamma$-enolase (8, 9). These isoforms share 85% homology, and it is not yet known which specific epitopes are recognized by particular patients or patient groups. We also found that NSE is expressed in the placenta and postulated that the presence of NSE in both the placenta and pituitary could explain the strong link between autoimmune pituitary disease and pregnancy (9). In the present cohort of Sheehan's syndrome, the strongest correlation with pituitary antibodies was found in patients with significant PPH. Whether PitAb against NSE, the 49-kDa autoantigen, detected in 63.1% of patients with Sheehan's syndrome, would bind to the active epitopes responsible for enzymatic activity of the enolase is not known. However, the occurrence of such a process after PPH and pituitary necrosis might lead to added damage and progressive hypopituitarism. Thus, to conclude, patients with Sheehan's syndrome have serological evidence of pituitary autoimmunity even up to 8 yr after the onset of disease.

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No Evidence for Autoimmunity as a Major Cause of the Empty Sella Syndrome

Abstract

Objective: The cause of empty sella syndrome (ESS) remains largely unknown. We measured eleven organ-specific autoantibodies in serum in order to evaluate possible autoimmune components in ESS.
Patients: Thirty patients with ESS and 50 healthy blood donors participated in the study.
Measurements: Detection of pituitary autoantibodies was performed by immunoblotting with human pituitary cytosol as antigen. Thyroid peroxidase (TPO) and TSH receptor (TRAK) autoantibodies were analysed by radioimmunoassay. The remaining eight autoantibodies were detected by *in vitro* transcription and translation of the autoantigens and immunoprecipitation.
Results: The majority of the ESS patients (18/30) exhibited no immunoreactivity at all. None of the remaining 12 ESS patients reacted against more than one autoantigen. No immunoreactivity was found more frequently among ESS patients than healthy blood donors. Pituitary autoantibodies were not correlated to the ESS patients' pituitary function or sellar size, although the results indicated a tendency of increased autoimmunity in patients with hypopituitarism and normal sella size respectively.
Conclusion: Detection of autoantibodies is a valuable tool in the diagnostic work-up of autoimmune diseases. By analysing a large number of organ-specific autoantibodies we found no evidence of ESS being associated with any specific autoimmune disease. The pathogenesis of ESS is believed to be heterogeneous and our findings suggest autoimmune components to be of minor importance. In some selective cases, ESS in combination with hypopituitarism may be the result of an autoimmune disease in the pituitary gland but this needs further investigation.

Key words
Empty sella syndrome · autoimmunity · autoantibodies

Introduction

The empty sella is defined as a significant herniation of the suprasellar cistern into the sella turcica. An empty sella is primary when seen without prior irradiation or surgery to the sellar region. Provided that no signs of a pituitary adenoma are present, the primary empty sella is called the empty sella syndrome (ESS). Patients with ESS frequently present with neurological and endocrine malfunctions such as headache, visual defects and pituitary hormonal disturbances (Brismar and Efendic, 1981; Gallardo et al., 1992). The cause of ESS remains largely unknown, although chronic or intermittent increase of intracranial pressure (Brismar and Bergstrand, 1981) and partial pituitary apoplexy (Wakai et

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al., 1981; Bjerre et al., 1986) are believed to be important in the pathogenesis.

An autoimmune origin of ESS has also been proposed since it has been reported as an end stage of lymphocytic hypophysitis (Beressi et al., 1999), a disease of the pituitary gland that is considered to be autoimmune (Goudie and Pinkerton, 1962; Asa et al., 1981). Furthermore, there is a female preponderance as in many autoimmune disorders and ESS has also been reported to coexist with other autoimmune endocrine disorders (Brismar and Efendic, 1981; De Bellis et al., 1995). Only a few experimental studies have so far addressed the question of endocrine autoimmunity in ESS. Autoantibodies to ACTH-secreting mouse AtT_{20} and PRL-secreting rat GH_3 cells have been reported in 75% and 47%, respectively, of patients with ESS, supporting an autoimmune aetiology (Komatsu et al., 1988). In a recently published paper (Keda et al., 2002) elevated titers of autoantibodies to cell surface antigens of human somatotropinoma, human prolactinoma and rat adenohypophysis were found. Mau and colleagues did not, however, find an increased prevalence of pituitary hormone autoantibodies in ESS patients compared to patients with pituitary tumours (Mau et al., 1993).

Detection of autoantibodies is a valuable tool in the diagnostic workup of autoimmune diseases. The aim of this study was to evaluate the prevalence of autoantibodies, known to be associated with lymphocytic hypophysitis and other autoimmune endocrine diseases, in sera from ESS patients and healthy subjects. In contrast to earlier studies we wanted to use normal human pituitary tissue as antigen for the detection of pituitary autoantibodies. In addition, we wanted to correlate the presence of pituitary autoantibodies in ESS patients to sella turcica size and pituitary hormonal function.

Materials and Methods

Subjects

Thirty patients, 25 women and 5 men, diagnosed with ESS at the Department of Endocrinology and Diabetology, Karolinska Hospital, participated in the study. The mean age of the patients was 55.5 years (range 33 – 80 years) and the duration of disease ranged from 0 to 30 years. The diagnosis of empty sella was based on pneumoencephalography, computerised tomography (CT) or magnetic resonance tomography (MRT), depending on the method available at the time of diagnosis. Most patients showed an intrasellar cisternal herniation exceeding 50% of the sella turcica volume, and no patient exhibited a herniation that was less than 30% of the sellar volume at diagnosis. The patients' sellar size was evaluated and defined as normal or enlarged if exceeding 1.092 cm^3 (Di Chiro and Nelson, 1962). All patients had been evaluated regarding pituitary hormonal status. Neurological and endocrine abnormalities documented in the patients' medical records are shown in Table **1**. Laboratory investigation included the measurement of autoantibodies to human pituitary cytosol, Thyroid peroxidase (TPO), TSH receptor (TRAK), Glutamic acid decarboxylase isoform 65 (GAD65), Islet cell antigen (IA-2), 21-hydroxylase (21-OH), 17α-hydroxylase (17-OH), Side-chain cleavage enzyme (SCC), Aromatic L-amino acid decarboxylase (AADC), Tryptophan hydroxylase (TPH), and Tyrosine hydroxylase (TH). Sera from 50 healthy blood donors, between 18 – 65 years of age, with no documented allergic or endocrine disease or medication were used as controls in the autoantibody analysis. The study was approved by the Ethics Committee of the Karolinska Hospital, Stockholm. All subjects participated after giving informed consent.

Table **1** Frequency of clinical symptoms, signs and endocrine diagnosis in 30 patients with ESS

	Prevalence	
	n	***(%)***
Neurological symptoms and signs		
– *Headache*	28	93
– *Dizziness*	26	87
– *Fatigue*	25	83
– *Memory dysfunction*	13	43
– *Impairment of concentration*	11	37
– *Visual field defect*	9	30
– *Papilloedema/increased intracranial pressure*	9	30
Endocrine diagnosis		
– *Type 2 diabetes mellitus*	11	37
– *Hypothyroidism*	9	30
– *Impaired glucose tolerance*	4	13
– *Pituitary dysfunction*	4	13
– *Hyperparathyroidism*	1	3

Analysis of pituitary autoantibodies

The analysis of pituitary autoantibodies was performed as previously described (Crock et al., 1993; Crock, 1998). Normal human autopsy pituitary tissue was homogenized in phosphate-buffered saline (PBS) (pH 7.4) with protease inhibitors (leupeptin, pepstatin, phenylmethylsulfonylfluoride, ethylenediamine tetraacetate and ethyleneglycol tetraacetate) and centrifuged at $400 \times g$ and then $100{,}000 \times g$ to give a cytosolic fraction. The cytosol was depleted of immunoglobulin G using protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and fractionated (50 µg/well) on sodium dodecylsulphate (SDS)-polyacrylamide gels (10% running gel, 4% stacking gel) by electrophoresis under reducing conditions. Separated proteins were transferred by wet blotting to polyvinylidene difluoride membranes (NEN Life science Products, Boston, MA, USA). After blocking with 5% nonfat milk in PBS (pH 7.4) for 1 h, lanes were incubated with patient or blood donor serum diluted at 1 : 50 in PBS (pH 7.4) containing 1% nonfat milk on a shaker overnight at 4 °C. Membranes were washed first in PBS containing 0.05% Tween 20 and then PBS, followed by incubation with alkaline phosphatase-conjugated sheep anti-human IgG antiserum (Amrad Biotech, Victoria, Australia) diluted at 1 : 4000 in PBS containing 1% nonfat milk for 1.5 h at room temperature on a shaker. After repeated washing, membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NEN Life science Products, Boston, MA, USA) until colour development was optimal. All sera were analysed twice and results reported by an observer blinded

to the status of the serum samples. Serum from a patient with known high titer of pituitary autoantibodies was used as a positive control in each experiment.

Analysis of thyroid autoantibodies

TPO autoantibodies were determined using DYNOtest® anti-TPO_n, a competitive radioimmunoassay with enzymatic active native TPO from human thyroids as antigen (BRAHMS Diagnostica GmbH, Berlin, Germany). Results were expressed as U/ml of TPO autoantibodies and the upper level of normal was 60 U/ml. TRAK autoantibodies were determined by TRAK-Assay®, a radioimmunoassay with TSH receptor isolated from porcine thyroid as antigen (BRAHMS Diagnostica GmbH, Berlin, Germany). TRAK values were expressed as U/l and the upper level of normal was 9 U/l.

Analysis of autoantibodies to 21-OH, 17-OH, SCC, AADC, TPH, TH, GAD65 and IA-2

Detections were performed by *in vitro* transcription and translation of the autoantigens and immunoprecipitation as previously reported (Falorni et al., 1995; Husebye et al., 1997). Each autoantigen cDNA was transcribed and translated in vitro with ^{35}S-methionine in a TNT® coupled reticulocyte-lysate system (Promega, Madison, WI, USA). The size of the radioactive product was verified by SDS-polyacrylamide gel electrophoresis (BioRad, Richmond, Ca, USA). Microtiter plates (96 wells) with filter bottoms (MABV N12, Millipore, Bedford, MA) were used for immunoprecipitation experiments. After preincubation with a buffer containing 150 mM NaCl, 20 mM Tris-HCL, and 0.02% NaN_3, (pH 8.0) (buffer A) for 1 hour, the wells were coated with 1% bovine serum albumin (BSA) in buffer A for 2 hours. The plate was then washed twice with 0.05% Tween-20 in buffer A and finally, once with 0.1% BSA and 0.15% Tween-20 in buffer A (buffer B). The ^{35}S-radiolabeled autoantigen were mixed with patient or blood donor serum (1 : 20 dilution) in buffer B and incubated overnight at 4 °C. The mixtures of sera and autoantigen were then transferred to the wells, and a 50% slurry of protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) in buffer B was added to each sample. The plate was shaken on a rotating platform for 45 min at 4 °C and washed three times in buffer B using a vacuum manifold. After drying, scintillation fluid was added and the plate counted in a MicroBeta counter (Wallac Oy, Turku, Finland). The GAD65 and IA-2 assays have been validated by participation in international workshops for the standardization of islet antibody determination, and the upper levels of normal were 0.09 for the GAD65 assay and 0.064 for the IA-2 assay. The remaining autoantibody detection results were expressed as an index (cpm sample-cpm negative control)/(cpm positive control-cpm negative control) × 100. All sera were run in duplicates. The upper normal level for each autoantigen index was calculated as mean + 3 SD of blood donors. Sera from patients with a known high titer of autoantibodies against the different autoantigens were used as positive controls and serum from a blood donor served as a negative control in each assay.

Statistical method

Fisher's exact test was used in the statistical analyses. A p-value of less than 0.05 was considered statistically significant.

Table **2** Immunoreactivity to eleven different autoantigens in sera from ESS patients and healthy blood donors

Autoantigen	*Patients with ESS (n = 30)*	*Healthy blood donors (n = 50)*	*p-value**
49-kDa pituitary protein	6 (20%)	11 (22%)	ns
TPO	3 (10%)	6 (12%)	ns
GAD65	3 (10%)	7 (14%)	ns
TRAK	0	0	ns
IA-2	0	4 (8%)	ns
21-OH	0	0	ns
17-OH	0	0	ns
SCC	0	0	ns
AADC	0	0	ns
TPH	0	0	ns
TH	0	0	ns

P < 0.05 considered statistically significant; ns, no significant difference between groups.

Results

The occurrence of autoantibodies in ESS patients and healthy blood donors are shown in Table **2**. Six patient sera reacted to a 49-kDa pituitary cytosolic protein. In addition, three patients showed immunoreactivity to TPO, and three other patients towards GAD65. No patient reacted to more than one autoantigen. 18 ESS patients exhibited no immunoreactivity at all. No autoantibody was found more frequently in ESS patients than in healthy blood donors. Eleven ESS patients (37%) had a normal sized sella turcica whereas 19 patients (63%) had an enlarged sella (data not shown). All five men with ESS showed an enlarged sella turcica. No significant relation between the sellar size and immunoreactivity to the 49-kDa pituitary protein was found; 3/6 patients with 49-kDa pituitary protein immunoreactivity had an enlarged sella turcica and 3/6 had a sella of normal size. Pituitary dysfunction was present in four patients (Table **3**), and correlated neither to sellar size nor immunoreactivity against the 49-kDa pituitary protein or any other autoantigen (data not shown). All three patients with TPO autoantibodies had been diagnosed with primary hypothyroidism and were on thyroid hormone replacement therapy (Table **2**). One of the three patients with GAD65 autoantibodies had been diagnosed with type 2 diabetes mellitus prior to our study. The remaining two patients with GAD65 immunoreactivity did not suffer from any altered glucose metabolism. The absence of autoantibodies in 18 ESS patients (60%) excluded additional relationships between any immunoreactivity and endocrine dysfunction (Table **2**). There was no significant gender difference regarding pituitary dysfunction, sellar size or any immunoreactivity.

Discussion

The ESS was defined by strict radiological criteria in our study, but its pathogenesis may be heterogeneous. In accordance with previous reports, the majority of the patients were women, most

Table **3** Data on four ESS patients with pituitary dysfunction

Sex/Age at time of study	*Pituitary function*					*Sella turcica size*		*Immunoreactivity*
	GH	*PRL*	*ACTH*	*TSH*	*LH/FSH*	*Normal*	*Enlarged*	
F/37	↓	↓	↓	↓	↓	–	+	49-kDa pituitary protein
F/53	↓	N	↓	↓	↓	–	+	–
F/40	↓	↑	N	N	N	+	–	–
M/80	N	N	↓	↓	↓	–	+	49-kDa pituitary protein

↓, hormonal insufficiency; ↑, hormonal hypersecretion; N, normal hormonal secretion.

of them lacking symptoms and signs of abnormal pituitary function (Buchfelder et al., 1989). Our initial hypothesis was that ESS might be the end stage of an autoimmune process. Immunoreactivity to a 49-kDa pituitary cytosolic protein has previously been shown to be associated with biopsy-proven lymphocytic hypophysitis (Crock, 1998), idiopathic hypopituitarism (Strömberg et al., 1998) and a useful marker for neuroendocrine autoimmunity (O'Dwyer et al., 2002). This 49-kDa pituitary autoantigen has recently been identified as enolase (O'Dwyer et al., 2002). The study by Crock (1998) showed that 70% of patients with biopsy-proven lymphocytic hypophysitis had autoantibodies to the 49-kDa pituitary protein. Only 20% of the ESS patients in our study showed immunoreactivity against this pituitary autoantigen. In contrast to Komatsu et al. (1988) and Keda et al. (2002), we did not find an increased incidence of pituitary autoantibodies in ESS patients. The explanation for this diversity in results may be the choice of human pituitary cytosol as antigen in our study and rodent cell lines in the study of Komatsu et al. Similarily, the study by Keda et al. (2002) measured autoantibodies to *cell surface* antigens on human somatotropinoma, prolactinoma and rat adenohypophysisis, which may explain their elevated titers in ESS compared to our results.

We found no correlation between pituitary autoantibodies and the ESS patients' sellar size or any other immunoreactivity. Two of 4 (50%) patients with hypopituitarism were found to have autoantibodies against the 49-kDa pituitary protein. 3 of 11 (27%) with normal sellar size showed immunoreactivity to this pituitary protein, but only 3 of 19 (16%) with enlarged sellar size did so. Even if not statistically significant, the results indicate a tendency of increased autoimmunity in patients with hypopituitarism and normal sellar size respectively. We believe that larger series of patients with ESS, related to hypopituitarism and sellar size should be evaluated for the presence of pituitary autoantibodies to further explore possible associations.

A striking feature of the patient cohort was that nearly half suffered from type 2 diabetes (11/30) or impaired glucose tolerance (4/30) which is in accordance with previous findings (Brismar and Bergstrand, 1981). No patient suffered from type 1 diabetes mellitus (Table **1**). In accordance with these clinical findings, immunoreactivity to GAD65 and IA-2 were not more frequent among ESS patients than healthy blood donors.

Clinical hypothyroidism has been observed in 15/145 (10%) cases of lymphocytic hypophysitis (Beressi et al., 1999). In our study the prevalence of hypothyroidism in ESS patients was higher, 30%. However, only 3/9 ESS patients with a clinical diagnosis of primary hypothyroidism had positive titers of TPO autoantibodies supporting an autoimmune cause of their thyroid dysfunction. In addition, all sera tested negative for TRAK autoantibodies, commonly seen in patients with autoimmune hyperthyroidism.

The major autoantigen in autoimmune adrenal failure is 21-OH (Winqvist et al., 1992) but a minor proportion of patients also have additional autoantibodies against SCC and 17-OH (Betterle et al., 1999). In our study, no immunoreactivity to these enzymes or any clinical signs of primary adrenal failure were found.

Autoimmune polyendocrine syndrome 1 (APS1) is a rare monogenetic disorder, whose major disease components are primary adrenal failure, hypoparathyroidism and mucocutaneous candidiasis. However, other endocrine manifestations such as hypopituitarism have been described in APS1 patients (Ward et al., 1999). Interestingly, target autoantigens in APS1 are often enzymes involved in neurotransmitter synthesis such as AADC, TPH and TH (Husebye et al., 1997; Ekwall et al., 2000). Since the pituitary closely interacts with the hypothalamus and partly consists of neuronal tissue, we believed it to be relevant to analyse autoantibodies against these enzymes. However, no immunoreactivity against AADC, TPH or TH was found.

Thus, none of 11 immunoreactivities tested was more frequently found among ESS patients than in healthy blood donors. In addition, no ESS patient showed immunoreactivity against more than one autoantigen. By analysing autoantibodies we found no significant evidence of ESS being associated with any autoimmune endocrinopathy. Thus, ESS must mainly be regarded *not* as a primary autoimmune disease but the result of other causes such as intermittent increased intracranial pressure or suboptimal circulatory conditions. In some selective cases, ESS in combination with hypopituitarism may be the result of an autoimmune disease in the pituitary gland but this needs further investigation.

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Autoantibodies against pituitary proteins in patients with adrenocorticotropin-deficiency

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Abstract

Background An autoimmune cause of adrenocorticotropin (ACTH)-deficiency is presented, as it is known to be a characteristic feature of lymphocytic hypophysitis, a disease of the pituitary gland considered to be autoimmune.

Materials and methods The aim of this study was twofold: (1) to evaluate the occurrence of pituitary autoantibodies and (2) to correlate it to clinical and immunological features in a large group of patients with ACTH-deficiency of possible autoimmune aetiology. Sixty-five patients with ACTH-deficiency and 57 healthy subjects participated in the study. Pituitary autoantibodies were measured by an immunoblotting assay with human pituitary cytosol as antigen.

Results Autoantibodies to a novel 36-kDa pituitary autoantigen were seen in sera from 18·5% (12/65) patients and only 3·5% (2/57) of control subjects ($P = 0{\cdot}0214$). When taking only those subjects with strong immunoreactivity into account, the significance was lost; $P = 0{\cdot}3642$. Immunoreactivity to a 49-kDa pituitary autoantigen was observed in 21·5% (14/65) of ACTH-deficient patients compared with 8·8% (5/57) of control subjects ($P = 0{\cdot}0910$). This 49-kDa pituitary autoantigen has recently been identified as neurone-specific enolase and a candidate marker for neuroendocrine autoimmunity. Clinical parameters in patients with positive versus those with negative pituitary immunoreactivity did not differ. However, autoantibodies to thyroglobulin were positively correlated to immunoreactivity against the 36-kDa pituitary autoantigen ($P = 0{\cdot}014$).

Conclusions Our findings of pituitary autoantibodies in patients' sera support the theory that an autoimmune destruction of corticotrophs may be the underlying cause of hormonal deficit in some patients with ACTH-deficiency.

Keywords ACTH-deficiency, immunoblotting, lymphocytic hypophysitis, pituitary autoantibodies.
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Introduction

Adrenocorticotropin (ACTH)-deficiency may be caused by genetic abnormalities or may be secondary to destructive pituitary lesions such as trauma, tumours, surgery, irradiation and infiltrative diseases. However, the origin of ACTH-deficiency is uncertain in most cases. An autoimmune cause of ACTH-deficiency has been proposed, as it is known to be a characteristic feature of lymphocytic hypophysitis, a disease of the pituitary gland considered to be autoimmune [1,2]. Furthermore, as in many autoimmune disorders, there is a female preponderance and isolated ACTH-deficiency is also known to coexist with several other autoimmune endocrine disorders [3–6].

Detection of autoantibodies is a valuable tool in the diagnostic workup of autoimmune diseases. Several reports have described pituitary autoantibodies in patients with lymphocytic hypophysitis and isolated ACTH-deficiency. Conventional immunofluorescence methods detected pituitary autoantibodies in ACTH-deficient patients that reacted with rat pituitary gland and AtT20 murine cells [7–9].

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A drawback of immunofluorescence in regards to corticotrophs relates to the detection of nonspecific binding of autoantibodies by Fc receptors [10,11]. An immunoblotting technique has allowed more detailed characterization of targeted pituitary autoantigens by molecular weight [12,13]. Recently, a 22-kDa and a 49-kDa pituitary autoantigen have been identified as growth hormone and neurone-specific enolase, respectively [14–16]. Using a radioligand assay, Tanaka and colleagues identified autoantibodies against human growth hormone, pituitary gland specific factor 1a (PGSF 1a) and PGSF2, in patients with lymphocytic hypophysitis, isolated ACTH deficiency or idiopathic thyroid-stimulating hormone (TSH) deficiency [17].

In previous reports, only limited numbers of patients with isolated ACTH-deficiency have been tested for pituitary autoantibodies. The aim of our study was to examine pituitary autoantibodies in a large number of patients with ACTH-deficiency of possible autoimmune aetiology. We have used an immunoblotting assay with human pituitary cytosol as antigen in the detection of pituitary autoantibodies, which enables identification of target autoantigens by molecular weight and avoids the potential problems of species specificity. We also aimed to correlate the occurrence of pituitary autoantibodies to the clinical and immunological features of the patients.

Subjects

Sixty-five patients, 63 women and two men diagnosed with ACTH-deficiency of unknown origin at the Department of Endocrinology, Centre for Postgraduate Medical Education (Warsaw, Poland), participated in the study. At sampling, the mean age of the patients was 49 years (range 18–76) and the median duration of disease from diagnosis was 1 year (range 0–16). However, in many cases a long-term period of subclinical complaints, comprising weakness and fatigability, mainly following stressful conditions, preceded diagnostic procedures.

Sera from 57 healthy Swedish subjects, 52 women and five men, mean age 44·4 years (range 19–65), with no documented allergic or endocrine disease or medication were used as controls in the pituitary autoantibody analysis. The study was approved by the Ethics Committees of the Centre for Postgraduate Medical Education (Warsaw, Poland) and the Karolinska Hospital (Stockholm, Sweden). All subjects participated after giving informed consent.

Methods

Clinical and immunological investigations in patients with ACTH-deficiency

The diagnosis of ACTH-deficiency in patients was based on clinical characteristics and hormonal investigation including simultaneous plasma ACTH (by immunoradiometric method) and cortisol (by radioimmunoassay) determinations as well as 17-hydroxycorticosteroids (17-OHCS) urinary excretion (by the Silber and Porter method) in basal conditions and during 2 days of synthetic ACTH administration, as described previously [6,18]. ACTH and cortisol levels, below the lowest normal limit (normal range, 4·5–13·6 pmol L^{-1} at 08·00 h, 2·2–6·4 pmol L^{-1} at 22·00 h and 193–690 nmol L^{-1} at 08·00 h, 110–386 nmol L^{-1} at 22·00 h, respectively) were characteristic for all the patients. Basal 17-OHCS below the normal range (6·1–19·3 μmol 24 h^{-1}) rose significantly (7–30-fold) during stimulation testing.

Pituitary function was further evaluated in all patients by measurements of serum levels of prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and (TSH), while the thyroid function was evaluated by free thyroxin determinations. In women, serum estradiol levels were measured, and in men testosterone levels. Patients were asked about coexisting endocrine and autoimmune disorders as well as any previous pregnancies. Data relating to all previous pituitary imaging examinations were collected.

In all patients' sera, autoantibodies against thyroglobulin (aTg) and thyroid microsomes (aMIC) were examined (Thymune T and Thymune M, respectively, Murex Diagnostics, Dartford, UK) (normal values: up to 1/120). Autoantibodies against thyroid peroxidase (aTPO) were determined as described previously [19] (pathological values: equal or greater than 1/800).

Analysis of autoantibodies to pituitary cytosolic proteins

The analysis of pituitary autoantibodies was performed as previously described [12,13]. Normal human autopsy pituitary tissue was homogenized in phosphate-buffered saline (PBS) (pH 7·4) with protease inhibitors (leupeptin, pepstatin, phenylmethylsulphonylfluoride, ethylendiamine tetraacetate and ethyleneglycol tetraacetate) and centrifuged at 400 × *g* and then 100 000 × *g* to give a cytosolic fraction. The cytosol was depleted of immunoglobulin G using protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and fractionated (50 μg $well^{-1}$) on sodium dodecylsulphate (SDS)-polyacrylamide gels (10% running gel, 4% stacking gel) by electrophoresis under reducing conditions. Pre-stained molecular weight standards were used in every experiment (Biorad, Hercules, CA, USA). Separated proteins were transferred by wet blotting to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA, USA). After blocking with 5% nonfat milk in PBS (pH 7·4) for 1 h, lanes were incubated with patient or control subject serum diluted at 1 : 50 in PBS (pH 7·4) containing 1% nonfat milk on a shaker overnight at 4 °C. Membranes were washed first in PBS containing 0·05% Tween 20 and then PBS, followed by incubation with alkaline phosphatase-conjugated sheep antihuman IgG antiserum (Sigma-Aldrich Inc, Saint Louis, MO, USA) diluted at 1 : 4000 in PBS containing 1% nonfat milk for 1·5 h at room temperature on a shaker. After repeated washing, membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue

tetrazolium (NEN Life science Products, Boston, MA, USA) until colour development was optimal. Serum from a patient previously shown to have strong immunoreactivity against a 49-kDa pituitary autoantigen was used as a positive control in each experiment. All sera were analyzed twice and results reported by one observer blinded to the status of the serum samples. Immunoreactivity was reported as *negative*, *positive* or *strongly positive*. Negative result: no band of immunoreactivity observed. Positive result: a distinct, sharp band of immunoreactivity detected above background staining. Strongly positive result: a distinct, sharp band of immunoreactivity detected above background staining but of greater width and colourintensity, indicating a relatively high autoantibody titre.

Statistical analysis

Statistical analyses were performed using Statistica, StatSoft Inc (version 6·1), Tulsa, OK, USA. χ^2-test with Yates' correction was used when comparing immunoreactivities to pituitary autoantigens in patients vs. control subjects. The two-tailed Fisher exact test was used when comparing clinical and immunological parameters in patients with positive pituitary immunoreactivity vs. patients with negative pituitary immunoreactivity. Statistical significance was set at $P < 0{\cdot}05$. Clinical parameters included gender (male/female), age at diagnosis, duration of disease, pregnancy in the past (presence/absence), other pituitary insufficiency (presence/absence) and other endocrine disorders. Immunological parameters included a clinical history of other autoimmune diseases and circulating aTPO, aTG or aMIC autoantibodies (presence/absence).

Results

Clinical and immunological findings in patients with ACTH-deficiency

Pituitary function tests in patients revealed prolactin deficiency in a 21-year-old female, low TSH in a 40-year-old woman and low gonadotropin levels in two elderly women. Thus in 61/65 patients, isolated secondary adrenal insufficiency was diagnosed.

Coexisting autoimmune diseases (n = 45) were found in 33/65 (51%) patients (Table 1). Ten of the 33 patients suffered from multiple coexisting autoimmune diseases. Thyroid autoimmune diseases were most common. Positive results for the thyroid autoantibody analysis were found in 55/65 (85%) patients (Table 1). In 31 patients thyroid autoantibodies were present without laboratory signs of hypothyroidism.

A history of previous pregnancy was noted in 53/63 (84%) of the female patients.

Magnetic resonance imaging of the pituitary performed in 18 patients revealed a partially empty sella in six cases, an empty sella in one patient and a slightly enlarged pituitary in three patients. In the remaining eight patients no abnormalities were visualized.

Table 1 Coexisting autoimmune diseases and immunoreactivity against thyroid autoantigens in 65 patients with adrenocorticotropin-deficiency

	Prevalence
Coexisting autoimmune disease	
Hypothyroidism	22
Subclinical hypothyroidism*	4
Vitiligo	5
Hyperthyroidism in past	4
Pernicious anaemia	3
Premature ovarian failure†	2
Alopecia areata	2
Celiac disease	2
Type 1 diabetes mellitus	1
Thyroid autoantigen	
Thyroid peroxidase	50
Thyroglobulin	10
Thyroid microsomes	10

Prevalence, number of patients with the disease/immunoreactivity.

*Free thyroxin values within normal limits and no clinical features of thyroid disease but slightly increased thyroid-stimulating hormone.

†Ovarian failure appearing before 35 years of age.

Analysis of autoantibodies to pituitary cytosolic proteins

Duplicate immunoblotting runs revealed positive immunoreactivity against a 36-kDa pituitary cytosolic protein in 12 of 65 (18·5%) patients and two of 57 (3·5%) control subjects. The difference between patients and control subjects was statistically significant $P = 0{\cdot}0214$ (χ^2-test with Yates' correction). When taking only those subjects with strong immunoreactivity on both runs into account (six patients vs. two control subjects) the significance was lost, $P = 0{\cdot}3642$. Table 2 (patient nos 11–22) shows the clinical data of the 12 patients with positive 36-kDa immunoreactivity. Statistical analysis revealed no differences in the clinical parameters between patients testing positive or negative for this immunoreactivity; except, the group of patients with positive 36-kDa immunoreactivity had a higher frequency of aTG autoantibodies ($P = 0{\cdot}014$) (Fisher's exact test, two-tailed, not shown in the tables).

Positive immunoreactivity to a 49-kDa pituitary cytosolic protein was observed in sera from 14 of 65 (21·5%) patients with isolated ACTH deficiency and in five of 57 (8·8%) control subjects. Statistical calculation demonstrated that patients with ACTH deficiency had a tendency toward a higher frequency of immunoreactivity against this autoantigen compared with control subjects, $P = 0{\cdot}0910$ (χ^2-test with Yates' correction). When only subjects with strong

Table 2 Data on 22 patients with adrenocorticotropin-deficiency and positive immunoreactivity against pituitary cytosolic autoantigens

Patient	Sex/age	Pregnancy in past	Other pituitary deficits	Other endocrine/autoimmune diseases	Pituitary Immunoreactivity 49-kDa	Pituitary Immunoreactivity 36-kDa	Other autoantibodies
1	F/21	+	PRL	–	+		
2	F/34	+	–	Hyperthyroidism in past Subclinical hypothyroidism	++		aTPO 1/256 000
3	F/40	+	–	–	+		aTPO 1/16000
4	F/42	+	–	Hypothyroidism	++		aTPO 1/8000
5	F/44	+	–	Hyperthyroidism in past	+		aTPO 1/6400
6	F/48	+	–	–	+		
7	F/50	+	–	–	+		
8	F/61	+	–	–	+		aTPO 1/1600
9	F/66	+	–	–	++		aTPO 1/64 000
10	F/76	–	–	Subclinical hypothyroidism	++		aTPO 1/1000
11	F/40	+	–	–	++	+	
12	F/46	+	–	–	++	++	aTPO 1/4000
13	F/47	+	–	–	++	+	aTg 1/2160 aMic 1/2160
14	F/58	+	–	Vitiligo	++	++	aTPO 1/3200
15	F/30	+ recent	–	Hypothyroidism		++	aTPO 1/256 000 aTg 1/660
16	F/38	–	–	–		++	aTg 1/240 aMic 1/360
17	F/40	+	–	Hypothyroidism		+	aTPO 1/3200
18	F/41	+ recent	–	Hypothyroidism		++	aTPO 1/16 000
19	F/42	+	–	Hyperthyroidism in past Hypothyroidism		+	aTPO 1/3200
20	F/43	+	–	–		++	aTPO 1/3200 aTg 1/360 aMIC 1/720
21	F/68	–	–	Adrenal incidentaloma		+	aTPO 1/4000
22	F/69	+	–	Subclinical hypothyroidism		+	aTPO 1/8000 aTg 1/240 aMIC 1/360

F, female; age, years at serum sampling; PRL, prolactin; ++, strongly positive immunoreactivity; +, positive immunoreactivity; aTPO, thyroidperoxidase autoantibodies; aTg, thyroglobulin autoantibodies; aMIC, thyroidmicrosomal autoantibodies.

immunoreactivity in both runs were analyzed (eight patients vs. three blood donors) the tendency was lost, $P = 0{\cdot}2990$. Clinical data from the 14 patients with autoantibodies against the 49-kDa pituitary protein are shown in Table 2 (patient nos 1–14). Comparison of clinical and immunological parameters of these 14 patients to the 51 patients with no immunoreactivity against this protein showed no statistical differences.

Four patients reacted against both the 36-kDa and 49-kDa pituitary autoantigens in duplicate immunoblotting runs (patient nos 11–14, Table 2), while 43 patients exhibited no immunoreactivity against these pituitary autoantigens. A number of other pituitary cytosolic proteins were also detected by individual sera, but there were no significant patterns nor differences between the patients and the control subjects. Figure 1 shows immunoreactivity against the 36-kDa and 49-kDa pituitary autoantigens in some patients' sera.

MRI of the pituitary region was performed in 18/65 (28%) of patients. Statistical analysis of this subgroup of patients revealed no differences in imaging findings between the patients with or without 36-kDa and 49-kDa immunoreactivity, respectively.

Discussion

The anterior pituitary gland consists of five different cell types with six individual hormone synthesis pathways. Therefore, it is likely that patients with a range of pituitary hormone deficiencies due to lymphocytic hypophysitis exhibit various spectrums of pituitary autoantibodies. To date, only four autoantigens have been identified in a very limited number of patients with isolated ACTH deficiency: enolase (α and γ isoforms) [13,16,20,21], human growth hormone, PGSF1a and PGSF 2 [17]. Sauter *et al*. detected positive immunostaining, which was concentrated in secretory granules in rat pituitary corticotrophs in a man

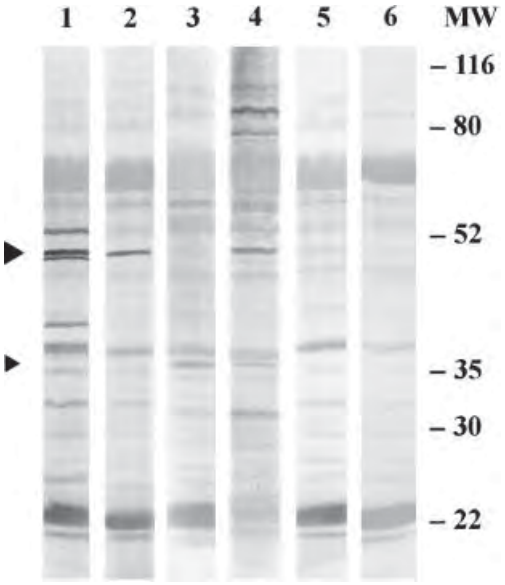

Figure 1 Immunoblotting assay for pituitary autoantibodies. Human pituitary cytosolic proteins were separated on 10% sodium dodecylsulphate (SDS)-polyacrylamide gels, transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes, and probed with sera. Lane 1: incubated with serum from a patient with known high titres of pituitary autoantibodies. Lanes 2–4: sera from patients with adrenocorticotropin-deficiency. Lane 5: serum from a control subject. Lane 6: nonspecific binding by second antibody (sheep antihuman IgG conjugated to alkaline phosphatase) alone. MW, molecular mass markers in kilodaltons (kDa). The large arrow indicates positive reactivity to a 49-kDa autoantigen and the small arrow indicates positive reactivity to a 36-kDa autoantigen.

with isolated ACTH-deficiency [9], but this protein is still uncharacterized.

In the current study, we have examined pituitary autoantibodies in a large group of patients with possible autoimmune ACTH-deficiency. We have identified a 36-kDa pituitary protein as a minor target of autoantibodies in ACTH-deficiency, with a small fraction (12/65, 18·5%) of the ACTH-deficient patients studied being positive. To our knowledge, this is the first report of immunoreactivity against a pituitary protein of this molecular weight. Until more careful characterization of this protein is performed, we can only speculate on the nature of this autoantigen. As many known endocrine autoantigens are intracellular enzymes involved in hormone synthesis [22], it is tempting to suggest that this is also the case with the 36-kDa pituitary autoantigen. The finding of 36-kDa immunoreactivity in sera from a couple of control subjects may indicate that the specificity of this autoantibody is not 100%. To clarify the relevance for this autoantibody as a marker for autoimmune ACTH-deficiency, further studies are needed. Complete identification of the 36-kDa pituitary autoantigen is necessary, and the cut-off level for autoantibody titres in healthy subjects needs to be defined. The occurrence of 36-kDa immunoreactivity in patients with different pituitary diseases also needs to be investigated.

We have also shown that some patients with ACTH-deficiency have autoantibodies against a 49-kDa pituitary cytosolic protein; however, not significantly more frequent than control subjects. Immunoreactivity against this autoantigen has been shown previously in 70% of biopsy-proven lymphocytic hypophysitis patients and in 55% of patients with suspected lymphocytic hypophysitis, including cases of isolated ACTH-deficiency [13], as well as in idiopathic hypopituitarism [23]. The 49-kDa pituitary autoantigen has been identified as neurone-specific enolase, but contradictory data on the importance of enolase autoantibodies as markers for neuroendocrine autoimmunity have been published [16,20,21].

Sixty-six percent (43/65) of the patients with ACTH-deficiency in our study did not show any immunoreactivity against the 36-kDa or 49-kDa pituitary autoantigens. There may be several reasons for this observation. First, the ACTH-deficiency in these patients may not be caused by autoimmune destruction of corticotrophs but other causes such as genetic abnormalities. Second, it is possible that some pituitary autoantibodies in ACTH-deficient patients cannot recognize the denatured form of antigen used in our immunoblotting assay. This phenomenon has been recognized for antiglutamic acid decarboxylase (GAD) autoantibodies in type 1 diabetes mellitus patients who rarely recognize denatured GAD. Instead, GAD autoantibodies are detectable by immunoprecipitation under nondenaturing conditions [24]. We chose to use human pituitary cytosol as the substrate in our study, as most endocrine autoantigens are known to be cytosolic. However, it is possible that additional target autoantigens are bound to pituitary cell membranes [12,25].

Many authors have suggested a relationship between the development of pituitary autoimmune disease and pregnancy [16,26,27]. As the features of secondary adrenal insufficiency are not as dramatic as in Addison's disease, it is probable that subclinical ACTH deficiency could follow autoimmune hypophysitis developed during pregnancy. The present series of patients with ACTH-deficiency includes a striking dominance of women: 63 females vs. two males. Fifty-one of 63 women had been pregnant 5–40 years before the study but only two women had pregnancies within the last 2 years. These two women both showed positive immunoreactivity to the 36-kDa pituitary autoantigen. Thus, a probable cause of the patients' lack of immunoreactivity against pituitary autoantigens could be the long duration of disease, resulting in complete destruction of corticotrophs (i.e. antigen material) and subsequent reduction in autoantibody titres. Selective loss of corticotroph cells in patients with lymphocytic hypophysitis has been reported [28–30].

A striking feature of this cohort of ACTH-deficient patients is the high frequency of associated autoimmune disease, in particular hypothyroidism. Co-existent autoimmune disease is also a feature of patients with biopsy-proven lymphocytic hypophysitis [31,32]. Patients with autoantibodies to the 36-kDa pituitary autoantigen did show a higher frequency of aTG autoantibodies than patients without immunoreactivity against this pituitary autoantigen, but we could not correlate immunoreactivity to the 36-kDa

or 49-kDa pituitary autoantigen to symptomatic hypothyroidism or any other specific autoimmune disease.

In conclusion, a 36-kDa pituitary protein has been shown in this study to be a novel target of autoantibodies in ACTH-deficiency. The precise role of autoantibodies in the pituitary disease process, and their possible clinical utility, is still to be determined.

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Lecture

Pituitary autoantibodies in autoimmune polyendocrinopathy – candidiasis - ectodermal dystrophy (APECED)

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Abstract. Autoimmune polyendocrinopathy - candidiasis - ectodermal dystrophy (APECED) is an autosomal recessive disease due to mutations in the *AIRE* (AutoImmune REgulator) gene. The role of pituitary autoimmunity in APECED is not known. We determined the prevalence of pituitary autoantibodies in a cohort of 67 Finnish patients with APECED from 217 serum samples collected over 26 years by one investigator. Overall, autoantibodies to the 49 kDa cytosolic autoantigen, human pituitary enolase were detected in 39 of the 67 patients (58%). On their first sample, 25 patients had autoantibodies compared to 5 of 68 controls (chi-square, 1df=17.11, p< 0.001; OR=7.32), but subsequently 14 patients seroconverted between 10 and 53 years of age. Once seropositive, all but two of the patients maintained their positive autoantibody status, even over many years. In the current study all but 7 of the 19 patients known to have high titre anti-candidal enolase antibodies had developed autoantibodies directed against human pituitary enolase. Other pituitary autoantibody reactivities were detected against cytosolic proteins of molecular weights 40-, 45-, 60- and 105 kDa in 15%, 16%, 12% and 3% of patients respectively. Autoantibodies to pituitary enolase are markers of neuroendocrine autoimmunity but seem not to be associated with clinical hypopituitarism in APECED patients. (www.actabiomedica.it)

Key words: Autoimmune polyendocrine syndrome type 1, autoimmune polyendocrinopathy, candidiasis, pituitary autoantibodies, enolase, molecular mimicry

Introduction

Endocrine organs are often the target of organ-specific autoimmune destruction, particularly pancreatic beta cells and the thyroid and less commonly the adrenal, pituitary and parathyroid gland. Susceptibility to such autoimmunity is usually linked to HLA loci (1). In contrast, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also called autoimmune polyendocrine syndrome type I) (2, 3), is caused by mutations in a single gene pair *AIRE* (AutoImmune REgulator) on chromosome 21q22.3 (3, 4). The AIRE protein is expressed in cells regulating immune tolerance in the thymic medulla and is a regulator of transcription (5, 6, 6a).

The classical evolution of APECED begins with mucocutaneous candidiasis in early childhood, followed by hypoparathyroidism and then Addison's disease (2). However, its clinical spectrum and course are widely variable (7). It may include destruction and hypofunction of up to six endocrine glands, the least common one being the pituitary gland. Growth hor-

mone deficiency has been diagnosed in four of 89 Finnish patients (2, 7), and four others are on record (8, 9) one of them with clinical and radiological evidence of hypophysitis and pituitary autoantibodies (10).

Other organ-specific autoimmune components may occur, from vitiligo and parietal cell atrophy to keratopathy, hepatitis and intestinal dysfunction. Mucocutaneous candidiasis appears in most of the patients. Dental enamel hypoplasia, punctate dystrophy of nails, and dystrophy of the tympanic membranes are hallmarks, which help in recognition of the disease (2).

The autoimmune destructive process is presumed to be T-cell mediated. Recent studies in AIRE knockout mice have shown an exaggerated proliferation of peripheral T cells when mice are challenged with immunization (11). Other studies with AIRE deficient mice have demonstrated a defect in the expression of self-antigens and in the deletion of self-reacting T-cell clones in thymus, establishing failure in central tolerance mechanisms as a major cause of APECED (12, 13). In association with this process, there is multiorgan lymphocytic infiltration and circulating autoantibodies. These autoantibodies are frequently directed against intracellular enzymes. The role of such autoantibodies in the destruction remains unclear, but they are important as diagnostic markers of the autoimmune process and appear commonly before clinical hormone deficiency.

Although rare, APECED seems to occur in most populations. Three populations have an exceptionally high prevalence: Iranian Jews (14), the Sardinians (15), and the Finns. More than 40 mutations of AIRE have been reported, and the number keeps growing, see http://bioinf.uta.fi/AIREbase/. The Iranian Jewish patients have been reported to have much less candidiasis and adrenal insufficiency than other patients (14), which may relate to their unique mutation.

The aim of this study was to determine the prevalence of serum pituitary autoantibodies in a cohort of Finnish patients with APECED, using an immunoblotting method (16). The immunoblotting assay enables the characterisation of pituitary target autoantigens by molecular weight. Pituitary autoantibody results were correlated with the clinical phenotypes of the patients.

Materials and Methods

Patients and methods

Two hundred and seventeen serum samples were collected from 67 APECED (36 female) patients in Finland between 1974 and 2000. Two to 4 samples were obtained from 30 of the patients over 2 to 25 years. Ages at sampling ranged from 4.3 to 57.4 years (mean 23.4 years). Sera were also obtained from 68 non-APECED controls, including 16 Finnish endocrine patients (12 female) with non-autoimmune conditions (age range 9.4 to 28.2 years; mean age 19.7 years), and 52 normal subjects (32 women; age range 19 to 60 years; mean age 29 years whose results have been reported previously) (17). Approval was obtained from the Ethics Committee, The Hospital for Children and Adolescents, University of Helsinki and the Human Research Ethics Committees of the Hunter Area Health Service and University of Newcastle.

Immunoblotting assay

Serum samples were screened for pituitary autoantibodies by immunoblotting as previously described (16, 17). IgG-depleted human autopsy pituitary cytosolic proteins were loaded on 10% SDS-polyacrylamide gels and separated under reducing conditions. Separated proteins were transferred to polyvinyl difluoridine membranes (Polyscreen 0.2 μm PVDF: NEN Life Sciences, Boston, MA, USA) by a wet electrotransfer technique in the "Mini Trans-Blot" unit (Bio-Rad, Hercules CA). Non-specific protein interactions were blocked using 5% BLOTTO (5% skim milk powder in PBS). PVDF membranes were placed in a Deca-probe™ apparatus (Hoefer, San Francisco, CA, USA). Patient and control sera were used at a dilution of 1:50 in 1% BLOTTO and incubated at 4°C overnight. Well-characterized serum from a patient with clinically suspected lymphocytic hypophysitis and with high titre autoantibodies (>1:1,000) to human pituitary enolase, was used as a positive control. Post-incubation, membranes were washed three times with 0.05% Tween 20 in PBS followed by PBS. Autoantibody reactivity was detected using alkaline phosphatase-conjugated goat anti-human IgG (Silenus,

Melbourne, Australia) secondary antibody at a dilution of 1:2,000 in 1% BLOTTO and incubated for 1.5 hours at room temperature. The PVDF membranes were then washed as above and bands of autoantibody reactivity detected by a color reaction system. Antibodies to candidal enolase were detected as previously described (18).

Analysis

Two independent observers assessed the bands of positive reactivity. Sera were coded and tested blind, and the code only broken after results had been tabulated. Results between the subject groups were analyzed by chi-square test, and the autoantibody reactivities were correlated with specific clinical manifestations by chi-square or Fisher's Test if n <5. Odds ratios (OR) were also calculated.

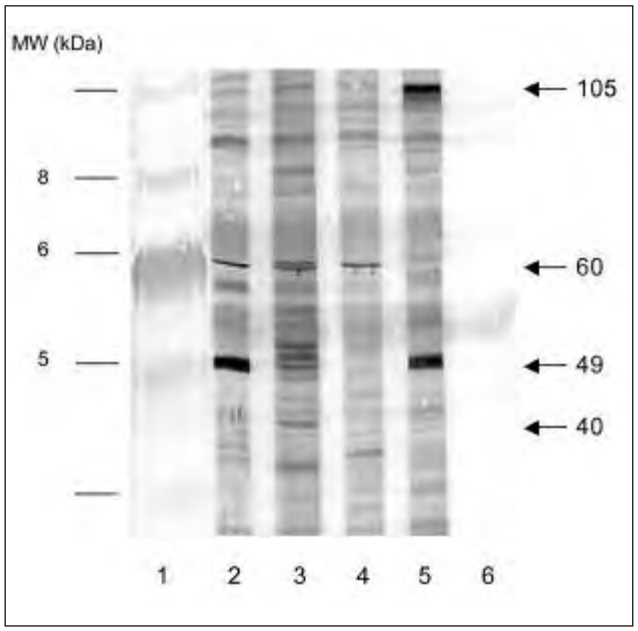

Figure 1

Results

Pituitary autoantibodies against human pituitary enolase (49 kDa cytosolic protein)

Autoantibodies to human pituitary enolase were detected in 25 of the 67 APECED patients in the earliest (i.e. initial) sample of their series, compared to 5 of 68 controls (chi-square, 1df = 17.11, p<0.001; odds ratio (OR)=7.32). The youngest age at which this reactivity was seen was 5.6 years. Fourteen APECED patients had negative early samples but seroconversion was detected subsequently, as early as 10.3 years of age and as late as 53.2 years. With the exception of two patients, once autoantibodies were demonstrated in a patient, all subsequent samples in that patient remained positive (Figure 1). These latter two patients had only two samples each, at intervals of 6.9 and 15.3 years. Thus in total, 39 of the 67 APECED patients (58%) were positive for pituitary enolase autoantibodies (Table 1).

In an earlier study, 24 of our patients were examined for antibodies against candidal enolase. Nineteen of them were found to have a high titre of antibodies in their serum to recombinant candidal enolase expressed from *Candida albicans* (18). Studying the same sera we now found 12 of these 19 patients positive for human enolase antibodies, and seven negative (Table 2). While all these seven were positive for candidal enolase antibodies, only one of them had a high titre. Of the total of eight patients with such high titre candidal enolase antibodies, the other seven were also positive for autoantibodies to human enolase. Conversely, human enolase autoantibodies were detected in all the five of the 19 patients that were negative for the candidal enolase antibodies, or had only low titres.

Autoantibodies against other pituitary cytosolic proteins

Amongst the 67 APECED patients, 19 had no serum reactivity against human pituitary cytosolic proteins. Some of the sera identified several other pituitary target autoantigens, with 18 patients displaying reactivity to more than one pituitary cytosolic autoantigen. Although the molecular weights of these autoantigens are known, they have yet to be characterized further.

Two patients had antibodies directed against a 105 kDa pituitary cytosolic protein, and eight patients to a 60 kDa cytosolic protein (Table 1 and Figure 2). The youngest age at which reactivity against the 105

Table 1. Endocrine and non-endocrine components of APECED and serum pituitary autoantibodies

Age, years	0-5	5-10	10-15	15-20	20-30	30-40	40-50	Over 50	All
No. of endocrine components per patient (percentages)									
1 or more	33.8	77.9	92.1	95.6	98.5	100	100	100	100
2 or more	4.4	27.9	50.0	66.2	82.4	91.2	94.1	94.1	73.5
3 or more	1.5	5.8	23.5	38.2	45.6	50.0	54.4	55.9	57.4
None	66.2	22.1	8.8	4.4	1.5	0	0	0	0
Newly-Diagnosed Endocrine components									
Hypoparathyroidism	21	22	9	4	1	0	1	0	58 85.3%
Adrenal failure	5	21	17	4	5	1	1	0	54 79.4%
Diabetes mellitus	1	0	2	3	2	2	2	1	13 19.1%
Hypothyroidism	0	0	0	4	3	2	1	0	10 14.7%
Ovarian failure (of 36 females)	0	0	11	4	3	2	0	0	20 55.6%
Testicular failure (of 32 males)	0	0	1	1	0	2	0	0	4 12.5%
Growth hormone deficiency									3 4.4%
Newly-Diagnosed Non-endocrine components									
Alopecia	1	7	8	3	2	1	0	0	22 32.4%
Vitiligo	2	1	5	2	2	2	0	0	14 20.6%
Keratopathy	6	8	5	0	0	0	0	0	19 27.9%
Hepatitis	2	3	4	1	0	0	0	0	10 14.7%
Intestinal malabsorption	3	1	2	2	5	1	1	0	15 22.1%
Nephritis	0	0	0	1	1	1	0	0	3 4.4%
Iritis	0	0	0	1	1	0	0	0	2 2.9%
Vasculitis	1	1	0	0	0	0	0	0	2 2.9%
Serum pituitary autoantibodies detected against human pituitary cytosolic proteins									
No. Patients tested in age group	2	13	24	30	38	24	10	3	67
Anti-human pituitary enolase	0	4	9	12	22	11	4	2	36
No. Patients positive	0	30.8	37.5	40.0	57.9	45.8	40.0	66.7	58
Percentages (%)									
40 kDa autoantigen (%)	0	7.7	4.2	6.7	10.5	12.5	40.0	33.3	14.7
45 kDa autoantigen (%)	0	7.7	8.3	10.0	18.4	12.5	10.0	33.3	16.2
60 kDa autoantigen (%)	0	0	8.3	10.0	7.9	8.3	20.0	33.3	11.8
105 kDa autoantigen (%)	0	0	4.2	0	5.3	4.2	0	0	2.9

kDa autoantigen was detected was 12.1 years, and for the 60 kDa pituitary cytosolic autoantigen, 13.7 years.

Autoantibodies to 40 kDa (Figure 2) and 45 kDa cytosolic proteins were detected in sera from 10 and

Table 2. Correlation of antibodies against two Enolases in the Finnish APECED cohort

Patient No	Age	Candida Enolase Antibodies	Human Pituitary Enolase Antibodies
1	16.5	-	-
	25.4	+	+
2	49.7	++	+
	51.6	++	+
	58.3	+++	+
3	21.1	+	+
4	27.1	+	-
	37.3	+	+
5	25.8	+	+
	29.4	+	+
6	34.3	+++	-
	44.2	++	-
7	41.7	-	-
	48.9	-	+
8	16.5	+	-
	30.7	+	-
9	23.6	-	-
	31.3	+	-
10	25.6	++	+
	35.4	+++	+
11	28.3	+	-
	41.5	-	-
12	24.0	-	+
	24.7	-	+
	34.7	-	+
13	12.3	-	+
	27.5	-	+
14	19.3	++	-
	24.3	+	+
	30.0	+	+
	32.6	++	+
15	23.4	-	+
	30.3	-	+
16	18.7	++	-
	33.9	+	+
17	57.4	+	-
18	22.9	+	-
19	17.8	++	+
20	32.2	+	-
21	9.0	-	+
22	8.8	++	+
23	12.5	+++	-
	15.7	+	-
	24.7	+	+
24	30.0	-	-
	31.9	-	+
	35.2	+	+
	42.7	-	+

11 patients, respectively. The youngest ages at detection were 8.6 and 5.5 years, respectively. Four control

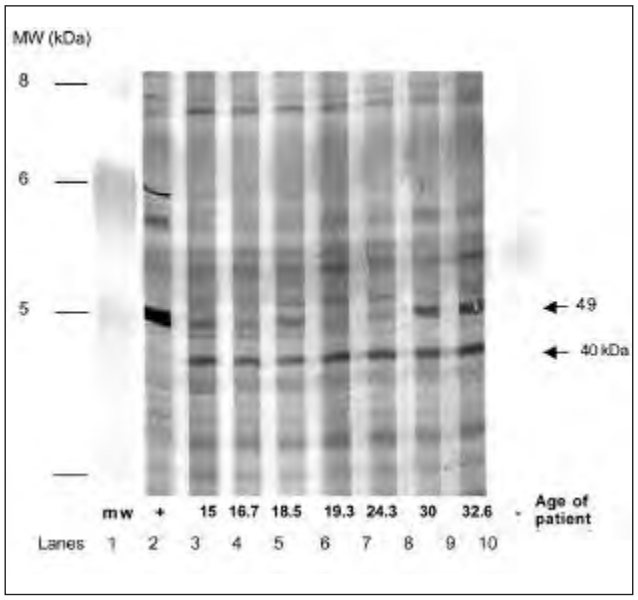

Figure 2

subjects had low titre antibodies directed against the 40 kDa cytosolic pituitary protein. No anti-45 kDa antibodies were found in control subjects.

Discussion

The detection of a range of pituitary autoantibodies in the sera of many APECED patients indicates that the pituitary can be part of the multi-organ involvement in this disease. The most remarkable feature of this study is the length of follow-up of the cohort over 26 years by one clinician. This has enabled us to demonstrate seroconversion in consecutive samples and the surprising stability of positive pituitary autoantibody status once achieved. Circulating organ-specific antibodies in APECED are highly predictive of adrenal and ovarian failure, but not of insulin dependent diabetes nor hypothyroidism (2). Although pituitary reactivity was relatively common in our series, hypopituitarism was very uncommon. The implication is that these autoantibodies are rarely pathogenic and may be epiphenomena. Immunoblotting is known to detect multiple bands of autoantibody reactivity, but some may be low titre "natural antibodies" and others "true" autoreactivity. We have characterized the 49 kDa cytosolic protein (discussed below) but

until the other relevant target autoantigens are further identified, it would be speculative to comment on their significance.

The predominant immunoreactivity was against a 49 kDa cytosolic protein. We have reported the association of 49 kDa pituitary cytosolic autoantibodies in 70% of cases of biopsy proven lymphocytic hypophysitis, to a lesser extent in other endocrinopathies (17), and in 28% patients with idiopathic hypopituitarism and 28% of their relatives (19). We have since identified the 49 kDa protein as enolase (20), a ubiquitous glycolytic enzyme that is highly conserved in evolution, with yeast enolase sharing 65% homology with human enolase (21). In the human, enolase exists as a homodimer or heterodimer of three isoforms, α-, β- and γ-enolase. We have recently shown by two-dimensional gel electrophoresis that autoimmune sera from patients with lymphocytic hypophysitis recognise both αα-and γγ-enolase in the human pituitary (22). γγ-enolase is neuron and neuroendocrine cell specific but shares 80% homology with the ubiquitous αα-enolase. It is likely that there is significant cross reactivity in patient sera between these forms and also potentially candidal enolase.

A number of the APECED patients in the current study were also involved in a research study looking at their antibody responses to *Candida albicans* infections, which are almost universal in this syndrome. Patient sera were used to immunoscreen a candidal cDNA expression library (18). Four cDNA clones were identified and found to be enolase, heat shock protein 90, pyruvate kinase and alcohol dehydrogenase. The reactivity to these antigens was studied by immunoprecipitation assays with in vitro-transcribed and translated proteins. Several cDNA clones encoding enolase were expressed in *E. coli* and studied by immunoblotting, with 84% of sera reacting with the recombinant candidal enolase. These results indicate that candidal enolase is a major antigen in APECED patients. It is possible that reactivity to human pituitary enolase is an epiphenomenon related to mucosal candidiasis, yet not all patients with candida antibodies had pituitary antibodies and vice versa.

However, the intriguing question is whether this cross-reactivity could represent a possible mechanism for autoimmunity in APECED patients through molecular mimicry. Some evidence already exists that antibodies directed against Candida can cross-react with mammalian tissues (23). Patients with thyroid, ovarian and adrenal, tissue-autoantibodies, exhibited significantly higher levels of Candida antibodies (60%), compared with tissue antibody negative sera (7.5%) and sera from healthy controls (10%). When sera containing high levels of Candida antibodies were pre-absorbed with tissue antigens, a 10-15% reduction in antibody titres was noted. Similarly, pre-absorption of thyroid antibody-positive sera with *C. Albicans* caused a reduction in thyroid antibody levels (23). The current study may offer a further demonstration of immunological cross reactivity between Candida and human endocrine tissues. Ultimately, further studies could indicate a possible pathogenic role of *Candida Albicans* in the development of autoimmune diseases in the genetically susceptible individuals affected by APECED.

The detection of pituitary autoantibodies, in patients with APECED suggests that the pituitary may be part of the multiorgan involvement and/or it shares antigens or cross-reactive epitopes with other target tissues in this disease. The one well characterised autoantigen so far is neuron specific enolase but it is not organ-specific, even if it is a marker of neuroendocrine autoimmunity. A possible link with candidal enolase is intriguing. The clinical significance of these autoantibodies in APECED remains to be determined as hypopituitarism is uncommon. Although pituitary autoimmune disease could develop over time in autoantibody-positive patients, the 26 year follow-up in our study makes this quite unlikely.

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Identification of the 49-kDa Autoantigen Associated with Lymphocytic Hypophysitis as α-Enolase

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Lymphocytic hypophysitis is part of the spectrum of organ-specific autoimmune diseases, and although its histopathology is well documented, its pathogenesis is unclear. Serum autoantibodies directed against a 49-kDa cytosolic protein are detected by immunoblotting in 70% of patients with biopsy-proven lymphocytic hypophysitis. Here we report the purification and identification of this first target autoantigen in lymphocytic hypophysitis. The autoantigen has a molecular mass of 49 kDa, a cytosolic localization, and a ubiquitous tissue distribution. The 49-kDa protein was purified from monkey brain and human placental cytosol. Limited amino acid sequencing after proteolytic digestion of the human placental protein showed identity with α-enolase. The identification was confirmed using sera from patients with pituitary autoimmunity, which strongly reacted with recombinant human α-enolase and yeast enolase, but not with rabbit muscle β-enolase. This indicates that the immunoreactive epitopes are largely conserved from yeast to human, but are not present in β-enolase. α-Enolase autoantibodies are not specific to pituitary autoimmune disease and have been reported in other autoimmune diseases. However, this study is the first to indicate a role for α-enolase as an autoantigen in lymphocytic hypophysitis. (*J Clin Endocrinol Metab* 87: 752–757, 2002)

LYMPHOCYTIC HYPOPHYSITIS is the term used for autoimmune disease of the pituitary, and it is part of the spectrum of organ-specific autoimmune endocrinopathies (1). The disease is characterized by infiltration of the pituitary by immune cells (2, 3) and is often associated with hypopituitarism (4). Approximately 85% of patients are women (5, 6). Over half of the female cases in the literature presented in late pregnancy or within 18 months postpartum (7).

One of the hallmarks of autoimmunity is the presence of autoantibodies that target organ-specific proteins (called autoantigens). Traditional pituitary autoantibody assays using immunofluorescence (8) were problematic (9). Serum pituitary autoantibodies were demonstrated by immunofluorescence in only a few cases of hypophysitis (10, 11). The development of an immunoblotting assay (12) has now enabled the detection of a number of pituitary target autoantigens (1) and their characterization by mol wt. Immunoblotting has detected pituitary autoantibodies in lymphocytic hypophysitis (1), isolated ACTH deficiency (13), and other autoimmune endocrine disorders (14). However, the identity, function, and possible role of these autoantigens are unknown.

Serum autoantibodies directed against a 49-kDa pituitary cytosolic protein were detected in 70% of patients with biopsy-proven lymphocytic hypophysitis and in 55% of patients with the clinical picture of hypophysitis, compared with 9.8% of normal subjects (1). However, a significant percentage of patients with other autoimmune diseases also had antibodies to this protein (1). In addition, 28% of Swedish patients with idiopathic hypopituitarism and 28% of their relatives were found to have the same autoantibody reactivity compared with 6.8% of controls (15).

We have shown in previous studies that the 49-kDa cytosolic protein is conserved across species. It is present in pituitary tissue from monkeys, sheep, and rats and appears enriched in the mouse AtT20 corticotroph cell line (1). Most major target autoantigens have been shown to be conserved through evolution. In endocrine autoimmunity, they are often tissue-specific enzymes, such as 21-hydroxylase in Addison's disease (16) and thyroid peroxidase in Hashimoto's thyroiditis (17). An ideal situation for diagnostic purposes would be if the 49-kDa autoantigen were pituitary specific, enabling the development of a disease-specific pituitary autoantibody assay. However, this is not the case for the 49-kDa protein; it is distributed in all tissues tested with the exception of skeletal muscle (1).

The aim of this study was to purify and identify the 49-kDa cytosolic target autoantigen associated with lymphocytic hypophysitis as a starting point to determine why pituitary autoimmune disease may occur. Monkey brain and human placenta were chosen as tissue sources because of the tissue volume required and their ready availability.

Materials and Methods

Preparation of cytosolic proteins

Cynomologous monkey brain was obtained in accordance with animal ethics guidelines of the Commonwealth Serum Laboratories (Melbourne, Australia). Human placenta was collected soon after delivery with the informed consent of the donor in accordance with the guidelines of the human research ethics committees of the Hunter Area Health Service and University of Newcastle. Monkey brain and human placental tissues were homogenized in PBS, 250 mM sucrose, and a cocktail

Abbreviations: CM, Carboxymethyl; DEAE, diethylaminoethyl.

of protease inhibitors [Roche Molecular Biochemicals (Indianapolis, IN) Complete protease inhibitor tablets (two per 50 ml), 1 mM EDTA, 1 mM EGTA, and 1 mM fresh phenylmethylsulfonylfluoride] using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY)], followed by a Teflon-glass tissue grinder. The cytosolic fraction was obtained by centrifugation at 100,000 × *g* at 4 C for 30 min.

Purification of the 49-kDa target protein

A carboxymethyl (CM)-Sepharose column (XK-26, with 150 ml resin, Pharmacia Biotech, Uppsala, Sweden) was linked in tandem to a diethylaminoethyl (DEAE)-Sepharose column (XK-26, with 150 ml resin). Both columns were preequilibrated with buffer A (20 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.4). Monkey brain cytosol or human placental cytosol was loaded onto the tandem columns with buffer A at 5 ml/min. The columns were disconnected, and proteins bound to the DEAE column were eluted with a linear gradient of 0–400 mM NaCl (in buffer A) at a flow rate of 5 ml/min. Forty 5-ml fractions were collected. All fractions were assayed for the 49-kDa target protein by immunoblotting, and positive fractions were pooled for further purification. After this point, monkey brain and human placenta were processed slightly differently, as outlined below.

For the placental preparation the DEAE-Sepharose fraction was next applied to a Poros-Q column preequilibrated with buffer A, and the 49-kDa protein was found in the unbound fraction. This fraction was then loaded onto phenyl-Sepharose.

Partially purified fractions of the 49-kDa target protein from DEAE-Sepharose (for monkey brain) or from the Poros-Q column (for human placenta) were applied to a phenyl-Sepharose CL-4B column (5 ml; Pharmacia Biotech) in 20 mM Tris-HCl, pH 7.4, containing 2 M NaCl. The column was eluted in batches with 150, 100, 50, and 0 mM NaCl in buffer A. A final elution was performed with 1% Triton X-100 in buffer A to strip the column. All batch-eluted fractions were immunoscreened for the 49-kDa target protein by immunoblot.

The Triton-eluted fraction from monkey brain was concentrated by vacuum centrifugation to about 2 ml and applied to a Mono-Q column (XK5, 5-ml column, Pharmacia Biotech) preequilibrated with 10 mM phosphate buffer, pH 7.4. Bound proteins were eluted with a NaCl gradient from 0–200 mM at 1 ml/min. Forty 1.5-ml fractions were collected, and immunoreactive fractions were concentrated to 200 μl using a centrifugal filter concentrator with a 30-kDa molecular mass cut-off (Millipore Corp., Bedford, MA). A silver-stained gel (18) showed a single protein in Mono-Q fractions. Direct N-terminal amino acid sequencing of the purified target antigen was attempted on an automatic amino acid sequencer (model ABS-470A Protein Sequencer with an on-line model 120A PTH analyzer, PE Applied Biosystems, Foster City, CA).

Microsequencing

The 49-kDa protein was excised from a Coomassie-stained 15% polyacrylamide gel containing the human placental phenyl-Sepharose fractions (Fig. 3B, lane 7). The protein was digested with trypsin, and the peptides were extracted into trifluoroacetic acid and acetonitrile and separated on a HP1090 LH microbore HPLC apparatus (Hewlett-Packard Co., Palo Alto, CA) with a Poly LC C_8 1 × 100-mm column (Millipore Corp.) at 50 C. Microsequencing of the peptides was performed on the ABS-470A sequencer as described above.

Peptide maps

The proteins from partially purified fractions of monkey brain cytosol (Mono-Q fractions) and human placental cytosol (phenyl-Sepharose fractions) were separated by electrophoresis on 7.5–15% gradient SDS-polyacrylamide gels at 110 V for 16 h. The gels were Coomassie stained, and the bands corresponding to the immunoreactive 49-kDa protein were excised and subjected to in-gel partial proteolysis by sequencing grade endoproteinase Glu-C (Promega Corp., Madison, WI). The digests were loaded onto 20% polyacrylamide gels and run at 160 V for 2.5 h until the proteins had run into the resolving gels. The current was stopped for 20 min to allow further digestion of the protein and recommenced at 185 V for 15 h. The gel was then silver-stained.

Fractions were immunoscreened for the presence of the 49-kDa target protein with positive control patient sera (1:100 dilution in 1% BLOTTO) as previously described (12). Goat antihuman IgG conjugated to alkaline phosphatase (Silenus, Melbourne, Australia) was used as the second antibody (1:2500 in 1% BLOTTO), and detection was performed with 5-bromo-4-chloro-3-indolyl phosphate toluene salt and *p*-nitro blue tetrazolium chloride substrates (Bio-Rad Laboratories, Inc., Richmond, CA) (12).

Western blots of purified enolase preparations

Bakers' yeast enolase and rabbit skeletal muscle enolase were purchased from Sigma (St. Louis, MO). Histone-tagged recombinant human α-enolase (r-α-enolase) expressed from the ENO1 gene product in JM109 (*Escherichia coli*) cells, was partially purified on a histone-Ni^{2+} affinity column (19), resulting in a 46-kDa protein (Fig. 5A, lane 3). The purified enolase enzymes and human pituitary cytosol were run on SDS-PAGE, transferred to polyvinylidene difluoride, and probed with goat polyclonal antienolase antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The second antibody was rabbit antigoat IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The polyclonal antienolase antibodies are known to recognize α-, β-, and γ-enolase subunits. The different forms of enolase were also probed with normal control sera and sera from patients with lymphocytic hypophysitis known to have pituitary autoantibodies directed against the 49-kDa cytosolic protein.

Results

Purification of the 49-kDa protein from monkey brain cytosol

The main autoantigen detected by serum autoantibodies in patients with lymphocytic hypophysitis is a 49-kDa cytosolic protein that is not pituitary specific (1). Our initial aim was to purify it from monkey brain cytosol. The 49-kDa protein did not bind to CM-Sepharose (data not shown), but bound to DEAE-Sepharose (Fig. 1). The immunoreactive protein eluted from DEAE-Sepharose between 210 and 270 mM NaCl (Fig. 1A). Normal control serum did not react specifically with this protein (Fig. 1B). The corresponding Coomassie blue stain revealed a 49-kDa protein of comparatively low abundance that correlated with the immunoreactivity (Fig. 1C).

To further purify the antigen, the DEAE fractions were applied to hydrophobic interaction chromatography on phenyl-Sepharose. The immunoreactive 49-kDa target protein did not elute until the column was stripped with 1% Triton X-100 (results not shown). This fraction was applied to a Mono-Q column and was only detected in fractions that passed directly through (Fig. 2A). Silver staining of a 10% polyacrylamide gel run under the same conditions revealed that the target protein was purified to apparent homogeneity, but the total amount of protein in these fractions was very low (Fig. 2B). The concentrated 49-kDa protein sample was loaded directly onto the amino acid sequencer, but no sequence data were obtained, suggesting that it was N-terminally blocked.

Partial purification of the 49-kDa protein from human placental cytosol

In parallel experiments, the 49-kDa immunoreactive protein was partially purified from human placenta (Fig. 3). It did not bind to CM- or DEAE-Sepharose, or to Poros-Q columns (Fig. 3, lanes 3–5). Therefore, unlike the monkey brain protein, the human placental protein did not bind to DEAE-resin under these conditions. The unbound fraction

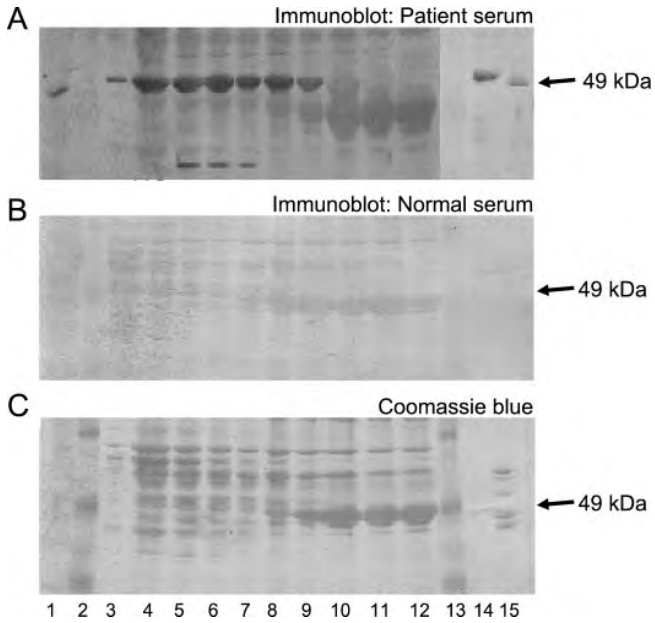

FIG. 1. Monkey brain cytosolic protein fractions eluted from DEAE-Sepharose. Monkey brain cytosol was passed through CM-Sepharose and bound to DEAE-Sepharose. Proteins were eluted from the DEAE column with an NaCl gradient, and 40 fractions were collected. Aliquots were run on gels and immunoblotted with patient serum positive for anti-49 kDa autoantibodies (A), immunoblotted with normal control serum (B), or stained with Coomassie blue total protein stain (C). The lanes contain total pituitary cytosol (lane 1), prestained mol wt markers (lane 2), column fractions 21–30 (lanes 3–12), mol wt markers (lane 13), monkey brain cytosol (lane 14), and the proteins that did not bind to either CM- or DEAE-Sepharose at pH 7.4 (15).

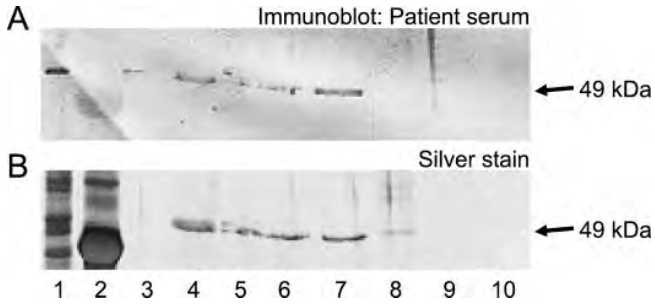

FIG. 2. Monkey brain cytosolic protein fractions eluted from Mono-Q. The phenyl-Sepharose fraction found to contain the partially purified 49-kDa protein from monkey brain cytosol was applied to a Mono-Q column. Aliquots of the eluted proteins from the Mono-Q column at the initial conditions were run on 10% SDS-PAGE and immunoblotted with patient serum positive for anti-49 kDa autoantibodies (A) or stained with silver stain (B). The lanes contain monkey brain cytosol proteins eluted from phenyl-Sepharose with 1% Triton X-100 (lane 1), mol wt markers (lane 2), and Mono-Q column fractions 1–8 eluted in the void volume (lanes 3–10).

was then applied to phenyl-Sepharose and bound well. The 49-kDa protein was eluted from phenyl-Sepharose with 500 mM NaCl (Fig. 3A, lane 6).

The 49-kDa proteins from monkey brain and human placenta are highly related

Despite the fact that the 49-kDa protein purified from monkey brain or human placenta both react with autoantibodies in

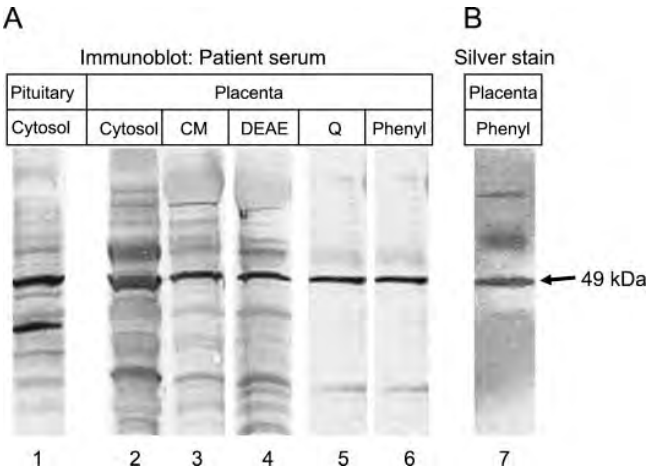

FIG. 3. Purification of the 49-kDa autoantigen from human placental cytosol. A, Immunoblot with patient serum of various fractions from the purification scheme (see *Materials and Methods*). The lanes contain human pituitary cytosol (lane 1), human placental cytosol (lane 2), or purification fractions from placental cytosol (lanes 3–7). The lanes with placental fractions contain samples that passed sequentially through CM-Sepharose (lane 3), through the DEAE column (lane 4), and through the Poros Q (lane 5). The Poros Q flow-through was finally eluted from phenyl-Sepharose with 500 mM NaCl (lane 6). B, Silver stain of the sample in A, lane 6 (lane 7). This sample was used for internal amino acid sequencing.

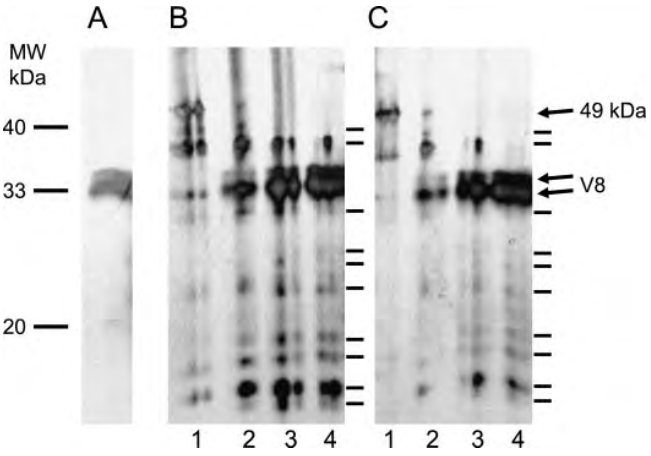

FIG. 4. Peptide maps of the 49-kDa target autoantigen digested with endoproteinase Glu-C (V8 protease). V8 protease (A) was used to digest the 49-kDa target protein from human placenta (B) or monkey brain (C). The protein in B and C were incubated with 2 ng (lane 1), 5 ng (lane 2), 10 ng (lane 3), or 20 ng (lane 4) V8 protease. A silver-stained gel is shown. Marks on the *right* indicate V8-derived peptides identical in B and C. Note that the gel did not run evenly on the *bottom right side* (C, lanes 3 and 4), producing a slight upward shift in some of the peptides. However, all samples were run on the same gel.

the same patient's serum, these proteins behaved differently on chromatography. It was important to determine whether they are indeed the same protein. Peptide mapping was used to illustrate the degree of homology between these proteins. Partial proteolysis with V8 protease (also called endoproteinase Glu-C) gave the same distinctive digestion patterns for the 49-kDa protein purified from monkey brain or human placenta (Fig. 4), indicating that they are related, if not identical.

Microsequencing of the 49-kDa placental protein

As the monkey brain and human placental 49-kDa proteins were shown to be similar in size, immunoblot, and peptide mapping, the placental protein was chosen for internal microsequencing. The 49-kDa band in the phenyl-Sepharose fractions enriched for the target protein was excised from a polyacrylamide gel (Fig. 3B, lane 7). Trypsin-digested fragments were separated by microbore HPLC, and five peptides were sequenced. Peptide 1 fully matched human α-enolase (residues 16–18, Table 1). Note that it did not match the closely related β- or γ-enolases, both of which differ by two amino acids in this region. Peptides 2–5 contained more than one sequence. To ensure an unbiased database search, the entire sequence data obtained for these peptides was used in a regular expression format to search the nonredundant protein database (using ProteinInfo version 2.0.1 at Proteometrics: www.proteometrics.com). Peptide 2 contained two sequences that matched 100% to human enolase (α-enolase 249–260 and α- or β-enolase 279–290; Table 1) and not to any other protein. Note that the former sequence also matched with a region of shared sequence identical in both α- and β-enolase, but which is not found in γ-enolase. This sequence also matched 100% to Myc promoter-binding protein-1, which is an alternative translation initiation product of the α-enolase gene ENO1 (20, 21). Peptides 3 and 4 each matched 4 of 6 amino acids to α-enolase or to a sequence common to all enolases, respectively. The sequence from peptide 5 matched 7 of 12 amino acids to sequences common to human α- and γ-enolase (positions 33–44 in α-enolase). Note that the sequences derived from peptides 1 and 3 are located in the N-terminal region of enolase and do not match Myc promoter-binding protein-1, which is a form of α-enolase that is truncated after Ile^{95}. This demonstrates that the 49-kDa target protein is α-enolase.

Patient serum reacts to enolase

Finally, we confirmed the identification of enolase and investigated which enolase isoforms were recognized by sera from patients with lymphocytic hypophysitis. Enolase was obtained from three sources: rabbit muscle β-enolase, recombinant human α-enolase, and bakers' yeast enolase (Fig. 5A). These proteins were probed with antibodies in human sera. Patient serum autoantibodies strongly recognized recombinant human α-enolase and weakly recognized yeast enolase (Fig. 5B). Normal control serum did not strongly recognize any of the three enolase sources or any 49-kDa protein in pituitary cytosol (Fig. 5C). An immunoblot with an antienolase polyclonal antibody that detects α-, β-, and γ-enolase isoforms confirmed the presence of enolase in all lanes (Fig. 5D). These data support the sequence data and suggest that patient sera recognize evolutionarily conserved sequences within α-enolase.

Discussion

This report is the first to identify a target autoantigen in pituitary autoimmune disease. We have identified the 49-kDa cytosolic autoantigen in lymphocytic hypophysitis as the ubiquitous glycolytic enzyme, α-enolase. Initially using monkey brain cytosol, an enriched source of the 49-kDa protein (1), we purified the 49-kDa autoantigen to homogeneity, but direct N-terminal amino acid sequencing failed. In parallel, the autoantigen was purified from human placenta, which expresses many proteins found in the brain and pituitary. It proved to be a particularly abundant source. The proteins from monkey brain or human placenta were shown to be highly related through digestion of the 49-kDa proteins with the same protease, producing an almost identical peptide map. The human placental form of the 49-kDa autoantigen was identified as α-enolase by amino acid microsequencing of two tryptic peptides.

Enolase is a ubiquitous enzyme whose amino acid sequence is highly conserved through evolution (22). Its main recognized role is to catalyze the conversion of phosphoglycerate to phosphoenolpyruvate in glycolysis (23), but it has other functions, which will be discussed below. Mammalian enolase exists as homodimeric or heterodimeric isoenzymes of a combination of three subunits: α-, β-, and γ-enolase. α-Enolase is expressed ubiquitously, β-enolase is predominantly expressed in muscle tissue, and γ-enolase is largely restricted to neuronal and neuroendocrine tissue (24).

TABLE 1. Amino acid sequence data for peptides derived from trypsin digestion of the 49 kDa human placental protein

Peptide no.	Sequence	Match (human database)	% Identity	Predicted M_r (kDa)
1	GNPTVEVDLFTSK	GNPTVEVDLFTSK α-enolase (residues 16–28)	100	46–49
2	[LVY][VGI][ESI][GHP][MDN][DQI][VLG]A[LDA][LEPS][YEG][FNK]	1) (R)-YISPDQLADLYK α-enolase (residues 279–290)	100	46–49
		2) (K)-VVIGMDVAASEF α- or β-enolase (residues 249–260)	100	46–49
3	[AS]GEHNI	(K)-AVEHII α-enolase (residues 70–75)	67	46–49
4	[VS][AL][TS][LN][PS][GNR]	(K)-KDATNVG α-, β-, or γ-enolase (212–217)	67	46–49
5	[AS]TV[YP][WF]L[AY]P[LT]LIY	(R)-AAVPSGASTGIY α- or γ-enolase (residues 33–44)	58	46–49

Note that peptide 2 contained three amino acid sequences, represented by the three amino acids (single letter code) enclosed in square brackets. Using regular expression searching of peptide 2 against the non-redundant database the sequence matched two different enolase tryptic peptides. Similarly, peptides 3–5 contained 2 sequences. All the residue numbers listed in brackets refer to the human α-enolase sequence.

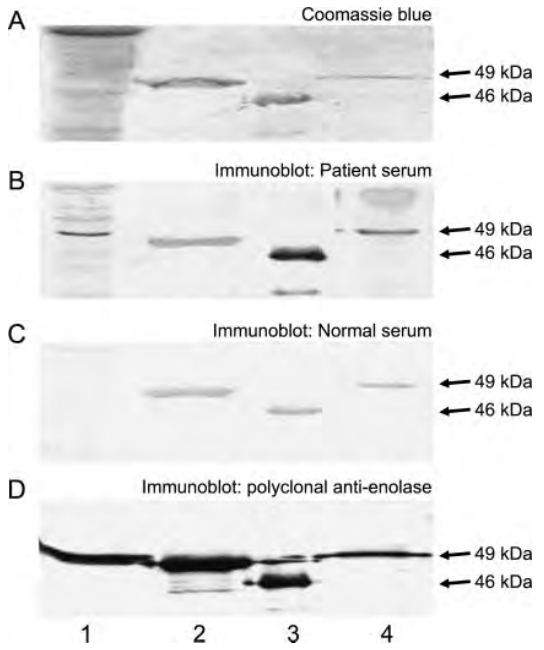

FIG. 5. Antibodies in patient sera recognize α-enolase. Enolase from three sources was run on SDS-PAGE and stained with Coomassie blue (A), or immunoblotted with patient sera (B), normal control sera (C), or a polyclonal antibody recognizing all forms of enolase (D). Human pituitary cytosol was used in lane 1, whereas the other lanes contained rabbit muscle β-enolase (lane 2), recombinant human α-enolase (lane 3), or bakers' yeast enolase (lane 4).

Each subunit consists of 434 amino acids, of which 336 (77%) are identical. As the 49-kDa protein from monkey brain behaved slightly differently on column chromatography compared with human placental α-enolase, this may suggest that the protein in brain could be neuron-specific enolase. Neuron-specific enolase is made up of two γ-enolase subunits (25) and is known to be abundant in brain (24).

Commercial enolase preparations were immunoblotted with sera from patients with lymphocytic hypophysitis to confirm antienolase antibody reactivity. Patient sera recognized recombinant α-enolase and yeast enolase, but not rabbit muscle β-enolase. This suggests that the epitope recognized by sera from patients with hypophysitis is not present in β-enolase. Other researchers reported similar findings. Serum from a patient with discoid lupus erythematosus recognized yeast enolase, but not rabbit muscle β-enolase (26). The exclusion of β-enolase as a target autoantigen is further supported by previous immunoblotting assays by our group showing that patient sera did not recognize the 49-kDa target autoantigen in skeletal muscle cytosolic preparations (1). In the current study we were unable to directly test γ-enolase, but subsequently we have shown by two-dimensional gel electrophoresis that sera from patients with peripartum hypophysitis recognize both α- and γ-enolase in pituitary tissue and placenta (27). Yeast enolase shares a high degree of homology with human enolase (22). As a result, antienolase antibodies in our study recognized evolutionarily conserved epitopes in yeast enolase.

Usually, organ-specific autoimmune diseases have tissue-specific enzymes as target autoantigens (28). Classic examples include thyroid peroxidase in thyroid autoimmunity (17) and 21-hydroxylase in Addison's adrenalitis (16). However, like enolase, other ubiquitous enzymes have been associated with organ-specific autoimmunity. The multimeric enzyme complex, dihydrolipoamide acetyl-transferase, is an autoantigen in primary biliary cirrhosis (29).

Patient serum reactivity to α-enolase is not specific for lymphocytic hypophysitis. The ever-growing list of organ-specific and nonorgan-specific autoimmune conditions associated with autoantibodies to α-enolase includes systemic rheumatic diseases (30), anti-neutrophil cytoplasmic antibody-positive vasculitis and systemic lupus erythematosus with renal disease (31), endometriosis (32), cancer-associated retinopathy (33), discoid lupus erythematosus (26), mixed cryoglobulinemia or arthritis with kidney involvement (34), primary biliary cirrhosis and autoimmune hepatitis (35), primary sclerosing cholangitis (36), inflammatory bowel disease (37), primary membranous nephropathy (38), and, finally, systemic sclerosis and rheumatoid arthritis (39). We have demonstrated anti-49-kDa autoantibodies, which we now recognize as enolase, in patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (40). The presence of anti-α-enolase antibodies in lymphocytic hypophysitis is therefore consistent with the findings of other studies of autoimmunity, but renders their detection nonspecific. There has been no explanation for why α-enolase is an autoantigen in so many autoimmune diseases. It may relate to its other properties. In addition to its role in glycolysis, α-enolase functions as a plasminogen receptor (19, 41), as a controller of cell growth and differentiation by down-regulation of c-*myc* protooncogene expression (21), as a structural protein in the lens of some species (42), and possibly as a suppressive lymphokine (43).

The plasminogen-binding property of streptococci is implicated in the bacteria's tissue invasion processes. Studies of pathogenic streptococcal bacteria have shown expression of α-enolase on the bacteria surface, displaying strong plasminogen binding activity (44). Streptococcal antibodies that cross-react with human α-enolase could be part of the molecular mimicry that contributes to acute rheumatic fever and other autoimmunity related to streptococcal infection (45). In lymphocytic hypophysitis, some patients have presented with a clinical picture of meningoencephalitis before the development of a pituitary mass (4). It is not unreasonable to suggest enolase as the link between the infective trigger and the development of autoimmunity.

Although studies have shown that some normal control subjects have circulating tissue-specific autoantibodies [*e.g.* against thyroid peroxidase (46)], they usually target different epitopes on the autoantigen compared with patient sera. In one study of cancer-associated retinopathy, one epitope of α-enolase recognized by patient sera caused more *in vitro* cytotoxicity and apoptosis of retinal cells than the sera of control subjects (47). Similarly, epitope mapping could determine specific regions of the enzyme that lead to pathogenic antienolase reactivity against the pituitary in lymphocytic hypophysitis patients.

In summary, this study identifies the 49-kDa autoantigen in lymphocytic hypophysitis as α-enolase. Although not disease specific, it is now clear that these autoantibodies are

common markers of autoimmune disease. They may be helpful in the diagnosis of patients with a clinical picture of lymphocytic hypophysitis who would otherwise not need pituitary biopsy.

Acknowledgments

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Pituitary Autoantibodies in Lymphocytic Hypophysitis Target Both γ- and α-Enolase – A Link with Pregnancy?

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Abstract

The first target autoantigen to have been identified in lymphocytic hypophysitis is a 49 kDa protein, identified as alpha-enolase. Pituitary autoimmunity is strongly associated with pregnancy and we have shown that pituitary autoantibodies from patients with peripartum lymphocytic hypophysitis also recognise enolase in the placenta. Enolase exists in different forms as a number of isoenzymes, which are homo- or heterodimers of three subunits, α, β and γ. αα-enolase is ubiquitous, ββ-enolase is muscle-specific and γγ-enolase, which is restricted to neuronal tissue and neuroendocrine cells, is known as neuron-specific enolase (NSE). NSE is expressed in normal human pituitary and pituitary neoplasms. The current study investigated which isoforms of enolase in pituitary and placenta reacted with the sera of patients with lymphocytic hypophysitis. Immunoblotting of two-dimensional gels of human pituitary cytosolic proteins showed that autoantibodies in patient sera react with both an acidic form, and more neutral forms of enolase. Immunoblotting with a monoclonal antibody to NSE confirmed the identity of the acidic enolase isoform as the γγ-isoform in both pituitary and placental samples. Gamma-enolase, i.e. NSE, was detected by immunohistochemistry in term placenta in decidua, syncytiotrophoblasts, anchoring villi and terminal villi. Our study is the first to describe the cellular localisation of NSE in normal human placenta, thus establishing a direct link between pituitary and placental autoantigens. This link provides a theoretical basis for the strong prediliction of lymphocytic hypophysitis to occur during or after pregnancy.

Keywords: Pituitary autoimmunity, pituitary autoantibodies, enolase, neuron-specific enolase, lymphocytic hypophysitis, immunohistochemistry.

Introduction

Lymphocytic hypophysitis is a pituitary autoimmune disease that often presents in the peripartum period. Women constitute approximately 85% of cases (Beressi et al., 1999) and over half the female cases are pregnancy-related (Hashimoto et al., 1997 and Crock, 1997). We hypothesised that one cause of this relationship with pregnancy could be the sharing of target autoantigens between the pituitary and placenta. Up to 70% of patients with biopsy-proven lymphocytic hypophysitis have autoantibodies to a 49 kDa pituitary cytosolic protein (Crock, 1998). We have recently isolated this same immunoreactive protein from placenta and identified it as αα-enolase by amino acid sequencing (O'Dwyer et al., 2001).

Enolase (2-phospho-D-glyceratehydrolase; EC 4.2.1.11) is a dimeric glycolytic enzyme encoded by three genes: alpha (α), beta (β), and gamma (γ) enolase. These give rise to three subunits which combine to form five homodimeric or heterodimeric isoenzymes. αα-Enolase is ubiquitous and is known as nonneuronal enolase (NNE), ββ-enolase is muscle-specific enolase (MSE), and γγ-enolase is called neuron-specific enolase (NSE) (Marangos et al., 1987). The γγ-enolase-isoenzyme has been detected in neuronal and neuroendocrine cells as well as neuroendocrine tumours (Cunningham et al., 1992). Two heterodimeric enolase isoenzymes have also been described, αγ and αβ (Royds, 1982).

The pituitary has been shown to contain NSE by immunohistochemistry (Asa et al., 1984). We have previously reported the 49 kDa autoantigen in monkey pituitary, brain, thyroid and adrenal (Crock, 1998), all of which express NSE or γγ-enolase (and the ubiquitous αα-enolase) and in lung, liver and spleen (Crock, 1998), which only express αα-

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enolase (Pancholi, 2001). Thus autoantibodies against the 49 kDa-protein could be recognising either or both of these enolase isoforms. The relationship between pregnancy and the development of hypophysitis suggests that the placenta may express a key autoantigen also present in the pituitary. We sought to identity the enolase isoforms in the pituitary recognised by the sera of patients with hypophysitis and to determine whether similar forms were expressed and recognised in the placenta.

Materials and methods

Cytosolic proteins were prepared from human pituitary and placenta as previously described (O'Dwyer et al. 2001; Crock et al. 1993) and in accordance with guidelines from the Human Research Ethics Committees of the Hunter Area Health Service and the University of Newcastle.

Two-dimensional gel electrophoresis of placental and pituitary cytosol extracts

Human pituitary and placental cytosol preparations were enriched for proteins between 30-kDa and 100-kDa by centrifugal ultrafiltration (Millipore, Bedford, MA USA). Samples were acetone precipitated to desalt and then re-dissolved in the same rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG Buffer (Amersham-Pharmacia-Biotech, Uppsala, Sweden), 40 mM Tris, 0.3% dithiothreitol (DTT), bromophenol blue) used to equilibrate the Immobilin IPG Drystrip™ (Amersham-Pharmacia-Biotech, Uppsala, Sweden). The samples were then subjected to isoelectric focusing (IEF).

IEF was run on 11-cm IPG strips, pH 3–10 (Pharmacia Multiphor apparatus). Following IEF, the IPG strips were pre-equilibrated in the SDS-PAGE sample buffer, laid on a 10% polyacrylamide gel, and overlayed with 1% agarose. Second-dimension gels were run at 8 mA for 16 h. The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels were used in immunoblotting experiments and were pre-stained with zinc to check the quality of the resolution before electro-transfer to nitrocellulose.

Immunoblotting of 2D-gels to identify enolase subunits in human placenta and pituitary

2D-PAGE from human pituitary and placental cytosol preparations were incubated with the following antibodies diluted in 1% BLOTTO (Skim milk powder in PBS): (A) polyclonal goat anti-enolase antibody 1 : 2500 (Santa Cruz Biotechnology Inc, CA, USA), that recognises all enolase isoenzymes, (B) monoclonal mouse anti-human NSE antibody 1 : 5000 (NSE-1G4 clone, Zymed Laboratories, San Francisco, CA, USA), that recognises only the γ-enolase subunit, and (C) sera from patients with peri-partum lymphocytic hypophysitis with high titre autoantibodies to the 49 kDa pituitary autoantigen we now know as αα-enolase. Primary antibody incubations were for 2.5 hours at 25°C, after which blots were washed with three changes of 0.05% Tween 20 in phosphate buffered saline (PBS) and three changes of PBS.

The blots were then incubated for 1.5 h at 25°C with the following secondary antibodies, diluted 1 : 2500 in 1% BLOTTO: (A) rabbit anti-goat IgG-conjugated to alkaline phosphatase (AP) (Southern Biotechnology Associates Inc. Birmingham, Alabama, USA); (B) AP-conjugated rabbit anti-mouse IgG antibodies (Silenus, Melbourne, Australia); and (C) AP-conjugated sheep anti-human IgG (Silenus, Melbourne, Australia), respectively. The blots were washed as above, and immunoreactivity detected with BCIP (5-bromo-4-chloro-3-indolyl-phosphate toluene salt) and NBT (p-nitro blue tetrazolium chloride) substrates (Bio-Rad, Richmond, CA, USA).

Immunohistochemical localisation of NSE in the human placenta

Tissues were formalin fixed and paraffin embedded. Sections of placenta were mounted on slides coated with 3-amino-propyltriethoxy-silane (AAS) (Sigma Chemical Co. St Louis, MO, USA). The anti-γ-enolase monoclonal antibody (Zymed) and the polyclonal goat anti-enolase antibody (Santa Cruz) were used in conjunction with avidin-biotin-peroxidase reagents (Universal LSAB+, DAKO. USA). Briefly, the sections were deparaffinised and endogenous peroxidase activity was quenched with hydrogen peroxide (1% in PBS) for 10 minutes. Non-specific binding was reduced by blocking sections with 20% normal horse serum in a moist chamber, at 4°C. The sections were then incubated overnight with the primary antibody or the appropriate substitute control. All antibodies were diluted in 1% bovine serum albumin (BSA) in PBS buffer. Following incubation, sections were washed with PBS (10 min) and incubated for 2 h at room temperature with a biotinylated anti-goat secondary antibody, washed for 10 min in PBS and then incubated with avidin-biotin peroxidase complex solution for 2 h at room temperature. Specific immunostaining was visualised using diaminobenzidine (Sigma Immunochemicals, DAB peroxidase substrate tablet set). The sections were counterstained with Carazzi's haemotoxylin (0.5 g haemotoxylin, 100 mL glycerol, 25 g aluminium potassium sulfate, 0.1 g potassium iodate and 400 mL distilled water) for 3 min and washed in running tap water for 5 min. Sections were then dehydrated in ethanol and mounted using DePex (BDH Chemicals).

Negative controls were conducted in which placental sections were incubated with non-immune goat serum or antibody dilution buffer alone or antibody pre-absorbed with the homogenised rat brain. Positive controls consisted of rat brain that were stained for enolase and γ-enolase. All experimental sections were stained simultaneously to allow direct comparison between samples. All sections were examined by light microscopy and qualitatively assessed. Sections were examined by at least two individuals with ten sites examined

on each section. A positive result was recognised when at least 80% of sites examined contained positive, preabsorbable staining.

Results

Immunoblotting of 2D-gels identifies enolase subunits in human placenta and pituitary

The polyclonal anti-enolase antibody detected two different enolase isoforms on 2D-PAGE from human pituitary and human placental cytosol, a single protein at acidic pH 4.9 but a number of proteins around pH 6.5 (Fig. 1A and 1D). The most acidic enolase isoform was identified as γ-enolase by monoclonal anti-NSE antibodies (Fig. 1B and 1E). The isoelectric point (pI) of γ-enolase estimated from the 2D-array as 4.9 is identical to the theoretical pI of 4.94 (using ExPASy, Compute pI/Mw tool). A number of immunoreactive enolase-subunits were detected around pI = 6.5, which is close to the theoretical pI for α-enolase of 6.99. α-enolase has a number of potential phosphorylation sites (Cooper et al., 1984), and it is assumed that the subunits detected in this region represent different phosphorylation states.

Patient sera react to both α- and γ-enolase subunits in human placenta and pituitary

The 2D-PAGE immunoblot using patient serum showed reactivity directed against both the γ- and α-enolase subunits in the pituitary. The corresponding Coomassie blue-stained gel

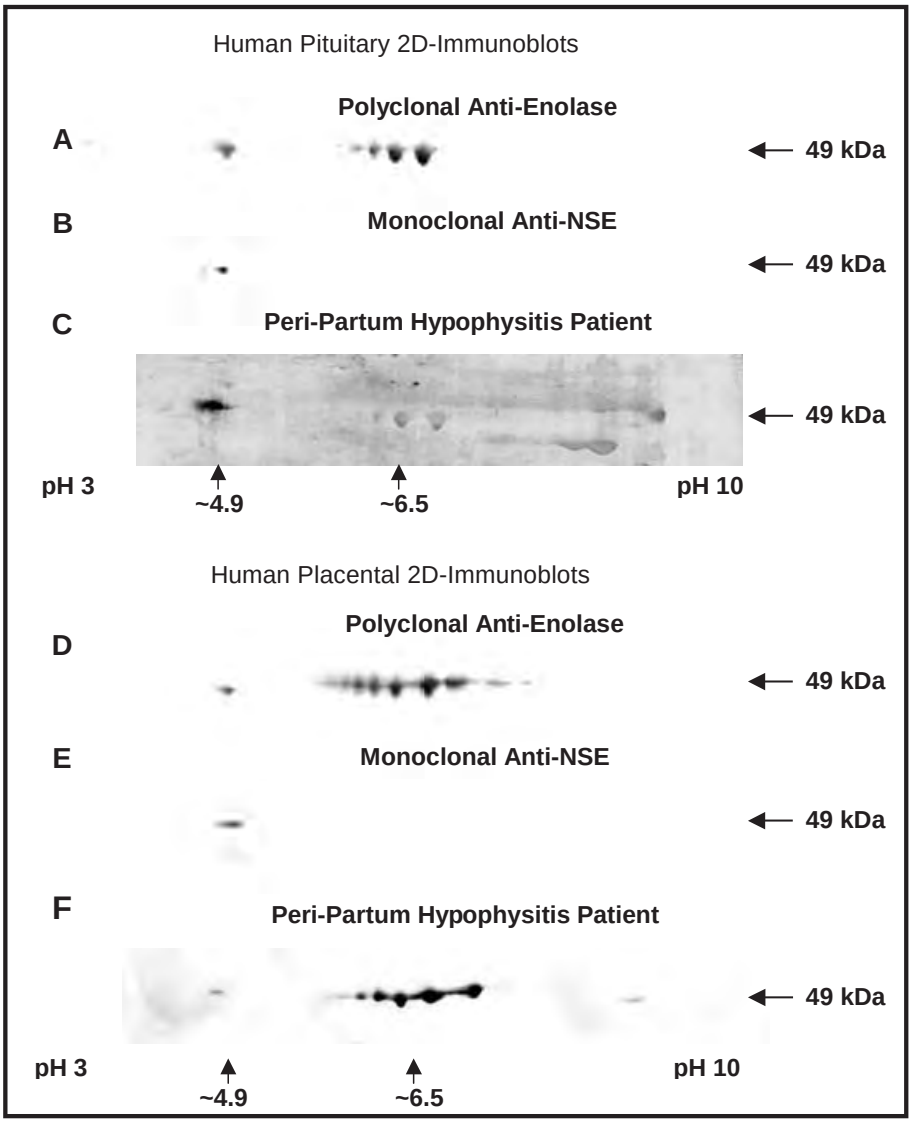

Fig. 1. Two-dimensional western blots of cytosolic proteins from human pituitary and placenta.
2D arrays of pituitary (panels A to C) and placental (panels D to F) cytosolic proteins were transferred to nitrocellulose and immunoblotted. Panels A and D were probed with polyclonal goat anti-enolase antibody; B and E with monoclonal mouse anti-enolase antibody and; C and F probed with positive control patient serum. Secondary antibodies were conjugated to an alkaline phosphatase detection system.

(not shown) showed that the acidic enolase species (ie the γ-subunit) was relatively less abundant than the more basic enolase species (α-subunit). Patient serum appeared to preferentially react with γ-enolase in human pituitary. A different result was found for the placental preparation. Although the placenta contained the same enolase subunits as the pituitary (Fig. 2, panels A and D) and the acidic isoform (at pI 4.9) was identified as such by the anti-γγ-enolase monoclonal antibody (panel E), patient sera only reacted weakly to the γ-enolase subunit on the placental 2D-PAGE (panel F).

Immunohistochemical localisation of NSE in the human placenta

Immunoreactive (IR) enolase and NSE were detected in the control tissue, rat brain. Preabsorption of the sections with the polyclonal anti-pan-enolase and the monoclonal anti-NSE antibodies resulted in a significant reduction in specific staining for enolase and NSE in the rat brain sections. This method was used to detect the enolase isoforms in term placentae with an anti-enolase polyclonal antibody and an anti-NSE monoclonal antibody. The immunoreactivity demonstrated by the anti-enolase polyclonal antibody reinforced the ubiquitous expression of enolase (mainly due to α-enolase reactivity). Using the monoclonal antibody to γ-enolase, NSE reactivity was localised to the decidua, vascular smooth muscle and vessels of anchoring and terminal villi, and especially to the syncytiotrophoblast cells. Pre-absorption of the monoclonal antibody to NSE on rat brain prior to application to the placental section resulted in a significant reduction in specific staining for NSE in the placentae (Fig. 2).

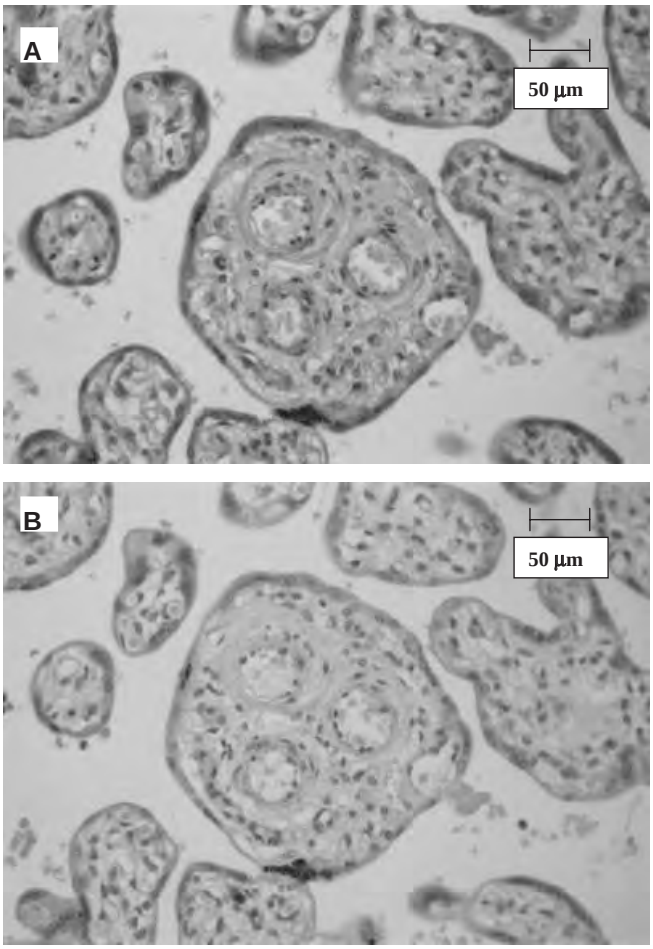

Fig. 2. Immunohistochemical localisation of γ-enolase (NSE) in human term placenta, 400 × magnification. Anti-NSE monoclonal antibody localised to syncytiotrophoblast cells (A), Anti-NSE antibody preabsorbed on rat brain prior to staining (B).

Discussion

The striking clinical association of lymphocytic hypophysitis and pregnancy was noted even in the first case report by Goudie and Pinkerton in 1962 (Goudie & Pinkerton,1962). They hypothesised that 'the changes (of Hashimoto's thyroiditis and anterior hypophysitis) are auto-immune reactions to the pituitary and thyroid antigens released during the puerperal involution of these glands'. Our aim was to explain this link in light of the current concept of shared tissue autoantigens and molecular mimicry in autoimmunity. We have identified one of the target autoantigens in lymphocytic hypophysitis as enolase, a glycolytic enzyme with a number of isoforms. The isoform we purified from placenta was αα-enolase, the ubiquitous form (O'Dwyer et al., 2001). However, patient autoantibodies may be recognising other isoforms.

Two-dimensional PAGE of pituitary cytosolic proteins enabled us to identify γ-enolase as the main target antigen of autoantibodies in the sera of patients with peripartum lymphocytic hypophysitis. The sera also recognised the α-subunit but the reaction was weaker. This is intriguing, as α- and γ-subunits share 85% sequence homology. The γ-subunit, which dimerises as γγ- or neuron specific enolase (NSE), is a cell-type-specific subunit and a marker of neuroendocrine tissue. This may explain the relatively high incidence of pituitary autoantibodies to the 49 kDa protein (we now know as enolase) reported by our group in Hashimoto's thyroiditis (19%) and Addison's disease (43%) (Crock, 1998). We were interested to know if γγ-enolase had been described in the human placenta, and to our knowledge it had not.

Probing two-dimensional PAGE of placental cytosolic proteins with polyclonal anti-enolase antibodies, we were able to identify α-enolase subunits as expected. A monoclonal antibody to γ-enolase identified this isoform clearly, although the corresponding Coomassie gel showed that this protein was far less abundant. Interestingly, the same patient serum which had bound strongly to γ-enolase in the pituitary 2D gels, now reacted more weakly to the placental protein. It is probable that the patient autoantibodies interact differently with the conformational epitopes in the placenta.

We confirmed our findings by immunohistochemistry. The current report is the first to describe the presence of γ-enolase

in placenta. It was localised to decidua, the vascular smooth muscle of anchoring and terminal villi and syncytiotrophoblast cells. Placental syncytiotrophoblasts are known to produce the peptide-hormones corticotrophin releasing hormone (CRH) and adrenocorticotrophin (ACTH), so the detection of γ-enolase in these cells is consistent with their neuroendocrine role. This finding highlights the concept of the 'diffuse' endocrine system (Carlei & Polak, 1984) in contrast to organ-specific autoimmune endocrinopathies with specific target enzymes such as thyroid peroxidase in Hashimoto's disease (Czarnocka et al., 1985) and 21-hydroxylase in Addison's disease (Winqvist et al., 1992). It has been suggested that one of the target autoantigens in autoimmune pituitary disease could be an enzyme involved in the production, or processing of pro-opiomelanocortin (POMC-derived) peptides (Sauter et al., 1990; Thodou et al., 1995). By definition such an enzyme would be in amine precursor uptake and decarboxylation (APUD) cells. The identification of γγ-enolase (present in APUD cells) as a major autoantigen in lymphocytic hypophysitis could provide an explanation for why hypophysitis is often seen in association with adrenalitis and/or thyroiditis (Beressi et al., 1999).

We have demonstrated that γγ-enolase, is a target autoantigen shared by the pituitary and placenta and that it is recognised by sera from patients with peripartum lymphocytic hypophysitis. Placental γγ-enolase was localised in syncytiotrophoblasts, which are the multi-nucleated cells on the maternal side of the placenta, and therefore exposed to the maternal circulation. It is conceivable that peri-partum lymphocytic hypophysitis may develop if the maternal immune system develops autoantibodies against placental γγ-enolase, which may then react against γγ-enolase containing cells in the pituitary. Alternatively, there may be cross-reactivity between αα- and γγ-enolase epitopes as they are 85% homologous and antibodies initially elicited against αα-enolase could then react with γγ-enolase by the phenomenon of 'epitope spreading'. The immunological milieu of pregnancy is unique and we have now shown a link between the placenta and pituitary, which could explain the association between lymphocytic hypophysitis and pregnancy.

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Pituitary autoantibodies in autoimmune polyendocrine syndrome type 1

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Contributed by Tomas Hökfelt, November 14, 2006 (sent for review October 5, 2005)

Autoimmune polyendocrine syndrome type 1 (APS1) is a rare autosomal recessive disorder caused by mutations in the autoimmune regulator (*AIRE*) gene. High titer autoantibodies (Aabs) toward intracellular enzymes are a hallmark for APS1 and serve as diagnostic markers and predictors for disease manifestations. In this study, we aimed to identify pituitary autoantigens in patients with APS1. A pituitary cDNA expression library was screened with APS1 sera and a tudor domain containing protein 6 (TDRD6) cDNA clone was isolated. Positive immunoreactivity against *in vitro* translated TDRD6 fragments was shown in 42/86 (49%) APS1 patients but not in patients with other autoimmune diseases or in healthy controls. By using immunohistochemistry, sera from 3/6 APS1 patients with growth hormone (GH) deficiency showed immunostaining of a small number of guinea pig anterior pituitary cells, and 40–50% of these cells were GH-positive. No such immunostaining was seen with sera from healthy controls. The APS1 Aab-positive, GH-negative cells may represent a novel subpopulation of anterior pituitary cells. In addition, 4/6 patient sera showed staining of a fiber-plexus in the pituitary intermediate lobe recognizing enzymes of monoamine and GABA synthesis. Thus, we have identified TDRD6 as a major autoantigen in APS1 patients and shown that several sera from GH-deficient patients stain specific cell populations and nerves in the pituitary gland.

autoantigens | growth hormone deficiency | pituitary intermediate lobe | tudor domain containing protein 6

Autoimmune polyendocrine syndrome type 1 (APS1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (OMIM 240300), is a rare autosomal recessive disorder caused by mutations in the autoimmune regulator (*AIRE*) gene encoded on chromosome 21q22.3 (1, 2). The autoimmune regulator is an important mediator of central tolerance by promoting the expression of organ-specific antigens in the thymus (3–5).

The classical triad of APS1 include hypoparathyroidism, adrenal failure and chronic mucocutaneous candidiasis (6). In addition, other organ-specific autoimmune diseases such as gonadal failure, intestinal dysfunction, type 1 diabetes mellitus, hypothyroidism and pituitary insufficiency as well as ectodermal manifestations are found with variable penetrance.

High titer autoantibodies (Aabs) against intracellular key enzymes, primarily involved in the synthesis of steroids or neurotransmittors, is a hallmark of APS1 (7–10). These Aabs constitute important diagnostic markers and are sometimes predictive of disease manifestations (11, 12).

Hypopituitarism is a rare manifestation of APS1. Isolated growth hormone (GH)-deficiency has to the best of our knowledge only been reported in 10 APS1 patients (6, 13–17), isolated hypogonadotroph hypogonadism in one patient (13) and central diabetes insipidus in three (18–20). Three siblings with APS1 and partial adrenocorticotropic hormone deficiency have been described (21). One APS1 patient with multiple pituitary hormonal insufficiencies has also been reported (22).

Pituitary Aabs against prolactin secreting cells have been detected by conventional immunofluorescence in sera from a few APS1 patients (6, 23). Immunoreactivity against median eminence dopaminergic nerve terminals and pituitary gonadotrops have been shown in an APS1 patient with GH deficiency (24). By using an immunoblotting method with pituitary homogenate, Aabs to a 49-kDa antigen was detected in 39/67 (58%) APS1 patients (25). The 49-kDa pituitary protein has been identified as α-enolase (26), but the importance of enolase Aabs as markers for neuroendocrine autoimmunity has been debated (27). Moreover, no correlations between pituitary Aabs and pituitary dysfunction in APS1 have been described.

In this study, we aimed to identify novel pituitary autoantigens in patients with APS1 by immunohistochemistry and immunoscreening of a human pituitary cDNA expression library.

Results

Identification of Tudor Domain Containing Protein 6 (TDRD6) as an Autoantigen. A pituitary cDNA expression library was constructed and screened with sera from two APS1 patients. Twenty-seven cDNA clones were isolated and partially sequenced. Four of the clones encoded tryptophan hydroxylase (TPH) isoform 1, a well known APS1 autoantigen (9). *In vitro* transcription and translation (ITT) of other cDNA clones resulted in 10 recombinant proteins that were used for immunoprecipitation with a test panel of sera from six APS1 patients and five healthy blood donors. Most of these recombinant products were recognized solely by the screening serum, by both APS1 sera and control sera or by none of the sera. A protein encoded by a tudor domain containing protein 6 (TDRD6) cDNA clone was, however, efficiently immunoprecipitated by two of the

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The authors declare no conflict of interest.

Abbreviations: Aab, autoantibody; AADC, aromatic-L-amino acid decarboxylase; APS1, autoimmune polyendocrine syndrome type 1; GAD, glutamic acid decarboxylase; GH, growth hormone; ITT, *in vitro* transcription and translation; TDRD6, tudor domain containing protein 6; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

Data deposition: The TDRD6 mRNA reference sequence reported in this paper has been deposited in the GenBank database (accession no. EF 185284).

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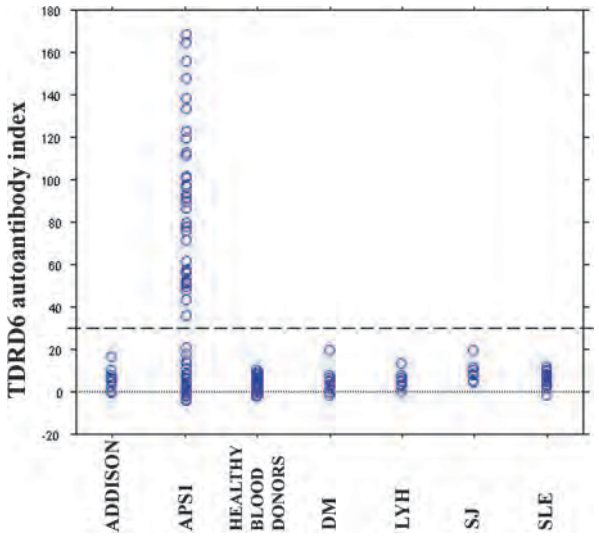

Fig. 1. Scattergram showing the immunoreactivity against TDRD6 fragment in sera from patients with APS1 ($n = 86$), lymphocytic hypophysitis (LyH) ($n = 11$), Addison's disease ($n = 17$), type 1 diabetes mellitus (DM) ($n = 20$), Sjögren's syndrome (SJ) ($n = 20$), systemic lupus erythematosus (SLE) ($n = 25$), and healthy blood donors ($n = 90$). The broken line indicates a cut-off value (30) of positive results.

APS1 sera but not by any of the healthy blood donor sera and was therefore selected for further studies.

The *TDRD6* gene which is located on chromosome 6p12.3 consists of four exons and spans over a region of 14 kb. The TDRD6 mRNA reference sequence reported in GenBank (accession no NM_001010870.1) is 6.8 kb long and encodes a 2,096-aa protein. Several different TDRD6 cDNA sequences with different putative transcriptional start sites and alternative splicing have been reported. The TDRD6 cDNA clone identified in this study spans from the middle of exon 1 to intron 3 and retains intron 1. Because of a termination codon, 6 bp into intron 1, the protein is truncated, and this cDNA is predicted to encode a 925-aa protein. Details on TDRD6 cDNA clones are in supporting information (SI) Fig. 5.

Immunoprecipitation of *in Vitro* Translated TDRD6 Fragment with Patient Sera. To determine whether immunoreactivity against TDRD6 was APS1 related, sera obtained from 86 APS1 patients, 93 patients with other autoimmune diseases and 90 healthy blood donors were tested for immunoreactivity against TDRD6. Forty-two of the 86 (49%) APS1 patients showed positive immunoreactivity against the *in vitro* translated TDRD6 fragment. No TDRD6 Aabs were found in sera from patients with isolated lymphocytic hypophysitis, Addison's disease, type 1 diabetes mellitus, Sjögren's syndrome, systemic lupus erythematosus, or healthy blood donors (Fig. 1). We found no associations between immunoreactivity against TDRD6 and clinical manifestations of APS1 (Table 1). Two of six APS1 patients with GH-deficiency showed positive immunoreactivity against TDRD6, but the limited number did not allow statistical interpretation.

Immunohistochemical Findings. Three of the six (50%) APS1 patients with GH-deficiency (Table 2, patients 1, 3, and 6) showed immunoreactivity against a small cell population in the guinea pig anterior pituitary lobe. We selected the patient serum that showed the strongest immunoreactivity (Table 2, patient 3) and performed double-staining for the following pituitary hormones: follicle stimulating hormone, luteinizing hormone, thyroid stimulating hormone, prolactin, adrenocorticotropic hormone or GH. No coexistence was found (Fig. 2 *A–E*), the GH antiserum being an exception (Fig. 2 *F* and *G*). Thus, 40–50% of the APS1-positive cells were GH-positive with serum no. 3. Conversely, <1% of the GH cells stained with the APS1 serum. Subsequently, about the same percentage were observed for patients 1 and 6. The APS1 immunofluorescence pattern showed aggregates of staining, distinctly different from the small, singular granules containing GH, suggesting that the targeted autoantigen(s) is not colocalized or cosecreted with GH (Fig. 2*G*). None of the ten sera from healthy control subjects showed any staining.

The APS1 sera were preadsorbed with TDRD6 or enzymes (glutamic acid decarboxylase, GAD; aromatic-L-amino acid decarboxylase, AADC; tyrosine hydroxylase, TH; or TPH). Preadsorption with AADC, but with none of the other proteins, abolished the staining in one of three APS1 patients (Table 2, patient 1) (compare Fig. 3 *B* with *A*).

In the rat intermediate lobe, four APS1 sera (patients 1, 3, 5, and 6, Table 2) showed a distinctly stained fiber plexus (Fig. 4*A*). Adsorption of one APS1 serum (Table 2, patient 5) with GAD alone caused a strong reduction in the number of fluorescent terminals (compare Fig. 4 *B* with *A*), whereas the effect of AADC or TH was less pronounced (compare Fig. 4 *C* and *D* with *A*) and that of TPH negligible (compare Fig. 4 *E* with *A*). No

Table 1. Immunoreactivity against *in vitro* expressed TDRD6 correlated to clinical manifestations in 86 patients with APS1

Clinical manifestation	Number with manifestation/total	Number with autoantibodies to TDRD6/total: With manifestation	Number with autoantibodies to TDRD6/total: Without manifestation	*P**
Mucocutaneous candidiasis	83/86	40/83	2/3	0.612
Hypoparathyroidism	71/86	37/71	5/15	0.258
Adrenal insufficiency	69/86	35/69	7/17	0.591
Alopecia areata	29/86	15/29	27/57	0.820
Gonadal failure	28/86	17/28	25/58	0.168
Intestinal dysfunction	22/86	12/22	30/64	0.624
Vitiligo	18/86	8/18	34/68	0.793
Chronic active hepatitis	16/86	10/16	32/70	0.274
Pernicious anemia	13/86	7/13	35/73	0.769
Type 1 diabetes mellitus	10/86	5/10	37/76	1.000
Pituitary insufficiency	6/86	2/6	40/80	0.677

*Calculated by use of two-tailed Fisher's exact test.

Table 2. Clinical characteristics and autoantibodies in six APS1 patients with GH deficiency

	Patient					
Variables	1	2	3	4	5	6
Characteristic						
Sex	M	M	F	F	M	F
Age (years) at evaluation	18	6	18	6	15	16
Mucocutaneous candidiasis	+	+	+	+*	+	+
Adrenal insufficiency	+	−	+	+*	+	+
Hepatitis	+	+	−	−	+	+
Hypoparathyroidism	+	−	+	+	−	+
Intestinal dysfunction	+	+	−	−	−	+
Gonadal failure	−	−	+	−	−	+
Alopecia	−	+*	−	−	+	−
Vitiligo	+	+*	+	−	−	−
Iritis	+	+	−	−	−	−
Keratitis	−	−	−	−	+	−
Thyroiditis	−	−	−	−	−	+
Failure of exocrine pancreas	+	−	−	−	−	−
Autoantigen						
Glutamic acid decarboxylase (GAD)	+	−	+	−	+	+
Aromatic-L-amino acid decarboxylase (AADC)	+	−	+	−	+	+
Tyrosine hydroxylase (TH)	−	+	+	−	+	−
Tryptophan hydroxylase (TPH)	+	+	+	−	+	+

*Manifestation diagnosed after blood sampling for this study.

fluorescent structures were seen after incubation with a mix of all four enzymes (compare Fig. 4 *F* with *A*).

Table 2 shows presence of Aabs against GAD, AADC, TH, and TPH among the six APS1 patients with GH-deficiency as detected by immunoprecipitation. No sera from healthy controls showed immunoreactivity against any of the four enzymes.

Discussion

By immunoscreening of a human pituitary cDNA library, we have identified TDRD6 as a major autoantigen in APS1. TDRD6 is known to be mainly expressed in the testis and only at very low levels in other endocrine tissues like the pituitary, adrenal gland, and pancreas (http://expression.gnf.org) (28). The function of TDRD6 is unexplored, but the protein is known to contain seven so-called tudor domains, that is, ≈60-aa repeats initially found in developmentally important proteins and first ascribed a putative RNA-binding function (29). More recent studies have revealed, however, that tudor domains do not bind nucleic acids but instead proteins containing methylated arginines (30, 31). No other protein motifs were detected.

In the present study, TDRD6 Aabs were frequently found among APS1 patients (42/86; 49%) by using an immunoprecipitation assay. These Aabs seem to be highly APS1 specific, because no immunoreactivity against *in vivo* translated TDRD6 was detected in a large number of sera from patients with organ-specific and systemic autoimmune diseases or healthy blood donors. TDRD6 has previously been identified as an autoantigen in a single patient with colon cancer (32); however apparently not cancer-related, because sera from 29 patients with colorectal cancer, in addition to 16 normal blood donors, tested negative for TDRD6 Aabs (32).

We were unable to correlate TDRD6 immunoreactivity with any APS1 manifestation; perhaps not unexpected, because the number of APS1 patients with hypopituitarism is very small. Notably, the lowest *P* value was seen for correlation between TDRD6 Aabs and gonadal failure ($P = 0.168$). This observation is interesting considering the TDRD6 expression pattern. The APS1 patients in the present report are all well characterized, but there is always a possibility of unrecognized manifestations, presence of subclinical disease or appearance of Aabs before the onset of clinical disease. To fully elucidate the possible correlations between gonadal failure and TDRD6 Aabs, more studies on TDRD6 immunoreactivity in patients with isolated and combined gonadal failure are needed. Also, it remains to be verified whether or not TDRD6 Aabs are related to other so far unrecognized APS1 manifestations.

Pituitary insufficiency is a rare manifestiation of APS1 and diagnosed in only 6 of our 86 patients. All these patients had an isolated GH-deficiency, but only two of which tested positive for TDRD6 Aabs, not allowing statistical interpretation.

The prevalence of TDRD6 immunoreactivity among APS1 patients (49%) is comparable with the high rates of Aabs against side–chain cleavage enzyme (52%), AADC (51%), and TPH (45%) (11). However, TDRD6 is not structurally related to any of these APS1 autoantigens and sharing of antigen epitopes is not obvious.

In this study, Aabs against guinea pig anterior pituitary cells were detected in sera from 3/6 APS1 patients with GH-deficiency. Double-staining for pituitary hormones revealed a partial colocalization with GH-producing cells, somatotrophs. Recent publications suggest that Aabs against somatotrophs, when present in high titers, may be considered a good diagnostic marker for autoimmune forms of GH-deficiency (33, 34).

To what extent presence of APS1 autoantigen, apparently only in a small number of GH cells and apparently not in the GH storage granules, may contribute to the GH-deficiency in these patients is unclear. It also remains to be established whether the APS1 autoantigen-positive cells seemingly not expressing any of the the classical anterior pituitary hormones represent a novel cell subpopulation.

Several of the major APS1 autoantigens are key enzymes in neurotransmitter synthesis, such as GAD, AADC, TH, and TPH which are all present in the pituitary, and one of our screening sera did identify clones encoding TPH. Because of this finding, we performed preadsorption tests with GAD, AADC, TH, and TPH. In one APS1 patient preadsorption with AADC abolished the staining, whereas preadsorption with the other enzymes or TDRD6 did not alter the immunostaining seen with patient sera.

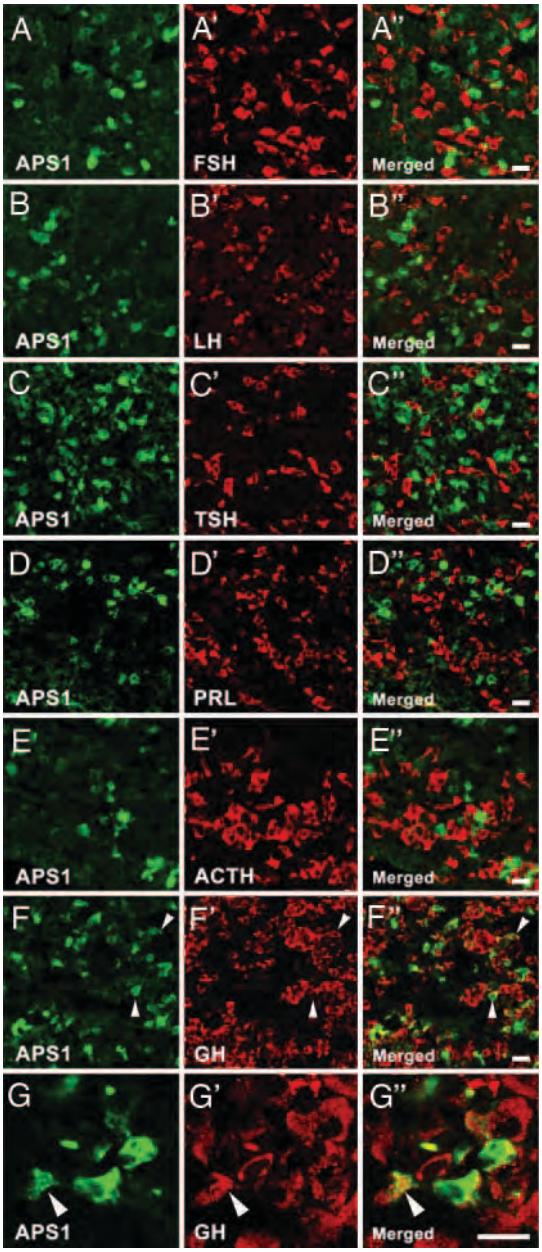

Fig. 2. Double-staining of guinea pig anterior pituitary lobe with serum from an APS1 patient (green) and antisera against pituitary hormones (red). No colocalization of the APS1 serum immunoreactivity and reactivity against follicle stimulating hormone (*A*), luteinizing hormone (*B*), thyroid stimulating hormone (*C*), prolactin (*D*), or adrenocorticotropic hormone (*E*) was found. The APS1 serum and GH antiserum revealed colocalization (yellow) within some cells (*F* and *G*, arrowheads). The APS1 staining was distinctly different from the GH-positive granules (*G*, arrowheads). (Scale bars: 20 μm.)

These results indicate the presence of further target autoantigen(s) than TDRD6 and AADC in anterior pituitary cells.

The three sera also stained a nerve plexus in the intermediate pituitary lobe (compare ref. 24), which is known to receive a dopaminergic (35, 36) and a GABAergic (37) innervation, both of central origin. These terminals are at least in part identical, in agreement with coexistence of TH and GAD in arcuate neurons (38). In addition, there is a central serotonergic innervation of the intermediate lobe (39) which should contain TPH. Serotonin can also be present in dopamine terminals, but in this case after uptake by the dopamine transporter (40), that is these terminals

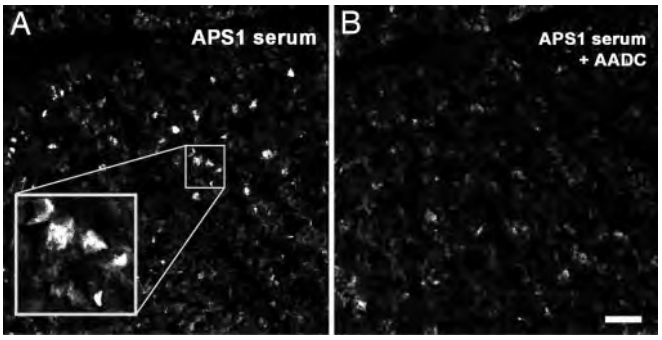

Fig. 3. Preadsorption with AADC of an APS1 serum that stains cells in the guinea pig anterior pituitary lobe. Shown are before (*A*) and after (*B*) adsorption. (Scale bar: 50 μm.)

do not contain TPH. Taken together, in the intermediate lobe, there are presumably nerve terminals containing TH plus AADC (dopamine), TH plus AADC plus GAD (mixed dopamine-GABA), GAD alone, and TPH alone. The preadsorption results seem to reflect that distribution, GAD enzyme being most efficient in reducing staining.

In conclusion, TDRD6 is a major autoantigen in APS1 patients, and 3/6 sera from GH-deficient patients stain specific cell populations and nerves in the guinea pig pituitary gland.

It may be speculated that presence of APS1 autoantigen in GH cells may contribute to the GH-deficiency. Alternatively, autoantibodies directed against the transmitter-synthesizing enzymes, acting at the level of the median eminence/arcuate nucleus, could alter brain control of the pituitary, contributing to this disorder.

Materials and Methods

Patients. Serum samples from 86 APS1 patients, 42 men and 44 women, of Swedish (n = 10), Norwegian (n = 17), and Finnish (n = 59) origin were analyzed. We also included sera from 11 patients with lymphocytic hypophysitis (3 biopsy-proven, 8 suspected), 17 with autoimmune Addison's disease, 20 with type 1 diabetes mellitus, 20 with Sjögren's syndrome, and 25 with systemic lupus erythematosus. Ninety healthy Swedish blood donors served as controls. All subjects gave their informed consent to the study, which was approved by the local ethical committees at Uppsala University and Karolinska Institutet.

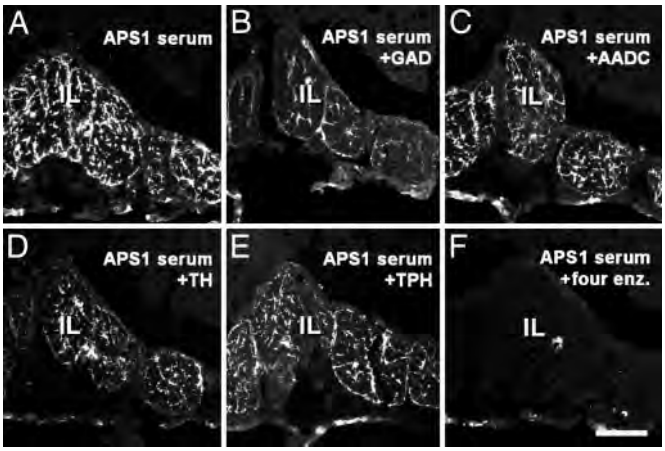

Fig. 4. Preadsorption with recombinant enzymes of an APS1 serum, which stained intermediate lobe (IL) of rat pituitary. Shown are before adsorption (*A*) and after adsorption with GAD (*B*), AADC (*C*), TH (*D*), TPH (*E*), and all four enzymes (*F*). (Scale bar: 100 μm.)

Immunohistochemistry. Experiments were designed in accordance to guidelines on animal care. Male and female guinea pigs (weight 250–300 g) and male Sprague–Dawley rats (weight 250–350 g) (all animals from B & K, Stockholm, Sweden), housed under controlled environmental conditions, were deeply anesthetized and perfused via the ascending aorta with formalin/picric acid in phosphate buffer.[§§] The pituitaries were immersed in the same fixative, rinsed with 10% sucrose in phosphate buffer, snap-frozen, cut at 14-μm thickness on a cryostat (Microm, Heidelberg, Germany) and thaw-mounted on chrome alum-gelatin-coated glass slides.

Sera from 6 APS1 patients diagnosed with GH-deficiency (2 Swedish and 4 Finnish, Table 2) and 10 healthy blood donors (diluted 1:2,000–1:10,000) were processed according to the tyramide signal amplification (TSA) immunohistochemical technique (41), that is, incubation overnight followed by horseradish peroxidase-conjugated rabbit or donkey anti-human IgG (1:200; Dako A/S, Copenhagen, Denmark; Jackson ImmunoResearch, West Grove, PA) by using the TSA-Plus Fluorescein System (PerkinElmer Life Science, Boston, MA).

For double labeling, the TSA technique was followed by conventional immunohistochemistry (42) with rabbit antisera against luteinizing hormone (1:1,600; Biogenesis, Poole, England), thyroid stimulating hormone (1:5,000; Chemicon International, Temecula, CA), or prolactin (1:400; a generous gift from N. M. György), mouse monoclonal antibodies against follicle stimulating hormone (1:500; Abcam, Cambridge, England), adrenocorticotropic hormone (1:1,000; Peninsula Laboratories, Belmont, CA), or a sheep antiserum against GH (1:5,000; Biogenesis). Appropriate secondary antibodies conjugated with FITC (Jackson ImmunoResearch) were used at (1:40–1:80) dilution.

The specificity of the binding was tested by preadsorption of human sera (diluted 1:2,000–1:4,000) with 60,000–120,000 cpm [^{35}S]-radiolabeled GAD, AADC, TH, TPH, or TDRD6 expressed *in vitro* as described below.

Sections were mounted in a mixture of glycerol and PBS (3:1), containing 0.1% *para*-phenylenediamine as anti-fading agent (Sigma–Aldrich, Stockholm, Sweden). The sections were examined in a Zeiss confocal laser scanning system (Model 510) or a Bio-Rad Radiance Plus confocal laser scanning system (Bio-Rad, Hemel Hemstead, U.K.) installed on a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) equipped with appropriate objectives and excitation and emission filters. Digital images were optimized for image resolution, and images with double labeling were merged in Adobe PhotoShop 9.02 (Adobe Systems, San Jose, CA).

***In Vitro* Transcription and Translation (ITT) of Enzymes and Immunoprecipitation.** cDNA clones corresponding to GAD, AADC, TH and TPH were subcloned into a pSP64 poly(A) vector as described (9, 10, 43, 44). Recombinant [^{35}S]-radiolabeled enzymes were produced by ITT in a TnT SP6 Quick coupled reticulocyte lysate system (Promega, Madison, WI). The correct size of the radioactive product was verified by SDS/PAGE (Bio-Rad) and [^{35}S]methionine incorporation was measured by trichloroacetic acid precipitation, followed by scintillation counting. The [^{35}S]-radiolabeled enzymes were used in immunohistochemistry experiments described above. In addition, enzymes were used for immunoprecipitation with the 16 sera selected for immunohistochemistry experiments, essentially as described elsewhere (44). The results were expressed as an index [(cpm sample − cpm negative control) / (cpm positive control − cpm negative control) × 100]. Serum samples were run in duplicates. APS1 patients with known high titers of GAD, AADC, TH, or TPH Aabs, were used as positive controls; and 4% bovine albumin (Sigma, St. Louis, MO) as negative control. The upper normal limit of the Aab index was set to the mean value for blood donors plus 3 standard deviations.

Construction and Screening of a Human Pituitary cDNA Library. A cDNA expression library was constructed from 5 μg of human pituitary gland Poly(A)$^+$ RNA (Clontech, Alto, CA) by using the ZAP Express cDNA synthesis kit and ZAP Express cDNA Gigapack III Gold cloning kit (Stratagene Cloning Systems, La Jolla, CA). The library, containing 1.7×10^6 unique cDNA clones, was then amplified once. Sera from two APS1 patients (diluted 1:1,000 and 1:3,000) were used for immunoscreening of the library as described (8). *In vitro* excision of pBK-CMV phagemid vectors from the ZAP express vector were performed according to the manufacturer's protocol. Isolated cDNA clones were analyzed by 5′and 3′sequencing by using a dye-terminator-sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 377 or 3700 sequencers (PerkinElmer Applied Biosystems, Foster City, CA). The sequence data were compared with available databases by using the Basic Local Alignment Search Tool (BLAST) (45). In addition, the cDNA clone encoding TDRD6 was completely sequenced by primer walking with internal primers (CyberGene, Huddinge, Sweden).

***In Vitro* Transcription and Translation (ITT) of TDRD6 and Immunoprecipitation.** Plasmids containing the cDNA fragment encoding TDRD6 were purified by using the Giagen midiprep kit (Qiagen, Hilden, Germany). Recombinant [^{35}S]-radiolabeled TDRD6 was produced by ITT in a TnT reticulocyte lysate system (Promega) and used for immunoprecipitation with sera in a 96-well plate assay, essentially as described elsewhere (44). The results were expressed as an index [(cpm sample − cpm negative control)/(cpm positive control − cpm negative control) × 100] with serum samples run in duplicates. The APS1 serum identifying the TDRD6 clone in immunoscreening was used as positive control and 4% bovine albumin (Sigma chemicals) as negative control. An arbitrary upper normal limit of the Aabs index was set to 30 as this value separated the APS1 cohort into those with clearly elevated values and those with normal values. Recombinant [^{35}S]-radiolabeled TDRD6 was also used in the immunohistochemistry experiments described above.

Statistical Analysis. Statistical analysis was performed with Statistica version 7.1 (StatSoft, Tulsa, OK). Two-tailed Fisher's exact test was used to compare the frequencies of different disease manifestations in APS1 patients with and without TDRD6 Aabs. $P < 0.05$ was considered significant.

We thank Lars Berglund and Agneta Hilding for help with statistical analyses and Åsa Hallgren for technical assistance. The prolactin antibody was a generous gift from Dr. Nagy M. György, Semmelweis University, Budapest, Hungary. This work was supported by the European Union FP6 Program on Rare Diseases; the Karolinska Institutet; the Knut and Alice Wallenberg, Marianne and Marcus Wallenberg, Magnus Bergvall, and Torsten och Ragnar Söderberg Foundations; and the Swedish Research Council.

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CLINICAL STUDY

Identification of TPIT and other novel autoantigens in lymphocytic hypophysitis; immunoscreening of a pituitary cDNA library and development of immunoprecipitation assays

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Abstract

Background: Lymphocytic hypophysitis is an organ-specific autoimmune disease of the pituitary gland. A specific and sensitive serological test currently does not exist to aid in the diagnosis.
Objective: To identify target autoantigens in lymphocytic hypophysitis and develop a diagnostic assay for these proteins.
Design/methods: A pituitary cDNA expression library was immunoscreened using sera from four patients with lymphocytic hypophysitis. Relevant cDNA clones from screening, along with previously identified autoantigens pituitary gland-specific factor 1a and 2 (PGSF1a and PGSF2) and neuron-specific enolase (NSE) were tested in an *in vitro* transcription and translation immunoprecipitation assay. The corticotroph-specific transcription factor, TPIT, was investigated separately as a candidate autoantigen.
Results: Significantly positive autoantibody reactivity against TPIT was found in 9/86 hypophysitis patients vs 1/90 controls ($P=0.018$). The reactivity against TPIT was not specific for lymphocytic hypophysitis with autoantibodies detectable in the sera from patients with other autoimmune endocrine diseases. Autoantibodies were also detected against chromodomain-helicase-DNA binding protein 8, presynaptic cytomatrix protein (piccolo), Ca^{2+}-dependent secretion activator, PGSF2 and NSE in serum samples from patients with lymphocytic hypophysitis, but at a frequency that did not differ from healthy controls. Importantly, 8/86 patients with lymphocytic hypophysitis had autoantibodies against any two autoantigens in comparison with 0/90 controls ($P=0.0093$).
Conclusions: TPIT, a corticotroph-specific transcription factor, was identified as a target autoantigen in 10.5% of patients with lymphocytic hypophysitis. Further autoantigens related to vesicle processing were also identified as potential autoantigens with different immunoreactivity patterns in patients and controls.

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Introduction

Lymphocytic hypophysitis is part of the spectrum of organ-specific autoimmune endocrine diseases and is characterised by the infiltration of self-reactive T-lymphocytes into the pituitary gland. The disease is more frequently seen in females than males at a ratio of 6:1 and has a striking association with pregnancy (1). In the acute phase, patients usually present with headaches and visual disturbances due to an upwardly expanding pituitary mass that mimics an adenoma (2). Corticotrophs are often the first cell type to be affected, in contrast to pituitary adenoma where they are usually the last to fail (3, 4, 5). The ensuing secondary adrenal insufficiency is potentially fatal. In chronic cases, the ongoing autoimmune process can cause post-inflammatory fibrosis leading to pituitary gland atrophy and an empty sella syndrome (6, 7, 8).

The pituitary mass in lymphocytic hypophysitis can be indistinguishable from that of a pituitary adenoma on MRI. Pituitary biopsy has been used to both alleviate the symptoms of the pituitary mass and to diagnose the disease on histological grounds. Pituitary biopsy can, however, lead to permanent pituitary failure and

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therefore a conservative approach using corticosteroids to reduce the size of the mass has been recommended in suspected cases.

High-titre autoantibodies are a characteristic feature of many autoimmune diseases. They can often be detected years before the onset of the disease and can be good predictors of disease progression and outcome (9, 10). Pituitary autoantibodies have been studied in various autoimmune diseases by a number of techniques including immunofluorescence (IF) (11, 12, 13, 14, 15), immunoblotting (16) and more recently immune screening of cDNA expression libraries followed by radioligand immunoprecipitation assay (17) to identify the autoantigens targeted. Indirect IF has the potential to detect autoantibodies to as yet uncharacterised autoantigens, although the method is probably not sensitive enough in many cases (1). Immunoblotting recognises linear epitopes and identifies autoantigens by molecular weight (16) but not cellular localisation (as in IF), whereas immunoprecipitation requires a tertiary structure.

A number of potential autoantigens have been proposed in lymphocytic hypophysitis including α-enolase (18, 19, 20), neuron-specific enolase (NSE) (20), GH (21, 22), pituitary gland-specific factors 1a and 2 (PGSF1a and PGSF2) (17), secretogranin II (23) and most recently chromosome 14 open reading frame 166 and chorionic somatomammotropin (24). Although some are undoubtedly markers of an underlying autoimmune process, they are not always specific to pituitary disease. The major target autoantigens in lymphocytic hypophysitis remain unknown.

This study aimed to identify potential target autoantigens in lymphocytic hypophysitis by screening a pituitary cDNA expression library. The cDNA clones identified by sera from patients with lymphocytic hypophysitis were subsequently evaluated using *in vitro* transcription and translation (ITT) followed by immunoprecipitation with patient and healthy control serum. We also tested the previously identified pituitary autoantigens NSE, PGFS1a, PGFS2 and a potential novel candidate, TPIT, a pituitary-specific transcription factor essential for development of the corticotroph lineage (25, 26).

Methods

Patients

Serum samples were collected for analysis from 86 patients with lymphocytic hypophysitis, including 21 biopsy-proven patients and 65 suspected cases of lymphocytic hypophysitis. The suspected cases were further sub-classified into groups consisting of 43 patients with 'suspected lymphocytic hypophysitis', ten patients with isolated ACTH deficiency, six patients with lymphocytic hypophysitis that had progressed to empty sella, two patients with isolated ACTH deficiency and an empty sella and four patients with diabetes insipidus (neuro-infundibulo-hypophysitis). In the spectrum of suspected cases, the diagnosis was considered likely by the referring endocrinologist, usually on the basis of clinical history, examination and MRI scan appearance. Serum samples were also obtained from 144 patients with other autoimmune endocrine diseases comprising 14 patients with Addison's disease, 20 with autoimmune polyendocrine syndrome type 1 (APS1), 20 with Graves' disease, 20 with Hashimoto's thyroiditis, and 20 with type 1 diabetes mellitus. A separate group of 50 patients with isolated ACTH deficiency was also used for comparison. This latter group has been investigated extensively (27). Serum samples collected from 90 healthy Australian blood donors served as controls in all experiments.

Ethical approval was obtained from the Committee of Ethics, Faculty of Medicine, Uppsala University, the Human Research Ethics Committees of the Hunter Area Health Service and University of Newcastle (9706183.13) and the Australian Red Cross Blood Bank Ethics Committee, with informed, written consent from all patients and controls.

Immunoscreening of a human pituitary cDNA library

Serum samples from four patients with lymphocytic hypophysitis (one biopsy proven and three suspected cases) were chosen for immunoscreening of a pituitary cDNA expression library on the basis of high-titre pituitary autoantibodies detected by an immunoblotting assay of pituitary cytosolic proteins (4) and a classical clinical history. The pituitary cDNA expression library (28) was immunoscreened separately with all four patient sera (diluted 1:200) as described previously (29). *In vitro* excision of the pBK-CMV phagemid vectors from the ZAP express library vector was performed according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The isolated cDNA clones were partially sequenced using a dye-terminator sequencing kit (Amersham Pharmacia Biotech) and ABI 3730 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). cDNA clones were identified by comparison of the sequencing data against available databases using BLAST.

Potential candidate autoantigens

The corticotroph-specific transcription factor, TPIT (also referred to as T-box 19) has been identified as the causative gene in isolated ACTH deficiency of neonatal onset. Hence, it was chosen for study as corticotroph cells tend to be preferentially targeted in lymphocytic hypophysitis resulting in isolated ACTH deficiency. A full-length cDNA TPIT clone was kindly donated by Dr Jacques Drouin (Montreal, Canada).

Previously reported candidate autoantigens

Full-length cDNA clones PGSF1a and PGSF2 were kindly donated by Dr Ke-ita Tatsumi (Japan) and NSE was purchased from the clone database (Image Clone 3629603).

ITT of autoantigens and immunoprecipitation

All library cDNA clones identified by immunoscreening that were of interest, as well as TPIT, PGSF1a, PGSF2 and NSE, were subcloned into the pTNT vector (Promega) by double-restriction-enzyme digestion for improved efficiency of ITT. Inserts were re-verified by sequencing as above. A full-length clone encoding rat Ca^{2+}-dependent secretion activator (rCADPS) protein was kindly provided by Dr Tom Martin (Michigan), which was also subcloned into the pTNT vector.

Autoantigens were expressed using an ITT assay to determine the frequency and specificity of immunoreactivity against these proteins. Recombinant ^{35}S-radiolabelled proteins were produced by ITT in an Sp6 Quick coupled reticulocyte lysate system (Promega) and used for immunoprecipitation with patient sera as described previously (30). The patient serum from which the respective cDNA clone was isolated by immunoscreening the pituitary cDNA library was used as the positive control and 4% BSA (Sigma) was used as the negative control. Positive and negative controls were run in triplicate, whereas all other sera were analysed in duplicate. Results were expressed as an antibody index (c.p.m. sample/mean c.p.m. of healthy controls). The upper normal autoantibody index was set at the mean of the unequivocally negative healthy blood donors plus 3 S.D. for all autoantigens tested.

Statistical analysis

A χ^2-test was performed to determine the probability of analysed autoantigens as significant target autoantigens in lymphocytic hypophysitis. Yates' correction was applied due to consistently small group sizes. A P value of <0.05 was considered significant.

Results

Isolation and identification of potential autoantigens from immunoscreening of a pituitary cDNA library

A pituitary cDNA expression library was immunoscreened with sera from four patients with lymphocytic hypophysitis: one biopsy proven and three suspected cases, previously shown to have high-titre pituitary autoantibodies on immunoblotting (4). A total of 58 individual cDNA clones were isolated and partially sequenced. A single cDNA clone encoding chromodomain-helicase-DNA binding protein 8 (CHD8) was independently identified on separate screenings by two different patients' sera. On comparison with the GenBank database (GenBank accession NM_020920.2), the partial cDNA sequence isolated from the library from both patients was found to encode the carboxyl-terminal region of the 2302 amino acid, 260 kDa CHD8 protein. The recombinant protein produced by ITT was efficiently immunoprecipitated by the two screening sera and hence was selected for additional analyses.

From the remaining cDNA clones, a subset with interesting functional characteristics was chosen for testing with ITT. When transcribed and translated into the immunoprecipitation assay system, most of the recombinant proteins were recognised solely by the screening serum or no sera at all. Two proteins encoding Piccolo (presynaptic cytomatrix protein) and CADPS were each immunoprecipitated by the screening serum as well as additional lymphocytic hypophysitis patients, but not by any of the healthy controls and therefore were selected for further investigation.

Two cDNA clones isolated from the biopsy-proven patient sera used for immunoscreening encoded a small portion of the 5′ end of the piccolo gene, the full length of which is reported to encode a 5142 amino acid protein, with a 4935 amino acids transcript variant also being identified (GenBank accessions NM_033026.4 and NM_014510.2 respectively).

Three transcript variants for the human *CADPS* gene located on chromosome 3p14.2 have been reported to differ in the 3′ nucleotide sequence of the gene. When compared with the GenBank mRNA reference sequences (variant 1: accession ID NM_003716, variant 2: accession ID NM_183394 and variant 3: accession ID NM_183393) the partial cDNA clone encoding CADPS extracted from the pituitary library matches the entire sequence of all three variants from nucleotide position 951 (the end of exon 4). The N-terminal protein sequence is crucial for correct protein folding of the CADPS protein, therefore a full-length rCADPS was obtained and used for all further analyses. The rat homologue is located on chromosome 15p16 and shares 98% homology with human CADPS protein.

Autoantibody analysis of library autoantigens CHD8, piccolo and rCADPS

To ascertain the specificity of CHD8, piccolo and rCADPS (full length) as autoantigens, ^{35}S-methionine-labelled proteins were immunoprecipitated against sera from 86 patients with lymphocytic hypophysitis and 90 healthy blood donors (Table 1).

CHD8 autoantibodies were detected in the sera from seven of the 86 (8.14%) patients with lymphocytic hypophysitis including two of the 21 (9.52%) patients with biopsy-proven disease, four of the 43 (9.30%)

Table 1 Immunoreactivity specificity against lymphocytic hypophysitis candidate autoantigens.

	Autoantibody (positive/total (%))		
Autoantigen	Lymphocytic hypophysitis	Healthy controls	***P****
TPIT	9/86 (10.47)	1/90 (1.11)	0.0186†
CHD8	7/86 (8.14)	3/90 (2.22)	0.2932
Piccolo	3/86 (3.49)	2/90 (2.22)	0.9563
CADPS	12/86 (13.95)	11/90 (12.22)	0.9058
PGSF2	5/86 (5.81)	2/90 (2.22)	0.4048
NSE	2/86 (2.33)	0/90 (0.00)	0.4571
Any 2 AutoAg	8/86 (9.30)	0/90 (0.00)	0.0093†

*Calculated by χ^2-test with Yates' correction, †$P<0.05$.

suspected cases and in one of the four (25%) patients with suspected hypophysitis with diabetes insipidus as the single presenting symptom. Positive immunoreactivity was also observed in three of the 90 (3.33%) healthy controls (Supplementary Figure 1, see section on supplementary data given at the end of this article, panel A). The frequency of autoantibodies in lymphocytic hypophysitis patients was not significantly different from healthy controls, $P=0.2932$ (χ^2-test with Yates' correction).

Piccolo autoantibodies were found in three of the 86 (3.49%) patients with lymphocytic hypophysitis comprising one of the 21 (4.76%) biopsy-proven patients, one of the 43 (2.33%) patients with suspected lymphocytic hypophysitis and a single patient of ten (10.0%) with isolated ACTH deficiency. Autoantibodies were also detected in two of the 90 (2.22%) healthy controls, of which one had extremely high-titre autoantibodies in comparison with the control screening serum (Supplementary Figure 1, panel B).

Low-titre autoantibodies against the recombinant rCADPS protein were identified in the sera of 12 of the 86 (14%) lymphocytic hypophysitis patients. Of these 12, three were biopsy proven cases (three of 21, 14.3%), six were suspected hypophysitis (six of 43, 14%), and single cases with isolated ACTH deficiency (one of ten, 10%), empty sella as a late stage of hypophysitis (one of six, 16.7%) and isolated ACTH deficiency with empty sella (one of two, 50%). None of the four patients with diabetes insipidus had these autoantibodies. However, a similar frequency of CADPS autoantibodies was also observed in healthy controls with 11 of the 90 (12.2%) considered positive (Supplementary Figure 1, panel C).

Potential candidate autoantigen TPIT

A positive autoantibody index was seen against TPIT in nine of the 86 (10.5%) patients with lymphocytic hypophysitis. These included one of the 21 (4.76%) biopsy-proven patients, four of the 43 (9.30%) patients with suspected lymphocytic hypophysitis, one of the ten (10.0%) patients with ACTH deficiency, one of the six (16.7%) patients with empty sella and two of the four (50%) patients with diabetes insipidus. Immunoreactivity was only detected in one of the 90 (1.11%) healthy controls (Fig. 1). Patients with lymphocytic hypophysitis had a significantly higher frequency of autoantibodies against TPIT than that of healthy controls, $P=0.0186$ (χ^2-test with Yates' correction).

To determine whether TPIT autoantibodies were specific for lymphocytic hypophysitis, the autoantibody status of patients with other autoimmune endocrine diseases was investigated. Autoantibodies against TPIT were detected in the serum from two patients with Addison's disease, one patient with APS1, one patient with Graves' disease, two patients with Hashimoto's thyroiditis, one type 1 diabetes patient (six of the 94 patients vs nine of the 86 patients with hypophysitis, $P=0.47$ (χ^2-test with Yates' correction) (Fig. 1).

Two of the fifty patients with isolated ACTH deficiency had TPIT autoantibodies (Fig. 1).

Previously reported candidate autoantigens

The frequency and specificity of autoantibodies in lymphocytic hypophysitis sera were also determined with the previously described pituitary autoantigens PGSF1a, PGSF2 and NSE against the panel of 86 patients with lymphocytic hypophysitis and 90 healthy blood donors (Table 1).

Insufficient ^{35}S-methionine incorporation was obtained with PGSF1a despite subcloning into the pTNT vector. Therefore, no immunoprecipitation experiments were conducted with this autoantigen.

PGSF2 autoantibodies were detected in the sera of two of the 21 (9.52%) biopsy-proven patients, one of the 43 (2.33%) suspected cases, one of the six (16.7%) patients with empty sella and one of the four (25%) patients with diabetes insipidus compared with two of

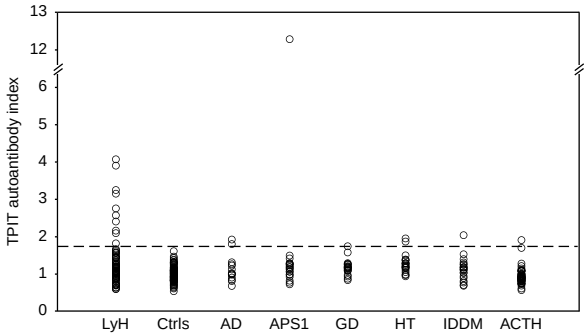

Figure 1 Analysis of autoantibodies against TPIT in sera from patients with lymphocytic hypophysitis (LyH; $n=86$), healthy controls (Ctrls; $n=80$), Addison's disease (AD; $n=14$), APS1 ($n=20$), Graves' disease (GD; $n=20$), Hashimoto's thyroiditis (HT; $n=20$), type 1 diabetes mellitus (IDDM; $n=20$) and isolated ACTH deficiency (ACTH; $n=50$). The broken line indicates the upper limit of the normal range calculated by the average autoantibody index of the negative healthy controls plus 3 s.d. (limit =1.62).

the 90 (2.22%) healthy blood donors (Supplementary Figure 1, panel D). The difference was not statistically significant.

Autoantibodies were found against the NSE recombinant protein in two of the 86 (2.33%) patients with lymphocytic hypophysitis, one patient (one of the six, 16.7%) with empty sella and a single patient (one of the two, 50%) with empty sella syndrome combined with isolated ACTH deficiency. No autoantibodies against NSE were detected in the sera from healthy controls (Supplementary Figure 1, panel E).

Patients with multiple autoantibody reactivity

In addition, eight of the 86 patients with lymphocytic hypophysitis had positive autoantibodies against two autoantigens in comparison to none of the 90 controls, $P=0.0093$ (χ^2-test with Yates' correction). Two patients (one biopsy proven and one 'suspected') had positive immunoreactivity to both CHD8 and PGSF2. Two further patients (one biopsy-proven case and one patient with diabetes insipidus) had a positive autoantibody index for CHD8 and TPIT. A single biopsy-proven patient had both piccolo and CADPS autoantibodies, while a 'suspected' lymphocytic hypophysitis patient showed immunoreactivity against CHD8 and CADPS. A patient diagnosed with empty sella syndrome had autoantibodies against TPIT and NSE and a further patient with empty sella syndrome and isolated ACTH deficiency was positive for CADPS and NSE autoantibodies. None of the healthy controls with immunoreactivity were positive for more than a single autoantigen.

Discussion

This study identified TPIT as a minor target autoantigen in lymphocytic hypophysitis when tested in an immunoprecipitation assay. A number of other potential autoantigens were found, including CHD8 (a DNA-binding protein), Piccolo (a presynaptic cytomatrix protein associated with the active zone) and a CADPS. These proteins are involved in vesicle processing that is fundamental to pituitary peptide hormone release.

The transcription factor TPIT was chosen as a candidate pituitary autoantigen as it is essential for the terminal differentiation of pituitary pro-opiomelanocortin-expressing cells and is cell specific (25, 26). Corticotrophs are often the first cell type to be affected in lymphocytic hypophysitis and some cases of isolated ACTH deficiency are probably autoimmune (4, 27). Mutations in the TPIT gene cause neonatal isolated ACTH deficiency (25, 26, 31). TPIT was identified as a significant autoantigen in 10.5% of patients with hypophysitis. So far, from our data, we could not link TPIT reactivity to specific clinical subtypes of hypophysitis. Interestingly, autoantibodies were not only confined to those patients with isolated ACTH deficiency but were also detected in two patients with diabetes insipidus as the presenting symptom of presumed infundibuloneurohypophysitis. In one patient, MRI scan showed stalk thickening and enlargement of the anterior pituitary with uniform enhancement. Diabetes insipidus has been associated with vasopressin cell antibodies in the hypothalamus using IF (14, 32). TPIT autoantibodies were also detected in patients with other autoimmune endocrine diseases and hence may not be specific for lymphocytic hypophysitis. It cannot be assumed that TPIT autoantibodies are non-specific in this setting as coexistent hypophysitis has been described at autopsy in patients with Addison's disease and up to 10% of patients with Hashimoto's thyroiditis have been shown to have pituitary antibodies by ELISA (33) and IF (34). Although a transcription factor as an autoantigen appears counterintuitive and the mechanism of autoantibody formation is not known, there is a precedent in SOX9 and SOX10 found in vitiligo (35). Recently, autoantibodies to the pituitary transcription factor PiT-1, detected by immunoblotting and antibody-specific ELISA, have been described in patients with late onset hypopituitarism and polyglandular autoimmunity (36). Interestingly, mutations in both TPIT and PiT-1 give rise to hypopituitarism, i.e. monogenic disorders that can be mimicked by acquired autoimmune hypophysitis.

CHD8 was isolated independently by the sera from two patients with suspected lymphocytic hypophysitis on immunoscreening of the pituitary library. Given the rarity of this disorder, this would be an amazing coincidence; therefore, the protein was considered a strong candidate as a pituitary autoantigen. The protein is a chromatin remodelling ATPase of the SNF2 family, that regulates gene expression. Its specific role is to bind p53 and suppress its function, thereby acting as an anti-apoptotic factor (37). Autoantibodies were detected in an additional five patients with lymphocytic hypophysitis (two biopsy proven); however they were not significantly more frequent than in healthy controls.

The secretion of hormones, neurotransmitters and peptides from neurons, neuroendocrine and endocrine cells is regulated by the Ca^{2+}-dependent fusion of secretory vesicles with the plasma membrane (38, 39). Two types of secretory vesicles, small clear synaptic vesicles and large dense-core vesicles, are essential to the secretion process of packaging, docking, priming and fusion. This is a fundamental route for the secretion of pituitary peptide hormones in the pituitary, the disruption of which could lead to hormonal insufficiencies. Secretogranin II, which is involved in this process, has previously been isolated from screening of the same pituitary library used in this study (23). The protein is abundantly expressed in gonadotrophs, thyrotrophs and corticotrophs (40) and is believed to mediate the packaging or sorting of peptide hormones and neuropeptides into granules of neuroendocrine cells and the

vesicles of selected neurons (41). In this study, we have isolated and identified two further candidate autoantigens, CADPS and piccolo, both related to vesicle processing.

Piccolo is a presynaptic cytomatrix protein associated with the active zone of neurons and neuroendocrine cells (42), which is a specialised region where synaptic vesicles dock and fuse to release neurotransmitter. piccolo and bassoon (a homologous protein) function as tethering proteins that mediate efficient synaptic vesicle clustering but do not directly participate in neurotransmitter release (43). In particular, piccolo functions as a Ca^{2+} sensor in exocytosis of this process (44). Autoantibodies against piccolo were detected in 8% of patients with lymphocytic hypophysitis; however, this was not statistically different from controls. The reason for the extremely high titre in one healthy control is unexplained.

CADPS plays a fundamental role in the Ca^{2+}-regulated exocytosis of dense-core vesicles in neuroendocrine cells and in the secretion of a subset of neurotransmitters (45, 46). The protein is a phosphatidylinositol 4,5-bisphosphate (PIP_2)-binding protein that acts after vesicle docking and priming (47), yet before calcium-triggered fusion and facilitates large dense-core vesicle exocytosis (48). Northern blot analysis has confirmed mRNA expression of CADPS in the pituitary (47). The CADPS isolated from the pituitary library did not possess the 5′ end of the gene sequence, essential for the correct folding of the protein. Immunoreactivity to rCADPS was detected at a similar frequency in both lymphocytic hypophysitis patients and healthy controls.

We have also confirmed that a minority of patients with lymphocytic hypophysitis have autoantibodies against PGSF2. Immunoreactivity against this protein was previously reported in a small number of patients with lymphocytic hypophysitis, isolated ACTH deficiency, idiopathic TSH deficiency and other autoimmune diseases. Tanaka *et al.* (17) also reported immunoreactivity against the pituitary-specific protein PGSF1a in a similar cohort of patients. Immunoreactivity against this protein has since been detected at a high frequency in patients with rheumatoid arthritis, suggesting it is more likely to be an autoantigen in rheumatoid arthritis than lymphocytic hypophysitis (49).

α-Enolase antibodies in lymphocytic hypophysitis have been well studied by immunoblotting (19) and ITT and immunoprecipitation techniques (18). Autoantibodies are believed to be more of a prognostic marker of autoimmunity itself rather than a specific diagnostic marker. Lymphocytic hypophysitis patient serum has also been shown to recognise the γ isoform of the enolase protein, NSE, on immunoblotting (20). In this study, autoantibodies were only detected in four patients, with patient sera previously positive on immunoblotting, not positive in the ITT system. This highlights the hazards of comparing results across different assays.

ITT and immunoprecipitation have been employed to analyse many autoantigens across multiple autoimmune diseases. It has the advantage over other techniques of high-affinity autoantibodies recognising three-dimensional conformational epitopes of the expressed autoantigen, rather than the linear epitopes of denatured proteins as with immunoblotting. The method also provides a quantitative analysis of high-throughput samples, with only small amounts of both protein and serum sample required. However, immunoprecipitation is not achievable with all proteins. Adequate protein levels may not be produced in some instances, as in the case of PGSF1a, and additionally autoantibodies may not recognise the autoantigen when not in its native *in vivo* form. Indeed with CHD8 and piccolo, addition of DTT was required for effective immunoprecipitation with the patient sera. Finally, the results of ITT with NSE as an autoantigen were totally different from those with the immunoblotting technique. Alternative techniques and approaches may therefore be required to validate results in certain cases.

There are several ongoing challenges in this field. The anterior pituitary has five hormone-secreting cell types, each of which could have their own specific target autoantigen(s) and whose autoimmune destruction may present a different clinical picture. Our data show that even immunoreactivity to a cell-specific transcription factor, TPIT, is neither confined to isolated ACTH deficiency, nor does it seem completely hypophysitis specific. Secondly, we have identified several proteins involved in vesicle trafficking as potential autoantigens. Finally, as no control serum recognised more than one protein, we suggest that a panel of target autoantigens be developed and reactivity patterns be compared across different clinical scenarios of autoimmune hypophysitis. Even then, it may not be possible to differentiate the different types of hypophysitis on the basis of autoantibodies alone.

We have identified TPIT as a minor autoantigen in lymphocytic hypophysitis, using a candidate approach. We have also shown that immunoscreening of a pituitary cDNA expression library is an effective way of identifying candidate autoantigens in lymphocytic hypophysitis, with ITT and subsequent immunoprecipitation assays being a valuable method for their evaluation. While the major autoantigen(s) were not identified, re-screening with additional patient sera holds the potential for isolating major autoantigens and the development of an essential serological test for lymphocytic hypophysitis.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-11-1015.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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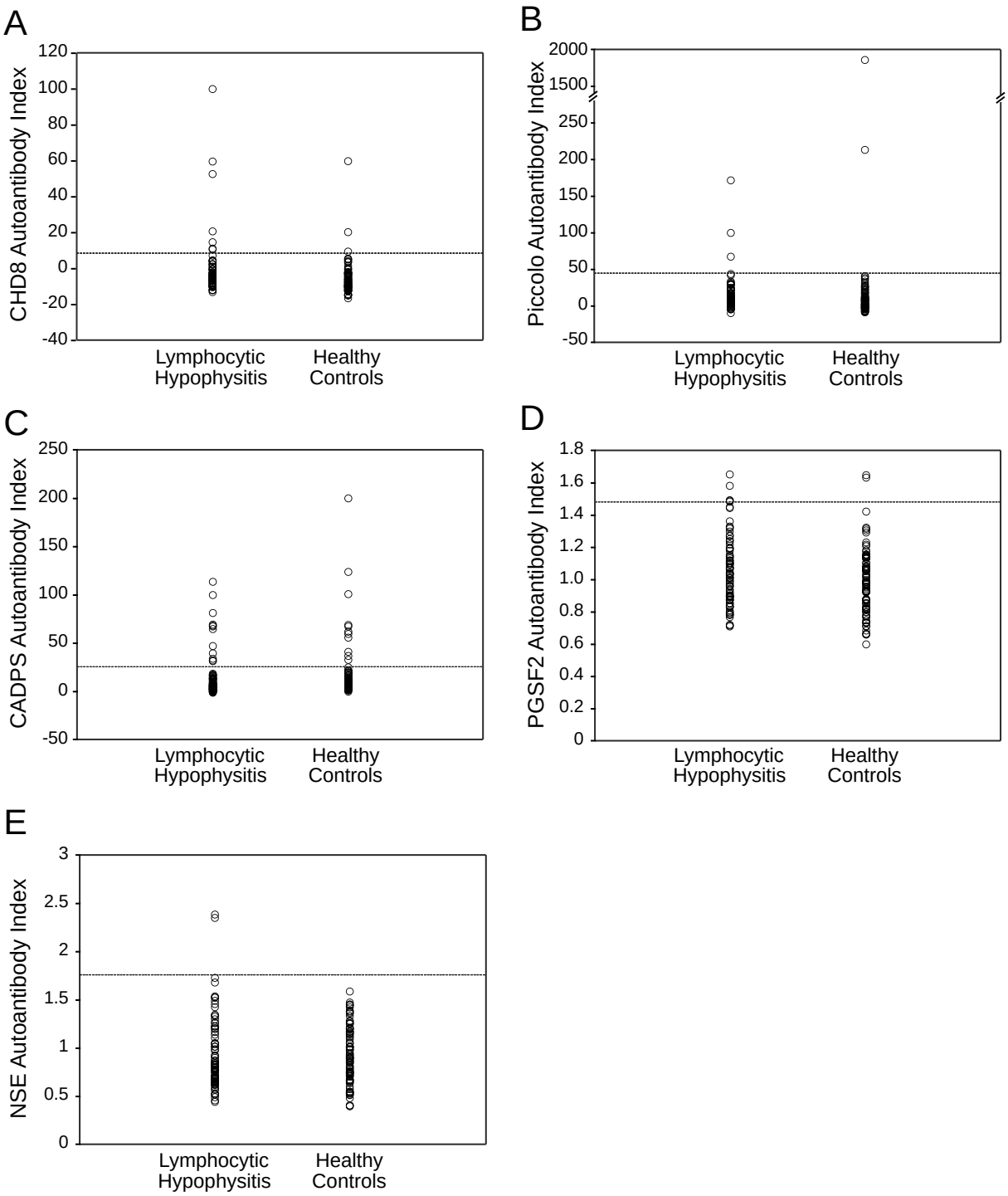
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Case Report

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Pituitary Disease in Chronic Hepatitis C Infection and Interferon-alpha Related Therapy: Two Case Reports

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Abstract

Pituitary dysfunction in chronic hepatitis C infection treated with interferon-α is a rare condition with 4 case reports world wide. We hereby report two cases of pituitary dysfunction in HCV patients, with and without interferon-α therapy. Case 1: A 34-year-old man co-infected with HIV and HCV presented with a 3 month history of lethargy, listlessness and a general lack of energy. Past medical histories include inactive neurosyphillis, chronic schizophrenia and seizure. His HCV is genotype 1 without cirrhosis and he completed a 48-week course of combination IFN-α and RBV for 48 weeks uneventfully 3 months prior. Examination and investigation found him to isolated ACTH deficiency. His condition improved markedly with corticosteroid replacement therapy. Case 2: A 45 year-old and treatment naive man with chronic HCV infection presented with a 20 kg weight loss, lack of energy and the occasional dizziness. Examination and investigation found him to have panhypopituitarism. Replacement therapy was initiated including hydrocortisone, testosterone and hydrocortisone. He made a slow but steady recovery and regained about 15 kg of weight but unfortunately was lost to follow up. It concluded that hepatitis C infection on its own or in conjunction with interferon-α based therapy can result in pituitary failure. The condition is readily treatable and hence should be considered in the appropriate clinical setting.

Keywords: Pituitary; Hypophysitis; Hepatitis C; Interferon-alpha

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Introduction

Pituitary pathology associated with interferon-α (IFN-α) therapy is an uncommon condition which so far has been poorly described and reported. Most are anecdotal with few unconvincing case reports. The mechanism as a result then is poorly understood but perhaps and similar to IFN-α related thyroid disease, immune-modulation is the major underlying pathogenesis. We hereby describe two cases of hepatitis C and IFN-α related disease where pituitary failure developed.

Case Report

Case 1

A 34-year-old man co-infected with Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) presented with a 3 month history of lethargy, listlessness and a general lack of energy. Past medical histories include treated and inactive neurosyphilis, chronic schizophrenia and seizure. His HCV is genotype 1 without cirrhosis and he completed a 48-week course of combination IFN-α and RBV for 48 weeks uneventfully 3 months prior.

Clinically he was unwell with BP of 110/70 sitting and 100/60 standing and PR of 89 beats per minute (bpm). General examination was unremarkable and there was no pigmentation. A baseline serum cortisol was 36nmol/L at 07:05 hrs with Adrenocorticotropic (ACTH) level of 3.3 pmol/L (Reference Range (RR), < 10). His Thyrotropin (TSH) level was 0.96 mIU/L (RR, 0.4 - 4.0), free tetra-iodothyronine (fT4) of 19.1 pmol/L (RR, 10.8 - 21.0), Luteinising Hormone (LH) 13.8 IU/L (RR, 5.5 - 11.5), Follicular Stimulating Hormone (FSH) 7.7 IU/L (RR, 2.1 - 8.0), Testosterone 13.9 nmol/L (RR, 8.0 - 25.9), Growth Hormone (GH) < 0.2mIU/L, Insulin-like Growth Factor 1 (IgF-1) 0.73 U/mL (RR, 0.5 - 2.0), Prolactin 402 mIU/L (RR, < 410). A 250 µg Synacthen stimulation test showed a rise from baseline of 70 to 304 nmol/L at 60 minutes. His electrolytes were normal with Na of 137 and K 4.1 mmol/L. His pituitary Magnetic Resonance Imaging was normal. Pituitary antibodies were

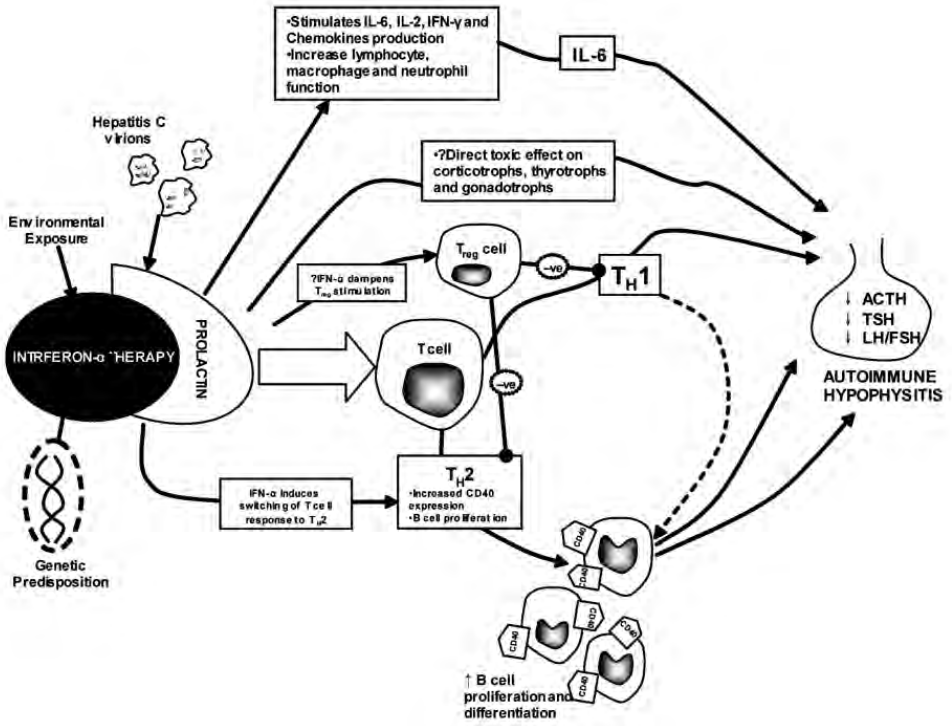

Figure 1. The proposed hypothesis for the development of autoimmune hypophysitis in HCV infection and IFN-based therapy. IL-6: Interleukin-6; IFN: Interferon; MHC-II: major histocompatability complex-II; T_H: T helper.

not available.

The patient was started on Hydrocortisone with marked improvement. Mineralocorticoid replacement therapy was not indicated as this is likely to be secondary adrenal insufficiency. The patient was to be followed up for an assessment of possible pituitary recovery.

Case 2

A 45-year-old man presented with cachexia, unintentional 25 kg weight loss over 6 months, recurrent nausea and vomiting on a background of chronic hepatitis C infection which he had acquired 20 years before from intravenous drug use. His past medical history included type 2 diabetes which recently became labile. He also developed recurrent hypoglycaemia without any major changes in his routine dietary and oral intake. There was no change or non-compliance with his oral hypoglycaemic regimen. Clinically he was unwell, cachectic with weight of 56.2 kg and height of 1.65 m, body mass index of about 20 kg/m^2. His BP was 120/70 sitting and 100/60 standing with pulse rate of 88 bpm. He appeared hypo-androgenic with sparse body hair distribution and an absence of pubic and axillary hair. His testes were 6 and 8 mL in size bilaterally. Further investigations are as follow: TSH 1.58 mU/L, fT4 9.1 pmol/L, fT3 4.7 pmol/L, ACTH < 1.1 pmol/L, Cortisol 326 nmol/L, LH 0.6 IU/L, FSH 0.3 IU/L, Testosterone < 0.7 nmol/L, Prolactin 252 mIU/L. A short-synacthen test revealed a rise from 326 to 430 nmol/L over 60 minutes consistent a sub-optimal response. On the basis of these results, no dynamic stimulation test was warranted.

The patient was given triple replacement therapy including thyroxine, cortisone acetate and testosterone isocaproate (Sustanon) injections. He made a rapid recovery and great symptomatic improvement. His hypoglycaemic crises resolved. Indeed, he became hyperglycaemic. He gained about 5 kg and was referred for treatment consideration with IFN-α therapy. However, he did not return for review and was subsequently lost to follow up.

Discussion

These two cases highlight the immuno-modulating effects of the hepatitis C viral particles and IFN-α therapy individually, especially in regards to pituitary pathology. Due to its rarity, the condition is poorly understood [1]. Theoretically however, it is thought that prolactin plays a major part in the pathogenesis of the condition [2]. The presence of HCV particles and IFN-α, both of which are potent immuno-modulators, further inflame the condition. The three combine to act through a similar pathway which was previously proposed for IFN-α related thyroid disease [3]. Prolactin is thought to activate the JAK/STAT pathways which lead to the activation of Interferon Regulatory Factor 1 (IRF1). Prolactin also

Table 1. Summary of Our Cases and Available Published Reports, Please Note Case 3 Involved Hepatitis B Infection

Authors and Year of publications	Gender:Age	Pituitary Antibody status	MRI findings	Treatment modality	Panhypopituitarism and therapy	Reversibility
1. Sakane et al, 1995	F:44	YES: GH3 cell	Normal	IFN-α monotherapy for 3 months	Y: Hydrocortisone and Thyroxine	Yes, after 11 months
2. Concha et al, 2003	M:39	NO:Normal Human Pituitary Tissues	Normal	IFN-α and RBV for 1 year	Y: Testosterone and Growth hormone	No
3. Chan et al, 2004	F:30 (HBV infection)	Not done	Anterior pituitary cyst	IFN-α monotherapy for 3 years	Y: Hydrocortisone, Oestrogen and Thyroxine	No
4. Ridruejo et al, 2006	F:54	Not done	Not done	IFN-α and RBV for 48 weeks	Y: No therapy	Yes, transient
5. Our case 1	M:34	Not available	Normal	IFN-α and RBV for 48 weeks	Y: Hydrocortisone	Unknown. Lost to follow up
6. Our case 2	M:45	Not available	Normal	Untreated	Y: Hydrocortisone, Testosterone and Thyroxine	No

activates T_H1 and T_H2 cytokine activities which lead to the development of autoimmunity. In addition, the T helpers are further in turn regulated by T regulator cells (T_{reg}). The latter function is dampened in the presence of IFN-α therapy, amplifying the PRL response, leading to the clinical expression of anterior pituitary deficiency [3]. The PRL hypothesis is probably more relevant in post-partum nursing mothers where hyperprolactinaemia predominates. However, only 50% of the discussed cases are females and none was breastfeeding. Genetic predisposition must play a part, as is the vascular supply. The anterior pituitary has an extensive vascular supply, exposing the pituitary cells to the HCV particles, IFN-α and associated antibodies, Figure 1. It remains unknown if the condition is reversible, especially once the virus has been terminated or cured with IFN-α therapy. The extermination of the HCV particles also reduces the stimulating effect helping the reversibility of the condition.

Previous published cases in the literature were sparse. The first case was described by Sakane et al [4] in 1995 in which the endocrinopathies developed 2 months (out of six) after stopping IFN therapy. This case was shown to have pituitary antibodies against GH3 cells, a rat pituitary tumor cell line that secretes growth hormone and prolactin. Fortunately, the condition was reversible. In 2003, Concha et al [5] reported a second similar case. The proposed panhypopituitarism was detected 1 year after the completion of therapy although there was no evidence of antipituitary antibodies. Chan et al [6] described a case of panhypopituitarism but in the presence of hepatitis B infection. The patient developed amenorrhoea whilst on treatment and displayed permanent panhypopituitarism thereafter. Ridruejo et al [7] in 2006 reported a possible case of reversible or spontaneously recovered hypophysitis whilst on combination IFN and RBV therapy. The diagnosis was clinically based in all cases using the temporal relationship with treatment, pituitary hormonal profile, pituitary magnetic resonance imagings, all of which are normal or non-contributory, and the absence of thyroid and other autoimmune markers. Except for case 3, all demonstrated the typical sequence of deficiencies in autoimmune hypophysitis where ACTH is the first to be affected, followed by TSH and then LH/FSH [8]. Antipituitary antibodies are also not available in most case as these remain poorly defined and the test is not routinely available in practice [8]. Contrary to de novo cases, none developed headache and/or visual disturbance. These few published cases are summarized in Table 1.

In addition, INF-α can unmask previously undetected pituitary Sheehan's syndrome or syndrome of inappropriate antidiuresis [9-11] which can be fatal if unrecognized.

The prevalence of pituitary dysfunction in relation to hepatitis C infection and IFN-α therapy is poorly known and appears very rare. Our previous report in postmortem cases found no evidence of pituitary involvement in untreated HCV cases [12]. This is not surprising given the rarity of clinical panhypopituitarism in relation to HCV infection and suggests that the condition occurs in an ad hoc fashion, presumably in genetically susceptible individuals. Surveillance therefore is not recommended. However, the diagnosis of pituitary dysfunction, either partial or complete should be considered in HCV patients with the appropriate clinical symptomatology. Similarly and not evidence based, patients should be followed up to assess for reversibility of the condition.

Conclusion

Hepatitis C infection and IFN-α associated pituitary dysfunction is rare but should be considered in the appropriate clinical setting. The condition is readily treatable and is potentially reversible.

Disclosure Statement

All authors have no conflict of interests relevant to this work.

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Publication: Springer eBook
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