Autoantibody Targets in Autoimmune Polyendocrine Syndrome Type 1 and Lymphocytic Hypophysitis

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Declarations

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I hereby certify that the laboratory work embodied in this thesis has in part been carried out at the Department of Medical Sciences, Uppsala Universitet, Sweden under the supervision of Prof Olle Kämpe and Dr Sophie Bensing. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

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Contents

SYNOPSIS

Background: Autoimmune diseases arise from the breakdown of central tolerance resulting in the escape of self reactive T-lymphocytes from the thymus to the periphery. As a group of conditions, autoimmune diseases occur in approximately 5% of the general population and represent the third most common cause of morbidity, placing considerable expenses on the health care system and society. Understanding the underlying pathogenesis and pathophysiology of these diseases is therefore important for the correct diagnosis and treatment of these patients. While some autoimmune diseases have been paid particular attention, little is known about the pathogenesis of the pituitary autoantibodies.

Aims: To identify target autoantigens in the pituitary autoimmune disease lymphocytic hypophysitis and autoantigen(s) relating to pituitary manifestations in APS1 patients.

Methods: A pituitary cDNA expression library was immunocreened with lymphocytic hypophysitis and APS1 patient sera to identify target autoantigens. These were then tested in an ITT assay for autoantigen specificity to relating to the disorders. Immunofluorescence of pituitary tissue was performed to determine the cell types targeted in the disorders.

Results: Two APS1 autoantigens were identified, a major autoantigen ECE-2 and a minor autoantigen TSGA10, although neither apparently correlated to pituitary manifestations in APS1. T-box 19 was also identified as a significant minor autoantigen in 10.5% of lymphocytic hypophysitis patients. Immunoreactivity in a single lymphocytic hypophysitis patient against cells of the intermediate lobe of the guinea pig pituitary is also reported.

Discussion: Immunoscreening a target organ cDNA expression library is a valuable method for identifying novel autoantigens, with immunopreciptation assay a quick and reliable method for analysing a large cohort of patients for autoantibodies. We have identified another two APS1 autoantigens and the first significant autoantigen in lymphocytic hypophysitis. While further characterisation of these autoantigens are required, these novel findings broaden our current understanding of pituitary autoimmunity.

Abbreviations

21-OH	21-Hydroxylase
AADC	Aromatic L-amino acid decarboxylase
ACTH	Adrenocorticotropic hormone
ANA	Antinuclear antibodies
AIRE	Autoimmune regulator gene
APS1	Autoimmune Polyendocrine Syndrome Type 1
CADPS	Ca^{2+}-dependent secretion activator protein for secretion
CHD8	Chromodomain helicase DNA binding protein 8
CRH	Corticotrophin releasing hormone
DM	Type 1 diabetes mellitus
ECE-1	Endothelin Converting Enzyme-1
ECE-2	Endothelin Converting Enzyme-2
ET	Endothelin
FSH	Follicle-stimulating hormone
GAD	Glutamic acid decarboxylase
GH	Growth hormone
GHD	Growth hormone deficiency
IF	Immunofluorescence
ITT	*in vitro* transcription and translation
LH	Luteinizing hormone
LPH	Lipoprotein
MSH	Melanocyte stimulating hormone
mTECs	Medullary thymic epithelial cells
NALP5	NACHT leucine-rich-repeat protein 5
NEP	Neprilysin
NSE	Neuron specific enolase
PGSF1a	Pituitary gland specific factor 1a
PGSF2	Pituitary gland specific factor 2
POMC	Pro-opiomelanocortin
SCC	Side-chain cleavage enzyme
SLE	Systemic lupus erythematosus
TPH	Tryptophan hydroxylase
TSAs	Tissue-specific antigens
TSH	Thyroid-stimulating hormone / Thyrotropin

1. GENERAL INTRODUCTION

The human immune system is capable of reacting to an enormous array of microbes. In the development of the immune repertoire lymphocytes capable of recognising self antigens are constantly produced yet are rendered inactive or destroyed in the maturation process. This ability to discriminate self from non-self is vital to a healthy functioning immune system, the failure of which leads to the host's immune system attacking its own cells, a phenomenon known as autoimmunity. The concept of autoimmunity was first described over 100 years ago by Ehrlich and Morgenroth after the observation that goats injected with foreign hematopoietic cells elicited a potent immune response, whereas no reaction was elicited against their own cells (1). They hypothesised that….

> "*The organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from working against the organism's own elements and so giving rise to autotoxins. So, we might be justified in speaking of a* horror autotoxicus *of the organism. These contrivances are naturally of the highest importance to the individual."*

These "contrivances" are known today as immunological or self tolerance and the state of "horror autotoxicus" of self-reactivity has been recognised as the cause of autoimmune diseases. There are over 80 autoimmune diseases currently recognised which affect 3-10% of the world's population with the incidence increasing over the past three decades (2, 3).

1.1 Definition of an Autoimmune Disease

Originally conceived in 1957 by Witebsky (4) and revised by Rose in 1993 (5), a disease may be classified as autoimmune by the three criteria.

1. Direct Proof: Disease is induced by transfer of pathogenic antibodies and/or T cells to a healthy recipient
2. Indirect Proof: Inducible disease in an experimental animal model by immunisation with known autoantigen or autoantibodies and/or self reactive

T cells isolated from the major organs targeted in the autoimmune disease, or genetically engineered

3. Circumstantial Evidence: Autoimmune disease suspected from clinical evidence including; association with other defined autoimmune disease, lymphocytic infiltration of target organs, harbouring a particular susceptibility MHC haplotype, and favourable response to immunosuppressive treatment

Autoimmune diseases are classically divided into two categories: organ specific diseases e.g. thyroid disease, type 1 diabetes mellitus and coeliac disease, and systemic illnesses including systemic lupus erythematosus, rheumatoid arthritis and systemic sclerosis. The diseases are mostly chronic conditions that progress over the course of years and are characterised by the presence of autoantibodies towards target autoantigens (6) (Table 1). These autoantibodies can be used as predictors of underlying autoimmune disease as they are detectable long before the clinical onset of disease and if detected during this phase, disease manifestation may possibly be preventable (7-9). Furthermore, it is believed the presence of these antibodies may also be a predictor of the course of the autoimmune disease in a person with established disease manifestation (7).

1.2 Self Tolerance in Autoimmune Disease

Tolerance to self antigens is vital in maintaining a healthy immune system; the breakdown results in the pathogenesis of autoimmune disease. The exact mechanism of how self tolerance is achieved is not fully understood, hence several hypotheses from experimental data have been formulated, including:-

1. Clonal Deletion: Immature auto-reactive lymphocytes undergo programmed cell death during the development and differentiation process (10).
2. Clonal Anergy: Anergy is the state of nonresponsiveness to an antigen. Auto-reactive T and B cells, when exposed to certain antigenic peptides, become inactivated and unable to elicit an immune response (11).
3. Anti-idiotype Network: A network of antibodies naturally existing within the body, capable of neutralising auto-reactive lymphocytes by preventing the receptor from combining with antigen (12).

Table 1: Specific autoantibodies detected in various autoimmune diseases

	Autoimmune Disease	Autoantibodies Detectable
Organ Specific	Hashimoto's Thyroiditis	Thyroid peroxidase (TPO) Thyroglobulin Na+/I- symporter
	Graves' disease	Thyroid-stimulating-hormone receptor
	Pernicious Anaemia	H+/K+ ATPase pump Intrinsic factor
	Addison's disease	21-hydroxylase 17-hydroxylase P450 Side-chain cleavage enzyme (SCC)
	Myasthenia gravis	Acetylcholine receptors
	Type 1 Diabetes	Insulin Glutamic acid decarboxylase (GAD65) Insulinoma Associated antigen 2 (IA2)
	Multiple sclerosis	Myelin basic protein Oligodentritic glycoprotein
	Coeliac disease	Transglutaminase Gliadin
	Vitiligo	Tyrosinase SOX 9 SOX 10
	Crohn's disease	Ubiquitination factor E4A (UBE4A)
	Ulcerative colitis	Tropomyosin 5 (TM5) Peripheral anti-neutrophil nuclear antigen (pANNA)
Systemic Diseases	Sjogren's Syndrome	Sjogren's syndrome antigen A (SS-A/Ro) Sjogren's syndrome antigen B (SS-B/La) Antinuclear antibodies (ANAs) Ribonucleoprotein (RNP)
	Systemic Lupus Erythematosus	ANAs Double stranded DNA SS-A/Ro SS-B/La RNP Smith Antigen (Sm)
	Rheumatoid arthritis	Cyclic citrullinated peptides Peptidylarginine deiminase 4 (PAD4) BRAF Carbonic anhydrase III PGSF1a
	Scleroderma / Systemic sclerosis	ANAs Centromere (CENP-B) Topoisomerase I RNA polymerase III

4. Clonal Ignorance: Lymphocytes with affinity to self-antigens exist but do not bind the antigen to cause an effect because the antigen is either sequestered, is in low concentration or interaction is too weak to elicit a response.
5. Receptor editing: Immature B cells with strong affinity for self antigens undergo editing whereby the light-chain and sometimes the heavy-chain MHC peptide sequences are rearranged to form a new non auto-reactive receptor.
6. Suppressor cells: Reactivity to self antigens is down regulated or inhibited either directly by suppressor T cells or via the production of cytokines including TGF-beta and IL-10.

1.3 Genetic factors in Autoimmune Disease

Autoimmune diseases share a number of characteristics suggesting common etiologic pathways or mechanisms, including reactivity to self-antigens by the humoral and/or cellular immune systems, as well as genetic associations. From the observation that autoimmune diseases seem to cluster both with other autoimmune diseases and within families, autoimmunity is believed to be caused by a combination of common specific genes (13, 14). However, other influences such as environmental factors must also be involved in disease manifestation. The concordance rate between monozygotic twins is low so conferring genes alone are not enough to trigger disease (15, 16).

1.4 Major Histocompatibility Complex (MHC) genes

The MHC proteins encoded by the MHC class I and MHC class II genes are highly polymorphic glycoproteins involved in the presentation of peptide antigens to T cells. The MHC genes appear to be correlated with autoimmune disorders with an increased susceptibility seen in association with particular polymorphic regions of MHC alleles and haplotypes of the DRB1, DQB1 and DQA1 genes (17-22). A striking association is seen in Coeliac disease with 90% of patients possessing genetic variants of the HLA-DQ2 allele (23). The HLA-DQ8 is also commonly seen in individuals with biopsy proven coeliac disease patients. These two alleles are also reported in association with an increased propensity of Type I diabetes (24, 25) as well as other autoimmune diseases in general. Haplotypes of the HLA-DR3 allele have also been associated with multiple autoimmune disorders including Graves' disease (26), Addison's disease (27) and SLE (28). However, possession of these MHC genes

implies only a susceptibility for disease development and alone is not sufficient to cause disease. In addition, other HLA haplotypes have a protective effect against disease; DR7 is protective against Graves' disease (29), whereas DR11, DR15, DQ6 and DQ7 are protective against Type I Diabetes (30).

1.5 Non-MHC genes

As MHC genes alone are not sufficient to cause autoimmune disease, the focus has been shifted to identifying new non-MHC genes. A number of genes have been identified including the cytotoxic T lymphocyte antigen-4 (CTLA-4) and the protein tyrosine phosphatase nonreceptor 22 (PTPN22) gene.

CTLA-4 plays an important role in the negative regulation of T-cell activation (31). Additionally, mice lacking the CTLA-4 gene develop autoimmunity suggesting this gene may contribute to the pathogenesis of autoimmune disease in humans. The presence of a number of SNPs in the gene have been established in association studies of patients with Type 1 Diabetes and Graves' disease (32-37) as well as Addison's disease (38, 39), coeliac disease (40), autoimmune hypothyroidism (41), primary biliary sclerosis (42), multiple sclerosis (43), SLE (44), and rheumatoid arthritis (45, 46). Administration of CTLA-4 blocking antibodies in the immunotherapeutic treatment of patients with advanced melanoma may also induce the onset of autoimmune manifestations such as enterocolitis, dermatitis, and lymphocytic hypophysitis (47-49).

The PTPN22 gene is a protein tyrosine phosphatase expressed primarily in lymphoid tissues. The gene functions as a strong negative regulator of T-cell activation, hence has been a focus in the potential role in the development of autoimmune disease. The 1858T minor allele of a R620W (1858 C>T) polymorphism was first identified in association to patients with Type 1 diabetes mellitus (50-53). This minor allele has since been associated with rheumatoid arthritis (54-56), systemic lupus erythematosus (SLE) (55, 57-61), Graves' disease (62), Hashimoto thyroiditis (57), and autoimmune thyroid disease (63).

1.6 Environmental factors

Besides genetic influences, the manifestation of autoimmune diseases is dependant upon a complex interaction of environmental factors. Various chemical, dietary factors and lifestyle can all effect disease onset and outcome. Autoimmune diseases are far more common in females, yet the mechanism(s) behind the sex bias remain unknown. Theories to explain the phenomenon include difference in sex hormones (64-68), foetal microchimerism in pregnancy (69, 70), sex chromosome abnormalities (71-76) and skewed X chromosome inactivation (77, 78), although these remain to be confirmed.

Autoimmune diseases can also be induced by both bacterial and viral infections, or be drug induced. Interferon (IFN) alpha therapy used in the treatment of chronic viral infections is known to play a role in the pathogenesis and maintenance of certain autoimmune diseases including systemic lupus erythematosus (SLE), type 1 diabetes, autoimmune thyroid disease (79) and lymphocytic hypophysitis (80).

1.7 Monogenic Diseases

In general, multiple interacting factors including both genetic and environmental are involved in the development of autoimmune disease. However, there are a few rare diseases caused by mutations in a single gene. Immune dysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) is caused by defects in the FOX3 gene impairing the suppressive function of T-reg cells (81-83). Another classical example is autoimmune lymphoproliferative syndrome (ALPS) resulting from mutations in Fas, Fas ligand and caspase, genes involved in the control of apoptosis in lymphocytes which blocks the elimination of activated peripheral T-cells (84-89). A third monogenic autoimmune disease is autoimmune polyendocrine syndrome type 1 (APS1), characterized by mutations in the Autoimmune Regulator (AIRE) gene, an important gene involved in central tolerance in the thymus (90, 91). Although rare, these monogenic disorders provide the opportunity to study autoimmunity in a more simplified way and have provided invaluable information about the pathogenesis of autoimmunity.

2. APS1

Autoimmune polyendocrine syndrome Type 1 (APS1 – OMIM 240300) alternatively known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare monogenic autoimmune disease caused by mutations in the autoimmune regulator (AIRE) gene. The syndrome was first officially described in juvenile patients in 1956 (92), however the first description of hypoparathyroidism in association with candidiasis was published in 1929 (93) and with idiopathic adrenal insufficiency in 1946 (94).

APS1 is a rare disease with only an estimated 500 people affected worldwide, although it is more common in some populations with an estimated occurrence of 1:25000 in Finland (95), 1:14000 in Sardinia (96) and 1:9000 in Iranian Jews (97). APS1 is characterised by the classical triad of autoimmune diseases; Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. Chronic candidiasis is generally the initial manifestation to emerge, typically before the age of 5 years. Hypoparathyroidism usually manifests subsequently before the age of 10 years followed lastly by Addison's disease before 15 years of age (95, 98-100). The emergence of further autoimmune disorders, all of which are organ specific, continues until at least the fifth decade of life and include gastrointestinal dysfunction, type I diabetes mellitus, hypothyroidism, chronic active hepatitis, alopecia, vitiligo, pernicious anemia and gonadal failure with premature ovarian failure presenting more frequently than primary testicular failure. In addition, ectodermal manifestions are also frequently observed in APS1 patients with variable penetrance. The earlier the first manifestation of APS1 appears, the greater the likelihood of these secondary components developing, whilst the converse is also true, the later the initial symptom appears the fewer secondary autoimmune diseases that will develop (95, 99).

2.1 Genetics of APS1

The AIRE gene was identified as the causative gene in APS1 by positional cloning simultaneously by two groups in 1997 (90, 91, 95, 99). The gene is located on chromosome 21q22.3 and consists of 14 exons spanning over 13kb of genomic DNA encoding a 58kDa protein of 545 amino acids. To date, approximately 60 mutations have been reported including missense, nonsense and frameshift mutations. While

mutations have been detected throughout the entire coding sequence, three mutational hotspots have been observed which cluster in three of the functional domains of the protein; the HSR domain, the SAND domain and the first PHD domain (101-105). Furthermore, three distinct founder mutations have been ascertained which account for a high proportion of APS1 cases. The nonsense mutation, R257X, accounts for 83% of Finnish APS1 patients and is also common in patients of Italian, central and eastern European decent (90, 91, 103, 105, 106). A 13bp deletion in exon 8 of the AIRE gene, 967-979del13bp, is frequently detected in North American, British and Norwegian APS1 patients and accounts for 50-70% of cases (104, 107), while a Y85C missense mutation is common in Iranian Jews (103) and an R139X mutation in often observed in APS1 patients of Sardinian decent (96).

Correlations between the genotype and phenotype in APS1 patients are not clearly evident. Even siblings harbouring the same mutation can differ substantially in their clinical presentation, implying there are other modifier genes involved (95, 108). A gender association was determined with a reduced incidence and later onset of hypoparathyroidism in male patients irrespective of their particular AIRE mutation (109). Interestingly, candidiasis is rarely observed in Iranian Jews harbouring the Y85C missense mutation (97), while an R257X mutation has been associated with an higher incidence of mucocutaneous candidiasis (110).

With the identification of the AIRE gene, the diagnostic criteria for APS1 has been reviewed, as some patients with identified mutations in both alleles of the AIRE gene may not present with two of three main manifestations of adrenal insufficiency, hypoparathyroidism and mucocutaneous candidiasis (111). A study of a large Finnish cohort revealed only 22% of patients had 2 of the 3 cardinal manifestations at 5 years of age, 65% by age 10 and 93.5% by 30 (112).

The importance of the AIRE gene can be demonstrated by its highly conserved homology between species. The coding region of the human mouse AIRE gene share 77% homology while the proteins are 71% homologous, with the functional domains of the protein including the HSR, SAND and PHD domains, highly conserved between the species (113-115). Furthermore, a study has been conducted comparing Aire

among different phylogenetic groups and included human, mouse, opossum, chicken, xenopus, zebrafish and pufferfish. The study showed the PHD domain in human Aire is highly evolutionarily conserved throughout all groups. Moreover, they deduced the most highly conserved regions of the protein were those where point mutations are found in human APS1 patients. While there has been rapid evolutionary change in certain regions of the gene during evolution, the conserved homogenic regions support an Aire dependent mechanism of T cell tolerance which can be traced back to the emergence of the bony fish (116).

2.2 AIRE localisation

Tissue expression profiling has shown AIRE to be expressed in numerous tissues with the highest level of expression in the thymus. Northern blot analysis of human tissues showed AIRE mRNA to be expressed in the thymus, lymph node, foetal liver, appendix and peripheral blood lymphocytes, but not in any of the other tissues studied including the adrenals, adult liver and pancreas (90) plus spleen by in situ hybridisation (104). RT-PCR analysis of mouse tissue however, has shown Aire expression throughout the entire body tissues including the thymus, spleen, lymph node, adrenal gland, thyroid gland, lung, heart, liver, kidney, ovary, testis, brain, skeletal muscle, and fetal liver (113, 117, 118). Expression in the thymus has been localised to the medullary thymic epithelial cells (mTECs) (119) along with blood lymphocytes, neutrophilic granulocytes and monocytes as well as differentiated dentritic cells in the periphery (120-122). Expression in parenchymal tissue has been detected, although at low levels and with low consistency (119, 123), with the exception of the ovary (124).

The normal cellular distribution of AIRE has two distinct patterns; as speckled domains or “nuclear dots” excluding the nucleoli, and cytoskeletal filaments or microtubular staining within the cytoplasm (119, 120, 125). This cellular distribution of the protein was dramatically altered in mammalian cultured cells with in vitro expression of AIRE mutants, both naturally occurring and introduced (103, 125). Furthermore, the mutant polypeptides lost their function as transcriptional activators of reporter genes (103).

2.3 AIRE function in mouse models

How immunological tolerance to self antigens is achieved has long been speculated. To avoid autoimmunity, self reactive thymocytes need to be removed from the circulation. Immature lymphocytes reactive to thymic tissue are removed soon after generation in the thymus, yet how lymphocytes reactive to self proteins expressed in parenchymal tissues were identified and removed remained unexplained. Hence the theory of two mechanisms of removal was conceived: (1) central tolerance where tolerance to ubiquitously expressed or blood-borne antigens is achieved in the thymus, and (2) peripheral tolerance for eliminating or inactivating self-reactive lymphocytes encountered in the periphery.

Studying of monogenic autoimmune disease gives a unique insight into the mechanism by which the immune system achieves tolerance and in its breakdown how autoimmune diseases develop. The study of *Aire* knockout mouse models has provided invaluable insight into how autoimmune disease develops and has challenged the theories of autoimmune disease processes.

2.4 APS1 Animal Models

2.4.1 Mouse knockout models

The first mouse models of APS1 emerged independently from two groups in 2002 (124, 126). Knockout of the *Aire* gene in the C57BL/6 mouse strain leads to spontaneous development of a highly selective autoimmune attack directed towards multiple specific organs. Most noticeably, there was autoimmune destruction of salivary gland, retina, ovary and prostate all of which was confined within particular substructures of the organs. The number of organs infiltrated increased with age as seen in human APS1 patients. Serum autoantibodies were detectable in all knockout mice correlating to the organs with lymphocytic infiltration (124, 126). *Aire* knockouts have also been independently produced in NOD mice (127, 128) and in BALB/c X C57BL/6 mice (128, 129). All mice develop organ specific autoimmunity which differs in severity and in which organs are targeted, depending on the strain. No mouse strain develops any of the classical triad of Addison's disease, hypoparathyroidism and mucocutaneous candidiasis seen in human APS1 patients.

The immune system components were not deficient in comparison to their wild type counterparts with the exception of two components. Both the number of mTECs and activated/memory CD44hiCD62Llo T cells in both the $CD4^+$ and $CD8^+$ compartments of the peripheral lymphoid organs were observed at twice the frequency of wild type mice, alluding to Aire having a role in eliminating self reactive T cells in the periphery (124).

The accepted theory of central tolerance induction has been reviewed after the discovery of promiscuous gene expression in the thymus. In particular, mTECs have been shown to express a highly diverse set of genes essentially representing tissues from the entire body (130, 131). In addition to the increase in mTECs in Aire-deficient mice, microarray and expression studies have demonstrated the importance of Aire in the regulation and transcription of multiple tissue-specific antigens (TSAs) promiscuously expressed in mTECs (124, 131). Antigen presentation through the expression of a TSA by mTECs results in the deletion of all T cells self-reactive to that particular TSA. Aire-deficient mouse models demonstrate the key function of AIRE in the removal and inhibition of autoreactive T-cells in the thymus before they reach the periphery (124, 132, 133), a breakdown of which results in self-reactive T lymphocytes escaping to the periphery and the organ-specific autoimmune destruction seen in APS1 (Figure 1). Yet, Aire does not regulate all TSAs expressed in the mTECs as some TSAs including C-reactive protein and GAD are still expressed in the Aire-deficient mice (124). Furthermore, transplantation of a thymus from Aire-deficient mice depleted of all thymocytes is capable of producing autoimmune destruction in alymphoid recipient (124), confirming AIRE expression in thymic parenchymal tissue is not necessary for controlling peripheral autoimmune attack.

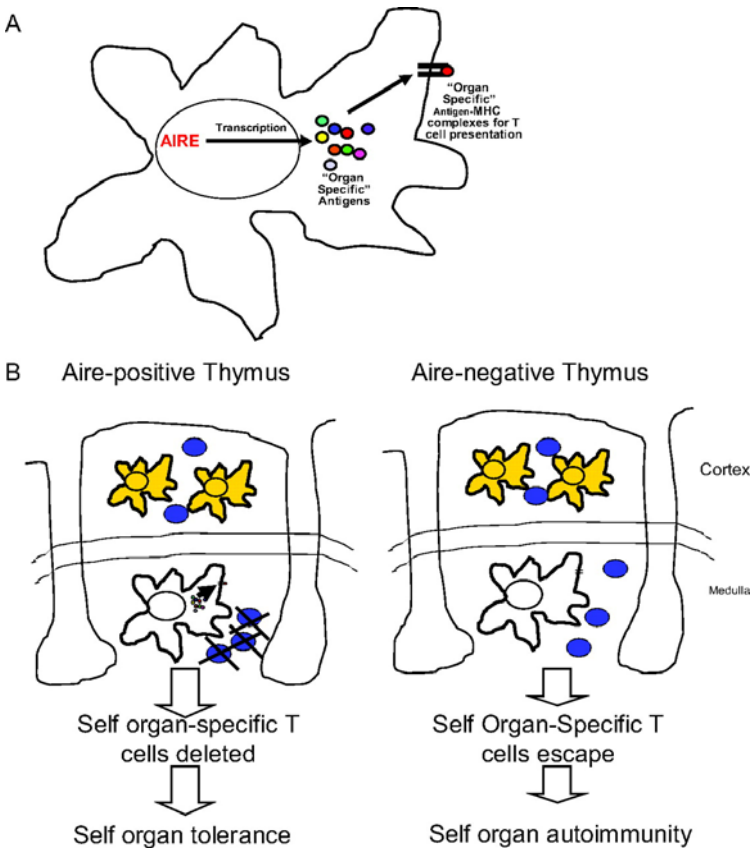


Figure 1. Model of the function of Aire in the thymus. **(A)** Aire appears to help mediate the transcription of many self-antigens in mTECs in the thymus. **(B)** Impact of Aire on T-cell selection. These self-antigens are then presented in the thymus to developing thymocytes (blue-colored cells) in the medulla, resulting in the deletion of self-antigen specific thymocytes in this compartment. In the absence of Aire, the self-antigens fail to be generated by these mTECs, and self-antigen specific T cells mature and escape the thymus and migrate into the periphery and promote autoimmune responses (Anderson *et al.* 2008) (134).

2.4.2 Mouse knockin models

A dominant AIRE mutation resulting in an APS1-like phenotype has also been reported in an Italian kindred. A G228W heterozygous genotype was associated with dominantly inherited hypothyroid autoimmune thyroiditis in this family. In addition, some affected members also have features of APS1 including hypoparathyroidism and mucocutaneous candidiasis, but not adrenal insufficiency (135).

To study this genotype further, Su *et al* produced a knockin mouse model, heterozygous for the G228W mutation. In these mice the mutation acted in a dominant-negative fashion, suppressing promiscuous gene expression in the thymus and inhibiting wild type Aire from reaching sites of active transcription and instead,

localising the protein within nuclear inclusion bodies of mTECs. This resulted in autoimmune infiltration and destruction of specific organs including salivary and lacrimal infiltration, but not of the entire organ spectrum seen in Aire-deficient mice (136). This demonstrated that a reduction in AIRE expression leads to a global defect in central tolerance with autoreactive T cells not being deleted and resulting in dominant autoimmune disease.

2.5 Autoantibodies in APS1

A breakdown in central tolerance mediated by AIRE leads to self-reactive lymphocytes reaching the periphery and consequently the progressive lymphocytic infiltration and autoimmune destruction of multiple organs throughout the body. Consequently, high titre autoantibodies are a characteristic feature of APS1 with many autoantigens being associated with a particular disease manifestation (Table 2). Autoantibodies can be detected years before the onset of disease, and therefore can be good predictors of future disease progression and useful in improved targeted treatment strategies.

In addition to these more frequent polyendocrinopathies, cholelithiais, hypo- or asplenism and ectodermal dystrophies including enamel and nail dysplasia and keratoconjuctivitis are also often described in APS1 patients. Some of the more uncommon features include exocrine pancreatic insufficiency, squamous cell carcinoma of the mouth or esophagus, chronic iridocyclitis and pituitary deficiency (137). Pituitary deficits are reported in up to 7% of APS1 patients. Deficits can manifest as either single or multiple pituitary hormone deficiencies. The most commonly reported pituitary deficit is isolated growth hormone (GH) deficiency, with partial adrenocorticotropin hormone deficiency, isolated hypogonadotroph hypogonadism and central/idiopathic diabetes insipidus also being described (95, 138-142). APS1 has also been reported in combination with lymphocytic hypophysitis in a French-Canadian patient with GH deficiency. An MRI scan showed the characteristic ring-enhancement seen in lymphocytic hypophysitis (143). Immunoreactive staining to the pituitary gland has been investigated in two separate studies on APS1 patients with GH deficiency. They showed staining of the median eminence of dopamine nerve terminals as well as gonadotropes (144). Staining of the fibre-plexus in the intermediate lobe and scattered cells within the anterior pituitary of which 40-50%

were GH positive have also been reported (145). Double labelling with known pituitary antigens including AADC, GAD, TH and TPH did not account for the entire staining seen in these patients (144, 145), suggesting there is another autoantigen corresponding to these pituitary manifestations that has not been identified. Pituitary manifestations are rarely investigated in APS1 patients unless overt symtoms are observed. It is therefore likely that a reported rate of pituitary manifestations of 7% among APS1 patients is lower than the actual rate.

Table 2. Association of the major autoantigens with the clinical manifestations of APS1

Clinical Manifestation	Frequency (percentage)	Autoantigen	Frequency (percentage)
Addison's disease	22-100	21-hydroxylase	66
Chronic candidiasis	18-100	N/A – T cell mediated	-
Hypoparathyroidism	76-100	NALP5	41
Gonadal failure	17-69	17-alpha hydroxylase SCC	44 52
Gastrointestinal dysfunction	6-26	Histidine decarboxylase (HDC) Tryptophan hydroxylase (TPH)	37 45
Autoimmune thyroid disease	2-36	Thyroglobulin (TG) Thyroid peroxidase (TPO)	36 36
Type I diabetes mellitus	2-33	Glutamic acid decarboxylase (GAD65) IA2 Insulin	37 9 7
Chronic active hepatitis	5-31	Aromatic L-amino acid decarboxylase (AADC) P450-IA2/CYP-IA2 P450-2A6/CYP-2A6	51 8 ?
Alopecia	13-72	Tyrosine hydroxylase (TH)	40
Vitiligo	0-26	AADC SOX9 SOX10	51 15 22
Pernicious Anaemia	0-31	Parietal cells (PCA)+ anti-intrinsic factor	?

3. Lymphocytic Hypophysitis

Lymphocytic hypophysitis is an organ specific autoimmune disease of the pituitary characterised by lymphocytic infiltration into the pituitary gland. As with the majority of autoimmune diseases, the disorder presents more commonly in females than males with a ratio of 6:1 (146) and also has a striking correlation with pregnancy with approximately 60% of women presenting in the third trimester or postpartum period. The average age of diagnosis in women is 34.5 years and 44.7 years in men (147). A

few cases of lymphocytic hypophysitis have also been reported in adolescents (148, 149) and also of elderly onset (150, 151).

The autoimmune infiltration of the pituitary may be localised to the particular sections of the pituitary and as such lymphocytic hypophysitis may be divided into three main subtypes:

1. Lymphocytic Adenohypophysitis: Lymphocytic infiltration is confined to the anterior portion of the pituitary
2. Lymphocytic Infundibuloneurophypophysitis: Both the pituitary stalk and posterior pituitary are targeted by lymphocytic infiltration.
3. Lymphocytic Panhypophysitis: The entire pituitary gland is affected by the autoimmune process.

3.1 History

Lymphocytic hypophysitis was first described as a distinct clinical entity by Goudie and Pinkerton in 1962 (152). They reported the case of a 22 year old woman presenting 14 months after the birth of her second child with severe lower abdominal pain radiating to the right iliac fossa, vomiting and diarrhoea, who died from shock eight hours after the removal of an unruptured gangrenous appendix. Autopsy revealed lymphocytic infiltration into both the thyroid and pituitary glands and severely atrophic adrenal glands. Noting the coexistence of Hashimoto's thyroiditis, a well characterised autoimmune disease, the authors concluded the existence of lymphocytic infiltration into both glands was not coincidental but more likely due to the onset of autoimmune reactions to both the pituitary and thyroid.

The first antemortem cases of lymphocytic hypophysitis were diagnosed from transphenoidal hypophysectomy 20 years later in 1980 simultaneously by Quencer and Mayfield (153, 154). Infundibuloneurohypophysitis was first described by Saito et al in 1970 (155) and lymphocytic panhypophysitis by Nussbaum et al in 1991 (156).

3.2 Epidemiology

The incidence of lymphocytic hypophysitis in the general population is unknown owing to the relative unawareness of the disease until fairly recently. Reported cases in

the literature have increased dramatically over the past years. Only 16 cases of lymphocytic hypophysitis were reported in the 20 years (1962-1981) following its initial description as an autoimmune disease. With the introduction of the MRI and greater clinical awareness among endocrinologists the number of patients diagnosed increased substantially in the ensuing 20 years (1982-2001) with 290 reported cases and a further 73 cases from 2002-2004 (146). The frequency of lymphocytic hypophysitis in the general population has been estimated at 1 in 9 million per year incidence (157) but with increasing awareness and with many subclinical cases the number is likely to be underestimated.

Pituitary biopsy still remains the gold standard for the diagnosis of lymphocytic hypophysitis. These patients generally represent acute cases whereas patients presenting for example with hypopituitarism in the post-partum period are far less likely to undergo a biopsy. A number of large surgical studies have been undertaken examining pituitary surgery sections in the UK (157) Germany (158, 159) and the USA (160). They have shown inflammatory conditions including both lymphocytic hypophysitis and granulomatous hypophysitis account for 0.24 to 0.8% of all pituitary surgeries performed.

3.3 Aetiology

Following the three criteria of direct proof, indirect proof and circumstantial evidence, there is much support that lymphocytic hypophysitis should be classified as a recognised autoimmune disease. While no direct proof is evident as with most autoimmune diseases, recent advances in establishing a successful animal model of hypophysitis have been made, providing the indirect proof of autoimmunity.

Since the original description in 1962, a plethora of circumstantial evidence has been reported cementing lymphocytic hypophysitis as indeed an autoimmune disease. The disorder is more frequently observed in the female population, and lymphocytic infiltration of the pituitary has been widely reported in these patients. Several patients also have responded well to glucocorticoid treatment, with symptoms of hypopituitarism improving or even resolving.

The coexistence of other autoimmune diseases in also frequently reported in lymphocytic hypophysitis patients in as many as 18-25% of cases (146, 161-163). The most commonly associated autoimmune condition is autoimmune thyroid disease, foremost Hashimoto's thyroiditis (152, 164-166) with Graves' disease also being reported in numerous cases (167, 168). Other autoimmune disease reported are both organ specific and systemic in nature and include SLE (169-172), Addison's disease (173, 174) type 1 diabetes mellitus (162, 167), atrophic gastritis (167, 175), Sjögren's syndrome (176), APS1 (143), primary biliary cirrhosis (151) and autoimmune hepatitis (177).

Limited studies have been done on HLA typing of hypophysitis patients. In the small series of lymphocytic hypophysitis that underwent MHC class II typing, the DR4 which is frequently observed in patients with various autoimmune diseases was present in approximately 41% of cases. Whereas, the DR5 often seen in Japanese patients with Graves' disease and Hashimoto's thyroiditis (178, 179), was found in 23% of lymphocytic hypophysitis patients (161).

The identification of pituitary autoantibodies in the sera of lymphocytic hypophysitis patients also strongly suggests the presence of an underlying autoimmune entity. Few autoantigens have been identified as potential targets in the manifestation of lymphocytic hypophysitis, yet the main autoantigen remains unidentified. Establishing a reliable diagnostic test for pituitary autoantigens in lymphocytic hypophysitis would be invaluable in the diagnosis of these patients and eliminate unnecessary surgical intervention.

3.4 Clinical spectrum

Lymphocytic hypophysitis patients usually present with chronic headaches and visual disturbances due to an upwardly expanding pituitary mass. Visual disturbances including diplopia and decreased visual acuity are caused by compression of the optic chiasm. Headaches result from the distension and distortion of the dura mater and diaphragma sellae by the expanding mass (163). The disease frequently presents in the second or third trimester of post partum period, the existence of the disease however,

does not necessarily confer secondary infertility with many subsequent pregnancies being reported (148, 180-182).

Autoimmune destruction of the pituitary cells results in a progressive disturbance in pituitary hormone production affecting ACTH, prolactin, and TSH production followed less frequently by somatotroph and gonadotroph cell function. ACTH deficiency caused by the destruction of the corticotrophs, is the most common hormonal deficit occurring in 65% of cases (183) and can lead to secondary adrenal failure if untreated. It is frequently the initial pituitary deficit observed and often the only symptom of hypopituitarism, in contrast to hypopituitarism due to tumours in which the initial symptoms relate to growth hormone (GH) or gonadotroph deficiency with ACTH dysfunction generally being the last component to develop (184-186).

The continuing autoimmune destruction progressively disrupts hormone production in the pituitary with thyrotropin (TSH) deficiency being a frequently reported manifestation (162). The effect on prolactin levels are however variable, with both deficiencies and over production of the hormone being described. Prolactin deficiency is observed in approximately 11% of hypophysitis patients most often diagnosed clinically by the inability to lactate post-partum. Hyperprolactinemia has been observed in as many as 23% of patients of all ages including men and elderly women. However diagnosis can be difficult as elevated levels are normal during pregnancy and while breast-feeding.

Infundibuloneurohypophysitis manifests with diabetes insipidus. It has been postulated this may be caused by the pressure exerted from the expanding pituitary mass, yet as diabetes insipidus is rarely observed pre-operatively in patients with adenomas, it is more likely due to autoimmune destruction of the posterior pituitary tissue and stalk.

Autoimmune destruction of the pituitary exists in both transient and chronic forms. Several transient cases have been reported in which a pituitary mass spontaneous resolves (165, 187). Chronic autoimmune attack is more common where the continuing autoimmune attack causes post-inflammatory fibrosis leading to pituitary gland atrophy and an empty sella.

3.5 Pathological and Radiological features

3.5.1 Macroscopic appearance

The pituitary gland of lymphocytic hypophysitis patients may appear either normal, grossly enlarged or atrophied. In most autopsy cases there was significant atrophy of the pituitary gland accompanied by secondary atrophy of the adrenal glands. At surgery, the gland is usually firm and tough both in its appearance and to the touch. The gland appears white to grey or yellow in colour and is adherent to the walls of the sella making it difficult to surgically remove (161).

3.5.2 Microscopic appearance/Histopathology

Lymphocytic hypophysitis is characterised by extensive, diffuse infiltration consisting mainly of lymphocytes with some plasma, mononuclear cells and occassionally eosinophils (166, 188). Mast cells (189) and dendritic-like pituitary folliculo-stellate cells (190) have also been identified in the infiltrate.

Various degrees of oedema and fibrosis have been observed as well as aggregation of lymphocytes to form lymphoid follicles and germinal centres (146). The autoimmune destruction of the gland does not appear to be confined to a particular pituitary cell type and foci of unaffected pituitary tissue remains morphologically normal (161).

The lymphocytic infiltrate has been shown to consist of activated T cells with a dominant expression of CD4+ cells (CD4+/CD8+, ratio 2:1) (166, 183, 186, 191-200) and macrophages expressing the MT1 marker (186, 196). This predominance of T cells over B cells is a characteristic finding of the infiltrate observed in other autoimmune diseases including IDDM and Hashimoto's thyroiditis (201, 202).

3.6 MRI findings

The first case of lymphocytic hypophysitis visualised by MRI was by Levine et al in 1988, who reported a mass in the sella turcica with homogenous signal intensity indistinguishable from a pituitary adenoma (203).

On T1-weighted pre-contrast images, the pituitary appears isointense relative to grey matter with a symmetric homogeneous sellar mass with suprasellar extension and an

intact and flat sella floor. Intense homogeneous enhancement of the entire gland is seen after the addition of gadolinium. Typical images in hypophysitis patients show the enhancement confined to the periphery of the lesion as “ring enhancement” (204) or extend along the dura mater as a “dural tail” (205).

The MRI findings in lymphocytic hypophysitis can vary with the stage and extent of the inflammatory process. The pituitary may appear enlarged from infiltration of lymphocytes in the active autoimmune destruction phase, through to an empty sella after the gland has atrophied.

3.7 Differences between adenoma and hypophysitis

One of the major issues in lymphocytic hypophysitis is to differentiate it from a pituitary adenoma and avoid the need for unnecessary surgery. While not completely diagnostic, there are various differences observed between lymphocytic hypophysitis and adenoma of the pituitary on MRI images summarised in Table 3. Classical MRI images from a lymphocytic hypophysitis and pituitary adenoma patient are shown in Figure 2.

3.8 Treatment

The treatment of lymphocytic hypophysitis is aimed at reducing the effects caused by pressure from the expanding pituitary mass and the hormonal replacement of impaired endocrine function where necessary. The major treatment strategies in lymphocytic hypophysitis in terms of immune modulation are the administration of glucocorticoids.

Surgery has been the most common form of treatment employed to reduce the pituitary mass in lymphocytic hypophysitis until recently. Transphenoidal hypophysectomy can provide a histologically proven diagnoses of lymphocytic hypophysitis, is effective in immediately decompressing the pituitary mass, resolving headaches and visual field defects. However, it does not improve endocrine deficiency problems experienced by patients and often leads to permanent hypopituitarism (157, 206). Surgical intervention is therefore only advised in cases where visual compromise cannot be rapidly improved with medical therapy, in individuals with recurrent mass effects despite

immunosuppressive therapy and cases where the diagnosis of a pituitary adenoma or other tumour cannot be excluded (163, 167).

A more conservative treatment is now more widely employed and accepted which utilises glucorticoids to reduce the size of the mass and/or the pituitary stalk and for the replacement of adrenal function. The most common corticosteroid used is prednisolone which was first tried successfully in 1980 by Mayfield et al (154). Kristoff et al. performed the only prospective trial of high dose methylprednisolone therapy on nine lymphocytic hypophysitis patients. In seven patients the therapy was successful in reducing the size of the sellar mass or pituitary stalk. Four of the nine patients had improvement in adenopituitary function and diabetes insipidus ceased or improved in all 4 patients (207). Dexamethasone has also been used with success (195).

Table 3: Comparison of typical findings in MRI of hypophysitis and non-functioning pituitary adenomas (including personal experiences of DK Ludecke with more than 3500 pituitary surgeries and 16 cases with lymphocytic hypophysitis) [*Personal communication]

	Hypophysitis	**Pituitary Adenomas**	**References**
T1-weighted images	Low signal, not cystic	Distinct mass or non-homogenous, may be cystic	Ahmadi *et al*. 1995 (208)
Posterior lobe	Hyperintense bright spot, which is lost when affected	Occasionally seen but is deformed or displaced	Ahmadi *et al*. 1995 (208) Miura *et al.* 1989 (209) Imura *et al.* 1993 (194)
Contrast enhancement (Gadolinium)	High – intense and homogenous enhancement	Moderate	Ahmadi *et al*. 1995 (208)
	Dural enhancement adjacent to an enlarged pituitary mass	Rarely seen in pituitary adenomas*	Ahmadi *et al*. 1995 (208)
	Early diffuse homogenous uptake	More likely to be heterogeneous*	Powrie *et al.* 1995 (210)
	Enhancement of the cavernous sinus	Also seen in tumours, often asymmetric *	Lee *et al.* 1994 (211)
	Delayed contrast enhancement of the whole pituitary	Also seen in adenomas, pituitary is often displaced enhancing more than the adenoma *	Sato *et al.* 1998 (212)
	Ring enhancement	Also with cystic adenomas*	Crock 1998 (167)
Sella turcica floor	Flat and intact	At least unilaterally depressed	Ahmadi *et al*. 1995 (208)
Pituitary enlargement	Symmetrical	Mostly asymmetrical	Ahmadi *et al*. 1995 (208) Caturegli *et al.* 2005 (146)
Pituitary stalk	Thickened but not usually deviated	Mostly deviated and thinned not thickened	Ahmadi *et al*. 1995 (208) Abe *et al.* 1995 (183) Crock 1998 (167)

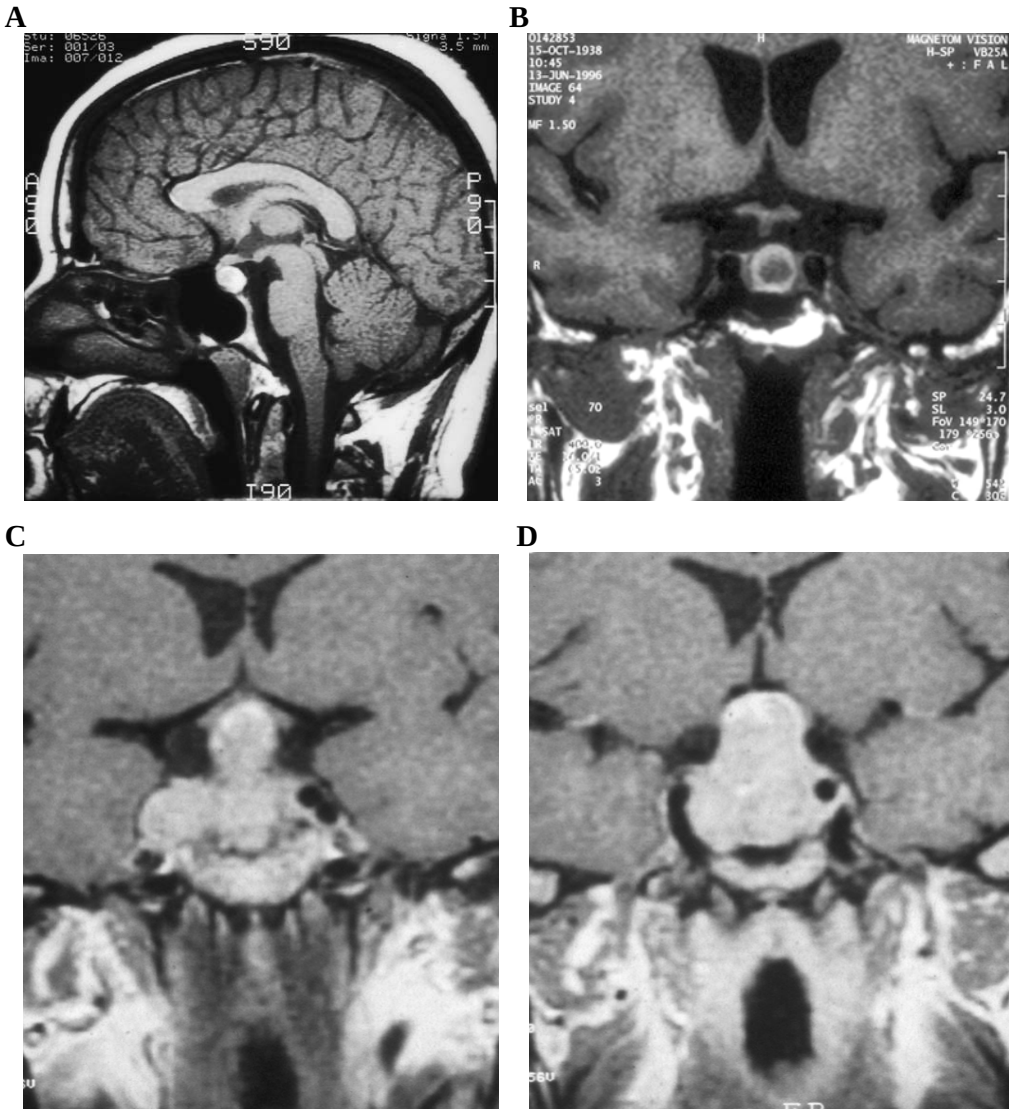


Figure 2. (A) Magnetic resonance imaging (MRI) scan of a classic case of lymphocytic hypophysitis in a 24-year-old woman who presented with symptoms of a pituitary tumor. Note the uniform enhancement with contrast with extension to the hypothalamus and **(B)** Coronal T1 weighted MRI scan of a 57-year-old man who presented with hypopituitarism and diabetes insipidus (DI) presumed to be due to lymphocytic hypophysitis. (From Crock et al. 2008) (213). **(C and D)** Coronal T1 weighted MRI with Gadolinium of a Non-functioning Pituitary Macroadenoma: Enhancement of the left-sided compressed pituitary and the non-infiltrated cavernous sinus; right cavernous sinus showing tumor invasion; optic chiasm is compressed dorsally from below (from personal surgical series of DK Ludecke, Hamburg University)

3.9 Animal models of autoimmune hypophysitis

Relatively few studies have attempted to establish an animal model of lymphocytic hypophysitis. An experimental model of lymphocytic hypophysitis would be invaluable to the understanding of the disease process and help explain how two structurally and developmentally distinct sections of the pituitary gland are open to autoimmune destruction. It would also be useful to give insight into strong link between pregnancy and disease onset in lymphocytic hypophysitis which could be extrapolated to help explain the immunological changes that occur in pregnancy both in women with and without autoimmune endocrine diseases.

Beutner and Witebsky endeavoured to establish the first working model of lymphocytic hypophysitis by immunising rabbits with suspensions and extracts of rabbit anterior pituitary in Freund's adjuvant. While immunofluorescent stainings showed isolated immunoreactivity to anterior pituitary cells, there was no evidence of pathological changes in the immunised animals (214). The first successful lymphocytic hypophysitis model was established by injecting Lewis rats with rat pituitary tissue homogenate. The injected animals showed diffuse infiltration of lymphocytes, monocytes and a few epithelioid cells into the anterior pituitary. Additionally, the autoimmune condition could be induced in pregnant rats and increased in severity in post-partum animals (215). Further studies showed only heterologous tissue from guinea pigs was effective in initiating the autoimmune destruction in rats and not pituitary tissue derived from cow, human, dog or rabbit (216).

Using the traditional approach of immunising animals with the desired antigen in Freund's adjuvant experimental lymphocytic hypophysitis has also been induced in a single rhesus monkey by multiple injections of human placental extracts (217), in rabbits (218) and again in rats (219). This approach is successful in producing focal lymphocytic infiltrate limited to the anterior pituitary. Autoantibodies in these animals against GH, TSH and LH, but not ACTH, FSH or prolactin have also been detected (219). No follow-up studies were performed on any of these animals.

Lymphocytic hypophysitis has also been induced in animals by immunising with different viral proteins. Hamsters developed autoantibodies against pituitary cells and

lymphocytic infiltration after being immunised with glycosylated, membrane-associated E1 and E2 rubella virus proteins (220). While establishing an experimental model of diabetes by infecting mice with a reovirus, autoantibodies to GH producing cells in the pituitary was observed. Experiments showed the S1 gene segment of reovirus type 1 but not type 3 was successful in inducing autoantibodies against GH (221, 222).

Pituitary autoimmunity has also been induced through a CD8 T cell response. Influenza nucleoprotein was expressed as a transgene under the control of the human GH locus control region in Rag1 knockout mice. This resulted in the expressed nucleoprotein being stored in secretory vesicles of somatotrophs and secreted like GH. A CD8 Tc cell-mediated response was triggered resulting in autoimmune destruction confined to the anterior pituitary gland seen by severe GH deficiency and a reduction in both prolactin and TSH. This showed an antigen expressed and secreted in the pituitary can gain access to CD8 T cells and elicit a CD8 T cell mediated autoimmune response. Adoptive transfer of T cells resulted in slight reduction of GH, however, a resulting autoimmune pathology appears to require an excessive number of CD8 T cells (223). Further to this study, the transgene was also expressed in a new mouse line where the nucleoprotein was confined to the cytoplasm of somatotrophs and did not reach the secretory pathway. Pituitary autoimmunity was triggered but delayed in onset in comparison to the nucleoprotein secreting mice. This delay in T cell activation suggested a longer time to reach critical antigen density needed to trigger an autoimmune response. Autoimmunity was also shown to be triggered by the introduction of a virus in these mice, which increased the nucleoprotein-specific CD8 T cell pool. This demonstrates the pituitary could be susceptible to T-cell mediated pathology after infection with a virus expressing a soluble pituitary antigen. The pituitaries of the control mice were unaffected (224).

A working experimental model of lymphocytic hypophysitis has recently been established in the SJL/J mouse strain most effectively by injection with mouse pituitary whole extracts. The induced disease closely resembled that of the human disease with injected mice showing an enlarged pituitary with marked mononuclear cell infiltration of the anterior pituitary. The autoimmune attack systematically

destroyed the pituitary gland as the disease progressed resulting in an empty sella. The disease was more frequently seen in female mice who were also more severely affected. Pituitary function studies were done to measure serum levels of corticosterone, thyroxine and insulin-like growth factor-1. Corticosterone levels proved the most reliable at reflecting the disease severity suggesting ACTH cells are an early target of the disease in these mice as also seen in the human disease. Pituitary autoantibodies were detectable by ELISA against mouse pituitary cytosolic proteins. Interestingly, autoimmune destruction of the pituitary could be induced in healthy mice through the passive transfer of T cells from affected mice (225). While autoimmune destruction of the anterior pituitary is inducible, no experimental lymphocytic infundibuloneurophypophysitis model has yet been established.

4. Pituitary Autoantibodies

Anti-pituitary antibodies (APAs) have been widely studied in numerous disorders both of the pituitary and various autoimmune diseases. A number of methodologies have been employed to study APAs which differential characterise the autoantigens. The most widely used techniques include:

1. Indirect immunofluorescence: useful for identifying the cell type autoantibodies are directed against
2. Immunoblotting: identifies by molecular size and linear epitopes
3. Radioligand assay: quantitative measurement of autoantibodies against a radiolabelled protein produced *in vitro*

4.1 Indirect Immunofluorescence

Indirect immunofluorescence was first utilised to study APAs by Bottazzo et al. in 1975 who found immunoreactivity against lactotrophs in 19 of 287 patients with various autoimmune endocrine disorders, none of whom had hypopituitarism (226). APAs have also been detected against thyreotrophs (227), gonadotrophs (228), corticotrophs (229, 230) and somatotrophs (145, 231) in various disorders.

APAs have been detected in only 16/62 lymphocytic hypophysitis patients studied by immunofluorescence (146) and also in a range of patients with pituitary disorders

including empty sella, pituitary tumours and ACTH deficiency. Furthermore, APAs have been detected in other autoimmune conditions such as diabetes mellitus (232-234), Graves' disease, Hashimoto's thyroiditis, APS1 and Cushings syndrome (230) most often in small numbers and at low titres. Interestingly, the presence of APAs does not always correlate with disease manifestations for example APAs have been detected in patients with APS1, but rarely in those patients with pituitary deficits (235).

One of the major issues with indirect immunofluorescence in studying APAs has been the selection of tissue substrate. Human foetal pituitaries and fresh frozen surgical material are considered the most ideal substrates (236). However, human adult pituitary ACTH cells express Fc receptors which react with virtually all human immunoglobulins producing non-specific diffuse cytoplasmic staining throughout the pituitary, unless the sera is first pretreated to cleave the immunoglobulin molecules to produce F(ab) fragments. Human foetal pituitaries lack the Fc receptor making it the ideal substrate (237, 238). Given the limited availability of both adult and foetal human pituitaries and the ethical issues concerning their use, a range of alternative substrates have been studied including primates, non-primates (including rat, guinea pig, bovine, porcine) as well as murine AtT_{20} and rat GH_3 pituitary cell lines with variable results.

Recent studies have focused on characterising APAs in various disorders by immunofluorescence on baboon pituitary sections from which, a link has been speculated between the presence of APAs and GH deficiency. High titre autoantibodies (diluted 1:32-1:64) were observed in 4/12 (33.3%) patients with isolated and apparently idiopathic GH deficiency who were treated with recombinant GH in childhood and also in 5/180 (2.78%) of patients with organ-specific autoimmune diseases, all of which were severely GH deficient. Low titer autoantibodies (serum diluted 1:2-1:8) were also detected in patients with organ-specific autoimmune diseases (35/180) in addition to 6/20 patients with pituitary adenoma and 2/50 controls, all with normal GH levels (239).

Secondary to this study, the serum from patients with idiopathic GH deficiency of childhood onset was shown to exclusively immunostain somatotrophs, whereas

idiopathic GH deficiency of adult onset stained not only somatotrophs but also prolactin secreting cells, corticotrophs, thyrotrophs and gonadotrophs. In contrast, sera from patients with autoimmune endocrine diseases without pituitary impairment stained mainly prolactin-producing cells but few somatotrophs (231). Immunoreactivity to the baboon pituitary section has also been seen the in patients with prepubertal idiopathic short stature (240), hypogonadotropic hypogonadism (241), idiopathic hyperprolactinemia (242) and Sheehan's syndrome (243).

APAs in patients with autoimmune thyroid disease have also been studied by immunofluorescence on pituitary baboon sections. Immunreactivity was reported in 92/707 (13%) of patients with Hashimoto's thyroiditis, 18/254 (7.1%) with Graves' disease and 3/329 (0.9%) of patients with non-autoimmune thyroid disease. Additionally, of the 102/110 autoantibody positive autoimmune thyroid disease patients who underwent functional pituitary assessment, 36 (35.2%) of patients were deemed to have mild or severe GH deficiency (244). However, evidence of positive immunostaining presented in the paper appeared of a more generalised non-specific background staining pattern than true immunoreactivity. Therefore, these results will not be considered further in the context of this thesis.

Together with their detection in non-autoimmune pituitary conditions and in numerous autoimmune diseases, the clinical significance and specificity of APAs in relation to disease manifestation remains limited. Immunofluorescence while invaluable in ascertaining the cell type autoantibodies are targetting can not be used to identify the specific target autoantigen.

4.2 Immunoblotting

The immunoblotting method was developed by Crock *et al.* in 1993 (245) for the further characterisation of pituitary antibodies. This technique has the advantage over indirect immunofluorescence of recognising single proteins by molecular weight which can be subsequently isolated and identified by other methods rather that just identifying the cell type autoantibodies are targeting. Human pituitary autopsy material overcomes the problem of species specificity and eliminates the problem of attaining

fresh human pituitary tissue. It can also be divided into cytosolic and membranous portions.

Using this method, immunoreactivity was detected against a 49kDa cytosolic protein (167), later identified as alpha-enolase (246, 247), at a high frequency in patients with biopsy proven lymphocytic hypophysitis (70%; 7/10) and also in 55% (12/22) of patients with suspected lymphocytic hypophysitis. Autoantibodies against enolase were also identified in 42% (6/14) of patients with Addison's disease, 20% (4/20) of patients with pituitary tumours, 15% (5/33) of patients with thyroid autoimmune disease (1/12 Graves' disease and 4/21 Hashimoto's patients), 13% (2/15) of patients with rheumatoid arthritis and 9.8% (5/52) normal controls (167). Similarly, immunoreactivity against the 49kDa enolase was observed at a higher frequency in patients with isolated ACTH deficiency (21.5%; 14/65) than in healthy controls (8.8%; 5/57) (184). A high incidence of immunoreactivity has also been detected in the serum of Sheehan's syndrome patients (63.1%, 12/19). Immunoblotting further identified enolase as a significant, if non-specific, pituitary cytosolic autoantigen in 58% (39/67) of APS1 patients (248).

Lymphocytic hypophysitis sera was further shown to react with the gamma isoform of enolase known as neuron-specific enolase (NSE), in both pituitary and placental tissue providing a possible link for the high rate of disease onset in pregnant and post-partum women (247). However, further analysis of alpha enolase in a radioligand assay suggests enolase may be a marker for autoimmunity in general and not specific to any particular autoimmune disease (see below) (249).

Autoantibodies against a 22kDa cytosolic protein, later identified as growth hormone (250), have been observed in patients with lymphocytic hypophysitis and idiopathic pituitary insufficiency (250, 251) as well as diabetes mellitus (252, 253).

Immunoreactivity has also been detected against a number of other proteins which have not yet been formally characterised. Reactivity was seen in 50% (5/10) biopsy proven and 27% (6/22) of suspected cases of lymphocytic hypophysitis against a 40kDa pituitary cytosolic protein but in only 7.7% (4/52) healthy controls (167).

Autoantibodies against pituitary membrane proteins of 43kDa and 68kDa have also been reported at low frequency in sera from lymphocytic hypophysitis patients (2/25 and 5/25 respectively) but not in the sera from patients with isolated ACTH deficiency, type 1 and 2 diabetes mellitus or in healthy blood donors (254).

A 36kDa protein has also been identified in the sera of isolated ACTH deficiency patients by immunoblotting with human pituitary cytosol. Autoantibodies were detected in 12/65 ACTH deficiency patients and only 2/57 healthy controls. This autoantigen is yet to be identified and characterised (184).

A recent study compared the sensitivity and specificity of immunoblotting on human autopsy tissue to immunofluorescence with baboon pituitary sections in patients with biopsy proven lymphocytic hypophysitis. The results showed immunoblotting had greater sensitivity and specificity than immunofluorescence with 64% versus 57% sensitivity and 86% versus 76% specificity respectively. Neither however proved adequate as a clinical diagnostic assay for lymphocytic hypophysitis. In addition, two further potential autoantigens were described; chromosome 14 open reading frame 166 and chorionic somatomammotropin, both recognised at a higher frequency in sera from lymphocytic hypophysitis patients than pituitary adenoma patients and healthy controls (255).

4.3 Radioligand/ITT Assay

More recent advances in identifying the autoantigen(s) in lymphocytic hypophysitis have come from a candidate autoantigen approach. The ^{35}S-Methionine labeled protein is produced by *in vitro* transcription and translation (ITT) using rabbit reticulocyte lysate then immunoprecipitated with patient serum using Protein A-Sepharose. The assay is a quantitative method and has the advantage over immunoblotting as the protein is kept in a more native 3D conformational form and not subjected to denaturation. The ITT assay, alternatively known as the radioligand assay, is detailed in Figure 3. For a more detailed description of the ITT method, see Appendix I.

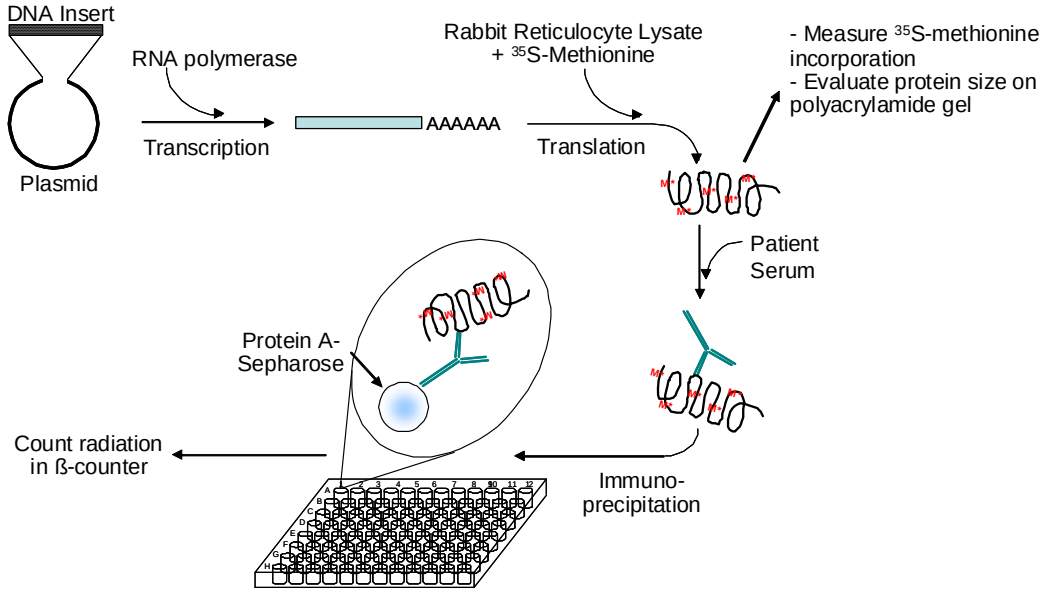


Figure 3: Schematic representation of the ITT assay (Crock *et al.* 2008) (213).

Tanaka et al 2002a identified two novel pituitary specific proteins, pituitary gland specific factor (PGSF) 1a and 2 by probing a pituitary cDNA library and examining the expression profile of the actively transcribed genes (256). The proteins were subsequently evaluated as autoantigens in lymphocytic hypophysitis with the radioligand assay along with growth hormone. Immunoreactivity against PGSF1a was detected in 33.3% (1/3) of biopsy proven lymphocytic infundibuloneurohypophysitis patients, 20% (2/10) patients with isolated ACTH deficiency and 3.2% (1/31) of patient sera with other autoimmune diseases. Autoantibodies against PGSF2 were detected in 20% (1/5) of lymphocytic adenohypophysitis patients, 11.1% (1/9) suspected lymphocytic infundibuloneurohypophysitis patients, 10% (1/10) of isolated ACTH deficiency cases, 50% (2/4) of patients with idiopathic TSH deficiency, and 6.5% (2/31) patients with other autoimmune diseases. Autoantibodies directed against GH were also seen in 20% (1/5) of lymphocytic adenohypophysitis patients, 33.3% (1/3) of biopsy proven lymphocytic infundibuloneurohypophysitis patients, and in the sera of 10% (1/10) of isolated ACTH deficiency cases, 25% (1/4) of idiopathic TSH deficiency cases and 6.5% (2/31) patients with other autoimmune diseases (257). Further to this study, immunoreactivity against PGSF1a was detected frequently in rheumatoid arthritis patients (43.4%; 20/46) and may therefore be more useful as a marker for rheumatoid arthritis than lymphocytic hypophysitis (258).

Contrary to previous immunoblotting results, Tanaka *et al.* studied alpha enolase in their radioligand assay detecting autoantibodies at similar frequencies in sera from patients with lymphocytic hypophysitis and pitutiary non-functioning adenomas (41.2%; 7/17 vs 46.2%; 6/13 respectively), indicating alpha enolase is not a suitable diagnostic indicator for lymphocytic hypophysitis (249). Furthermore, alpha enolase autoantibodies have been detected in a variety of autoimmune and infectious diseases including inflammatory bowel disease (259), rheumatoid arthritis (260, 261), systemic lupus erythematosus, mixed cryoglobulinemia, systemic sclerosis (262), Behçet's disease (263), multiple sclerosis (264) and Hashimoto's encephalopathy (265). This implies immunoreactivity against enolase may be a marker for underlying autoimmune disease in general and not specific to any one disease. Alpha enolase antibodies were detected in 0 - 8.5% of healthy controls depending on the methodology employed (243, 253, 255, 256, 257, 259).

Tatsumi *et al.* compared immunoreactivity against prohormone-processing enzymes in patients with nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis and other pituitary diseases. Autoantibodies against prohormone convertase (PC) 1/3 were detected in 45% (5/11) of nonfunctioning pituitary macroadenoma cases and 14% (2/14) of lymphocytic hypophysitis patients while immunoreactivity against 7B2 (also known as Secretogranin V) was identified in the sera from 55% (6/11) of nonfunctioning pituitary macroadenoma patients, 14% (2/14) of lymphocytic hypophysitis patients and 33% (1/3) of Sheehan’s syndrome patients. With PC 1/3 and 7B2 autoantibodies decisively more frequent in patients with pituitary adenoma than patients with lymphocytic hypophysitis and other pituitary disease, they could serve as novel tumour-associated antigens useful in the differential diagnosis of pituitary adenoma (266).

4.4 Immunoscreening of a pituitary cDNA expression library

An invaluable technique for isolating and identifying candidate autoantigens is immunoscreening of a cDNA expression library (made from the organ targeted by autoimmune destruction) with patient serum. The method, in brief, involves the isolation of mRNA from the desired organ, which is ligated into vectors. Bacteriophages containing the vectors are grown on agar plates and the proteins then

absorbed or impregnated onto overlaying nitrocellulose filters. Patient serum is then added and positive clones are isolated and identified by sequence analysis (Figure 4).

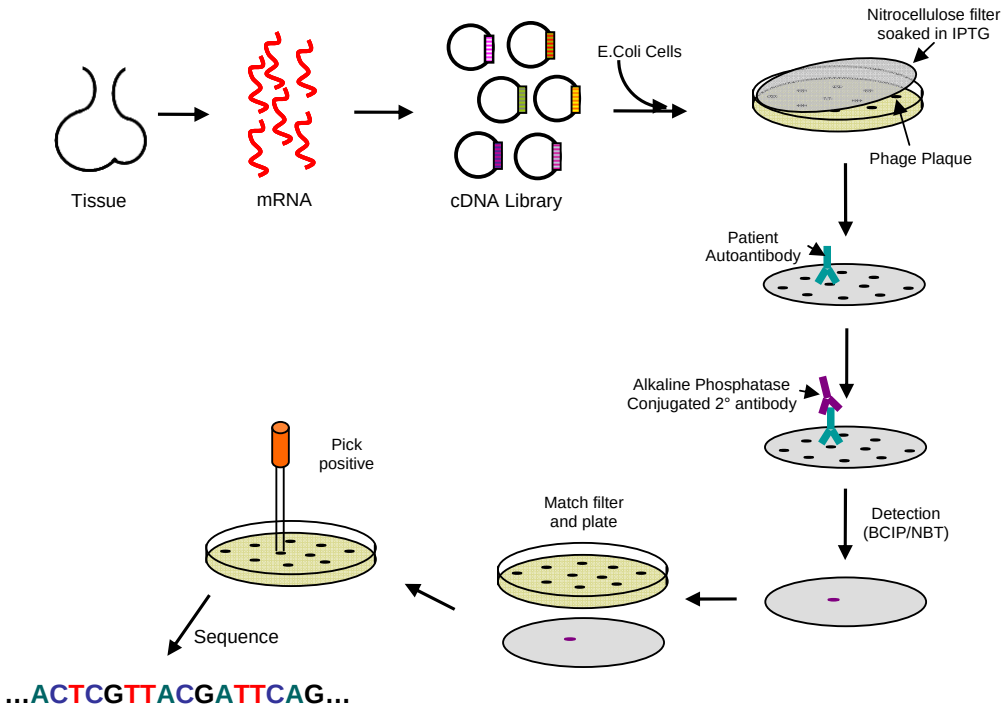


Figure 4: Schematic diagram of immunoscreening a cDNA expression library.

This methodology has been successfully utilised to identify the target autoantigens in numerous autoimmune diseases including several of the major autoantigens in APS1 (267-271), as well as SLE (272), primary sclerosing cholangitis and inflammatory bowel disease (273). Secretogranin II was isolated from a pituitary cDNA library using the serum of a 79 year old male patient with lymphocytic hypophysitis (274). The protein is abundantly expressed in pituitary tissue in gonadotrophs, thyrotrophs and corticotrophs (275) 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 (276-278), a vital process to hormone secretion in the pituitary. Secretogranin II however, has not been further assessed for antigenicity in lymphocytic hypophysitis.

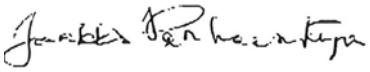
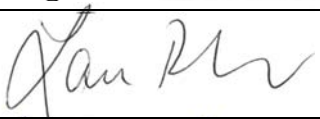
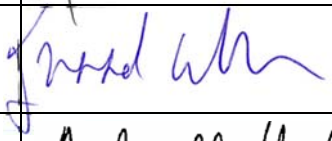
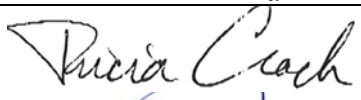
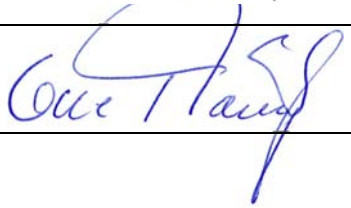
Using the sera from patients with APS1 and GH deficiency to immunoscreen a pituitary cDNA expression library, tudor domain containing protein 6 (TDRD6) was identified as a possible target autoantigen. The protein was expressed by ITT and the recombinant protein used for immunoprecipitated against APS1 sera. TDRD6 was found to be a major autoantigen in APS1 with autoantibodies against the protein detected in 49% (42/86) of the patients analysed. However, there was no correlation between TDRD6 and pituitary manifestations in APS1 suggesting as yet unidentified autoantigen(s) accountable for the pituitary deficits seen in these patients (145).

While there are many studies of APAs in various disorders both autoimmune and non-autoimmune in origin, few studies have focused on identifying the target autoantigens in lymphocytic hypophysitis. A small number of candidate autoantigens have been proposed yet only in a minority of patients and their clinical relevance is limited. Identification of the underlying pathogenic autoantigens would lead to the development a non-invasive serological test for the disease, negating the need for pituitary biopsy (which can lead to permanent pituitary failure) and aid in the understanding of disease onset and progression. In addition, identification of the autoantigen(s) relating to pituitary manifestations in APS1 patients would assist in the improved targeted treatment of patients with this disorder.

Paper I [redacted]

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

I hereby certify the proportion of my contribution to the following paper entitled "Identification of Endothelin Converting Enzyme-2 as an Autoantigen in Autoimmune Polyendocrine Syndrome Type 1"

Co-author	Contribution	Signature	Date
Casey Smith	86.5%		8/5/09
Sophie Bensing	2.0%		07.05.09
Mohammad AliMohammadi	2.0%		07.05.09
Mikael Oscarson	1.0%		May 11th 2009
Jaakko Perheentupa	1.0%		27.4.09
Eystein Husebye	0.5%		27 April 2009
Jan Gustafsson	0.5%		April 28, 2009
Lars Rönnblom	0.5%		11/05/09
Gunnel Nordmark	0.5%		11/5/09
Antonella Meloni	0.5%		20-05-09
Tomas Hökfelt	1.0%		April 2, 2009
Patricia Crock	2.0%		1/5/2009
Olle Kämpe	2.0%		07.05.09

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

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Abstract

Context: Autoimmune polyendocrine syndrome type 1 (APS1) is a rare monogenic autoimmune disorder caused by mutations in the autoimmune regulator (AIRE) gene. High titre autoantibodies are a characteristic feature of APS1 and are often associated with particular disease manifestations. Pituitary deficits are reported in up to 7% of all APS1 patients, with immunoreactivity to pituitary tissue frequently reported.

Objective: In this study, we aimed to isolate and identify specific pituitary autoantigens in patients with APS1.

Design/Methods: A pituitary cDNA expression library was immunoscreened using serum from two patients with APS1 and growth hormone deficiency and a cDNA clone encoding endothelin converting enzyme (ECE)-2 was isolated.

Results: Immunoreactivity against ECE-2 was detected in 48/104 (46%) of APS1 patient sera but not in 95 patients with other autoimmune disorders nor in 118 healthy controls. In addition, quantitative-PCR showed ECE-2 mRNA to be most abundantly expressed in the pancreas with high levels also in the pituitary and brain.
A fibrous network staining pattern of posterior and intermediate guinea pig pituitary tissue was seen with APS1 sera, irrespective of ECE-2 autoantibody status. Staining of isolated anterior pituitary cells was also seen.

Conclusion: ECE-2 is a highly specific major autoantigen in APS1, with no apparent correlation to the major recognised clinical phenotypes of the disease.

Introduction

Autoimmune polyendocrine syndrome type 1 (APS1; alternatively known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) is a rare monogenic autosomal recessive disease caused by mutations in the autoimmune regulator (AIRE) gene. APS1 is characterised by the combined presence of at least two of the classical triad of Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. In addition, other organ-specific autoimmune diseases as well as ectodermal manifestations occur with variable penetrance. High titer autoantibodies are a hallmark for APS1. These autoantibodies serve as diagnostic markers and are sometimes predictive of disease manifestations (1-4).

Pituitary manifestations are uncommon in APS1 patients being reported in approximately 7% of cases (5). Patients present with either single or multiple pituitary deficits, the most commonly reported being isolated growth hormone (GH) deficiency. Partial adrenocorticotropin hormone deficiency, isolated hypogonadotrophic hypogonadism and central diabetes insipidus have also been described (6-11).

Autoantibodies to prolactin secreting cells and gonadotrophs have been detected in APS1 patients using immunohistochemistry (5, 12, 13). APS1 sera have also been shown to immunostain a small number of guinea pig anterior pituitary cells of which 40-50% were GH positive (14). The exact autoantigens targeted by the APS1 patients´ autoantibodies in these pituitary cells remain unknown. Immunoblotting identified enolase as a significant, if non-specific, pituitary cytosolic autoantigen in 58% of APS1 patients (15). Screening of a pituitary cDNA library with sera from 2 APS1 patients with GHD identified TDRD6 as a major APS1 autoantigen to which autoantibodies are present in 49 % of patients but not apparently linked to pituitary deficiency (14).

This study aimed to identify pituitary autoantigens from immunoscreening of a human pituitary cDNA expression library in an attempt to delineate the connection between pituitary insufficiency in APS1 patients and pituitary autoantibodies.

Methods

Patients

Serum samples were obtained for analysis from 104 APS1 patients including 60 Finnish patients, 11 Swedish patients, 17 Norwegian patients, and 16 Sardinian patients. The diagnosis of APS1 was based on the presence of at least two of the classical triad of APS1 components; mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency. Patients with only one of these features who were homozygous or compound heterozygous for mutations in the AIRE gene were also included. Ten patients in this series were identified as exhibiting pituitary manifestations including eight with GH deficiency and two patients with hypogonadotrophic hypogonadism.

Serum samples were also collected from 95 patients with other autoimmune diseases comprising 15 patients with Addison's disease, 20 with Sjögren's syndrome, 20 with biopsy proven lymphocytic hypophysitis, 20 with type 1 diabetes mellitus and 20 with systemic lupus erythematosus. 118 healthy Australian blood donors served as controls.

Ethical approval was obtained from the Committee of Ethics, Faculty of Medicine, Uppsala University and the Human Research Ethics Committees of the Hunter Area Health Service and University of Newcastle with informed consent from all patients and controls.

Construction and Screening of a Human Pituitary cDNA Library

A pituitary cDNA expression library (14) was immunoscreened with sera from two Swedish APS1 patients with GH deficiency (diluted 1:5000 and 1:1000) as previously described (1). *In vitro* excision of the pBK-CMV phagemid vectors from the ZAP express library vector was performed according to the manufacturer's instructions (Stratagene). The isolated cDNA clones were partially sequenced using a dye-terminator sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 3730 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) and identified by comparison of the sequencing data against available databases using BLAST. The cDNA clone encoding ECE-2 was fully sequenced using additional internal primers (CyberGene, Huddinge, Sweden).

All library cDNA clones of interest were subcloned from the pBK-CMV vector into the pTNT vector (Promega) by double restriction enzyme digestion, for improved efficiency of *in vitro* transcription and translation. Inserts were re-verified by sequencing as above.

In Vitro Transcription and Translation (ITT) of autoantigens and Immunoprecipitation

Recombinant 35S-radiolabelled proteins were produced by ITT in an Sp6 Quick coupled reticulocyte lysate system (Promega) and used for immunoprecipitation with patient sera. In 96 well plates, 25000 to 30000 cpm of the radiolabeled protein and 2.5µL of undiluted patient serum were mixed in a buffer containing 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), and 0.02% NaN_3, 0.1% BSA and 0.15% Tween-20 (buffer B) in a total volume of 50 µL and incubated overnight at 4°C. The antibody complexes were then precipitated with 50 µL of a 50% (vol/vol) slurry of protein A-Sepharose (Pharmacia, Stockholm, Sweden) in buffer B in pretreated 96 well microtiter plates with filter bottoms (16) (MABV N12, Millipore, Bedford, MA) for 45 min at 4°C. The plates were washed ten times with Buffer B using a vacuum manifold. After drying, 70 µL OptiPhase SuperMix scintillation fluid (Perkin Elmer Life Sciences, Boston, MA) was added to each well and the plates counted in a beta counter (Wallac 1450 MicroBeta, PerkinElmer). The patient serum from which ECE-2 was isolated on immunoscreening the pituitary cDNA library, was used as the positive control and 4% bovine serum albumin (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 [(cpm of the unknown sample – cpm of the negative control)/(cpm of the positive control – cpm of the negative control) x 100] and the upper normal antibody index was set at 18, a value which clearly differentiated between positive and negative samples.

Association study

A two-tailed Fisher's exact test was performed to determine the association of major APS1 disease manifestations with immunoreactivity to ECE-2. All statistical analysis was done with Intercooled STATA 8.2 (Stata Corp, College Station, TX). A p value of <0.05 was considered significant.

Immunohistochemistry

Pituitary sections from three male and three female guinea pigs were prepared as previously described (14). Immunoreactivity to guinea pig pituitary was evaluated in 10 APS1 patients with positive and 10 APS1 patients with negative ECE-2 autoantibody indices. Sera diluted 1:5000-1:10000, were assessed using the tyramide signal amplification system (TSA-Plus; PerkinElmer Life Science, Boston, MA, USA) (17). To evaluate the specificity of patient sera binding to ECE-2 in the pituitary sections the staining patterns with untreated sera were compared to immunostainings after the sera had been preabsorbed with ECE-2 protein. Ten APS1 patient sera (diluted 1:5000) were immunoprecipitated overnight at 4°C with 80000-120000 cpm 35S-radiolabeled ECE-2 protein expressed in vitro, as described above, in order to first deactivate the ECE-2 autoantibodies in the patient sera before use on the pituitary sections. Furthermore, using the sera from two patients with a high ECE-2 autoantibody index and unequivocal immunoreactivity against the guinea pig pituitary gland, a dilution series ranging from 1:5000 – 1:2000000 was established to fully ascertain the binding specificity before and after preabsorption with ECE-2 protein produced by ITT. Pituitary sections were examined using a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) equipped with a Hamamatsu ORCA-ER C4742-80 digital camera and Hamamatsu photonics Wasabi 150 software (Hamamatsu, Hamamatsu City, Japan).

Tissue expression profiling of ECE-2

To establish the tissue expression profile of ECE-2 mRNA throughout various organs, quantitative PCR was performed on cDNA from human tissue. Primers were designed with Beacon Designer® version 5.11 software, which flanked the intron-exon junctions, hence avoiding amplification of genomic DNA. Quantitative PCR was carried out on human normalised multiple-tissue cDNA panels (BD Bio Sciences, Palo Alto, CA, USA) as well as pituitary, aorta (Stratagene) and adrenal cortex cDNA prepared from normal adrenal tissue removed during adrenal adenoma surgery, using a MyiQ iCycler (Bio-Rad, Hercules, CA, USA). Reactions were performed in a volume of 25 µl, with 200 nM of each primer using iQ™ SYBR®Green supermix (Bio-Rad, Hercules, CA, USA) as per the manufacturer's instructions. All samples were run in triplicate. Thermal cycles consisted of an initial denaturation of 95°C for 3min,

followed by 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. Standard curves were then established from the serial dilution of ECE-2 and control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) PCR templates. ECE-2 mRNA levels were deduced from the standard curve and normalised to the endogenous GAPDH tissue content.

Results

Identification and isolation of ECE-2

To isolate and identify potential pituitary autoantigens, a pituitary cDNA expression library was screened with sera from two APS1 patients with GH deficiency. A total of 46 positive cDNA clones were isolated and partially sequenced revealing seven different clones in addition to tryptophan hydroxylase (TPH) isoform 1, a well defined APS1 autoantigen. Radiolabelled recombinant proteins for each of these seven clones were produced by ITT and used for immunoprecipitation against a small test panel of APS1 and healthy control sera. One of the isolated clones encoding endothelin converting enzyme (ECE)-2 was immunoprecipitated by 5/8 APS1 sera, including the library screening patient, but no healthy controls. ECE-2 was hence selected for further analysis.

Five transcript variants of the ECE-2 gene located on chromosome 3q27.1 were identified in the GeneBank database (GeneBank Accessions: Variant 1: NM_014693.3, Variant 2: NM_001037324.2, Variant 3: NM_032331.3, Variant 4: NM_001100120.1, Variant 5: NM_001100121.1). The longest variant, variant 1, consists of 19 exons encoding a 883aa protein, and the shortest, variant 3, having 3 exons transcribing a 255aa protein. The partial ECE-2 cDNA sequence isolated from the pituitary cDNA expression library matched from within exon 8 of ECE-2 transcript variant 1 and differs only at nucleotide position 1605 with a silent t>c transition. This sequence is predicted to encode a 549 aa protein common to all transcript variants of the gene excluding variant 3 (Figure 1).

Identification of ECE-2 as an autoantigen in APS1

To determine the frequency and specificity of ECE-2 autoantibodies in APS1 sera, a 35S-methionine labelled ECE-2 protein produced by *in vitro* transcription and translation, was immunoprecipated against sera from 104 APS1 patients, 95 patients with other autoimmune diseases and 118 healthy blood donors. ECE-2 autoantibodies were found in 48 of the 104 (46%) patients with APS1 but not in the sera from patients with Addison's disease, systemic lupus erythematosus, Sjögren's syndrome, type 1 diabetes mellitus, biopsy proven lymphocytic hypophysitis or healthy blood donors (Figure 2). No associations were found between the major clinical manifestations of APS1 and the presence of ECE-2 autoantibodies (Table 1).

Immunohistochemistry

Ten APS1 patients with ECE-2 autoantibodies and 10 patients without autoantibodies, were studied to determine any staining patterns of the pituitary. Immunoreactivity against guinea pig pituitary tissue was found in 18/20 APS1 sera studied. One patient with a positive ECE-2 autoantibody index and a single patient with no detectable ECE-2 autoantibodies by ITT, exhibited no immunoreactivity to the guinea pig pituitary. The immunoreactivity in the remaining sera was visually unaffected by the preabsorption of the patient sera with recombinant ECE-2 protein at a serum dilution of 1:5000 in comparison to untreated sera. The specificity of the pituitary staining was further delineated in two ECE-2 autoantibody positive patient sera with strong staining of the pituitary. Immunoreactivity to guinea pig pituitary began to diminish between a serum dilution of 1:100000 and 1:200000. Preabsorption with ECE-2 recombinant protein at these dilutions did not affect the immunoreactivity to pituitary tissue.

A recurrent staining pattern of the pituitary was observed in the APS1 patients studied, irrespective of their antibody status (data not shown). A fibrous network staining pattern was frequently observed both in the posterior and intermediate lobe of the guinea pig pituitary. Immunoreactivity against the anterior lobe of the pituitary was also repeatedly observed, most often as staining against distinct singular cells. No immunoreactivity was observed with healthy control serum samples.

Tissue expression profile

The expression profile of ECE-2 in various organs was examined by quantitative PCR. ECE-2 mRNA was found to be predominantly expressed in pancreatic tissue, with high levels of expression also detected in the pituitary and brain. Low expression levels of ECE-2 mRNA were also detected in the small intestine, testis, ovary, prostate, colon, heart, thymus and the adrenal cortex (Figure 3).

Discussion

ECE-2 was isolated and identified as a major, highly specific APS1 autoantigen by immunoscreening a pituitary cDNA expression library and subsequent ITT and immunoprecipitation. ECE-2 autoantibodies were detected in 46% (48/104) of APS1 patients studied by immunoprecipitation. Autoantibodies were specific for APS1 as no detectable autoantibodies in were found any serum samples from patients with other autoimmune diseases including Addison's disease or in healthy controls.

The frequency of ECE-2 immunoreactivity in APS1 is comparable to the previously characterised major APS1 autoantigens including side-chain cleavage enzyme (SCC also known as CYP11A1; 52%), aromatic L-amino acid decarboxylase (AADC; 51%), tryptophan hydroxylase (TPH; 45%), and most recently NACHT leucine-rich-repeat protein 5 (NALP5; 41%) (3, 4). Nevertheless, ECE-2 is not structurally or functionally related to any of the previously identified autoantigens. ECE-2 is a zinc metalloprotease belonging to the M13 family, which also includes both ECE-1 and neprilysin (NEP) (18), with the highest homology to ECE-1 at 59% (19). The ECEs are named for their function in converting big endothelins (big ETs) to their biologically active form of potent vasocontrictors, endothelins (20, 21). ECE-2 is an intracellular protein, likely located in the trans-golgi network and secretory vesicles, with clearly differential expression throughout the brain and body compared to ECE-1. While ECE-1 shows a broad tissue expression pattern, ECE-2 exhibits a restricted neuroendocrine distribution (22, 23). Interestingly, in addition to the processing of big ET-1, ECE-2 has been proposed to be an important neuropeptide-processing enzyme capable of modulating levels of regulatory peptides (18, 24, 25).

High expression of ECE-2 mRNA was detected in brain tissue. Previous studies show ECE-2 is expressed throughout the brain with high levels in the cerebral cortex, cerebellum, hippocampus, hypothalamus, as well as in catecholaminergic nuclei (23, 26). We have recently shown that sera from APS1 patients can immunostain monoamine and GABA systems in the brain and that these autoantibodies sometimes are associated with neurological manifestations (27). Autoantibodies directed against ECE-2, acting at the level of the hypothalamus, could hypothetically alter brain control of the pituitary, contributing to the pituitary deficits seen in APS1 patients, but this remains to be explored. ECE-2 knockout mice develop normally and are healthy with a normal life span (22), but suffer minor deficits in learning and memory (28). It may be speculated that this may be due to amyloidosis as ECE-2 has been shown to be involved in the degradation of amyloid beta (29-32). To our knowledge, learning and memory impairment has not yet been recognised in APS1 patients. It would therefore be interesting to evaluate these functions in a large patient cohort and any associations with ECE-2 autoantibodies or amyloidosis.

ECE-2 autoantibodies were found at high frequency in patients with APS1 and pituitary insufficiency including 75% (6/8) of patients with GH deficiency and 50% (1/2) of patients with hypogonadotrophic hypogonadism. However, 43.6% (41/94) of APS1 patients without pituitary deficiencies were also determined to be reactive to ECE-2, inferring ECE-2 does not always function as a pituitary specific autoantigen in APS1. The number of APS1 patients with pituitary deficits in this series is small and maybe the true number of APS1 patients with pituitary manifestations is underestimated since all pituitary hormone levels are not routinely tested in APS1 patients and symptoms may be masked by the plethora of other endocrine deficiencies. The patients cited with hypopituitarism in this series may therefore represent only those with obvious pituitary deficiencies. Cases with subtle or subclinical symptoms or subjects with autoantibodies appearing before the onset of clinical disease could be overlooked. It may also be possible that the presence of ECE-2 autoantibodies precedes the manifestation of pituitary disease. Hence, ECE-2 should not be fully out ruled as a pituitary autoantigen in APS1.

In our study, antipituitary autoantibodies were detected by immunofluorescence against guinea pig pituitary in 90% (18/20) of APS1 patients examined in this series, irrespective of ECE-2 autoantibody status. While ECE-2 was shown to be abundantly expressed in pituitary tissue, preabsorption of APS1 patient sera with ECE-2 autoantibodies did not abolish or diminish pituitary autoreactivity, suggesting the presence of other, as yet unidentified, pituitary autoantigen(s). Furthermore, a consistent pituitary staining pattern of the fibrous network was observed in the APS1 sera studied, with distinct immunostaining visible in all three lobes of the pituitary. This is consistent with the pituitary staining observed in the few APS1 patients studied previously (12, 14). Several of the major APS1 autoantigens previously identified are involved in neurotransmitter synthesis and are expressed in pituitary tissue. It has been shown previously that APS1 sera targets AADC and TH in the anterior pituitary and GAD in the intermediate lobe (12, 14), yet these do not account for the entire immunostaining seen. Other pituitary autoantigens thus remain to be identified.

We have shown ECE-2 mRNA to be most abundantly expressed in the pancreas, suggesting a possible role of ECE-2 as a pancreatic autoantigen. Interestingly, a 90% transcriptional downregulation in ECE-2 mRNA expression in the kidneys has been shown in association with the early stages of autoimmune diabetes in NOD mice (33). However, no correlation between the presence of ECE-2 autoantibodies in APS1 patients and type 1 diabetes mellitus was seen in this study.

ECE-2 has also been implicated in the conversion of big ET-1 to its biologically active form ET-1, a potent vasoconstrictor peptide (23). ECE-2 knockout mice when crossed with ECE-1 null mice produced a mouse phenotype with more severe cardiac abnormalities than those of the ECE-1 null mice alone (22). As such, the expression of ECE-2 mRNA was examined in both the aorta and heart. No detectable levels of ECE-2 mRNA were detected in the aorta and only minimal levels in the heart, suggesting ECE-2 does not exert any cardiac or arterial affects at these sites.

In summary, this is the first report of ECE-2 being an autoantigen in man. However, ECE-2 autoantibodies did not define a clear clinical subset of APS1 and their possible clinical utility is yet to be determined. With its restricted neuroendocrine distribution

and proposed important role in the processing of regulatory peptides ECE-2 may indeed influence several endocrine and cognitive functions. Hence, it would be of great interest to elucidate the possible role of ECE-2 autoantibodies in Alzheimer's disease and other disorders of cognition.

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Table 1. Association of the major clinical manifestations of APS1 to ECE-2 autoantibodies				
Clinical Manifestation	**APS1**	**APS1 and ECE-2 Autoantibodies**		***p****
		With manifestation	Without manifestation	
	Number with manifestation/total (percentage)			
Mucocutaneous candidiasis	100/104 (96)	48/100 (48)	0/4 (0)	0.122
Hypoparathryoidism	87/104 (84)	41/87 (47)	7/17 (41)	0.792
Adrenal insufficiency	81/104 (78)	41/81 (51)	7/23 (30)	0.102
Alopecia areata	39/104 (38)	19/39 (49)	29/65 (45)	0.691
Gonadal failure	35/104 (34)	16/35 (46)	32/69 (46)	1.000
Intestinal dysfunction	31/104 (30)	17/31 (55)	31/73 (43)	0.286
Vitiligo	23/104 (22)	13/23 (57)	35/81 (43)	0.344
Chronic active hepatitis	19/104 (18)	12/19 (63)	36/85 (42)	0.129
Pernicious anaemia	15/104 (14)	5/15 (33)	43/89 (48)	0.402
Type 1 diabetes mellitus	12/104 (12)	7/12 (58)	41/92 (45)	0.540
Pituitary insufficiency	10/104 (10)	7/10 (70)	41/94 (44)	0.181

*Calculated by Fisher's two-tailed exact test.

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Figure Legends

Figure 1: Schematic diagram showing the alignment of the isolated ECE-2 protein with reported GeneBank transcript variants

Figure 2: Immunoreactivity to ECE-2 in sera from patients with APS1 (n=104), Addison's disease (n=15), systemic lupus erythematosus (SLE) (n=20), Sjögren's syndrome (n=20), type I diabetes mellitus (DM) (n=20), biopsy proven lymphocytic hypophysitis (n=20) and healthy controls (n=118). The broken line indicates the upper limit of the normal range (18).

Figure 3: Expression profile of ECE-2 mRNA in adult tissues analysed by quantitative PCR. *Adrenal cortex cDNA was measured in a second PCR run and its relative expression calculated from the expression levels of brain and pancreas mRNA in both runs and using heart and placenta as negative control values.

Figure 1


Exon 1
2
3
4,5a/b
8
10
11
12
14
15
17
20
22
NM_014693.3
NM_001037324.2
NM_032331.3
NM_001100120.1
NM_001100121.1
Isolated ECE2 cDNA

Figure 2

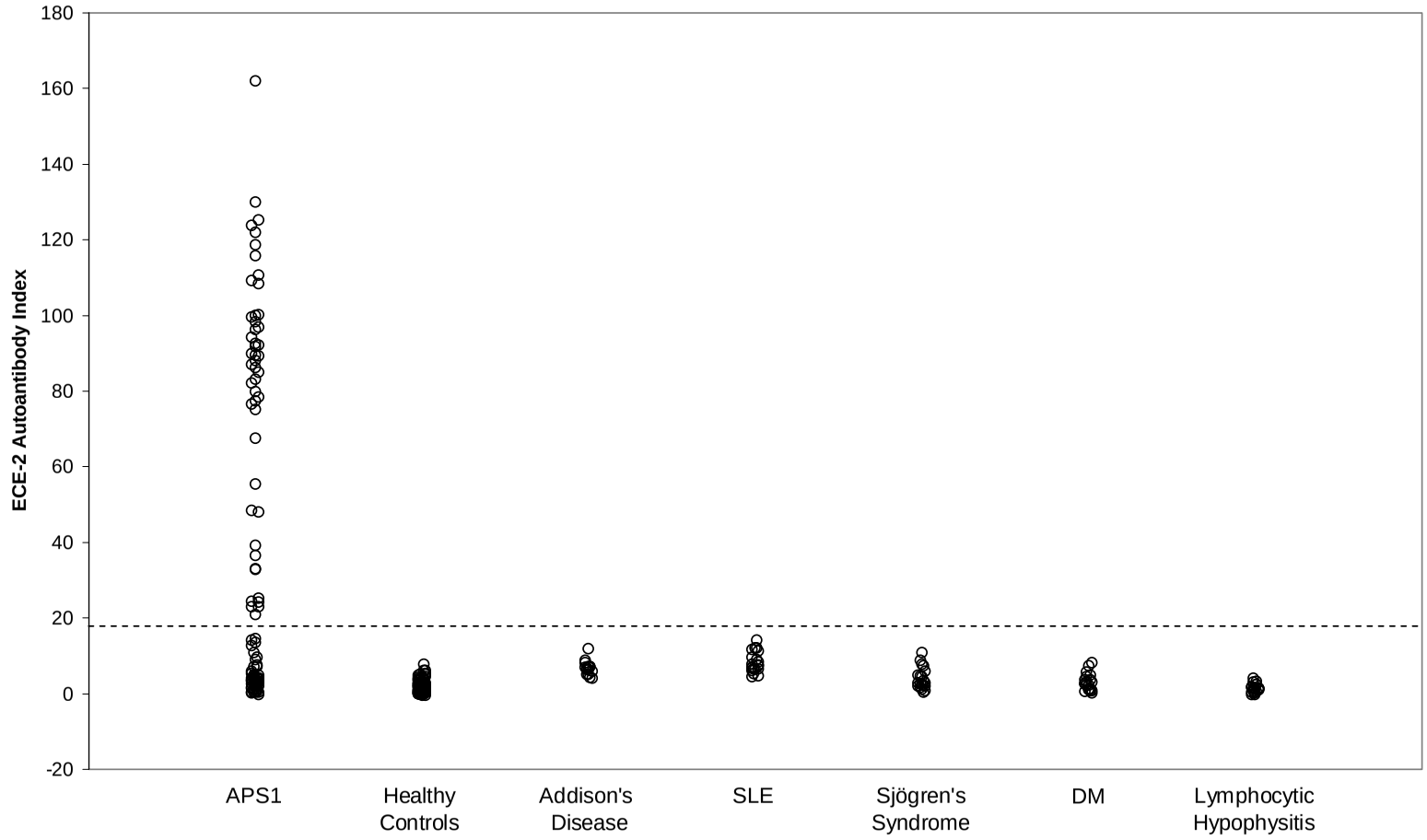
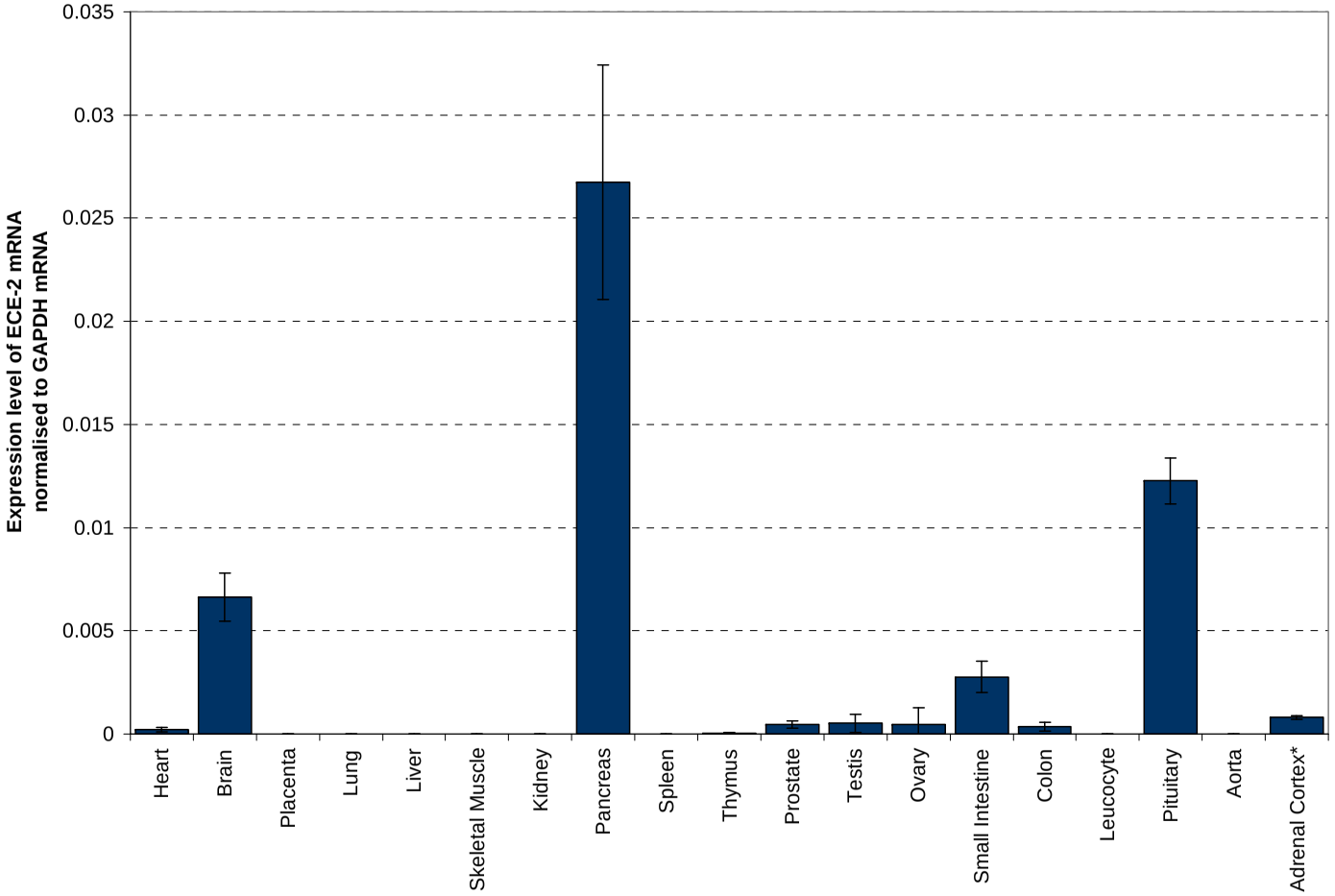

ECE-2 Autoantibody Index
180
160
140
120
100
80
60
40
20
0
-20
APS1
Healthy Controls
Addison's Disease
SLE
Sjögren's Syndrome
DM
Lymphocytic Hypophysitis

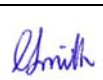
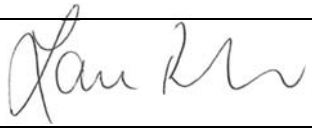
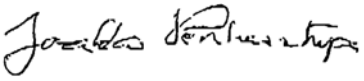
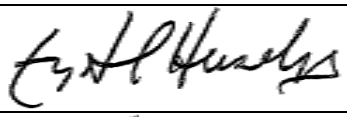
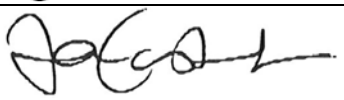
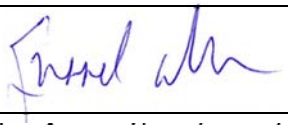
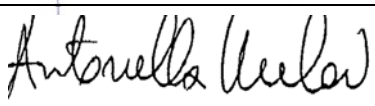
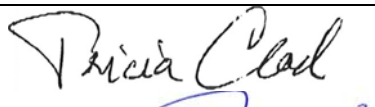
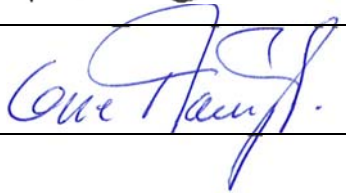
Figure 3


Expression level of ECE-2 mRNA normalised to GAPDH mRNA
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small Intestine
Colon
Leucocyte
Pituitary
Aorta
Adrenal Cortex*

Paper II [redacted]

Paper II: TSGA10 – a target for autoantibodies in Autoimmune Polyendocrine Syndrome Type 1 and Systemic Lupus Erythematosus

I hereby certify the proportion of my contribution to the following paper entitled "TSGA10 – a target for autoantibodies in Autoimmune Polyendocrine Syndrome Type 1 and Systemic Lupus Erythematosus"

Co-author	Contribution	Signature	Date
Casey Smith	84.0%		8/5/09
Sophie Bensing	5.0%		08.05.09
Mohammad AliMohammadi	2.0%		07.05.09
Mikael Oscarson	1.0%		May 11th 2009
Lars Rönnblom	1.0%		11/05/09
Jaakko Perheentupa	1.0%		27.4.09
Eystein Husebye	0.5%		27 April 2009
Jan Gustafsson	0.5%		April 28, 2009
Gunnel Nordmark	0.5%		11/5/09
Antonella Meloni	0.5%		20-05-09
Patricia Crock	2.0%		1/5/2009
Olle Kämpe	2.0%		070509

TSGA10 – a target for autoantibodies in Autoimmune Polyendocrine Syndrome Type 1 and Systemic Lupus Erythematosus

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Abstract

Autoimmune polyendocrine syndrome type 1 (APS1) is a rare monogenic autoimmune disorder caused by mutations in the autoimmune regulator (AIRE) gene. High titre autoantibodies are a characteristic feature of APS1 and are often associated with particular disease manifestations. Pituitary deficits are reported in approximately 7% of APS1 patients, with immunoreactivity to pituitary tissue frequently reported. Using APS1 patient serum to immunoscreen a pituitary cDNA expression library, TSGA10 was isolated. Immunoreactivity against TSGA10 was detected in 5/101 (4.95%) patients with APS1, all of Finnish origin, but also 2/72 (2.78%) SLE patients and 1/122 (0.82%) healthy controls. TSGA10 autoantibodies were not detected in the serum from patients with any other autoimmune diseases. In addition, TSGA10 autoantibodies were detectable from a young age in 4/5 positive APS1 patients with autoantibody titres remaining relatively constant over time. Furthermore, real-time PCR confirmed TSGA10 mRNA to be most abundantly expressed in the testis and also showed moderate and low expression levels throughout the entire body. TSGA10 should be considered as a minor APS1 autoantigen, evidently confined to patients of Finnish origin and also in a minority of SLE patients. No recognisable clinical phenotype could be found to correlate with positive autoantibody reactivity.

Introduction

Autoimmune polyendocrine syndrome type 1 (APS1; alternatively known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) is a rare monogenic autoimmune disease resulting from mutations in the autoimmune regulator (AIRE) gene. The AIRE gene plays a vital role in the removal and inhibition of self reactive T-cells in the thymus [1-3], a breakdown of which consequently leads to the development of the organ specific autoimmune disease APS1. The disorder is characterised by the classical triad of chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal failure, the combined presence of at least two of these are traditionally required for the diagnoses. These symptoms begin to manifest in the first decade of life followed by the progressive emergence of other organ-specific autoimmune diseases including gonadal failure, intestinal dysfunction, hypothyroidism, alopecia, vitiligo, chronic active hepatitis and insulin dependent diabetes mellitus as well as ectodermal manifestations, all with variable penetrance.

Pituitary manifestations are another lesser described component of APS1, being diagnosed in approximately 7% of all APS1 patients [4]. Patients present with single or multiple pituitary deficits, the most commonly reported deficit being isolated growth hormone (GH) deficiency [5]. Partial adrenocorticotropin hormone deficiency, isolated hypogonadotrophic hypogonadism and central/idiopathic diabetes insipidus have also being described [5-11]. A single case of APS1 has also been reported in combination with lymphocytic hypophysitis in a French-Canadian patient with GH deficiency. An MRI scan showed the characteristic ring-enhancement seen in lymphocytic hypophysitis [12].

Pituitary autoantibodies in APS1 sera have been detected against both lactotrophs and gonadotrophs using immunohistochemistry [5, 13, 14]. APS1 patients also have autoantibodies directed towards a small number of guinea pig anterior pituitary cells, 40-50% of which are GH producing cells [15]. In addition, a fiber-plexus staining pattern is observed in the pituitary intermediate lobe. Several of the major APS1 autoantigens previously identified are involved in monoamine and GABA synthesis and are expressed in pituitary tissue. APS1 patient sera targets aromatic L-amino acid decarboxylase (AADC) and tyrosine hydroxylase (TH) in the anterior pituitary and

GAD in the intermediate lobe [13, 15], yet these do not account for the entire immunostaining seen. A further two pituitary autoantigens, alpha enolase [16] and TDRD6 [15], have been proposed but could not be associated to pituitary insufficiency or account for the pituitary immunostaining seen in APS1 patients. Other pituitary autoantigens thus remain to be identified.

This current study aimed to identify potential pituitary autoantigens from immunoscreening of a human pituitary cDNA expression library to delineate the correlation between pituitary manifestations in APS1 patients and pituitary autoantibodies.

Methods

Patients

Serum samples from a total of 101 APS1 patients including 57 Finnish, 17 Norwegian, 16 Sardinian and 11 Swedish patients were collected for analysis. The clinical diagnosis of APS1 was based on the presence of at least two of the classical triad features of APS1; mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency. Patients with only one of these features who had confirmed mutations in both alleles of the AIRE gene were also included. In this series, nine patients had confirmed pituitary manifestations including seven with GH deficiency and two with hypogonadotrophic hypogonadism.

Serum samples were also obtained from 132 patients with other autoimmune diseases comprising of 15 patients with Addison's disease, 20 with Sjögren's syndrome, 20 with biopsy proven lymphocytic hypophysitis, 20 with type 1 diabetes mellitus and 57 with systemic lupus erythematosus (SLE). 122 healthy Australian blood donors served as controls.

Ethical approval was obtained from the Committee of Ethics, Faculty of Medicine, Uppsala University and the Human Research Ethics Committees of the Hunter Area Health Service and University of Newcastle with informed consent from all patients and controls.

Screening of a Human Pituitary cDNA Library

Two APS1 patients were selected for analysis, one with clinically reported GH deficiency and one without any known pituitary manifestations. The sera were used to immunoscreen a pituitary cDNA expression library as previously described [15]. In-vitro excision of the pBK-CMV phagemid vectors from the ZAP express vector was performed according to the manufacturer's instructions (Stratagene). Isolated positive cDNA clones were partially sequenced in both the 5' and 3' direction using a dye-terminator sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 3730 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). The cDNA clones were then identified by comparing the sequencing data against available databases using the BLAST program. The cDNA clone encoding TSGA10 was fully sequenced with additional internal primers (Cybergene, Huddinge, Sweden).

In Vitro Transcription and Translation (ITT) of autoantigens and Immunoprecipitation

Recombinant ^{35}S-methionine radiolabelled proteins were produced by ITT in a T3 coupled reticulocyte lysate system (Promega) and analysed for ^{35}S-methionine incorporation according to the manufacturer's instructions, before being used for immunoprecipitation with patient sera. In 96 well plates, 25000 to 30000 cpm of the recombinant protein and 2.5µL of undiluted patient serum were mixed in a buffer containing 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), and 0.02% NaN_3, 0.1% BSA and 0.15% Tween-20 (Buffer B) in a total volume of 50 µL and incubated overnight at 4°C. The antibody complexes were precipitated with 50 µL of a 50% slurry of protein A-Sepharose (Pharmacia, Stockholm, Sweden) in Buffer B in pretreated 96 well microtiter plates with filter bottoms [17] (MABV N12, Millipore, Bedford, MA) for 45 min at 4°C. The plates were washed ten times with Buffer B using a vacuum manifold. After drying, 70 µL OptiPhase SuperMix (Perkin Elmer Life Sciences, Boston, MA) was added to each well and the plates counted in a beta counter (Wallac 1450 MicroBeta, PerkinElmer). Results were expressed as an antibody index [(cpm sample – cpm negative control)/(cpm positive control – cpm negative control) x 100]. Patient sera were analysed in duplicate, whereas the positive control (the screening patient serum from which the clone was isolated) and the negative control (4% bovine serum albumin; Sigma) were run in triplicate. An upper normal antibody

index for TSGA10 was calculated as the average antibody index of the healthy blood donors plus four standard deviations.

A consecutive study was also performed on the archival serum samples from patients established to have a positive TSGA10 autoantibody index to determine the age at which these patients developed autoantibodies towards the protein. ITT was performed as above on all archive serum samples collected from the time of diagnosis. The same positive and negative controls were used for all ITT experiments.

Tissue expression profiling of TSGA10

The tissue expression profile of TSGA10 mRNA throughout various organs was studied by quantitative PCR performed on cDNA from human tissue. Primers were designed with Beacon Designer® version 5.11 software with one primer flanking an intron-exon junction to avoid amplification of genomic DNA. Quantitative PCR was carried out on human normalised multiple-tissue cDNA panels (BD Bio Sciences, Palo Alto, CA, USA) as well as pituitary, aorta (Stratagene) and adrenal cortex cDNA prepared from normal adrenal tissue removed during adrenal adenoma surgery. Reactions were performed on a MyiQ iCycler (Bio-Rad, Hercules, CA, USA) in a volume of 25 µl, with 200 nM of each primer using iQ™ SYBR®Green supermix (Bio-Rad, Hercules, CA, USA) as per the manufacturer's instructions. All samples were run in triplicate. Thermal cycles consisted of an initial denaturation step of 95°C for 3min, followed by 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. Standard curves were then established from the serial dilution of TSGA10 and control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) PCR templates. TSGA10 mRNA levels were deduced from the standard curve and normalised to the endogenous GAPDH tissue content.

Results

Cloning and identification of TSGA10

A total of 27 cDNA clones were isolated and identified from immunoscreening of a human pituitary cDNA expression library with the sera from two APS1 patients, one with clinical GH deficiency, and one with no reported pituitary manifestations. Five of

the clones isolated from the patient without any pituitary deficits encoded TSGA10, a gene located on chromosome 2q11.2. *In vitro* transcription and translation of two of the TSGA10 clones resulted in good quantities of recombinant proteins that were used for immunoprecipitation with a test panel of sera from six APS1 patients and five healthy controls. Both TSGA10 recombinant proteins were efficiently immunoprecipitated by the screening serum but not by any of the healthy controls and one of the corresponding TSGA10 clones was therefore selected for further studies. The TSGA10 gene consists of 19 exons spanning over 80kb of genomic DNA. Two transcript variants have been reported, differing in the 5' UTR. Both variants are transcribed from exon 6 to exon 21 and encode a 698 amino acid protein. In comparison to the GeneBank mRNA reference sequences (GeneBank accession nos. **NM_182911** and **NM_025244**), the TSGA10 cDNA clone used for the immunoprecipitation studies, extends from the middle of exon 9 to the end of the coding sequence, with exons 11, 12 and 13 omitted. This sequence is predicted to encode a 431 amino acid protein.

Analysis of TSGA10 as an APS1 autoantigen

To determine whether autoantibodies against TSGA10 were specific for patients with APS1, sera collected from 101 APS1 patients, 95 patients with other autoimmune diseases and 122 healthy blood donors were analysed for immunoreactivity against the TSGA10 recombinant protein. Five of 101 (4.95%) APS1 patients were found to have anti-TSGA10 antibodies. These five autoantibody positive patients consisted of one female and four male APS1 patients. The highest autoantibody index was observed in serum from the female patient (index: 130) whereas the male indexes ranged from 30 to 104. Two of 57 (3.51%) SLE patients and 1/122 (0.82%) healthy blood donors also had positive TSGA10 autoantibody indexes, with one of the positive SLE patients and the healthy blood donor both having low titre autoantibodies (index of 16.5 and 21 respectively) towards TSGA10. No autoantibodies were detected in the sera from patients with Addison's disease, Sjögren's syndrome, type 1 diabetes mellitus or biopsy proven lymphocytic hypophysitis (Figure 1).

The positive APS1 patients were analysed to ascertain any manifestations common to all five patients. All five patients immunoreactive against the recombinant TSGA10

protein were of Finnish origin yet no associations between the major clinical manifestations of APS1 and TSGA10 autoantibodies were evident in these patients. Aside from the classical triad components of mucocutaneous candidiasis and adrenal insufficiency, no major clinical feature was common to all five patients (Table 1). Furthermore, none of the nine patients in the series with pituitary defects were TSGA10 antibody positive.

The SLE patient with high TSGA10 autoantibody index was a woman who developed SLE at 72 years of age (74 when sampled). She had a very active disease with hemolytic anemia, serositis (both pleuritis and pericarditis), arthritis, oral ulcers, and fever without infections. In addition, she lost weight, which was interpreted as a result of the very active disease. She had very high titers of antinuclear antibodies (ANA) and double-stranded DNA antibodies (anti-dsDNA) and a very low titer of rheumatoid factor (RF). No other important autoantibodies were detected in routine analysis. She was treated with cytotoxic drugs and massive doses of steroids. To prevent osteoporosis she also received Calcium and D-vitamin supplement. She died 8 years after the diagnosis of SLE due to a severe pulmonary infection. The patient was not known to suffer from any malignant disease.

The SLE patient with a low TSGA10 antibody titer had her first disease manifestations at 19 years of age but obtained the SLE diagnosis at age 34 (serum sample obtained at 37). She has a rather mild disease with arthritis, sun sensitivity, oral ulcers and ANA with Ro52 antibodies. In addition, she has a secondary Sjögrens syndrome. During the last years the disease has been inactive.

Consecutive archive analysis of TSGA10 autoantibodies

To determine both the age at which TSGA10 autoantibodies manifest and if there are any fluctuations in TSGA10 autoantibody titers over disease duration, ITT was conducted on all serum samples collected from the five autoantibody positive patients collected from the time of APS1 diagnosis (Figure 2). Serum samples were available from a range of 4.5 years post diagnosis to 23.5 years post diagnosis with a median of 14.5 years for each patient.

Three of the five patients had autoantibodies against TSGA10 from the first available serum sample at ages 7, 9 and 14 years. Seroconversion to a positive TSGA10 autoantibody index was observed in the remaining two patients at age 8 and the second at 29 years of age. Autoantibody titres remained constant for each patient with every sample available with the longest follow-up time of 23.5 years.

Tissue expression profile

The tissue expression of TSGA10 was also examined in various organs by quantitative PCR. TSGA10 mRNA was predominantly expressed in testicular tissue (Figure 3), with expression also being detected in almost all tissues studied, albeit at very low levels in most organs. Virtually undetectable TSGA10 mRNA expression was observed only in the heart, skeletal muscle, leucocytes and adrenal cortex.

Discussion

Pituitary manifestations are a rare feature of APS1 presenting as either single or multiple hormonal deficiencies. Autoantibodies against pituitary tissue have been repeatedly shown by immunofluorescence in the sera of APS1 patients, yet no autoantigen has thus far been ascribed. Interestingly, anti-pituitary autoantibodies are infrequently detected in APS1 patients with pituitary manifestations [4]. To identify potential pituitary antigens in APS1, sera from one APS1 patient with clinically diagnosed GH deficiency and a patient with no known pituitary deficits were used to immunoscreen a human pituitary cDNA expression library. A cDNA clone encoding TSGA10 was isolated and identified as a minor autoantigen in APS1.

While conducting the present study, the TSGA10 autoantigen was also independently isolated from a human testis cDNA expression library and characterised using sera from within the same Finnish APS1 series [18]. In agreement with their findings, high titre antibodies against TSGA10 were found in a small percentage of Finnish patients (5 of 57 vs 5 of 66), yet we did not detect any autoantibodies in the additional patients of Swedish, Norwegian or Sardinian descent. Three of the five positive patients are common to both studies; we identified an additional two APS1 patients, one male and one female with autoantibodies against TSGA10. In contrast to the previous study, we

also detected TSGA10 autoantibodies in two patients with SLE of which one had high titre autoantibodies, and also at low titre in a single healthy blood donor.

We also studied the age at which TSGA10 autoantibodies are detectable using archived patient sera. Autoantibodies were present from a young age (≤14 years) in four of the five positive APS1 patients, whereas the fifth patients seroconverted at the age of 29 years. Moreover, once the patient has seroconverted, TSGA10 autoantibody titres remained fairly consistent for each patient over time, with the longest follow up period of 23.5 years.

TSGA10 is highly expressed in the testis [19] where it is processed to form a structural protein of the fibrous sheath in mature spermatozoa [20]. No expression of TSGA10 mRNA was detected in the testes of two infertile men [19] suggesting a link between TSGA10 and infertiliy. Gonadal failure is a common feature of APS1 seen in 17 to 60% of patients [4, 21]. Primary ovarian failure is frequently observed in approximately 65% of female patients, whereas male hypogonadism is far less common being reported in around 12% of male APS1 patients [22]. Of the five TSGA10 autoantibody positive APS1 patients, the four male patients were not diagnosed with hypogonadism, but the one positive female patient had primary ovarian failure. None of the remaining 34 patients with gonadal failure had detectable TSGA10 autoantibodies, suggesting this antigen does not function as a gonadal antigen in APS1 patients.

By quantitative PCR TSGA10 mRNA has been shown not only to be highly expressed in the testis, but is almost ubiquitously expressed throughout the body, albeit at low levels [23]. We have also confirmed these results and further shown TSGA10 mRNA to be moderately expressed in the pituitary gland and aorta, with very little expression in the adrenal cortex. It was been previously proposed the function of TSGA10 may not just be limited to spermatogenesis but have a more widespread role throughout the body being expressed in actively dividing or post-mitotic cells [24]. The finding of TSGA10 mRNA being expressed in many organs helps support this hypothesis. Its antigenic target in APS1 may therefore be in any organ where expression is seen. We isolated TSGA10 from a pituitary cDNA library and also showed moderate levels of

mRNA expression in this organ and therefore investigated TSGA10 as a potential pituitary antigen. No association however, was observed between TSGA10 autoantibody status and pituitary manifestations in APS1, with the protein being isolated from a patient with no pituitary defects and none of the other positive patients diagnosed with a pituitary deficit.

In addition, TSGA10 is considered to be a possible cancer/testis (CT) -like antigen as over-expression of the protein is seen in a range of cancers including cancers of the colon, ovary, prostate, bladder [25], brain, breast and skin [26] as well as in cutaneous lymphoma [23] and acute lymphoblastic leukemia [27]. Cancer is an infrequently reported complication of APS1. Chronic Candida albicans infections appear to predispose individuals to squamous cell carcinoma of the mouth or oesophagus. This has been seen in 10.5% of APS1 patients over the age of 25 years, with no other malignancies being reported in APS1 patients [28]. To our knowledge, none of the five APS1 patients or the single SLE patient were diagnosed with squamous cell carcinoma or any other cancer at the time of sampling.

None of the common associated features of APS1 besides the classical diagnostic triad of mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency, were common to all five TSGA10 autoantibody positive APS1 patients. These patients may possibly have a rare feature of APS1 that has not been clinically reported. Conversely, autoantibodies against TSGA10 may not result in a typical phenotype.

The explanation of the finding of a high TSGA10 autoantibody titer in one of the SLE patients is not evident since she did not suffer from any APS1 manifestation or malignant disease. APS1 is highly associated with organ-specific autoimmunity however the patients rarely present systemic autoimmune manifestations. To our knowledge no APS1 patient has co-presented with an SLE diagnosis. It has also been suggested that AIRE-mediated thymic negative selection of lymphocytes is not a relevant pathway in SLE pathophysiology [29]. Larger cohorts of SLE patients need to be evaluated to establish the possible role for TSGA10 as a minor autoantigen in SLE.

The identification of TSGA10 as an autoantigen in APS1 augments the growing list of autoantigens involved in the complex autoimmune progression of the disease. Independent isolation of this antigen from both a pituitary and testis cDNA library shows this technique is an effective was to identify autoantigens both specific to that target organ or more ubiquitously found in the body. In contrast to the earlier study, we have shown TSGA10 autoantibodies are not restricted to APS1 patients, but were also found in the sera from patients with SLE and a healthy control. While the exact functional role of anti-TSGA10 antibodies in disease manifestation remains to be clarified, TSGA10 should be considered as a minor APS1 autoantigen, evidently confined to patients of Finnish origin and also in a minority of SLE patients.

Table 1. Clinical characteristics of APS1 patients with TSGA10 autoantibodies					
	Patient				
Characteristic	**1**	**2**	**3**	**4**	**5**
Sex	M	M	M	F	M
Mucocutaneous candidiasis	+	+	+	+	+
Hypoparathyroidism	-	-	+	+	-
Adrenal insufficiency	+	+	+	+	+
Gonadal failure	-	-	-	+	-
Alopecia	-	-	-	+	+
Vitiligo	-	-	-	-	-
Type 1 diabetes mellitus	-	-	+	-	-
Intestinal dysfunction	+	-	-	+	-
Hepatitis	+	-	-	+	-
Pernicious anaemia	-	-	-	-	-
Pituitary dysfunction	-	-	-	-	-
Age (years) autoantibodies detectable	≤7	8	≤9	≤14	29

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Figure Legends

Figure 1. Analysis of autoantibodies against TSGA10 in sera from patients with APS1 (n=101), Addison's disease (n=15), systemic lupus erythematosus (SLE) (n=57), Sjögren's syndrome (n=20), type I diabetes mellitus (DM) (n=20), biopsy proven lymphocytic hypophysitis (n=20) and healthy controls (n=122). The broken line indicates the upper limit of the normal range calculated by the average autoantibody index of the negative healthy controls plus 3SD (limit = 12).

Figure 2: TSGA10 autoantibody titers in archive serum of patients determined to be autoantibody positive.

Figure 3. Expression profile analysis of TSGA10 mRNA in various human adult tissues analysed by quantitative real-time PCR.

Figure 1

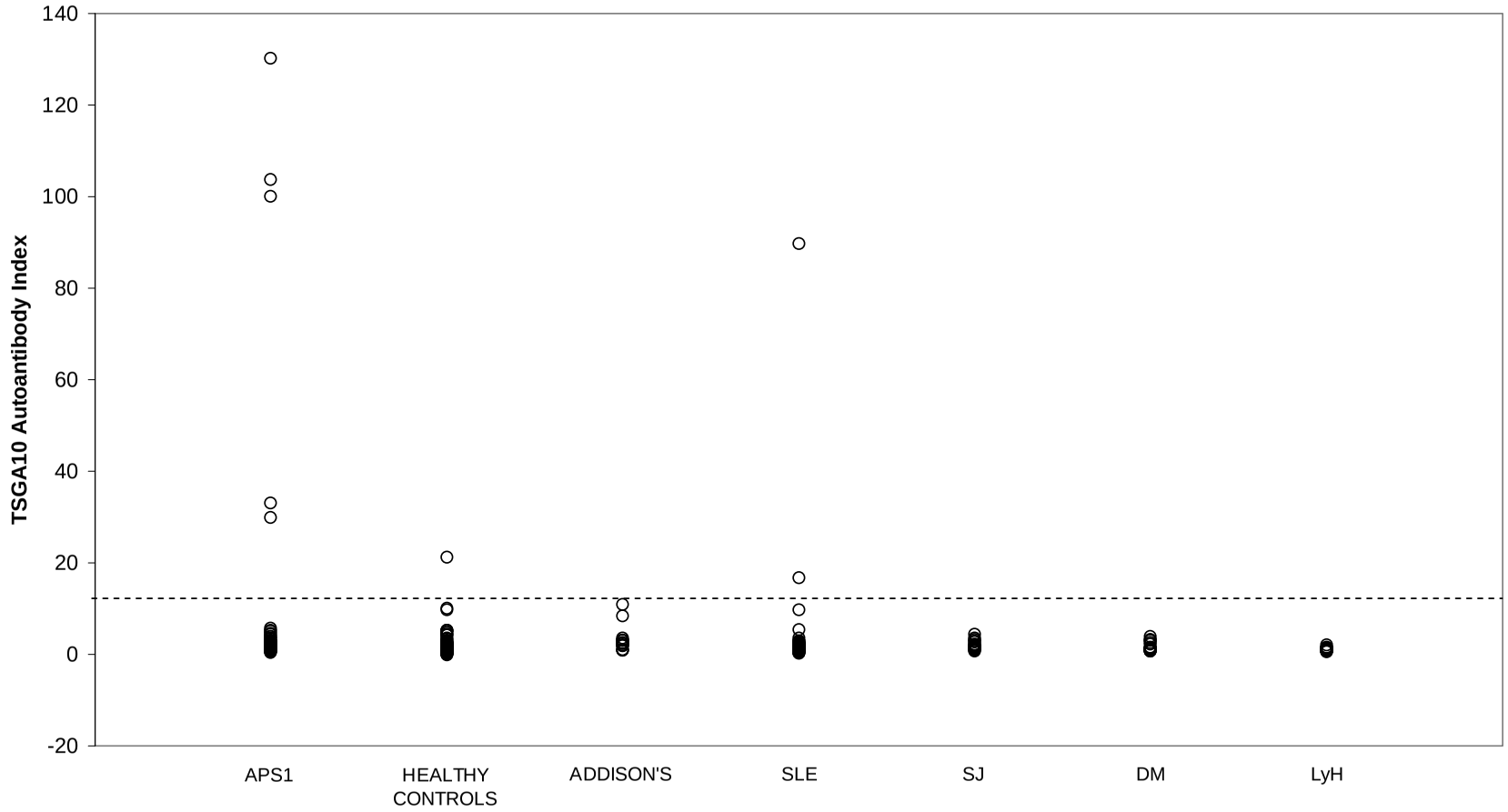

140
120
100
80
60
40
20
0
-20
TSGA10 Autoantibody Index
APS1
HEALTHY CONTROLS
ADDISON'S
SLE
SJ
DM
LyH

Figure 2

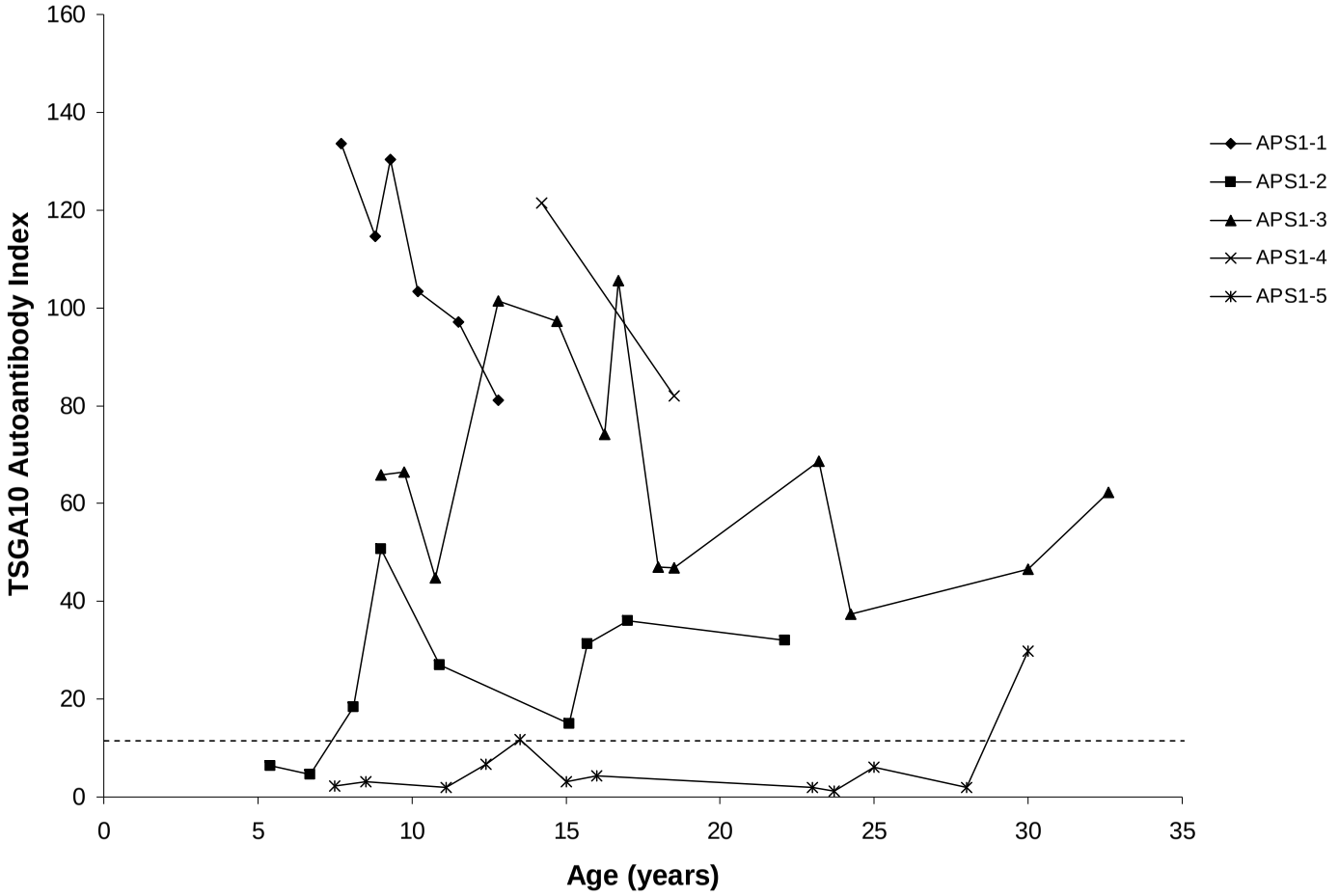
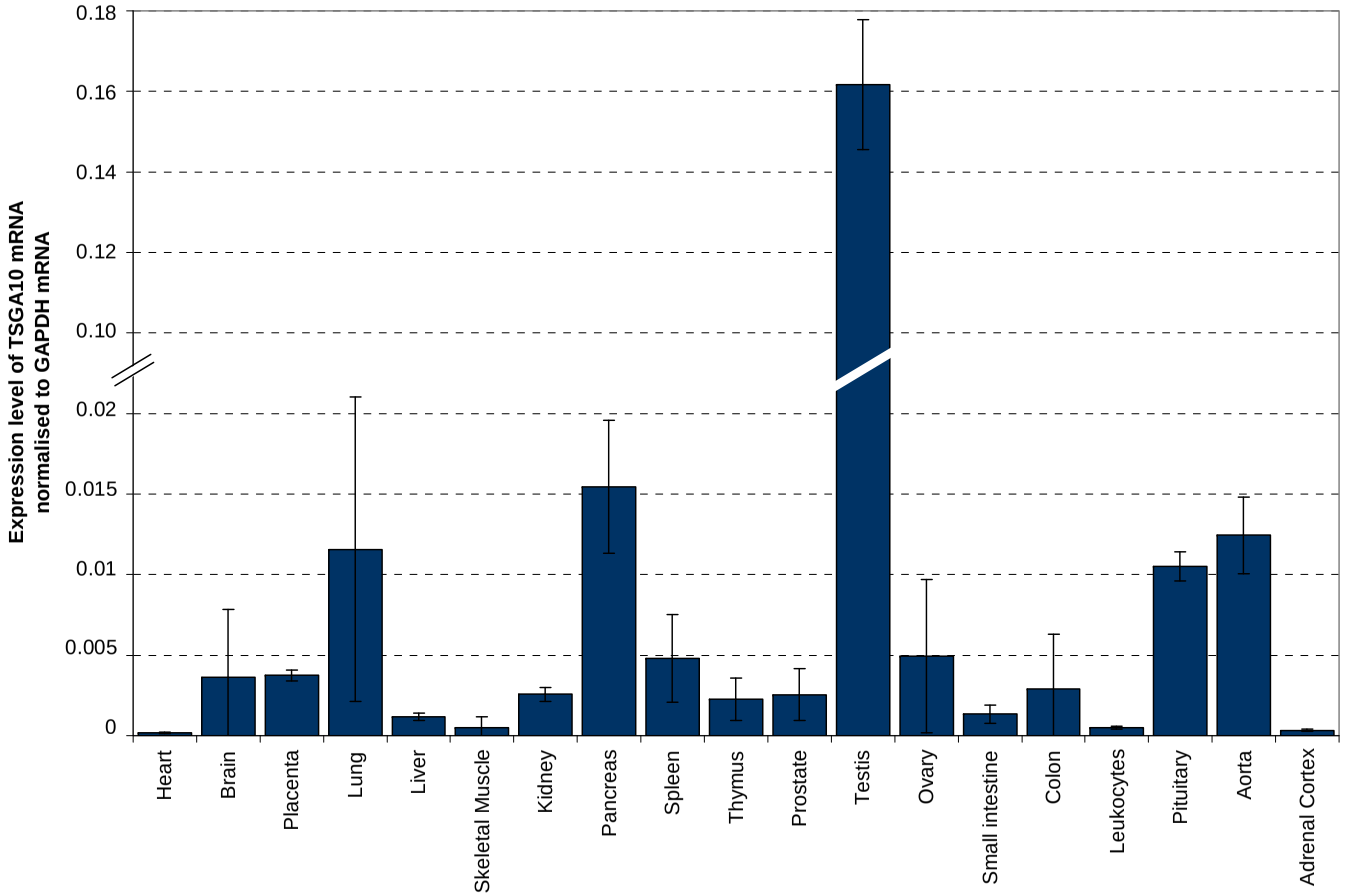

TSGA10 Autoantibody Index
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Age (years)
APS1-1
APS1-2
APS1-3
APS1-4
APS1-5

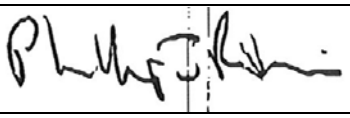
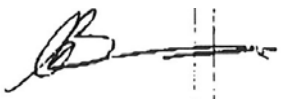
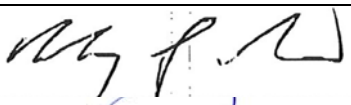
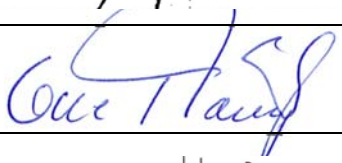
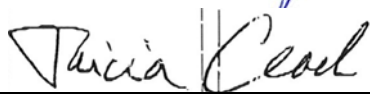
Figure 3


Expression level of TSGA10 mRNA normalised to GAPDH mRNA
0.18
0.16
0.14
0.12
0.10
0.02
0.015
0.01
0.005
0
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
Leukocytes
Pituitary
Aorta
Adrenal Cortex

Paper III [redacted]

Paper III: Identification of candidate autoantigens in lymphocytic hypophysitis; immunoscreening of a pituitary cDNA library and development of immunoprecipitation assays

I hereby certify the proportion of my contribution to the following paper entitled "Identification of candidate autoantigens in lymphocytic hypophysitis; immunoscreening of a pituitary cDNA library and development of immunoprecipitation assays"

Co-author	Contribution	Signature	Date
Casey Smith	92.0%		8/5/09
Sophie Bensing	2.0%		07.05.09
Phillip Robinson	1.0%		1/5/2009
Christine Burns	0.5%		1/5/2009
Rodney Scott	0.5%		1/5/2009
Olle Kämpe	2.0%		07.05.09
Patricia Crock	2.0%		1/5/2009

Identification of candidate 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 characterised by infiltration of lymphocytes into the pituitary gland. A specific and sensitive serological test currently does not exist to aid in the diagnosis of this disease.

Objective: To identify target autoantigens in lymphocytic hypophysitis and to develop a diagnostic assay to determine the frequency and specificity of immunoreactivity to these proteins.

Design/Methods: A pituitary cDNA expression library was immunoscreened using serum from four previously characterised patients with lymphocytic hypophysitis. Promising cDNA clones from the screening, along with previously identified autoantigens pituitary gland specific factor 1a and 2 (PGSF1a, PGSF2) and neuron-specific enolase (NSE) were tested in an ITT (*in vitro* transcription translation) immunoprecipitation assay. The corticotroph-specific transcription factor, T-box 19 was also investigated as a candidate autoantigen.

Results: Significantly positive autoantibody reactivity against T-box 19 was found in 9 of 86 (10.5%) hypophysitis patients versus 1 of 90 (1.11%) controls (p=0.018, χ^2 Yates correction). CHD8 autoantibodies were detected in 7 of 86 (8.14%) hypophysitis patients versus 3 of 90 (3.33%) of healthy controls (p=0.29 χ^2 Yates correction); piccolo autoantibodies were found in 3 of 86 (3.49%) patients versus 2 of 90 (2.22%) of healthy controls (p=0.96 χ^2 Yates correction); CADPS autoantibodies were found in 12 of 86 (14.0%) of patients versus 11 of 90 (12.2%) of healthy controls (p=0.91 χ^2 Yates correction). PGSF2 autoantibodies were seen in 5 of 86 (5.81%) patients and 2 of 90 (2.22%) controls (p=0.40 χ^2 Yates correction). NSE autoantibodies were seen in 2 of 86 (2.33%) patients but no controls (p=0.46 χ^2 Yates correction).

Conclusions: T-box 19, a corticotroph-specific transcription factor, was identified as a minor target autoantigen in 10.5% of lymphocytic hypophysitis patients. Immunoscreening of a pituitary cDNA expression library was found to be a powerful method in identifying multiple potential target autoantigens.

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 correlation with pregnancy, with approximately two thirds of women presenting in the third trimester or in the post partum period (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 adenomata where they are usually the last to fail (3-6). The ensuing secondary adrenal insufficiency can prove fatal if untreated. In chronic cases of the disease, the ongoing autoimmune process can cause post-inflammatory fibrosis leading to pituitary gland atrophy and an empty sella (7-9).

The pituitary mass in lymphocytic hypophysitis can be indistinguishable from that of a pituitary adenoma on MRI. Although some features on MRI are more suggestive of hypophysitis, such as ring enhancement and a “dural tail”, it may be impossible to differentiate the two. Pituitary biopsy has been used to both alleviate the symptoms of the pituitary mass and to diagnose the disease on histological grounds. However, pituitary biopsy can lead to permanent pituitary failure and as such a more 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. The autoantibodies can be detected years before the onset of the disease and can be good predictors of disease progression and outcome (10-12). Pituitary autoantibodies have been studied in various autoimmune diseases by a number of techniques including immunofluorescence, immunoblotting and most recently a radioligand (immunoprecipitation) assay to identify the autoantigens targeted.

A number of potential autoantigens have been proposed in lymphocytic hypophysitis including alpha-enolase (13-15), neuron specific enolase (15), growth hormone (16, 17), pituitary gland specific factors 1a and 2 (18), secretogranin II (19) and most

recently chromosome 14 open reading frame 166 and chorionic somatomammotropin (20). 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 at identifying 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 neuron specific enolase (NSE), PGFS1a, PGFS2 and a potential new candidate, T-box 19, a pituitary-specific transcription factor essential for development of the corticotroph lineage.

Methods

Patients

Serum samples were collected for analysis from 86 lymphocytic hypophysitis patients, 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" 10 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 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 (1 biopsy proven and 3 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 (21) and a classical clinical history. The pituitary cDNA expression library (22) was immunoscreened separately with all four patient sera (diluted 1:200) as previously described (23). *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). The isolated cDNA clones were partially sequenced using a dye-terminator sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 3730 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). cDNA clones were identified by comparison of the sequencing data against available databases using BLAST.

Potential Candidate Autoantigens

The corticotroph-specific transcription factor, T-box 19 (also referred to as TBX19 and TPIT) has been identified as the causative gene in isolated ACTH deficiency of neonatal onset. It was hence chosen for study as corticotroph cells tend to be preferentially targeted in lymphocytic hypophysitis resulting in isolated ACTH deficiency.

Previously reported Candidate Autoantigens

Full-length cDNA PGSF1a and PGSF2 clones were kindly donated by Dr Tatsumi (Japan) and neuron specific enolase (NSE) was purchased from the clone database (Image Clone 3629603).

In Vitro Transcription and Translation (ITT) of autoantigens and Immunoprecipitation

All library cDNA clones identified by immunoscreening that were of interest, as well as T-box 19, PGSF1a, PGSF2 and NSE were subcloned into the pTNT vector

(Promega, Madison, WI) by double restriction enzyme digestion, for improved efficiency of in vitro transcription and translation. 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 sublconed into the pTNT vector.

Autoantigens were expressed in 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, Madison, WI) and used for immunoprecipitation with patient sera. In 96 well plates, 25000 to 30000 cpm of the radiolabeled protein and 2.5μL of undiluted patient serum were mixed in a buffer containing 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), and 0.02% NaN_3, 0.1% BSA and 0.15% Tween-20 (Buffer B) in a total volume of 50 μL. In addition, dithiothreitol (DTT) was added to Buffer B at a final concentration of 0.1% for cDNA clones CHD8 and Piccolo to linearise the proteins and hence improve immunoprecipitation efficiency. Plates were then incubated overnight at 4°C. The antibody complexes were then precipitated with 50 μL of a 50% (vol/vol) slurry of protein A-Sepharose (Pharmacia, Stockholm, Sweden) in buffer B in pretreated 96 well microtiter plates with filter bottoms (24) (MABV N12, Millipore, Bedford, MA) for 45 min at 4°C. The plates were washed ten times with Buffer B using a vacuum manifold. After drying, 70 μL OptiPhase SuperMix scintillation fluid (Perkin Elmer Life Sciences, Boston, MA) was added to each well and the plates counted in a beta counter (Wallac 1450 MicroBeta, PerkinElmer).

The patient serum from which the respective cDNA clone was isolated on immunoscreening the pituitary cDNA library, was used as the positive control and 4% bovine serum albumin (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 [(cpm of the unknown sample – cpm of the negative control)/(cpm of the positive control – cpm of the negative control) x 100]. For the potential and previously reported candidate autoantigens where no positive controls were available (ie T-box 19, PGSF1a, PGSF2 and NSE), antibody indexes were calculated as [cpm sample / mean cpm of healthy controls]. The upper

normal autoantibody index was set at the mean of the unequivocally negative healthy blood donors plus 3 standard deviations for all autoantigens tested.

Statistical Analysis

A χ^2-test with 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 lymphocytic hypophysitis patients; 1 biopsy proven and 3 suspected cases, previously shown to have high titre pituitary autoantibodies on immunoblotting (JCEM 98). 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 to the GeneBank database (GeneBank 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, 260kDa 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 were chosen for testing with ITT against a test panel of patient and control sera. When 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 Ca^{2+}-dependent secretion activator (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 isolated 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 encoded a 5142 amino acid protein, with also a 4935 amino acids transcript variant being identified (GeneBank 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 differing in the 3' nucleotide sequence of the gene. On comparison with the GeneBank mRNA reference sequences (variant 1: accession ID NM_003716, variant 2: accession ID NM_183394, 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 initial nucleotide sequence is crucial for correct protein folding of the CADPS protein, therefore a full length rat CADPS (rCADPS) was obtained and used for all further analyses. The rat homologue is located on chromosome 15p16 and shares 98% homology to human CADPS protein.

Autoantibody analysis of library autoantigens CHD8, Piccolo and rCADPS

To ascertain the specificity of CHD8, Piccolo and rCADPS (full-length) as lymphocytic hypophysitis autoantigens, ^{35}S-methionine labelled proteins produced by *in vitro* transcription and translation were immunoprecipitated against sera from 86 lymphocytic hypophysitis patients and 90 healthy blood donors (Table 1).

CHD8 autoantibodies were detected in the sera from 7 of 86 (8.14%) lymphocytic hypophysitis patients including 2 of 21 (9.52%) patients with biopsy proven lymphocytic hypophysitis, 4 of 43 (9.30%) suspected cases and in 1 of 4 (25%) patients with suspected hypophysitis with diabetes insipidus as the single presenting symptom. Positive immunoreactivity was also seen in 3 of 90 (3.33%) healthy controls (Figure 1a). Statistical analysis of the data demonstrated the frequency of autoantibodies in lymphocytic hypophysitis patients was not significantly different to healthy controls, $p=0.2932$ (χ^2-test with Yates' correction).

Piccolo autoantibodies were found in 3 of 86 (3.49%) patients with lymphocytic hypophysitis comprising one (1 of 21, 4.76%) biopsy proven patients, one (1 of 43, 2.33%) suspected lymphocytic hypophysitis patient and a single patient (1 of 10, 10.0%) with isolated ACTH deficiency. Autoantibodies were also detected in 2 of 90 (2.22%) healthy controls, of which one had extremely high titre autoantibodies in comparison to the control screening serum (Figure 1b).

Autoantibodies against the recombinant rCADPS protein were identified in the sera of 12 of 86 (14.0%) lymphocytic hypophysitis patients of which there were three (3 of 21, 14.3%) biopsy proven cases, six (6 of 43, 14.0%) suspected cases, and single patients with isolated ACTH deficiency (1 of 10, 10.0%), lymphocytic hypophysitis that had progressed to an empty sella (1 of 6, 16.7%) and isolated ACTH deficiency with empty sella (1 of 2, 50%). A similar frequency of CADPS autoantibodies was however also seen in healthy controls with 11 of 90 (12.2%) considered positive (Figure 1c).

Potential Candidate Autoantigen

A positive autoantibody index was seen against T-box 19 in 9 of the 86 (10.5%) patients with lymphocytic hypophysitis. These included 1 of 21 (4.76%) biopsy proven patients, 4 of 43 (9.30%) patients with suspected lymphocytic hypophysitis, 1 of 10 (10.0%) ACTH deficiency patients, 1 of 6 (16.7%) empty sella patients and 2 of 4 (50%) patients with diabetes insipidus. Immunoreactivity was only detected in 1 of 90 (1.11%) healthy controls (Figure 1d). Statistical analysis showed patients with lymphocytic hypophysitis had a significantly higher frequency of autoantibodies against T-box 19 than healthy controls, $p= 0.0186$ (χ^2-test with Yates' correction).

Previously reported Candidate Autoantigens

The frequency and specificity of autoantibodies in lymphocytic hypophysitis sera, was also determined with the previously described pituitary autoantigens PGSF1a, PGSF2 and NSE against the panel of 86 lymphocytic hypophysitis patients and 90 healthy blood donors (Table 1).

Sufficient ^{35}S-methionine incorporation was not achieved with PGSF1a despite subcloning into the pTNT vector, presumably as the protein only contains three

methionine residues. Therefore, no immunoprecipitation experiments were conducted with this autoantigen.

PGSF2 autoantibodies were detected in the sera of 2 of 21 (9.52%) biopsy proven patients, 1 of 43 (2.33%) suspected cases, 1 of 6 (16.7%) patients with empty sella and 1 of 4 (25%) patients with diabetes insipidus compared to 2 of 90 (2.22%) healthy blood donors (Figure 1e).

Autoantibodies were also found against the NSE recombinant protein in 2 of 86 (2.33%) lymphocytic hypophysitis patients, one patient (1 of 6, 16.7%) with empty sella and a single patient (1 of 2, 50%) with empty sella syndrome combined with isolated ACTH deficiency. No autoantibodies against NSE were detected in the sera from healthy controls (Figure 1f).

Patients with multiple autoantibody reactivity

Additionally, eight lymphocytic hypophysitis had positive autoantibodies against two autoantigens. Two patients, one biopsy proven and one "suspected" case 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 T-box 19. 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 T-box 19 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 T-box 19 as a minor target autoantigen in lymphocytic hypophysitis when tested in an immunoprecipitation assay. A number of other potential autoantigens were found by immunoscreening a human pituitary cDNA library, including CHD8 (a DNA binding protein), piccolo (a presynaptic cytomatrix

protein associated with the active zone) and CADPS (a Ca^{2+}-dependent activator protein for secretion). These autoantigens, and three previously identified proteins, PGSF1a and 2 and NSE, were then tested in an immunoprecipitation assay system.

Isolated ACTH deficiency of neonatal onset has recently been attributed to mutations in the T-box 19 gene, a transcription factor essential for development of the corticotroph cell lineage (25, 26). Mutations in this gene account for 70% of reported cases (25, 27). As corticotrophs are often the first cell type to be affected in lymphocytic hypophysitis resulting in isolated ACTH deficiency, we investigated T-box 19 as a candidate pituitary autoantigen. T-box 19 was identified as a significant minor autoantigen in lymphocytic hypophysitis with autoantibodies against the protein detected in 10.5% (9/86) patients. Interestingly, autoantibodies were not confined only to those patients with isolated ACTH deficiency as part of their disease spectrum, and were also detected in lymphocytic hypophysitis patients with diabetes insipidus as the presenting symptom. T-box 19 was determined to be a significant minor autoantigen in lymphocytic hypophysitis.

CHD8 was isolated independently from the sera of two patients with suspected lymphocytic hypophysitis on immunoscreening of the pituitary library. Given the rarity of this disorder, 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 (28). Autoantibodies affecting its function could therefore have a negative effect on cell proliferation and potentially cause pituitary failure. Autoantibodies were detected in an additional five lymphocytic hypophysitis patients including two biopsy proven patients, however 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 $Ca2^{+}$-dependent fusion of secretory vesicles with the plasma membrane (29-31). Two types of secretory vesicles, small clear synaptic vesicles and large dense-core vesicles both essential to the secretion process of packaging, docking, priming and fusion. This is a fundamental

process in the secretion of pituitary peptide hormones in the pituitary, disruptions of which could lead to hormonal insufficiencies. Secretogranin II which has been discovered to be involved in this process, has previously been isolated from screening of the same pituitary library used in this study by another lymphocytic hypophysitis patient's sera (19). The protein is abundantly expressed in gonadotrophs, thyretrophs and corticotrophs (32) 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 (33-35). In the present study, we have isolated and identified two further candidate autoantigens, CAPDS and Piccolo, both related to vesicle processes.

Piccolo is a presynaptic cytomatrix protein associated with the active zone of neurons; a specialised region where synaptic vesicles dock and fuse to release neurotransmitter (36-38). The cytoskeletal matrix of active zone functions in the regulation and mobilisation of synaptic vesicles within this zone and hence in the establishment of neurotransmitter release sites (39). In particular, Piccolo functions as a Ca^{2+} sensor in exocytosis of this process (40). Autoantibodies against Piccolo were detected in a small population of lymphocytic hypophysitis patients (7/86, 8.14%) however, not significantly higher than those detected in healthy controls.

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 (41-43). The protein is a PIP_2 (phosphatidylinositol 4,5-bisphosphate) binding protein acting subsequent to vesicle docking and priming (44), yet prior to calcium triggered fusion and facilitates large dense core vesicle exocytosis (45). In addition to the mRNA expression by northern blot in the pituitary (44), CADPS was tested to determine its potential role as a pituitary-specific autoantigen in lymphocytic hypophysitis. Immunoreactivity to CADPS however, was detected at a similar frequency in both lymphocytic hypophysitis patients and healthy controls (12/86, 14.0% vs 13/90, 14.4% respectively).

We have also shown a small 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 (2/17, 11.8%) as

well as in the sera of patients with isolated ACTH deficiency (1/10, 10%), idiopathic TSH deficiency (2/4, 50%), and other autoimmune diseases (2/31, 6.5%). They also reported immunoreactivity against the pituitary-specific protein PGSF1a in a small number of patients with lymphocytic hypophysitis (1/17, 5.9%), isolated ACTH deficiency (2/10, 20%) and patients with other autoimmune diseases (1/31, 3.2%) (18). Immunoreactivity against this protein has since been detected at a high frequency in patients with rheumatoid arthritis (20/46, 43.4%) suggesting it is more likely to be an autoantigen in rheumatoid arthritis than lymphocytic hypophysitis (46). However, we also evaluated PGSF1a in our ITT system. Despite subcloning efforts into the most optimal ITT vector, adequate amounts of the recombinant protein could not be produced in out system, most likely due to the low presence of methionine in the PGSF1a protein. We could therefore, not test for immunoreactivity in our lymphocytic hypophysitis patients.

Alpha enolase antibodies in lymphocytic hypophysitis have been well studied in both immunoblotting (14) and ITT and immunoprecipitation techniques (13). While immunoreactivity is detectable in many lymphocytic hypophysitis patients, alpha enolase autoantibodies are believed to be more of a prognostic marker of autoimmunity itself rather than a diagnostic marker in a particular disease (13). Autoantibodies have been detected in a large variety of autoimmune and infectious diseases including inflammatory bowel disease (47), rheumatoid arthritis (48, 49), systemic lupus erythematosus, mixed cryoglobulnemia, systemic sclerosis (50), Behçet's disease (51), multiple sclerosis (52), Hashimoto's encephalopathy (53). Lymphocytic hypophysitis patient serum has also been shown to recognise the gamma isoform of the enolase protein; NSE, on immunoblotting (15). In the current study we tested for NSE autoantibodies by ITT and subsequent immunoprecipitation, which included the patient serum set previously studied by immunoblotting. Autoantibodies were only detected four patients (4/81, 4.9%), with patients previously identified as having NSE autoantibodies on immunoblotting, not positive in out system. This highlights the limitations of the technique with particular autoantigens.

ITT and immunoprecipitation has been employed to analyse many autoantigens across multiple autoimmune diseases. It has the advantage over other techniques by high

affinity autoantibodies recognising three-dimensional conformational epitopes of the expressed autoantigen, rather than 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, DTT was required for effective immunoprecipitation with the patient serum. Alternative techniques and approaches may therefore be required to validate results of certain cases.

Numerous studies have endevoured to identify antigen target in lymphocytic hypophysitis in aim of developing a non-invasive serological test for the disease. Such a test would negate the need for pituitary biopsy in suspected cases which can lead to permanent pituitary failure. One of the ongoing challenges in lymphocytic hypophysitis relates to the pituitary containing five different hormone secreting cell types, each of which may have a different set of target autoantigens and whose autoimmune destruction will present a different clinical picture. A clinically relevant assay must therefore not only be pituitary-specific, but must potentially cover each of these clinical scenarios from an isolated adenohypophseal hormone deficiency through to panhypopituitarism.

We have identified T-box 19 as a minor autoantigen in lymphocytic hypophysitis, using a candidate approach. We have also shown immunoscreening of a pituitary cDNA expression library is an effective way of identifying candidate autoantigens in lymphocytic hypophysitis as well as ITT and subsequent immunoprecipitation assays being a valuable method for their evaluation. While the major autoantigen(s) were not identified in this immunoscreening, ensuing re-screenings with additional patient serum, holds the potential of isolating major autoantigens and the development of an essential serological test for lymphocytic hypophysitis, in addition to other minor autoantigens such as T-box 19.

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Table 1: Immunoreactivity specificity against lymphyocytic hypophysitis candidate autoantigens

Autoantigen	**Lymphocytic Hypophysitis**	**Healthy Controls**	***p****
	Autoantibody Positive/Total (percentage)		
T-box 19	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

*Calculated by χ^2-test with Yates' correction

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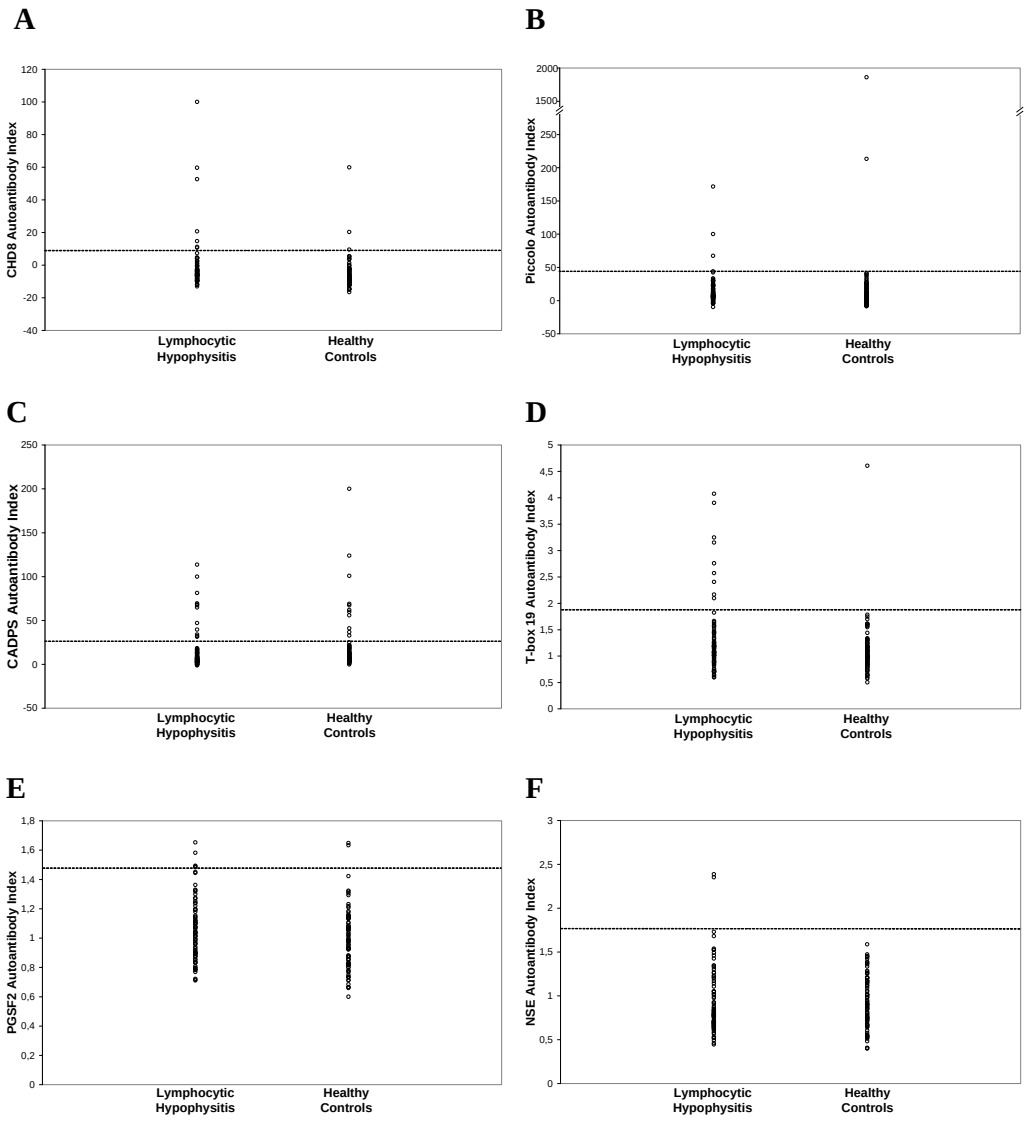
Figure Legends

Figure 1: Autoantibody indexes of pituitary autoantibodies in serum samples from patients with against (A) CHD8, (B) Piccolo, (C) CADPS, (D) T-box 19, (E) PGSF2 and (F) Anti-NSE antibodies against lymphocytic hypophysitis (n=86) and healthy controls (n=90). The broken lines in each diagram indicate the upper limit of the normal autoantibody range equal to the average of the negative healthy controls plus 3 standard deviations. CHD8, Piccolo and CADPS autoantibody indexes were determined in relation to the positive control sera whereas T-box 19, PGSF2 and NSE autoantibody indexes were calculated in relation to the negative healthy controls.

Figure 1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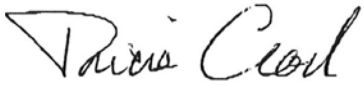
Paper IV [redacted]

Paper IV: Intermediate lobe immunoreactivity in lymphocytic hypophysitis; an immunofluorescence study

I hereby certify the proportion of my contribution to the following paper entitled "Intermediate lobe immunoreactivity in lymphocytic hypophysitis; an immunofluorescence study"

Co-author	Contribution	Signature	Date
Casey Smith	91.0%		8/5/09
Sophie Bensing	2.0%		07.05.09
Tomas Hökfelt	2.0%		090429
Roger Smith	1.0%		6/5/2009
Olle Kämpe	2.0%		070509
Patricia Crock	2.0%		1/5/2009

Intermediate lobe immunoreactivity in lymphocytic hypophysitis; an immunofluorescence study

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Abstract

Lymphocytic hypophysitis is an organ-specific autoimmune disease characterised by the infiltration of self reactive T-lymphocytes into the pituitary gland. The spectrum of autoantibodies characterised by indirect immunofluorescence (IF) in these patients have not been substantially defined. An IF study was hence undertaken to determine the spectrum of antipituitary autoantibodies in a series of 16 lymphocytic hypophysitis patients. A single patient was found to have high titer antipituitary autoantibodies against guinea pig pituitary tissue. Strong immunoreactivity was detected against distinct cells of the pituitary intermediate lobe, with no staining detectable in either the anterior or posterior lobe. The patient is a 24 year old woman who presented with headaches, polyuria and polydipsia. A uniformly enlarging pituitary mass was visible on MRI and a diagnosis of lymphocytic hypophysitis was made. We postulate this patient has an autoimmune process directed towards the major cell type in this lobe, the melanotroph, with autoantibodies against peptides involved in POMC processing consistent with the marked skin pallor and isolated ACTH deficiency evident in the autoimmune pathology of this patient.

Introduction

Lymphocytic hypophysitis is an organ specific autoimmune disease characterised by the infiltration of self reactive T-lymphocytes into the pituitary gland. The disorder presents more frequently in females than males with a ratio of 6:1 [1] and also has a striking correlation with pregnancy with approximately 60% of women presenting in the third trimester or postpartum period [2, 3]. In the acute phase of the disease, patients usually present with headaches and visual disturbances due to an upwardly expanding pituitary mass, often indistinguishable from that of a pituitary adenoma [4].

Autoimmune destruction of pituitary tissue results in the progressive disruption of pituitary hormone production affecting ACTH, prolactin, and TSH production followed less frequently by somatotroph and gonadotroph cell dysfunction. ACTH deficiency resulting from autoimmune destruction of corticotrophs is frequently the initial pituitary deficit and often the only symptom of hypopituitarism in lymphocytic hypophysitis patients in contrast to pituitary adenomata where ACTH deficiency is usually the last hormonal deficit to manifest [5-8].

Pituitary autoantibodies were originally studied by indirect immunofluorescence (IF), before the advent of immunoblotting [9] and more recently have been analysed by immunoprecipitation assays [10-14]. The immunofluorescence approach has the advantage of identifying the cell type targeted, as well as the cellular localization of the immunoreactivity. However, it cannot identify the protein(s) involved. Immunoreactivity has been observed against various pituitary hormone producing cells including lactotrophs [15], thyrotrophs [16], gonadotrophs [17], corticotrophs [18, 19] and somatotrophs [20-25]. In the early publications reporting IF results, autoantibodies were nearly always detected at low titre, using even undiluted serum or with the highest titre at 1 in 16 [15]. More recent studies have detected pituitary autoantibodies against growth hormone secreting cells in baboon pituitary, at higher titres ranging from 1 in 16 to 1 in 128. They also found that higher titre autoantibodies were predictive of growth failure due to GH deficiency [21, 22, 24, 25].

Limited studies have been conducted in patients with lymphocytic hypophysitis in order to define the cell types being targeted by pituitary autoantibodies in the disease. This study focused on identifying the cell types targeted by pituitary autoantibodies in lymphocytic hypophysitis patients assessed by IF against guinea pig pituitaries.

Methods

Patients

Serum samples from 13 patients with histologically proven lymphocytic hypophysitis and from three suspected cases previously evaluated for pituitary autoantibodies were collected for evaluation. For the 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 obtained from 13 healthy Australian blood donors served as controls.

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.

Immunohistochemistry

Pituitary sections were prepared from three male and three female guinea pigs as previously described [23]. Sera from 16 lymphocytic hypophysitis patients (13 biopsy proven and 3 suspected cases) and 13 healthy blood donors were evaluated for immunoreactivity to the pituitary tissue. An APS1 patient with previously defined high immunoreactivity to the guinea pig pituitary [23] was used as a positive control in all experiments to validate the protocol. The optimal serum dilution where immunoreactivity was visible with no background staining was determined by serial serum dilutions from 1:400 to 1:10,000 with nine lymphocytic hypophysitis patients. Immunoreactivity was assessed using the tyramide signal amplification system (TSA-Plus; PerkinElmer Life Science, Boston, MA, USA) [26]. Briefly, thaw-mounted

pituitary sections were equilibrated in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) followed by an overnight incubation at 4°C with patient sera diluted 1:5,000 in PBS. For two patients with high background staining, the patient serum was diluted 1:10,000 in PBS. The sections were washed in TNT buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20) for 15 min, incubated for 30 min with TNB buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent) at room temperature, followed by another 30 min incubation with horseradish peroxidase–conjugated donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in TNB buffer. After washing in TNT buffer, sections were incubated with tyramide-fluorescein diluted 1:100 in amplification diluent for 10 min and subsequently washed in TNT buffer. Pituitary sections were examined using a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) equipped with a Hamamatsu ORCA-ER C4742-80 digital camera and Hamamatsu photonics Wasabi 150 software (Hamamatsu, Hamamatsu City, Japan).

Results

Immunohistochemistry

Sixteen lymphocytic hypophysitis patients (13 histologically proven cases and 3 suspected patients) and 13 healthy blood donors were investigated to deduce a possible staining pattern of the guinea pig pituitary in lymphocytic hypophysitis patients. Serum from a single suspected lymphocytic hypophysitis patient showed immunoreactivity to a cell population located within the intermediate lobe of the guinea pig pituitary. No cells in the anterior or posterior pituitary exhibited any immunoreactivity with this patient's serum (Figure 1). None of the 13 biopsy proven cases, the further two suspected lymphocytic hypophysitis patients nor the healthy blood donors showed any immunoreactivity to guinea pig pituitary. The clinical background of this single patient (Patient A) was assessed to deduce a possible correlation between intermediate lobe autoantibodies and clinical phenotype.

CASE REPORT: Patient A

A 24 year old woman was referred for investigation of secondary infertility. She had a normal menarche at age of 14 years. At age 17 years, when she started the oral contraceptive pill, she had developed bilateral, throbbing headaches associated with photophobia and nausea. These headaches were attributed to migraine. At age 19 years she had a miscarriage. In spite of stopping the oral contraceptive pill, the headaches became more severe and more frequent. At age 20 years she delivered a healthy female baby. Re-starting the oral contraceptive pill in the post-partum period worsened the headaches, but they resolved when she stopped the oral contraceptive pill one year later in an attempt to fall pregnant. Secondary amenorrhoea was associated with mild hyperprolactinaemia. At age 22 years she was treated with Bromocriptine therapy, but her periods did not return. The desired pregnancy was not achieved.

At age 24 years she developed polyuria and polydipsia and was found to have diabetes insipidus with partial hypopituitarism. MRI scan of the pituitary showed a uniformly enlarged pituitary. Sarcoidosis and haemochromatosis were excluded. At age 25 years she had an episode of severe headache, photophobia and profound lethargy due to pituitary apoplexy. A repeat MRI was consistent with pituitary haemorrhage and pituitary testing showed total hypopituitarism, including ACTH deficiency.

Within six months of the episode of pituitary apoplexy, her diabetes insipidus and hypopituitarism had resolved and replacement therapy was stopped. However she again complained of recurrent, severe headaches associated with photophobia, lasting days to weeks. At this time, the patient was working as a mushroom picker. Specialist neurological opinion was that these headaches were migrainous. Four months later, panhypopituitarism recurred and she was given a course of high dose corticosteroids on the assumption that the headaches were related to the recurrence of an inflammatory process of the pituitary. The headaches improved considerably.

At age 27 years, she was once again pan-hypopituitary. She was not compliant with hormone replacement, except for desmopressin. At age 28 years she was noted to have mild optic atrophy. Repeat MRI at age 31 years showed generalised high intensity

signal from the pituitary, (without contrast), suggestive of an inflammatory process. At age 33 years she complained of worsening headaches, particularly behind the left eye. She was noted to be extremely pale with fine creases on her face as seen in severely hypopituitary patients.

Over the next few years, she had ongoing problems with compliance in spite of profound hormonal deficiencies associated with osteoporosis. She has had three hospital admissions with adrenal crises, due to severe respiratory infections including pneumonia and a staphylococcal abscess of the shoulder.

At age 36 years (2002), MRI showed a small pituitary gland, 1.5 mm in height. The posterior pituitary was slightly enlarged with normal high signal intensity and measured 5mm in height. Infundibular stalk enhanced and measured 1.5 mm in diameter.

Discussion

Indirect IF has been utilised widely in the study of APA's. This is the first study to show positive immunoreactivity in a patient with suspected lymphocytic hypophysitis to the guinea pig pituitary intermediate lobe by IF. Autoantibodies were detected at high titre and were confined entirely to the intermediate lobe of the pituitary.

While the intermediate lobe is present in most mammals, the lobe does not appear to be present in the adult human. The lobe is well developed in human foetal life but undergoes involution in the adult pituitary and has been shown to form a group of colloid-filled cysts that integrate with the posterior lobe [27]. The mammalian pituitary intermediate lobe is comprised of one major endocrine cell, the melanotrophs, in which the precursor POMC is first cleaved to release ACTH and β-lipoprotein (LPH), with ACTH being further processed to α-melanocyte stimulating hormone (α-MSH) and corticotrophin-like intermediate peptide (CLIP) and β-LPH being virtually completely cleaved to β-endorphin and γ-LPH [28]. This is in contrast to the anterior lobe where

POMC is cleaved to γ-MSH, ACTH and β-LPH which is further cleaved to β-endorphin.

The main studies of melanotrophs have focused on α-MSH. Melanocortin peptides have been shown to act via different melanocortin (MC) receptors 1-5 with α-MSH acting via receptors MC1, 3, 4 and 5 to affect pigmentation of the skin and hair, regulation of appetite and feeding behaviour, and sexual arousal [29, 30]. Also an anti-inflammatory role of α-MSH mediated through the receptor MC1 has also been proposed [31, 32].

Patient A in the present study has marked skin pallor consistent with autoantibodies seen against the melanotrophs of the intermediate lobe, which can be seen in patients with ACTH deficiency [33]. While the role of pituitary derived α-MSH in skin pigmentation is not fully elucidated due to the apparent absence of the intermediate lobe in adults, a distinct darkening of the skin is observed in disorders involving the overproduction of POMC including Cushing's syndrome [34], Addison's disease [35] and Nelson's syndrome [36]. Furthermore, the enzyme PC2 which cleaves ACTH to α-MSH and CLIP is present in human genome with expression seen in neuroendocrine tissues [37] suggesting some residual intermediate lobe function. Indeed, α-MSH cells have been found to be scattered throughout the normal pituitary gland by immunofluorescence, illustrating while there is no distinct anatomical location of the intermediate lobe in the adult pituitary, some functions of this lobe remain conserved [38]. However, given α-MSH is a processed product of the pre-cursor POMC through ACTH, all three of these proteins would invariably be detected by this technique.

The placenta also expresses POMC and secretes significant levels of ACTH, β-LPH, α-MSH and β-endorphin [39, 40] into the bloodstream albeit at a relatively low level in comparison to the pituitary. The levels of these peptides tend to increase towards term, presumably correlated to the elevated synthesis of corticotrophin releasing hormone (CRH) by the placenta towards term [41], although usually remain within the normal range [42]. Lymphocytic hypophysitis has a striking correlation to pregnancy with 60% of women presenting in the third trimester of post-partum period [2, 3]. We have

previously speculated that shared expression of proteins such as enolase in the pituitary and placenta is related to the triggering of pituitary autoimmunity with pregnancy [43]. In Patient A, it is possible that a similar mechanism explains the development of ACTH deficiency in the post-partum period.

Immunoreactivity has also been reported against the intermediate lobe of rat pituitary by IF in combination with staining against the anterior lobe in a man with hypocortisolism due to isolated ACTH deficiency. Immunostaining of stellate-shaped cells in both the anterior and intermediate lobe co-localising with ACTH was observed. However, immunoreactivity was not diminished by immunoabsorption of the patient sera with ACTH, γ-MSH, CLIP, β-endorphin or β-LPH [18], suggesting the possibility of another corticotroph derived peptide playing a pathogenic role. Serum from this patient was positive for enolase immunoreactivity by the immunoblotting method [44], further supporting an underlying autoimmune process.

Pituitary autoantibodies are infrequently detected by IF with lymphocytic hypophysitis patients. According to a literature review, immunoreactivity has been detected in only 16 of 62 (26%) of patients tested by IF [1]. In our series only one of 16 patients diagnosed with lymphocytic hypophysitis (including 13 biopsy proven patients) was immunoreactive against the guinea pig pituitary. The lack of apparent APA's in small minority of patients may be in part due to an incorrect classification of hypophysitis of an autoimmune aetiology. Recent examination of the histology and immunological make-up of two patients diagnosed with biopsy proven lymphocytic hypophysitis were shown to result from two distinct immune aetiologies. One patient presenting with the histology and clinical course reflecting the classical description of lymphocytic hypophysitis, was found to have an immune response driven by low T regulatory cells (T_{regs}) and a high IL-17 response as seen in other autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus as well as T_{reg} knockout mice. Further characterisation of the second patient with a less classically described onset of autoimmunity, showed the inflammatory process in the pituitary was driven by an overwhelming T_{reg} disease process. This pathogenesis does not correspond with the classical autoimmune pathology, but may more probably

reflect a homeostatic immune process of either infectious or infiltrative aetiology [45]. Therefore, it is possible that a minority of patients diagnosed with lymphocytic hypophysitis patients on biopsy have a process that is different to our classical idea of organ-specific autoimmunity. This process may not generate tissue autoantibodies.

Another major issue with indirect IF in the study of APA's is the choice of tissue substrate. Human foetal pituitaries and fresh frozen surgical material are considered the most ideal substrates [46]. However, human adult pituitary ACTH cells express Fc receptors which react with virtually all human immunoglobulins producing non-specific diffuse cytoplasmic staining throughout the pituitary unless the sera is first pretreated to cleave the immunoglobulin molecules to produce F(ab) fragments. Human foetal pituitaries lack the Fc receptor making it the ideal substrate [47, 48]. Given the limited availability of both adult and foetal human pituitaries and the ethical issues concerning their use, a range of alternative substrates have been studied including primates, non primate (rat, guinea pig, bovine, porcine) as well as murine AtT_{20} and rat GH_3 pituitary cell lines with variable results. Pathogenic autoantigens are considered to be highly conserved within the mammalian species. Guinea pig pituitary sections have proven a suitable/sufficient substrate for the detection of APA's in the monogenic autoimmune disease APS1 [23]. We therefore chose them for our current study.

The selected serum dilution is also an important factor in IF. APA's have been shown to be detected frequently at low titres [15, 25] and thus an appropriate dilution must be ascertained. High background staining was visible at a dilution 1:2000 in all nine patients tested at this titre. All background was eliminated at 1:5000 in the majority of sera, yet positive staining was only seen in one patient. There is a possibility of overlooking possible autoantibody titres within this range resulting in false negative results at 1:5000. However, the pathogenicity of autoantibodies at low titres is debateable and therefore only high titre autoantibodies should be considered for clinical relevance.

This study shows clearly a single patient with suspected lymphocytic hypophysitis with autoantibodies against the pituitary intermediate lobe by IF. The major endocrine cell type of this lobe is the melanotroph, suggesting this patient has autoantibodies towards peptides involved in POMC processing. Further studies are required to determine the exact autoantigen(s) being targeted.

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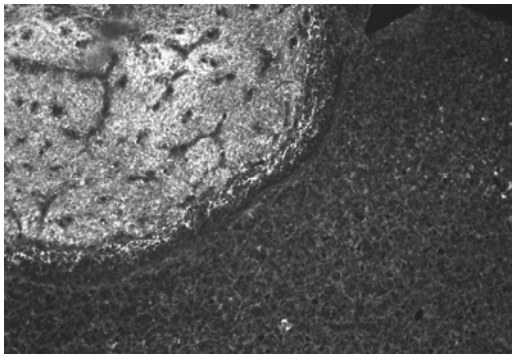
Figure Legends

Figure 1: Immunofluorescence of guinea pig pituitary sections. **(A)**: APS1 positive control showing a neural fibre staining pattern in the posterior and intermediate lobe with distinct cellular staining in the anterior lobe (10X magnification), **(B)**: Healthy blood donor (10X magnification), **(C)**: Patient A showing distinct cellular staining in the intermediate lobe at lower magnification (10X) and, **(D)**: higher magnification (20X).

Figure 1

A



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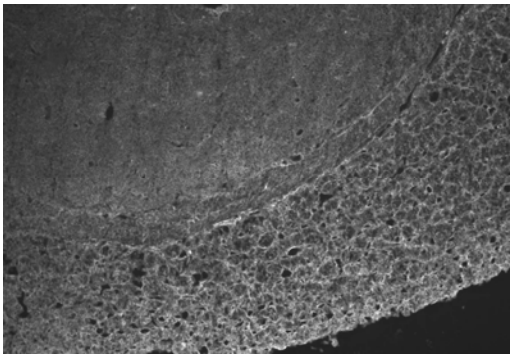

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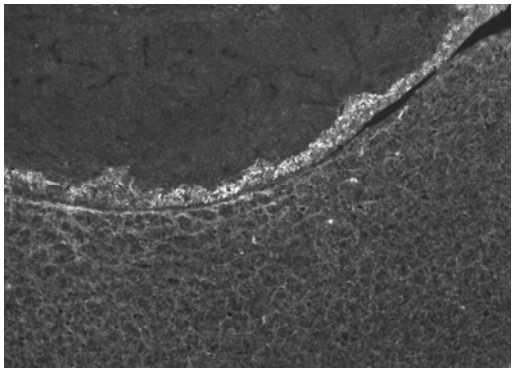
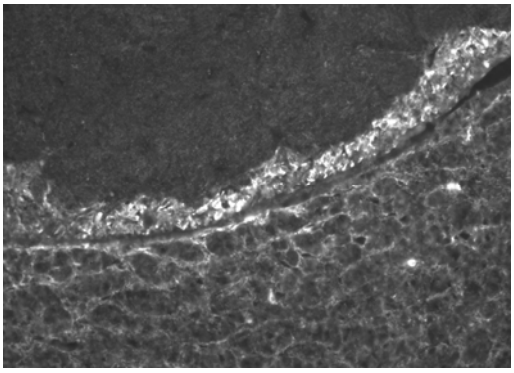


D



General Discussion

5. GENERAL SUMMARY AND DISCUSSION

Autoimmune diseases arise from the breakdown of central tolerance resulting in the escape of self reactive T-lymphocytes from the thymus to the periphery. As a group of conditions, autoimmune diseases occur in approximately 5% of the general population and represent the third most common cause of morbidity, placing considerable expenses on the health care system and society (2, 3). Understanding the underlying pathogenesis and pathophysiology of these diseases is therefore important for the correct diagnosis and treatment of these patients.

Autoimmune diseases are classically divided into two categories: organ-specific and systemic disorders. Organ-specific autoimmune diseases can be further divided into destructive (e.g. APS1) and non-destructive disorders (e.g. Graves' disease) (279). Autoimmune destruction of self-tissue is mediated by self-reactive T-lymphocytes in destructive autoimmune disorders, whereas autoantibodies confer pathogenicity in non-destructive autoimmune disease (279). Nevertheless, self-reactive antibodies are present in all autoimmune disorders. Over the last decade, serological tests for specific disease-related autoantibodies in affected patient have become widely accepted as diagnostic tools in favour of T-cell studies which are still in their infancy and prone to methodological problems.

Since the pioneering study demonstrating the correlation between thyroid antimicrosomal antibodies and post-partum thyroiditis (280), numerous studies have been undertaken to ascertain the role and risk factors associated with the presence of autoantibodies in both the general population and in patients with various autoimmune diseases. Autoantibodies have been found to be indicative of disease presence, disease course and the severity of the autoimmune response in a particular individual can be reflected in the type of autoantibody detected, as well as its titre and the number of antoantibodies present (7, 281). Additionally, autoantibodies have been found to precede the clinical onset of autoimmune disease by years and thus are predictive of the propensity of progression towards a given autoimmune disease.

In a large cohort of 130 persons diagnosed with SLE, serum samples obtained before the onset of disease showed at least one autoantibody was detectable up to 9.4 years

(average of 3.3 years) before the clinical onset of the disease. They found the appearance of SLE specific autoantibodies followed a predictable course with a progressive accumulation of autoantibodies in the pre-clinical asymptomatic phase. Furthermore, all autoantibodies were evident before the onset of disease and did not develop further after diagnosis. Thus, SLE specific autoantibodies are highly specific to predict the ensuing onset of the disease (282). The presence of predictive autoantibodies in the pre-clinical phase have been observed in many autoimmune diseases including rheumatoid arthritis (283, 284), postpartum autoimmune thyroid disease (285, 286) and type 1 diabetes mellitus (287-289).

Autoantibodies are also good indicators of the clinical course and prognosis of a particular autoimmune disease with emerging evidence they can predict both flares and remissions (279, 290). High levels of TSH receptor autoantibodies are associated with a greater probability of relapse of hyperthyroidism in Graves' disease following conventional therapy for the disease (291). Several studies have also evaluated the autoantibodies in rheumatoid arthritis given early intervention can prevent the irreversible and debilitating erosion of the joints in the disease. Patients with rheumatoid factor autoantibodies have a greater risk of earlier death, whereas anti-cyclic citrillinated peptide autoantibodies can be used to predict the severity of radiologic damage (292-294). However, while there is increased knowledge about the clinical relevance of autoantibodies in various autoimmune diseases, little is known about the role of pituitary autoantibodies in disease manifestations of pituitary autoimmune diseases such as lymphocytic hypophysitis.

5.1 Pituitary Autoantibodies

APAs have been widely studied in numerous disorders both pituitary and non-pituitary specific autoimmune disorders. APAs were originally studied by indirect immunofluorescence (IF), before the advent of immunoblotting (245) and more recently immunoprecipitation/radioligand assays (145, 249, 257, 258, 266, 274). The knowledge of pituitary autoantibodies remain in their infancy, while many target autoantigens have been proposed, their clinical relevance in disease manifestations remains to be evaluated. Some recent studies have shown immunoreactivity against somatotrophs of baboon pituitary sections by IF may be considered a good clinical

marker of autoimmune GHD in patients with idiopathic GH deficiency (231, 239), prepubertal idiopathic short stature (240), hypogonadotropic hypogonadism (241), idiopathic hyperprolactinemia (242) and Sheehan's syndrome (243).

However, limited studies have concentrated on evaluating APAs in APS1 and lymphocytic hypophysitis. Identification of the underlying pathogenic autoantigens could lead to the development of a non-invasive serological test for lymphocytic hypophysitis, negating the need for pituitary biopsy in suspected cases which can lead to permanent pituitary failure. Furthermore, it would aid in the understanding of disease onset and progression. In addition, identification of the autoantigen(s) relating to pituitary manifestations in APS1 patients could assist in the improved targeted treatment of patients with this disorder.

Immunoscreening of a pituitary cDNA expression library with patient serum is a very effective tool for identifying potential autoantigens in autoimmune diseases. Only two studies have used this approach for identifying target autoantigens in APS1 and lymphocytic hypophysitis. TDRD6 was identified as a major autoantigen in APS1 although, is not a pituitary specific autoantigen (145), while secretogranin II was identified from a single patient with lymphocytic hypophysitis (274). Further studies have not been conducted to establish the pathogenic role of this autoantigen in lymphocytic hypophysitis. Lupi *et al.* (255) identified a number of potential candidate autoantigens using 2D gel analysis, including chromosome 14 open reading frame 166 and chorionic somatomammotropin. The clinical relevance of these autoantigens also needs further characterisation to assess their role in the pathogenesis of lymphocytic hypophysitis.

5.2 Current Studies

The current studies were focused on the identification and characterisation of pituitary specific autoantibodies both in patients with APS1 and lymphocytic hypophysitis. High titre autoantibodies are a characteristic feature of APS1 and are often correlated to specific disease manifestations. Up to 7% of APS1 patients have diagnosed pituitary deficits including single and multiple hormone deficiencies (95, 139-142, 235). Immunoreactive staining to the pituitary gland is also detected in these patients (144,

145), yet no autoantigen corresponding to these pituitary manifestations has been ascribed. Patients with APS1 usually have very high autoantibody titres in comparison to patients with other autoimmune diseases; therefore, we decided to use this feature to increase the chances of detecting new pituitary autoantigens.

Lymphocytic hypophysitis is an autoimmune disease of the pituitary characterised by the infiltration of self-reactive T-lymphocytes into the gland. Some disease-specific autoantigens have been proposed including alpha-enolase (246), neuron specific enolase (247), growth hormone (250, 251), pituitary gland specific factors 1a and 2 (257), secretogranin II (274) and most recently chromosome 14 open reading frame 166 and chorionic somatomammotropin (255), yet none of these to date have proven clinically relevant in the diagnosis of the disease. Pituitary biopsy therefore currently remains the gold standard in the diagnosis of lymphocytic hypophysitis, which in itself can lead to permanent pituitary failure.

To identify potential autoantigens, a pituitary cDNA expression library was immunoscreened with both APS1 and lymphocytic hypophysitis patient serum samples. Positive cDNA clones were isolated and identified by sequencing analysis and evaluated for antigenicity in patient samples using an ITT and immunoprecipitation assay.

5.2.1 APS1 Findings

In APS1 two autoantigens were identified and characterised: ECE-2 a major autoantigen in APS1 and a minor autoantigen, TSGA10. ECE-2 autoantibodies were detected in 46% (48/104) of APS1 patients studied by immunoprecipitation and are specific to APS1 sera with no detectable autoantibodies in any sera from patients with other autoimmune diseases including Addison's disease, or in healthy controls. By quantitative real-time PCR we showed ECE-2 mRNA to be most predominantly expressed in the pancreas with moderate levels also detected in the pituitary and brain. However, statistically ECE-2 autoantibodies could not be correlated to any of the major components of APS1 including pituitary deficiency. The major issue is whether we are looking for the right clinical phenotype. It is very possible that in this complex

multi-system autoimmune disease we are missing the pertinent signs and symptoms attributable to ECE-2 autoantibodies.

Indirect IF studies on guinea pig pituitaries showed a specific staining pattern in APS1 sera from both ECE-2 autoantibody positive and negative patients. A neural fibrous network staining pattern was recurrently observed against both the posterior and intermediate lobe of the guinea pig pituitary sections with immunoreactivity against distinct cells population in the anterior lobe also detected. Pre-absorption of patient sera found to have a positive ECE-2 autoantibody index with ECE-2 recombinant protein produced by ITT, did not diminish the immunostaining seen in these patients. Hence, another unidentified autoantigen attributing to the pituitary deficits and pituitary immunostaining observed in these patients remains to be identified.

Additionally, TSGA10 was identified as a minor autoantigen in APS1 with 4.95% (5/101) of patients found to have a positive autoantibody index. Quantitative real-time PCR showed TSGA10 mRNA to be expressed at very high levels in the testis, although autoantibodies could not be correlated to gonadal dysfunction in APS1 patients. Low to moderate expression of TSGA10 mRNA was detected in almost all tissues studied with the exception of heart tissue, skeletal muscle, leucocytes and adrenal cortex. No common manifestation was present in all five TSGA10 autoantibody positive patients, suggesting a possibly minor symptom that may not be documented. Furthermore, archival serum available from all five positive patients showed autoantibody titres remained comparatively constant over time with the longest follow-up period of 23.5 years and in 4 of 5 cases were detectable from a young age (≤14 years).

Interestingly, TSGA10 autoantibodies were also detected at high titre in a single patient with SLE and at low titre in a second patient of the 77 serum samples tested. APS1 is a highly organ-specific disease and patients rarely present with systemic autoimmune manifestations. No APS1 patient, to our knowledge, has co-presented with an SLE diagnosis or SLE-like manifestations. A possible correlation in autoantibody status with APS1 and SLE patients is intriguing with evaluation of a

larger cohort of SLE patients required to establish any possible association in disease pathogenesis or manifestations in these patient cohorts.

5.2.2 Lymphocytic Hypophysitis Findings

Both a candidate and investigative approach were taken for the identification of potential autoantigens in lymphocytic hypophysitis. A pituitary cDNA expression library was immunoscreened to isolate and identify novel autoantigens, while three previously proposed autoantigens PGSF1a, PGSF2 and NSE were tested in our system. A candidate autoantigen, T-box 19 a pituitary-specific transcription factor essential for development of the corticotroph lineage which is associated with a genetic predisposition for isolated ACTH deficiency of neonatal onset (295-297) was also investigated as a potential lymphocytic hypophysitis autoantigen. T-box 19 was found to be a significant minor autoantigen in lymphocytic hypophysitis with 10.5% (9/86) patients positive for T-box 19 autoantibodies in comparison to 1.1% (1/90) healthy controls; p=0.0186 using χ^2-test with Yates' correction. CHD8 autoantibodies were detected in 7 of 86 (8.14%) hypophysitis patients versus 3 of 90 (3.33%) healthy controls (p=0.29 χ^2); piccolo autoantibodies were detected in 3 of 86 (3.49%) patients versus 2 of 90 (2.22%) healthy controls (p=NS χ^2); CADPS (rat full-length) autoantibodies were detected in 12 of 86 (13.95%) patients versus 11 of 90 (12.22%) healthy controls (p=NS χ^2). Previously proposed autoantigens PGSF2 and NSE were found not to be significant autoantigens in our assay (p=0.4048 and 0.4571 respectively) while previously reported results for PGSF1a were not replicable in our system.

Indirect IF is invaluable at identifying cell populations autoantibodies are recognising in the target organ. We studied 16 lymphocytic hypophysitis including 13 biopsy proven cases and 3 "suspected" patients used for immunoscreening the pituitary cDNA library, to establish a possible staining pattern in lymphocytic hypophysitis patients. Only one suspected patient was found to have autoantibodies against guinea pig pituitary. Strong immunostaining was detected solely against cells of the intermediate lobe. No staining was visible in the anterior or posterior lobe. No staining was observed in the remaining lymphocytic hypophysitis patients or in any healthy control.

5.3 Methodologies and Problems Encountered

5.3.1 ITT and immunoprecipitation

A direct approach of ITT and immunoprecipitation was taken to quantify APAs in the serum from patients with both APS1 and lymphocytic hypophysitis. This methodology was chosen as it has many characteristics superior to other techniques as 1) the antigen is rapidly and easily produced; 2) the incorporation of ^{35}S-methionine in the protein product means only minute amounts of antigen are required; 3) the antigenic protein produced has a closer native confirmation to the *in vivo* protein and 4) it is a high throughput and sensitive quantitative method.

The method of library screening in combination with ITT and immunopreciptation has successfully been utilised to identify multiple autoantigens in particular many organ-specific major autoantigens in APS1 including AADC (271), TH (269), TPH (268), and NALP5 (267). For this reason, we screened a pituitary cDNA library with APS1 patient sera in an attempt to isolate pituitary autoantigens in these patients.

Using the methods of immunoblotting and ELISA, some APAs have been proposed for lymphocytic hypophysitis including enolase (246) and GH (250). However, the results of these studies have not been convincing or adequate to identify major pituitary autoantigens involved in lymphocytic hypophysitis. A different approach to autoantibody characterisation was therefore undertaken, whereby potential autoantigens were identified by immunoscreening of a pituitary library followed by ITT and immunopreciptation of these antigens. This method negates the need for obtaining pituitary tissue need for immunoblotting and also the proteins do not need to be denatures as will immunoblotting. The technique is also far simpler than the ELISA as the antigen can be easily produced with Sp6, T7 or T3 polymerase, rather than obtaining the protein for coating of ELISA plates. Incorporation of ^{35}S-methionine makes the ITT method very sensitive with only small amounts of both antigen and patient sera required for immunoprecipitation. The immunoprecipitation is also highly specific with false negatives occurring very rarely and running samples in duplicate brings the false negatives to practically zero.

However, there are also some drawbacks with the ITT methodology. 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 (298); however, ITT has 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 autoantibodies in the sera of patients with Graves' disease. The addition of canine pancreatic microsomes had been used in the ITT reaction in order to obtain the mature glycosylated forms of the proteins being produced. This effectively improved the immunoprecipitation efficiency of autoantibodies against the calcium-sensing receptor in patients with acquired hypoparathyroidism (299) but was ineffective for TSH receptor (300). Expression of adequate amounts of bioactive the TSH receptor was obtained only after transfection into a leukaemia cell line (301).

In our current studies, we had some other autoantigens of interest (data not included), isolated from the pituitary library immunoscreening procedure which were not able to be effectively immunoprecipitated with patient sera. The addition of microsomes was tested, but with little to no effect. The trial of other methodologies, such as production of the protein in human cell lines, should be considered in future studies. However, the addition of DTT did substantially improve the immunoprecipitation efficiency of both CHD and Piccolo. While this technique relies on the autoantibodies binding to the antigenic epitope in a three dimensional fashion, the folding of some proteins in ITT may be incorrect and hide the epitope inhibiting any binding. In these cases the addition of DTT will linearise the proteins allowing in some cases, for the antibody to the antigen. This is most effective where the antibody recognises a linear epitope of the antigen.

Other problems encountered relate to the size of the proteins being produced. It was noticed that both very small and very large proteins were not optimal with this method of antibody detection. While there are no known studies relating to this, it is believed this could be related to a number of factors. Short proteins may contain only a small number of methionine residues, therefore even if the protein is produced, it may not be adequately detected in a beta counter. Similarly, with very large proteins only a small

copy number may be produced hence making detection once again difficult. In these cases a different methodological approach should be considered.

5.3.2 Immunohistochemistry

One of the major concerns with immunohistochemistry is the choice of tissue substrate. While the ideal substrate when looking for human autoantigens would be ideal, normal human pituitary tissue is not easily available. Additionally, human adult pituitary ACTH cells express Fc receptors which react with virtually all human immunoglobulins producing non-specific diffuse cytoplasmic staining throughout the pituitary (Gluck, M., 1993. *Autoimmunity* 14:299-305, Pouplard, 1976. *Nature* 261:142-144.). Additional steps are therefore required to block this binding before the immunostaining procedure.

Owing to these two factors we chose to use guinea pig pituitary tissue as our substrate. Previous studies have shown autoantibodies in APS1 patient sera bind very effectively to guinea pig tissue (145). Furthermore, it has been shown in many previous studies that the epitopes of autoantigens in autoimmune disease are highly conserved across mammalian species. The Tep69 epitope of ICA69 to which autoantibodies are directed against in insulin-dependent diabetes patients, is 100% homologous with the murine epitope (302). Autoantibodies have also been shown to cross react between species with alpha3 chain of type IV collagen autoantibodies in Goodpasture syndrome (303), antiguanosine autoantibodies in human lupus (304) and there is striking similarities in epitopes of human and mouse autoimmune gastritis autoantibodies (305). This is highly suggestive that the use of any mammalian tissue substrate for immunohistochemistry would be appropriate in detecting any major autoantibodies in human sera.

Autoantibodies in the serum of APS1 patients bind very effectively to guinea pig pituitary tissue, with 18 of 20 APS1 patients studied by immunohistochemistry showing immunoreactivity. The major autoantigens in APS1, AADC, GAD, TH and TPH, have also been previously shown to react with guinea pig pituitary tissue (145). However, the serum from lymphocytic hypophysitis did not show immunoreactivity to guinea pig pituitary with the exception of one single patient. Whether this is due just to

patients having very low autoantibody titres, or whether guinea pig pituitaries are not a sufficient substrate for these patients will need to be further examined. The use of baboon pituitary as a substrate or possible human pituitary tissue removed with an adenoma may prove a more suitable substrate.

5.4 Future Investigations

- To characterise ECE-2 further to find the pathological function of autoantibodies against the protein in the disease manifestations of APS1.
- To elucidate the role of TSGA10 autoantibodies in APS1 and their possible correlation with an SLE phenotype.
- To further evaluate T-box 19 as an autoantigen in lymphocytic hypophysitis investigating both its functional role in the disease and also evaluating the autoantibody status of patients by alternative methodologies to determine the true significance of the autoantigen.
- To determine the autoantigen being targeted by the patient sera against the guinea pig intermediate lobe by IF and determine its antigenicity in all lymphocytic hypophysitis patients.

5.4.1 APS1

Knockout animal models have shown ECE-2 to play a role in learning and memory integrity. While these mice have a phenotypically normal appearance, they display several minor deficits in learning and memory showing both poor short-term memory and a disturbance in long-term memory (306). ECE-2 has also been implicated in Alzheimer's disease with the enzyme involved in the degradation of amyloid beta (307-310). Disruption of this enzyme's function caused by autoantibodies directed against it could therefore result in amyloidosis in the brain of APS1 patients. We would therefore like to evaluate memory function in APS1 patients, a feature that has not been reported. Due to the severity of the disease in these patients, it is possible that mild memory deficits may be overlooked. It would indeed be interesting to evaluate learning and memory skills in a large patient cohort to determine any associations with ECE-2 autoantibodies or amyloidosis. An MRI study could also be useful in determining if these APS1 patients have amyloidosis.

Organ-specific and systemic autoimmune diseases rarely present in the same patient. To our knowledge SLE has never been reported in association with APS1, with the AIRE-mediated thymic negative selection of lymphocytes not apparently relevant in the pathogensis of SLE (311). It is therefore interesting that TSGA10 autoantibodies were detected in such distinct diseases as APS1 and SLE. Determining the functional manifestation of these autoantibodies may therefore provide a common link in the pathophysiology of autoimmune diseases in general. By analysis a larger cohort of SLE patients by ITT and immunoprecipitation we will be able to conceivably establish a better clinical picture and find a common feature in these patients and conceivably correlate this to APS1. We will also test the positive SLE patients against the known major autoantigens in APS1 by ITT including 21-OH, SCC, AADC, TPH and NALP5, to determine if these patients have an APS1-like syndrome.

5.4.2 Lymphocytic Hypophysitis

A clinically relevant autoantigen has not been previously described in lymphocytic hypophysitis. Using a candidate gene approach T-box 19 was found to be a significant minor autoantigen in the disease in comparison to healthy controls. Correct conformational binding is essential in the detection of autoantibodies against target autoantigens. ITT relies on the 3-dimensional binding of proteins therefore the accurate folding of the protein is essential to the methodology. Given the discrepancies in autoantibody results using different methods in various autoimmune diseases, it should be noted that no single autoantibody test is entirely specific and results are more often a reflection on the assaying method employed (7). We would therefore like to evaluate T-box 19 as a lymphocytic hypophysitis autoantigen by alternative methodologies including immunoblotting and ELISA, to establish the true clinical relevance of this protein in lymphocytic hypophysitis. We would also determine the specificity of T-box 19 as a lymphocytic hypophysitis autoantigen by testing serum samples from other organ-specific autoimmune diseases for autoantibodies against T-box 19.

The utilisation of indirect IF in lymphocytic hypophysitis has proven problematic with only a minority of patients (16/62) positively staining pituitary tissue (146). We have identified a single patient with intermediate lobe staining consistent with the patient's

clinical picture of marked skin pallor and prominent ACTH deficiency. The absence of staining in the further 15 patients studied confirms the finding in the literature that IF rarely detects autoantibodies in lymphocytic hypophysitis sera. Further evaluation of the autoantibodies in this patient could prove interesting. To try and identify the target autoantigen(s), we would perform double stainings with this patient's serum using POMC peptide products including α-MSH and β-endorphin. If a positive identification was made, we would proceed to test all lymphocytic hypophysitis patients in the ITT assay to see if they also have autoantibodies against this autoantigen.

In summary, this thesis has augmented our understanding of autoantibodies in the organ-specific autoimmune diseases APS1 and lymphocytic hypophysitis. Two new autoantigens were discovered for APS1: the major autoantigen ECE-2 and a minor autoantigen TSGA10, also present in a minority of SLE patients. This is also the first report of a significant minor autoantigen in the pituitary autoimmune disease lymphocytic hypophysitis. T-box 19, a corticotroph-specific transcription factor, was detected in 10.5% of patients ($p=0.0186$). This thesis also demonstrates immunoscreening of a target organ cDNA expression library is a valuable method for identifying potential candidate autoantigens in autoimmune diseases. Furthermore, we have reported positive staining of the intermediate lobe in a single lymphocytic hypophysitis patient, presumably against the melanotrophs. These novel findings broaden our current understanding of pituitary autoimmunity.

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Appendix [redacted]

6. METHODS

6.1 Pituitary cDNA expression library screening

A pituitary cDNA library was immunoscreened with the serum from patients with APS1 and lymphocytic hypophysitis to identify potential candidate autoantigens (Figure 1). For the initial screening, 50,000 plaque forming units (pfu) of the amplified library were infected into Escherichia coli XL-1 Blue MRF' and plated onto 10 large agar plates. To do this the equivalent of 50,000 pfu/plate of pituitary cDNA library phages was added to 600µl/plate of XL-1 Blue MRF' bacteria (OD_{600} of 0.5) and incubated at 37°C for 15 minutes. 7ml of NZY top agarose (5 g NaCl, 2 g $MgSO_4.7H_2O$, 5 g yeast extract, 10 g NZ amine, H_2O to 1L, pH 7.5) tempered to 48°C was added and immediately onto preheated (at 42°C) NZY agar plate. After incubation for 4-5hrs at 42°C, the plates were overlaid with nitrocellulose filters (Hybond C, Amersham) preabsorbed with 10 mM isopropyl f-Dthiogalactopyranoside (IPTG; Sigma) and cultured for a further 10-12hrs at 37°C. The filters were removed from the plates and washed three times for 5 min in 15 ml of a buffer containing 20 mM Tris-HCl (pH 7.5), 0.1% gelatin and 0.05% Tween-20 (TBS-GT). Nonspecific protein binding was blocked by incubating with 15 ml of 1% gelatin in 20 mM Tris HCl (pH 7.5) for 1 hr, followed by washing in TBS-GT as above. The filters were incubated for 30 min with normal goat serum diluted to 1:200 in TBS-GT, to reduce nonspecific binding of the secondary antibody during colour development. After an additional wash cycle, the filters were incubated overnight with patient sera diluted 1:200 in TBS-GT at room temperature. Filters were incubated with alkaline phosphatase-conjugated goat anti-human IgG 1:3000 in TBS-GT for 1.5 hr, washed as before, followed by an additional 5 min washing with TBS-GT without Tween-20 and developed using a p-nitrobluetetrazolium chloride (NBT)/5-bromo-4-chloro-3- indoyl phosphate/toluidine (BCIP) colour development system (Bio-Rad, Hercules, CA).

Positive clones were picked by matching each filter with the corresponding agar plate and extracting the positive plaques with a pasteur pipette. Agar plugs were placed in 1ml of SM buffer (0.58 g NaCl, 0.2 g $MgSO_4$ $7H_2O$, 5 ml 1 M Tris-HCl pH 7.5, 0.5 ml 2% (w/v) gelatine, H_2O to 100 ml: dilute 1:10 before use) with 20µl chloroform vortexed and left on a shaker at RT for 1-2 hours to eluate the phages from the agar.

Positive clones were rescreened in a secondary and tertiary immunoscreening until pure isolates were obtained.

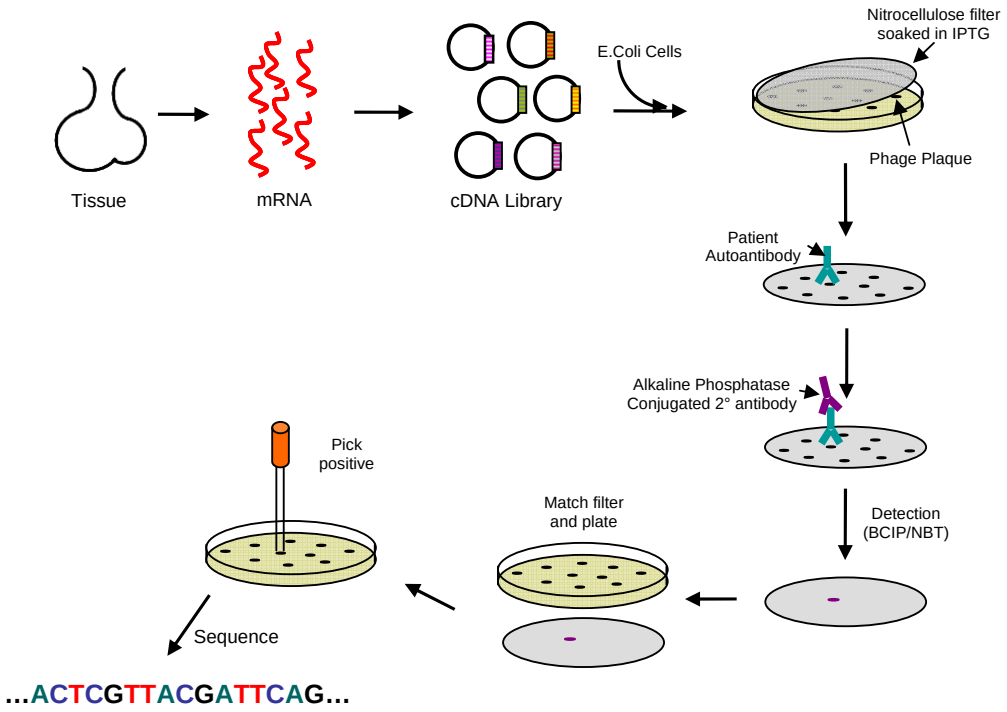

Figure 1: Schematic diagram of immunoscreening a cDNA expression library.

6.1.1 Library Titration: The concentration of pfu's in the cDNA library was determined by titering of the library. A dilution series was prepared with a start concentration of 1:1,000 in SM buffer and serially diluted by a factor of 10 up until $1:10^7$. 1µl from each dilution was mixed with 200µl of XL-1 Blue MRF' bacteria (OD_{600}: 0.5). The tubes were incubated at 37°C for 15 minutes and plated onto small agar plates as above, with the exception of using 3mL NZY top agarose for plating. The same method was used to titre positive clones using a dilution of 1:500 in SM buffer for five randomly selected clones. The average titer was used to determine the amount of phage mix for the secondary (500 phages/plate) and tertiary (50 phages/plate) immunoscreenings.

6.1.2 Preparation of Phage-competent Bacteria: XL1-Blue MRF' bacterial stock was streaked on an LB-agar plate with 12.5µg/ml of tetracycline and incubated overnight at 37°C. A single colony was then cultured in 100ml of LB medium with 10mM $MgSO_4$ and 0.2% maltose at either 30°C or 37°C with 250rpm shaking until the bacteria was at a concentration of $OD_{600} \approx 0.5$. The bacteria culture was then centrifuged at 500 x *g* for 10 minutes at 4°C and the pellet resuspended in 10 mM $MgSO_4$ to an $OD_{600} \approx 0.5$.

6.2 In vivo excision

An ExAssist® Interference-Resistant Helper Phage with XLOLR Strain kit and protocol (Stratagene, La Jolla, CA) was used for excision of the pBK-CMV phagemid from the ZAP Express® vector used in the pituitary cDNA library. To achieve this 250 µl of phage stock obtained from the tertiary library screening (containing >1 × 105 phage particles) was combined with 200µl of XL1-Blue MRF´ bacteria (OD_{600} of 1.0) and 1µl of ExAssist helper phage (>1 × 106 pfu/µl) and incubated at 37°C for 15 minutes to allow the phage to attach to the bacteria cells. To the bacterial suspension, 3ml of LB medium was added and incubated at 37°C for 3 hours with 250rpm shaking. The lambda phage particles were lysed at 70°C for 20 minutes and the cell debris removed by centrifugation at 1000 x *g* for 15 minutes. The excised pBK-CMV phagemids were plated by combining 100µl of the phage supernatant to 200µl pf XLOLR bacteria (OD_{600} of 1.0) and incubating at 37°C for 15 minutes and streaked on LB agar plated containing 50µg/ml kanamycin and incubated overnight at 37°C.

A single colony was cultured in 3ml of LB medium containing 50µg/ml kanamycin overnight at 37°C overnight with 225rpm shaking. The cDNA clone was then purified using a QIAprep spin miniprep kit (Qiagen: Cat # 27104).

6.3 DNA and sequencing analysis

Isolated cDNA clones from immunoscreening of the pituitary cDNA library with sera from patients with APS1 and lymphocytic hypophysitis were partially sequenced using a dye-terminator sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 3730 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Sequencing reactions were prepared with 10-30ng of cDNA template and 1.6pmol of primer in a total volume of 6µl and sent to the Uppsala Genome Center Sequencing Service

(Rudbeck Laboratory, Uppsala, Sweden) for sequencing. cDNA clones were identified by comparison of the sequencing data received against available databases using the Basic Local Alignment Search Tool (BLAST).

6.4 Subcloning

To improve ITT efficiency, all cDNA clones of interest (with the exception of TSGA10) were subcloned in the pTNT vector (Promega). The cDNA insert was cut from its primary vector using a double restriction digest and ligated into the multiple cloning site of the pTNT vector. The restriction enzymes used in the subcloning of cDNA clones are shown in Table 1.

Table 1: Restriction enzymes used for subcloning of cDNA inserts from their primary vectors into the pTNT vector.

cDNA Clone	Primary Vector	Primary Vector Digestion Enzymes	End Vector	pTNT Vector Digestion Enzymes
ECE2	pBK-CMV	SalI/NotI	pTNT	SalI/NotI
TSGA10*	pBK-CMV	N/A	N/A	N/A
CHD8	pBK-CMV	SalI/NotI	pTNT	SalI/NotI
Piccolo	pBK-CMV	SalI/NotI	pTNT	SalI/NotI
rCADPS	pBluescript SK	XhoI/XbaI	pTNT	SalI/XbaI
Tpit	pBluescript SK	XbaI/SalI	pTNT	XbaI/SalI
NSE	pOTB7	EcoRI/NotI	pTNT	EcoRI/NotI
PGSF2	pET-28a	EcoRI/NotI	pTNT	EcoRI/NotI
PGSF1a	pET-28a	EcoRI/XhoI	pTNT	EcoRI/SalI

*Translation efficiency using ITT was sufficient for TSGA10 and was therefore not subcloned into the pTNT vector.

Restriction enzyme digestions were prepared as follows:

10X React Buffer (Invitrogen)	2µl
Enzyme 1 (Invitrogen)	0.5µl
Enzyme 2 (Invitrogen)	0.5µl
DNA	500ng
H_2O	to 20µl

The vectors were digested in a 37°C waterbath for 3hrs and the entire digestion mix run on a 0.8% 1X TAE (242g Tris, 100mL of 0.5M EDTA pH 8.0, 57.1mL Glacial

Acetic Acid, H_2O to 1L: dilute 1:50 before use) agarose gel. The cDNA clone bands and digested pTNT vector were excised from the gel and the cDNA purified using a QIAquick Gel Extraction Kit (Qiagen: Cat # 28704).

The cDNA inserts were then ligated into the digested pTNT vector according to the manufacturer's instructions (Invitrogen) as follows:

5X ligase Buffer	4µl
Insert DNA	90-180fm
pTNT Vector	30fm
Ligase Enzyme	1µl (1unit)
H_2O	to 20µl

The tubes were incubated at room temperature for 1hr before being incorporated into electrocompetent XL1-Blue MRF' bacteria via electroporation. Ligation products (1µl≈10pg) were mixed with competent bacteria and pulsed at 1700 V with a resistance of 200 Ω and capacitance at 25 µF using an electroporator (Bio-Rad). Cells were resuspended in SOC medium (20.0 g of tryptone, 5.0 g of yeast extract, 0.5 g of NaCl, Add deionized H_2O to a final volume of 1 liter.) warmed to 37°C and incubated at 37°C with shaking at 225rpm for 1hr. 200µl of the transformation mixture was plated on LB agar plates containing ampicillin (100µg/ml).

Several colonies from each plate were cultured in 3mL of LB medium overnight and the plasmid extracted and purified using a QIAprep spin miniprep kit (Qiagen: Cat # -27104). The restriction enzyme digestion was then repeated as above to ensure the pTNT vector contained the target cDNA insert.

6.5 In vitro transcription and translation (ITT) and immunoprecipitation of autoantigens

Recombinant ^{35}S-methionine radiolabelled proteins were produced by ITT in an Sp6 Quick or T3 coupled reticulocyte lysate system and evaluated to determine the incorporation rate of ^{35}S-Methionine according to the manufacturer's instructions (Promega). The ITT reaction mixtures were prepared as follows:

Sp6 Quick Reaction Mix:

TNT® Sp6 Quick Master Mix	20µl
^{35}S-methionine 10µCi/µl	1µl
DNA-template 1µg/50µl	1µl
Nuclease free H_2O	3µl

T3 Reaction Mix:

TNT® reaction buffer	1µl
RNAse inhibitor	0.5µl
Amino acid mix minus methionine	0.5µl
^{35}S-methionine 10µCi/µl	1µl
DNA-template 1µg/50µl	1µl
TNT® T3 RNA polymerase	0.5µl
TNT® Reticulocyte Lysate	12.5µl
Nuclease free H_2O	8µl

The tubes were incubated at 30°C for 90min with shaking at 300rpm before the incorporation of ^{35}S-Methionine was measured.

6.5.1 Measuring the 35S-Methionine incorporation: A 2µl aliquot of the reaction mix was used to determine the percentage incorporation of radioactive labelling. The aliquot was mixed with 98µl of 1M NaOH/2% H_2O_2 and incubated at 37°C for 10mins. The translation product was precipitated by the addition of 25% TCA and incubated on ice for 30mins. Whatman® GF/A glass fiber filters (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were pre-wet with ice-cold 5% TCA, and the precipitated translation product added to the filter and washed 5 x 1ml 5% TCA followed by 2 x 1ml acetone using a 1225 sampling vacuum manifold (Millipore, Billerica, MA). Filters were allowed to dry at room temperature, before being added to a scintillation vial with 2.5ml of OptiPhase SuperMix scintillation fluid (Perkin Elmer Life Sciences, Boston, MA) and counted in a liquid scintillation counter (Wallac, WinSpectral 1414; PerkinElmer, Waltham, Massachusetts). 2µl of translation product was added directly to a Whatman® GF/A glass fiber filter and read as above in the beta counter to determine the total ^{35}S-Methione present in the sample. The percentage of

^{35}S-Methione was calculated as ([cpm of sample/cpm of the total ^{35}S-Methionine] x 100). An incorporation of >1% was considered necessary to use the product for immunoprecipitation experiments.

6.5.2 Protein size determination by SDS-PAGE electrophoresis: The size and integrity of the recombinant proteins were analysed by SDS-PAGE before use in the immunoprecipitation assay. A 1.5µl aliquot of the translated product was mixed with SDS sample buffer (50mM Tris-HCl (pH 6.8), 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 100°C for 2 mins to denature the proteins. The denatured sample was loaded on to a 4-20% Tris-Glycine Bio-Rad Ready Gel (Bio-Rad Cat # 161-0903) and run at 30mA until the dye front reach the bottom of the gel. The gel was washed in fixing solution (50% methanol, 10% glacial acetic acid, 40% water) for 30 mins with slow agitation then soaked in 10% glycerol for 5 mins to prevent the gel from cracking during the drying process. The gel was covered in plastic wrap and dried at 80°C for 90 minutes under vacuum in a gel dryer (Savant SGD4050; Artisan Scientific, Champaign, IL). The gel was then exposed to a phosphorimaging screen overnight and visualised on a PhosphorImager (Molecular Dynamic, Sunnyvale, CA)

6.5.3 Immunoprecipitation Assay: Those proteins with a higher incorporation than 1% were then used for immunoprecipitation with patient sera in 96 well microtiter plates with filter bottoms (MABV N12, Millipore, Bedford, MA) as shown in Figure 2. The plates were prepared for immunoprecipitation by firstly incubating the wells in a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 0.02% NaN_3 (Buffer A) for 1 hour before being coated with 1.0% bovine serum albumin (BSA; Sigma) in Buffer A for a further 2 hours. The wells were then washed twice with 0.05% Tween-20 in Buffer A and once with 0.1% BSA and 0.15% Tween-20 in Buffer A (Buffer B). 25000 to 30000 cpm of the recombinant protein and 2.5µL of undiluted patient serum were mixed in a Buffer B in a total volume of 50 µL. Additionally, dithiothreitol (DTT) was added to Buffer B to a final concentration of 0.1% for cDNA clones CHD8 and Piccolo to linearise the proteins and hence improve immunoprecipitation efficiency. Plates were incubated overnight at 4°C. The antibody complexes were transferred to the microtiter plates with filter bottoms and precipitated with 50 µL of a

50% slurry of protein A-Sepharose (Pharmacia, Stockholm, Sweden) in Buffer B for 45 min at 4°C. The plates were washed ten times with Buffer B using a vacuum manifold. After drying, 70 µL OptiPhase SuperMix scintillation fluid (Perkin Elmer Life Sciences, Boston, MA) was added to each well and the plates counted in a beta counter (Wallac 1450 MicroBeta, PerkinElmer). Results were expressed as an antibody index [(cpm sample – cpm negative control)/(cpm positive control – cpm negative control) x 100]. Samples were analysed in duplicate, whereas the positive control (the library screening patient) and the negative control (4% BSA) were run in triplicate. For the autoantigens without positive controls, antibody indexes were calculated as [cpm sample/mean cpm of healthy controls]. The upper normal antibody index cut off was set at the mean of the unequivocally negative healthy blood donors plus 3 standard deviations for lymphocytic hypophysitis autoantigens, a threshold which clearly differentiated positive from negative samples. Four standard deviations was deemed appropriate for the TSGA10 cut off, while the upper normal antibody index for ECE-2 was set at a value which clearly differentiated between positive and negative samples and not by means standard deviation.

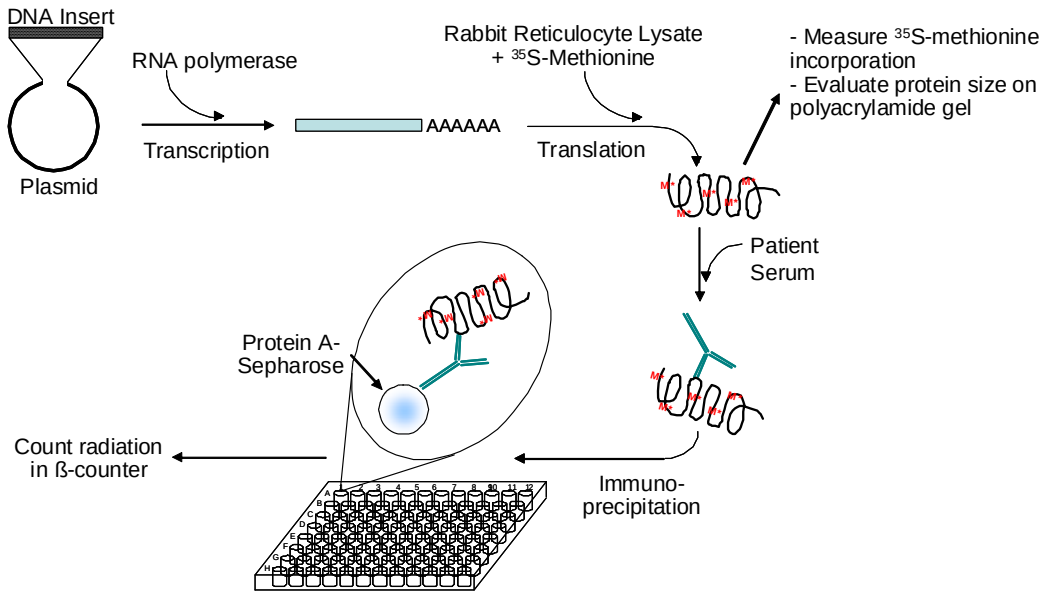

Figure 2: Schematic representation of the ITT assay.

6.6 Statistical Analysis

A two-tailed Fisher's exact test was performed to determine the association of major APS1 disease manifestations with immunoreactivity to ECE-2. All statistical analysis was done with Intercooled STATA 8.2 (Stata Corp, College Station, TX). A *p* value of <0.05 was considered significant.

A χ^2-test with Yates' correction was performed to determine the probability of candidate autoantigens as significant target autoantigens in lymphocytic hypophysitis. A *p* value of <0.05 was again considered significant.

6.7 Quantitative Real Time PCR

To establish the tissue expression profile of ECE-2 and TSGA10 mRNA throughout various organs, quantitative PCR was performed on cDNA from human tissue. Primers were designed with Beacon Designer® version 5.11 software, which flanked the intron-exon junctions, hence avoiding amplification of genomic DNA (Table 2).

Table 2: Primer sequences for Real-Time PCR

Gene	Forward Primer	Reverse Primer	Amplicon Length (bp)
ECE-2	tggatgatgtttatgacgggtacg	ggtagtaggcattcactgtctgg	162
TSGA10	aatcagaggcagataacaataccc	tttaagtgttgctcaacctctctg	115
GAPDH	agggctgcttttaactctggtaa	catattggaacatgtaaaccatgtagtt	250

Quantitative PCR was carried out on human normalised multiple-tissue cDNA panels (BD Bio Sciences, Palo Alto, CA, USA) as well as pituitary, aorta (Stratagene) and adrenal cortex cDNA prepared from normal adrenal tissue removed during adrenal adenoma surgery, using a MyiQ iCycler (Bio-Rad, Hercules, CA, USA). Reactions were performed in a volume of 25 µl, with 200 nM of each primer and iQ™ SYBR®Green supermix (Bio-Rad, Hercules, CA, USA) to a final concentration of 1X. All samples were run in triplicate. Thermal cycles consisted of an initial denaturation of 95°C for 3min, followed by 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. Standard curves were then established from the serial dilution of ECE-2 or TSGA10 and control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) PCR

templates. ECE-2 and TSGA10 mRNA levels were deduced from the standard curve and normalised to the endogenous GAPDH tissue content.

6.8 Immunofluorescence Stainings

Male and female guinea pigs (weight 250–300 g) (all animals from B & K, Stockholm, Sweden), housed under controlled environmental conditions, were deeply anesthetised 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.

Immunoreactivity to guinea pig pituitary was evaluated in 10 APS1 patients with positive and 10 APS1 patients with negative ECE-2 autoantibody indices as well as 16 lymphocytic hypophysitis patients, using the tyramide signal amplification system (TSA-Plus; PerkinElmer Life Science, Boston, MA, USA). The optimal serum dilution where immunoreactivity was visible with no background staining was determined by serial serum dilutions from 1:400 to 1:10,000 with nine lymphocytic hypophysitis patients. A predetermine optimal dilution of 1:5,000-1:10,000 was used for APS1 serum samples.Thaw-mounted pituitary sections were equilibrated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) for 15mins followed by an overnight incubation at 4°C with patient sera diluted 1:5,000-1:10,000 in PBS. The sections were washed in TNT buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20) for 15 min, incubated for 30 min with TNB buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent) at room temperature, followed by another 30 min incubation with horseradish peroxidase–conjugated donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in TNB buffer. After washing in TNT buffer, sections were incubated with biotinyl tyramide-fluorescein (BT-FITC) diluted 1:100 in amplification diluent for 10 min and subsequently washed in TNT buffer. Pituitary sections were mounted with 2,5% 1,4-diazabicyclo[2.2.2]octane (DABCO) in glycerol and examined using a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) equipped with a Hamamatsu ORCA-ER C4742-80 digital camera and Hamamatsu photonics Wasabi 150 software (Hamamatsu, Hamamatsu City, Japan).

To evaluate the specificity of patient sera binding to ECE-2 in the pituitary sections the staining patterns with untreated sera were compared to immunostainings after the sera had been preabsorbed with ECE-2 protein. Ten APS1 patient sera (diluted 1:5,000) were immunoprecipitated overnight at 4°C with 80,000-120,000 cpm ^{35}S-radiolabeled ECE-2 protein expressed in vitro, as described above, in order to first deactivate the ECE-2 autoantibodies in the patient sera before use on the pituitary sections. Furthermore, using the sera from two patients with a high ECE-2 autoantibody index and unequivocal immunoreactivity against the guinea pig pituitary gland, a dilution series ranging from 1:5,000 – 1:2,000,000 was established to fully ascertain the binding specificity before and after preabsorption with ECE-2 protein produced by ITT. Immunoreactivity was again assessed using the tyramide signal amplification system as above.

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.

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.

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.

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.

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- •• of outstanding interest

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15 Autoimmune Hypophysitis

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Contents

Summary

Autoimmune hypophysitis or pituitary autoimmune disease is now considered to be part of the organ-specific spectrum of endocrine autoimmunity. It is predominantly seen in women, often in association with pregnancy. The most common presentation in the acute phase mimics that of a pituitary adenoma with a mass and hypopituitarism. There is a predilection for adrenocorticotrophin (ACTH) and thyrotrophin deficiencies, in contrast to tumors and radiation-induced hypopituitarism where these are usually the last axes to be lost. Extension of the inflammatory process into the infundibulum causes diabetes insipidus and is termed infundibuloneurohypophysitis. Sub-acute cases classically present in the peripartum period, and the pituitary mass may resolve spontaneously. Chronic cases should be considered in patients with isolated ACTH deficiency or idiopathic hypopituitarism in the context of other autoimmune diseases and the empty sella syndrome (also with hypopituitarism). Magnetic resonance imaging usually shows a symmetrical pituitary mass with bright, homogeneous contrast enhancement. Biopsy is still considered the gold standard for diagnosis. Serological tests for pituitary autoantibodies are reviewed. Immunoblotting has identified a number of target autoantigens. The latest assay, based on the in vitro transcription translation of pituitary gland-specific proteins followed by an immunoprecipitation step, has identified pituitary gland-specific protein factor (PGSF) 1a and PGSF2 as probable target autoantigens. Management of potentially life-threatening adrenal insufficiency is of the utmost importance.

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A conservative approach has been recommended except in those patients with sight-threatening lesions or in those with recurrent disease despite a course of immunosuppression. Future directions are discussed.

Key Words: Lymphocytic hypophysitis, pituitary autoantibodies, pituitary autoimmunity, target autoantigens.

INTRODUCTION

Autoimmune endocrinopathies are classified as organ specific in the spectrum of autoimmune diseases. The classic example is autoimmune thyroiditis that was first described by Dr. Hakaru Hashimoto in 1912 *(1)* and still bears his name. Pituitary autoimmunity was not described until 50 years later by Goudie and Pinkerton *(2)* as "anterior hypophysitis." Interestingly, it was the association with Hashimoto's thyroiditis in this index case that led the pathologist and physician to make the etiological link. They also noted the link with pregnancy and the postpartum period that continues to hold true in over 50% of cases in the literature.

HISTORICAL PERSPECTIVE

It is tempting to surmise that there were some cases of end-stage hypophysitis among Sheehan's original series of patients who died of chronic hypopituitarism in the early 1900's *(3)*. Close examination of Carpenter's review of Schmidt's syndrome in 1964 *(4)* identified at least two cases of Addison's disease from the 1930s with lymphoid infiltration not just of the thyroid but also of the hypophysis *(5,6)*. However, the original case reported by Goudie and Pinkerton in 1962 *(2)* was the first time that the distinct entity of anterior hypophysitis or pituitary autoimmunity was formally enunciated.

Since then, autoimmune pituitary disease has seen a range of terminology, but the most common terms are currently lymphocytic hypophysitis and lymphocytic infundibuloneurohypophysitis, when there is associated diabetes insipidus (DI). The clinical spectrum of disease is increasingly recognized although the gold standard for diagnosis remains pituitary biopsy. The ability to detect pituitary autoantibodies, whether they are pathological or epiphenomena in the autoimmune process, would obviate the need for pituitary surgery in some cases. By analogy with Addison's disease *(7)* and type 1 diabetes *(8)*, autoantibodies could one day also be used to predict the risk of gland failure, in this case of hypopituitarism.

EPIDEMIOLOGY

The incidence and prevalence of lymphocytic hypophysitis are not known exactly, and population-based data are scarce. The nature and clinical course of cases that proceed to surgery ("biopsy proven") are quite different to those of cases that are "suspected" or represent the sub-acute and chronic ends of the spectrum *(9–14)*. These latter cases have become better defined by new imaging techniques and will be further defined as new, more specific and sensitive pituitary autoantibody assays evolve.

In the early histopathology literature, there was considerable discussion about what constitutes a "normal" pattern of lymphocytic infiltration and its significance. Simmonds and Brandes *(15)* described four types of lymphocytic infiltration in 200 unselected autopsy cases. Only two cases were found in "Group III," defined as

having diffuse lymphocytic infiltration of the anterior hypophysis, which would now be regarded as pathological. Shanklin's series of 100 autopsies *(16)* found no cases with lymphocytes in the anterior pituitary.

Sheehan and Summers *(3)* reviewed the pathology and clinical correlates of 95 autopsy cases of hypopituitarism. Some cases with end-stage fibrosis and scarring of the pituitary were clearly because of postpartum necrosis, named eponymously after Sheehan, but some may have been because of end-stage lymphocytic hypophysitis. Scheithauer et al. *(17)* examined the pituitary glands of 69 autopsies of women who had died in the peripartum period (during and after pregnancy or following abortion) on the assumption that undiagnosed cases of lymphocytic hypophysitis may be more common in this scenario. They found five pituitaries that had areas of lymphocytic infiltration on histopathology.

The incidence determined from large surgical series ranges from 0.24 to 0.88% *(18,19)* but, again, may only represent those cases that presented more acutely. In addition, neurosurgeons and their neuroendocrine teams who specialize in pituitary surgery may be more likely to suspect the diagnosis preoperatively and avoid surgery where possible. The largest series of 2500 surgical pituitary pathology cases, collected in Hamburg, Germany, between 1970 and 1996, were reported by Sautner et al. *(19)* and Fehn et al. *(20)*. Six cases (0.24%) of lymphocytic hypophysitis were identified. In another large German series from Erlangen, Honegger et al. *(21)* found 7 cases of hypophysitis among 2362 pituitary cases (0.3%). A review of 2000 case records from Charlottesville, Virginia, identified 16 patients, 13 of whom underwent surgery (0.65%), 10 with lymphocytic hypophysitis, and 3 with granulomatous hypophysitis *(22)*. The series of 5 patients from Nottingham in the United Kingdom, based on 619 consecutive cases, had a calculated incidence of 0.8% *(23)*. The preference of this surgical unit was for surgical decompression. The Johns Hopkins Hospital review of 905 pituitary surgical cases from the archives found 8 cases (0.88%) *(18)*.

GENETIC AND ENVIRONMENTAL FACTORS

Autoimmune diseases are often associated with particular major histocompatibility complex (MHC) alleles. A number of patients with hypophysitis have had haplotype analysis performed (*10,24–33*). The first two cases studied were black, female patients, both with the allele Bw35 *(24,27)*. This allele is associated with type 1 diabetes in non-Caucasians *(34)*. The two patients in Pestell et al.'s report *(30)* shared a number of human leukocyte antigen (HLA) alleles that have been associated with Hashimoto's thyroiditis and type 1 diabetes. As the number of patients investigated is small, no firm conclusions can be drawn *(9,10)*.

There are no known environmental triggers for hypophysitis, but the association with viral infections, particularly meningoencephalitis, may be relevant. Pituitary autoantibody testing, once sensitive and specific enough, would be helpful to answer this question.

DIAGNOSIS

Clinical Presentation

The diagnosis of lymphocytic hypophysitis should be entertained in a range of clinical scenarios that depend on the rapidity or otherwise of the disease process. It

has a striking female preponderance with a female to male ratio of 6:1 *(18)*, and its strong association with pregnancy should not be forgotten. It has been increasingly recognized in male patients *(14,18,26,30)*, but the recent preponderance of male cases probably reflects some reporting bias. Females tend to present at a younger age (34.5 years) than males (44.7 years) *(9)*. At the acute end of the spectrum, hypophysitis mimics the effects of a non-secretory pituitary tumor with adrenocorticotrophin (ACTH) deficiency predominating, and sub-acute cases have frequently been described as a resolving pituitary mass in the peripartum setting. The chronic spectrum is believed to include idiopathic hypopituitarism, sometimes in the context of other autoimmune endocrinopathies, and the empty sella syndrome.

Acute Presentations

Apart from some of the original autopsy cases *(11)*, the most dramatic, acute presentation was a case report of sudden death in labor of a young woman who presumably had undiagnosed adrenal insufficiency *(35)*. Another case involving sudden death was reported as recently as 1992 *(36)*. The first case to be diagnosed premortem was in 1980 by Mayfield et al. *(27)*, and the clinical presentation was indistinguishable from that of a pituitary adenoma. The symptoms are those of a pituitary mass lesion with or without suprasellar extension, including headaches, visual impairment with loss of visual acuity and field defects, and hypopituitarism *(14)*.

The predilection for corticotroph involvement and thus secondary adrenal insufficiency is unexplained. ACTH deficiency is usually the last component to develop in patients with hypopituitarism because of tumors, and yet in hypophysitis, it may be the first and only element of hypopituitarism *(12,37,38)*. Thyrotrophin (TSH) deficiency is also frequently seen *(13)*. There have been cases that presented with symptoms of pituitary apoplexy *(39)*, including three from our original Australian series *(40)* and one further Australian case *(41)*, but apoplexy is unusual and more suggestive of an underlying tumor or granulomatous or necrotizing hypophysitis. Hypercalcemia is another unusual manifestation of acute adrenal insufficiency that has been seen in a number of cases of hypophysitis *(14,38,42–48)*.

Sub-Acute Presentations

The classical sub-acute scenario is a young pregnant or postpartum woman who presents with symptoms and signs of a pituitary mass lesion that resolves with time *(25,40,49)*. Hypophysitis usually presents in the second or third trimester rather than the first, and a typical magnetic resonance imaging (MRI) scan is shown in Fig. 1. In the peripartum setting, some cases may be incorrectly attributed to Sheehan's syndrome even though there was no history of obstetric hemorrhage. Interestingly, the development of lymphocytic hypophysitis does not necessarily imply secondary infertility, and a number of cases of subsequent pregnancies have been reported *(50–53)*.

Prolactin levels may be high, low, or normal in approximately equal proportions *(18,54)*. High levels are normal in pregnancy and breast-feeding but may also suggest a prolactinoma. Alternatively, hyperprolactinemia may be because of stalk pressure, hypothyroidism, or in theory, autoantibodies that stimulate lactotrophs directly *(55)* or that cross-react in the prolactin assay, simulating macroprolactinemia. There is recent evidence that prolactin may have an immunomodulatory role *(56)*. Data on growth

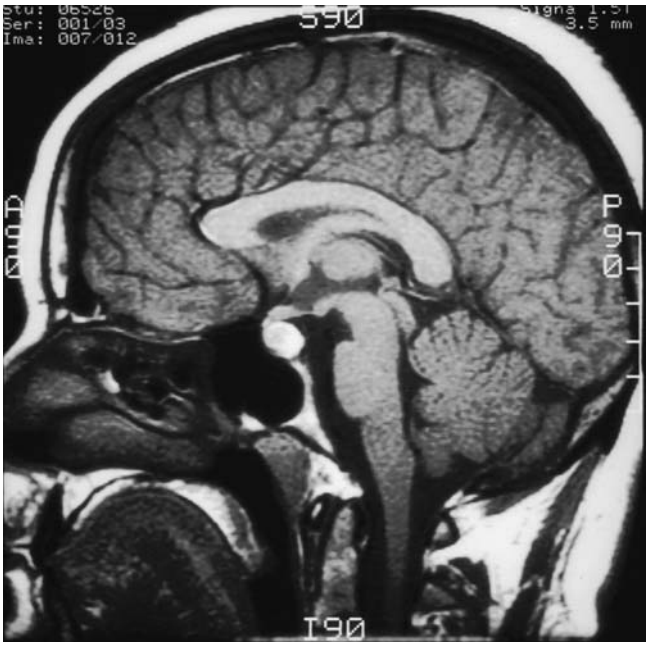

Fig. 1. Magnetic resonance imaging (MRI) scan of a classic case of lymphocytic hypophysitis in a 24-year-old woman who presented with symptoms of a pituitary tumor. Note the uniform enhancement with contrast with extension to the hypothalamus.

hormone (GH) status are less robust as this axis does not appear to be regularly assessed in adult patients *(11,13,18)*. In other causes of hypopituitarism such as traumatic brain injury *(57)* and cranial irradiation *(58)*, GH deficiency (GHD) is usually the first deficit detected, whereas in hypophysitis, it is the last or is even spared.

Extension of the Inflammatory Process

In both acute and sub-acute presentations, there may be extension of the inflammatory process into surrounding structures. This was evident as early as the original case (original histopathological sections reviewed by Prof. Goudie, personal communication). Involvement of the cavernous sinus has been reported, with patients complaining of persistent headaches and then developing diplopia with third, fourth, or sixth cranial nerve palsies *(32,59,60)*. The inflammation can even extend to cause bilateral internal carotid artery occlusion *(61)*.

Dural involvement is often seen on computed tomography (CT) or MRI scans *(62)* and can progress to the point that the diagnosis of hypertrophic cranial pachymeningitis is made *(63)*. It is likely that the association of aseptic meningitis with lymphocytic hypophysitis is part of this phenomenon of extension to surrounding structures *(59,64–67)*, although the alternative explanation is that the hypophysitis was triggered by viral meningitis. In their series of nine patients that were treated prospectively with high-dose methylprednisolone therapy, Kristof et al. *(68)* performed cerebrospinal fluid (CSF) analysis showing a significantly higher lympho-monocytic pleocytosis in patients with presumed lymphocytic hypophysitis [72 (SD = 64) cells/mm^3] compared with patients with pituitary adenomas [14 (SD 11) cells/mm^3].

Infundibuloneurohypophysitis

Extension of the inflammatory process into the posterior pituitary and up into the neurohypophysis will lead to DI. Imura et al. *(69)* described the first cases in 1993 and coined the term infundibuloneurohypophysitis. Since then, an increasing number of cases of DI in the context of hypophysitis have been reported, particularly by Japanese colleagues (*13,60,66,70–78*). In the review by Hashimoto et al. *(13)*, they found 30 of 152 cases (19.7%) with DI. Interestingly, this presentation appears to be more common in male patients.

Recently, the term lymphocytic panhypophysitis has been used to indicate that posterior pituitary dysfunction co-exists with anterior dysfunction *(18,79)*. It is extremely rare for pituitary adenomata to cause DI preoperatively, and so this is an important clue to an underlying inflammatory process and the chance to manage the patient conservatively *(14)*. The major concern is not to miss a dysgerminoma or Langerhans' cell histiocytosis *(80)* as well as the gamut of granulomatous conditions. Rathke's cysts can also present with DI but tend to have a characteristic appearance on T2-weighted MRI. A pericystic lymphocytic infiltrate in this situation is more likely to represent secondary hypophysitis than a primary event *(19)*.

Chronic Presentations

A sensitive and specific assay for pituitary autoantibodies is needed to delineate the chronic spectrum of the disease. This group of patients are far less likely to undergo pituitary biopsy unless there is a large fibrotic mass that resembles a tumor. In general, postinflammatory fibrosis leads to pituitary gland atrophy and an empty sella on CT or MRI scan. An example of this sequence of events is shown in Fig. 2.

The empty sella syndrome is almost certainly a heterogenous condition *(81)*. In a Swedish study of empty sella patients, we were unable to demonstrate a higher rate of pituitary autoantibodies by immunoblotting than in control patients *(81)*, but only 4 of 30 patients had pituitary dysfunction. Isolated ACTH and TSH deficiencies have been reported in association with autoimmune endocrinopathies and the empty sella syndrome. In the former, pituitary autoantibodies have been demonstrated by both immunofluorescence (IF) *(82–84)* and IB *(37,40)*. In contrast, pituitary tumors initially tend to cause GH or gonadotroph dysfunction.

Association with Other Autoimmune and Inflammatory Diseases

Autoimmune diseases have a tendency to cluster, and lymphocytic hypophysitis has been reported with both organ-specific and systemic autoimmunity in 25–50% of cases *(11,13,14,18,40)*. The most common association is Hashimoto's thyroiditis *(2,11,49,85)*. Other associations have included Addison's disease *(86,87)*, type 1 diabetes mellitus *(13,40)*, Graves' disease *(40,88)*, atrophic gastritis *(40,89)*, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) *(90)*, systemic lupus erythematosus *(72,91–93)*, Sjögren's syndrome *(63)*, autoimmune hepatitis *(94)*, and primary biliary cirrhosis *(95)*.

In the neurological and ophthalmological literature, there are a number of localized inflammatory conditions that seem to overlap with lymphocytic hypophysitis. Tolosa Hunt syndrome describes patients with painful ocular inflammation *(62,96,97)*. On closer examination of one case report, the patient was noted coincidentally to have

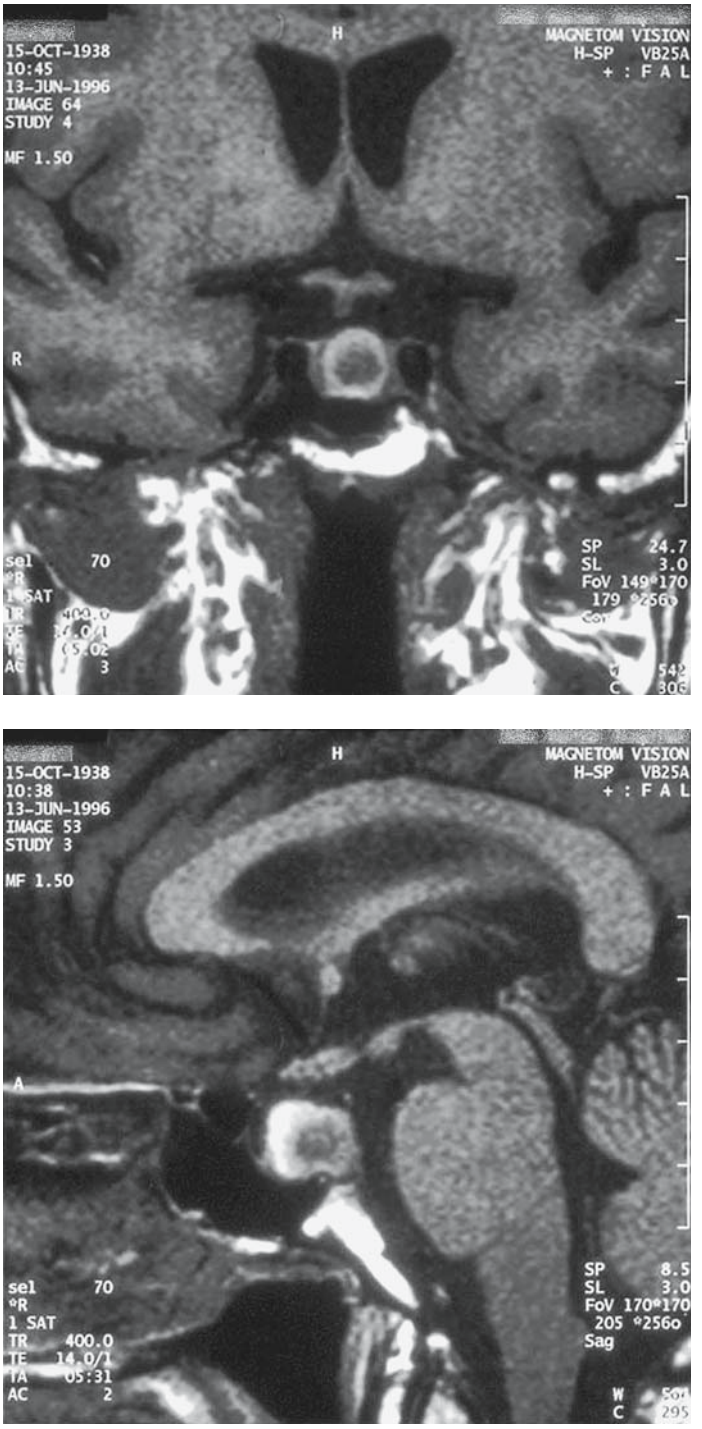

Fig. 2. *(Continued).*

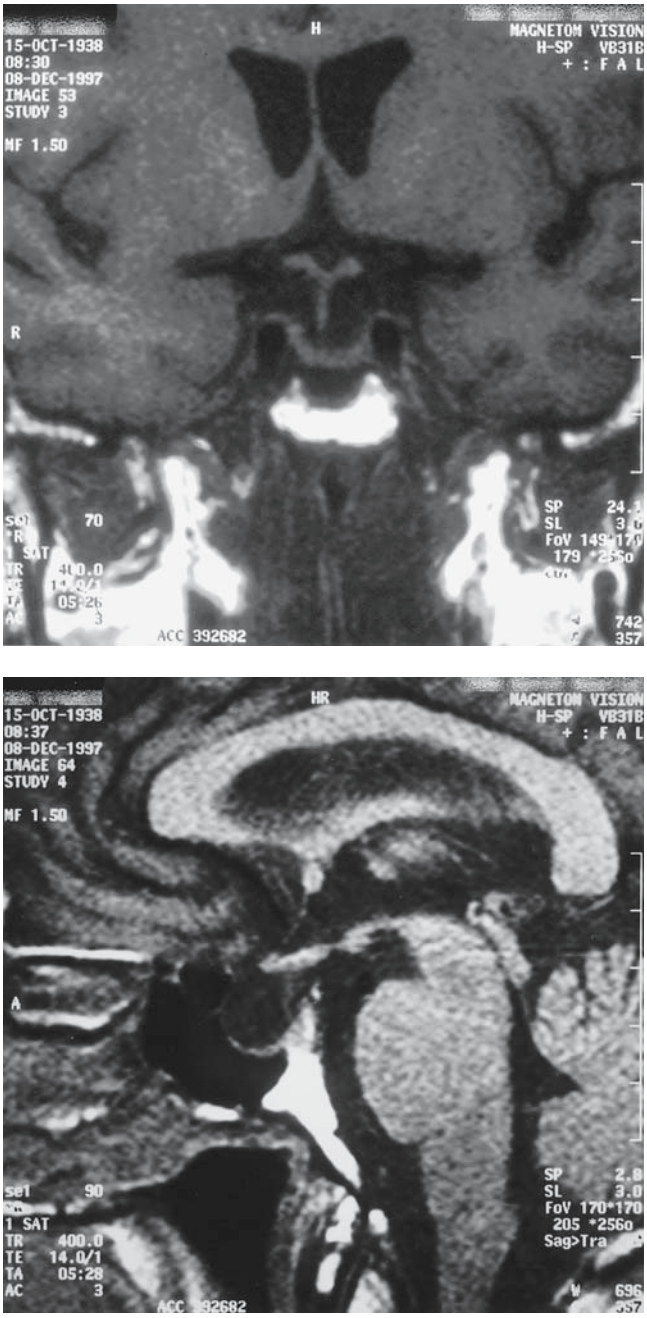

Fig. 2. Sequential magnetic resonance imaging (MRI) scans in a 57-year-old man who presented with hypopituitarism and diabetes insipidus (DI) presumed to be due to lymphocytic hypophysitis. Scans 1 (coronal section) and 2 (sagittal section) show a pituitary mass with ring enhancement. This appearance could also be sagittal granulomatous hypophysitis. Subsequent scans 3 (coronal section) and 4 (sagittal section), taken 18 months later, show progression to an empty sella.

an enlarged pituitary that resolved with steroid therapy for the ocular inflammation. Similarly, fibrosing pseudotumor *(22,98)*, inflammatory pseudotumor *(99)*, dacryoadenitis *(100)*, temporal arteritis *(22)*, and lymphocytic lacrimal and salivary gland involvement with Hashimoto's thyroiditis *(101)* have all been reported in association with hypophysitis, and some have been treated successfully with intravenous steroids. The conditions previously described are local, but lymphocytic hypophysitis has also been reported in patients with generalized inflammatory pathologies such as retroperitoneal fibrosis *(87,102)*. There is no unifying hypothesis to explain these associations other than some infectious trigger or the tendency for autoimmune conditions to cluster *(103)*.

As with other autoimmune conditions, there may be a relapsing and remitting course *(65)*. The longest time to full disease expression has been 8 years in two instances *(72,104)*. Spontaneous recovery has also been widely reported (*25,31,49,105–108*).

APECED

Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (OMIM 240300), also known as polyglandular autoimmune disease type 1, is a rare, autosomal recessive disorder caused by mutations in the autoimmune regulator (*AIRE*) gene (*see* Chap. 17). *AIRE* has an important role in central tolerance by promoting the expression of organ-specific antigens in the thymus *(109,110)*. Hypopituitarism is an uncommon feature. There has been one convincing case of clinical hypophysitis *(90)* in a French-Canadian patient with a severe phenotype where virtually no endocrine gland was spared. Autoantibodies to pituitary membrane proteins were demonstrated by IB but have not been further characterized *(90)*. Isolated GHD has been documented in another nine patients, isolated secondary hypogonadism in one case, and central DI in three *(111–113)*. Finally, three siblings with partial ACTH deficiency *(114)* and one patient with selective hypopituitarism *(115)* have been reported. Pituitary autoantibodies have been studied in the cohort of APECED patients from Scandinavia *(116)*, but their clinical significance is uncertain.

Special Case Scenarios

Pregnant Women with Type 1 Diabetes

A particularly interesting group of patients are pregnant women with type 1 diabetes *(40)*. The Australian prevalence study of postpartum thyroid dysfunction from Perth showed that 11.5% of normal women had evidence of hypothyroidism or hyperthyroidism 6 months postpartum *(117)*. It is well recognized that diabetic patients are at an even higher risk of postpartum thyroiditis (*see* Chap. 8). Therefore, these women may also be at higher risk of peripartum hypophysitis, which should be suspected in patients with peripartum headache and rapidly falling insulin requirements. In our experience, these patients can be easily overlooked and fatigue attributed to psychosocial factors and to the stress of caring for a new baby. Finally, hypopituitarism, presenting as the Houssay phenomenon (hypoglycemia because of hypopituitarism), has been attributed to microvascular disease because of the diabetes itself. In fact, some of these cases may have been autoimmune in etiology *(40)*.

Sheehan's Syndrome

The other interesting group of patients are those given a diagnosis of Sheehan's syndrome as a cause of their hypopituitarism when there was no history of postpartum hemorrhage or other obstetric calamity. Goswami et al. *(118)* used the IB method to study a large cohort of Indian women with severe peripartum hemorrhage leading to Sheehan's syndrome up to 8 years later. They found that 63.1% (12 of 19) developed anti-enolase antibodies compared with 17.8% (5 of 28) women with normal pregnancies and 14.2% (4 of 28) women who had never conceived. Despite near catastrophic hemorrhage at the time of delivery in all patients, some had a significant delay in the development of hypopituitarism. This delay supported the theory that an autoimmune process was triggered by the obstetric event.

Pediatric Cases

Pediatric cases of lymphocytic hypophysitis outside of APECED can be counted on one hand *(119–122)*. It is interesting that the third of the original three cases of xanthomatous hypophysitis was an adolescent female *(123)*. Granulomatous lesions have also been seen in adolescence *(124,125)*.

Patients with Cancer Treated with Cytotoxic T-Lymphocyte-Associated Antigen-4 Blockade

Cytotoxic T-lymphocyte-associated antigen (CTLA)-4 is a receptor expressed on activated T cells and a subset of regulatory T cells. It inhibits T-cell responses and is therefore important in the maintenance of peripheral tolerance against self-antigens *(126,127)*. Many malignant tumors are known to express self-antigens on their surfaces. Recent reports have established that administration of CTLA-4 blocking antibodies in patients with advanced melanoma mediates cancer regression *(128,129)*. Unfortunately, this kind of immunotherapy may also induce autoimmune manifestations such as enterocolitis, dermatitis, and hypophysitis *(128,130)*.

Blansfield et al. *(131)* reported six patients with melanoma and two patients with renal cell carcinoma that all developed hypophysitis during treatment with CTLA-4 antibodies. Before the immunotherapy was started, all patients had normal pituitary function and normal pituitary imaging on MRI, except for one patient who had an empty sella but no hypopituitarism. After CTLA-4 blockade, seven of eight patients had an evident increase in pituitary size, and all eight patients developed clinical signs of hypopituitarism. Low levels of cortisol and TSH were detected in the patients, and in seven of them, testosterone was also low. Once the immunotherapy was interrupted and hormonal replacement therapy started, all patients had resolution of their clinical symptoms. Based on these findings, it is recommended that patients treated with CTLA-4 antibodies be closely monitored for clinical and laboratory signs of hypopituitarism. Reversible hypopituitarism has also been reported after alpha-interferon therapy *(132)*.

INVESTIGATIONS

Imaging

Computed tomography scanning performed on the first case of hypophysitis diagnosed in a living patient *(27)* could not differentiate the mass from that of an adenoma. MRI is now the preferred technology.

The MRI findings of lymphocytic hypophysitis vary depending on the stage and extent of the inflammatory process. On T1-weighted precontrast images, lymphocytic hypophysitis appears isointense relative to gray matter. The sellar floor regularly appears flat and intact, whereas erosion that can be unilateral is more often seen in pituitary adenomata *(9)*. Postcontrast, acute cases often present as a symmetric homogeneous contrast-enhancing sellar mass with suprasellar extension *(133)*. Rarely, a heterogeneous cystic appearance can be found *(134–136)*. The intense enhancement may be confined to the periphery of the lesion as "ring enhancement" *(71)* or extend along the dura mater as a "dural tail" *(137)*. Interpretation of scans can be difficult in pregnant women as pregnancy itself leads to hyperintensity of the anterior pituitary on MRI *(138)*.

Dynamic MRI has displayed delayed contrast enhancement of the pituitary mass as a sign of abnormal hypophyseal vasculature *(139)*. A striking finding is that the pituitary stalk often is thickened and enhanced but rarely displaced *(70)*. If the inflammatory process involves the posterior pituitary lobe, its precontrast hyperintense bright spot may be lost *(69)*. Involvement of the cavernous sinus resulting in cranial nerve palsies as well as occlusion of one or both internal carotid arteries have been described *(32,61, 140)*. In its sub-acute and chronic presentations, lymphocytic hypophysitis may show a normal pituitary on MRI or signs of pituitary atrophy and empty sella *(141)*. Figure 2 shows the progression of hypophysitis from a mass with ring enhancement and stalk involvement causing DI to an empty sella over 18 months.

Features such as a homogeneous symmetrical mass, marked contrast enhancement, and stalk thickening with no deviation speak in favor of lymphocytic hypophysitis but can also be seen with other pituitary lesions. Therefore, it may be very hard to predict hypophysitis on MRI, and histopathological examination remains the definitive arbiter.

Immunopathogenesis

Gross Pathology

Inspection of the pituitary gland in the autopsy cases showed significant atrophy *(14)*, together with secondary atrophy of the adrenals in nearly all cases. The tissue at neurosurgery is often described as looking white-gray to yellowish with a consistency that may be soft but is more often firm, fibrous, and adherent to surrounding structures such as the dura mater. In occasional cases, a cystic appearance with yellow liquid is described, which raises the possibilities of a Rathke's cyst with secondary hypophysitis or of necrotizing or xanthomatous hypophysitis.

Histopathology

Lymphocytic hypophysitis is characterized by extensive, diffuse lympho-plasmacytic infiltration of the anterior pituitary as seen in Fig. 3. As in Hashimoto's thyroiditis, the lymphocytes can aggregate to form lymphoid follicles with germinal centers. The inflammatory infiltrate consists mainly of a polyclonal mixture of T and B lymphocytes with an admixture of plasma cells and occasional eosinophils, macrophages, and histiocytes *(19,20) (142–144)*. Recently, mast cells have been described *(145)* and also activation of the supporting dendritic-like pituitary folliculo-stellate cells *(146)*.

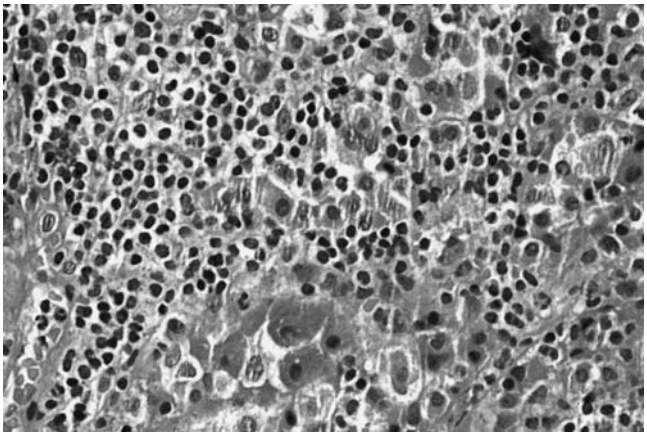

Fig. 3. Histological section of a classic peripartum case of lymphocytic hypophysitis stained with hematoxylin and eosin, showing the diffuse lymphocytic infiltrate with scattered plasma cells and eosinophils and islands of remnant pituitary cells.

In acute cases with large numbers of lymphocytes, it is important to exclude clonality, which would indicate an underlying lymphoma *(147)* or leukemia. In cases where the surgeon has found firm tissue, there is a correlation with significant fibrosis on light microscopy. Occasional neutrophils may be seen, but significant areas of necrosis or granuloma formation indicate other entities such as necrotizing infundibuloneurohypophysitis and granulomatous hypophysitis discussed under Differential Diagnosis.

Immunohistochemistry

Characterization of the T-cell and B-cell infiltrates has shown that the former predominate except in areas with lymphoid follicles. The tissue ratio of T-helper (CD4+) to T-suppressor (CD8+) cells has been described as 2:1 or greater in the majority of cases *(12,20,38,66,140,143,148)*. Gutenberg et al. *(143)* showed that the highest numbers of activated CD8+ T cells were observed in cases presenting in pregnancy and with a shorter duration of clinical symptoms.

Electron Microscopy

The first description of changes seen on electron microscopy (EM), by Asa et al. *(24)*, was of pituitary cells interdigitating with activated lymphocytes in those areas of the most dense inflammatory cell infiltration. Some pituitary cells were intact *(24,149)*, whereas others showed signs of oncocytic transformation or enlarged lysosomal bodies *(20)*. No immune complex deposits were seen. Jensen et al. *(38)* also noted phagocytosis of organelles from degenerating adenohypophyseal cells. In their case, isolated corticotropin deficiency was confirmed on EM by the selective loss of corticotrophs. Isolated loss of prolactin cells has also been shown *(146)*. Professor Ross McD. Anderson, an eminent neuropathologist in Melbourne, captured an exquisite example of an activated lymphocyte interacting with an adenohypophyseal cell in peripartum hypophysitis, and this is shown in Fig. 4 *(12)*.

Differential Diagnosis

In patients presenting acutely, the immediate concern is to treat the underlying hypopituitarism, to preserve vision, and then to exclude a pituitary tumor. If clinical

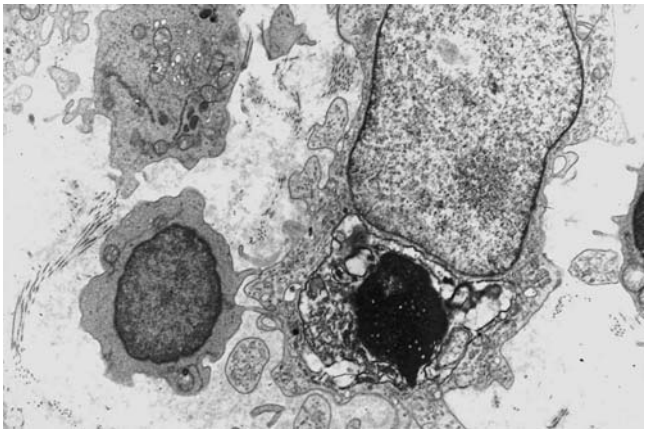

Fig. 4. Electron microscopy (EM) study of the case shown in Fig. 3. Note the lymphocyte "embracing" a degenerating adenohypophyseal cell. (Photograph reproduced with the kind permission of *Current Opinion in Endocrinology and Diabetes*.).

findings or imaging are suggestive of an inflammatory process, then there are a number of ancillary investigations, which help to narrow the diagnostic possibilities. However, biopsy may be needed to differentiate the types of hypophysitis.

Primary Hypophysitis

Hypophysitis can be classified as primary or secondary. Broadly, the primary inflammatory processes are lymphocytic hypophysitis, granulomatous hypophysitis, and the recently described xanthomatous hypophysitis. However, there are cases described as lympho-granulomatous hypophysitis *(21,29,150)*, necrotizing infundibuloneurohypophysitis *(151)*, and xantho-granulomatous hypophysitis *(144)*. It is unclear whether these are separate entities. Cheung et al. *(142)* illustrated the three major sub-types with classical case reports, as did Flanagan et al. *(135)*. Neither granulomatous and xanthomatous hypophysitis appear to have an autoimmune basis. They do not have a preponderance of female patients and are not usually associated with other autoimmune diseases *(143)*.

Granulomatous Hypophysitis

Granulomatous inflammation of the pituitary may be primary or secondary to an underlying systemic granulomatous disease (*see* Secondary Hypophysitis). Idiopathic primary granulomatous hypophysitis mainly affects the anterior pituitary but may also involve the neurohypophysis and infundibulum, particularly in male patients. Headache and visual disturbances are common, as are varying degrees of hypopituitarism with or without DI. The diagnosis is rarely made on clinical grounds but rather on the histological findings of granulomas with epithelioid histiocytes and multinucleated giant cells *(142)*. A variable number of lymphocytes and plasma cells may also be present. On radiological examination, an intrasellar mass, sometimes with parasellar extension, is seen *(21)*. There may also be stalk thickening and loss of the posterior pituitary bright spot on MRI *(152)* in those patients with DI.

Xanthomatous Hypophysitis

Xanthomatous hypophysitis is a recently described entity *(123)*, with less than 10 cases in the literature *(142–144,153,154)*. The mass lesions often appear cystic on MRI. The anterior pituitary is infiltrated with lipid-rich, foamy histiocytes strongly reactive to CD68 antibody (indicating monocyte–macrophage lineage), and some lymphocytes. There may be foci of liquefaction. The pathogenesis is not understood but felt to be infective.

Secondary Hypophysitis

It is important to consider whether there is an underlying treatable systemic condition that may manifest as primary hypophysitis. As intimated in the discussion on primary hypophysitis, this can be difficult when the two can co-exist. The list of conditions that can affect the pituitary is extensive *(155)*.

It was the observation of lymphocytic infiltration around craniopharyngiomas that led Puchner et al. *(19,156)* to coin the term "secondary hypophysitis." Hypophysitis secondary to tumors has been seen also with both GH-secreting *(157)* and prolactin-secreting *(158)* adenomata and in association with Rathke's cysts *(135,159–162)*.

In patients with DI, the entity of infundibuloneurohypophysitis needs to be differentiated from two major conditions—dysgerminoma and Langerhans' cell histiocytosis, especially in children and adolescents *(80)*. Even biopsy of pituitary stalk lesions can be misleading, as there may be a secondary lymphocytic infiltrate around a dysgerminoma *(163)*. This phenomenon was best illustrated by the case of a young patient from Germany whose initial biopsy suggested lymphocytic hypophysitis, but whose subsequent course was that of an aggressive dysgerminoma *(164,165)*. Langerhans' cell histiocytosis is S100 positive on biopsy, but preoperatively, the diagnosis can be suspected when there is a characteristic rash (more common in pediatric patients), an ear discharge, or a positive bone scan *(166)*.

Neurosarcoidosis can involve the infundibulum and extend into the pituitary. Serum angiotensin-converting enzyme (ACE) levels may be raised, and a chest radiograph may show the classical infiltrate. There has been a case report of pulmonary sarcoidosis being associated with biopsy-proven lymphocytic hypophysitis *(167)*. Granulomatous diseases including Wegener's disease *(168)*, sarcoidosis *(169)*, tuberculosis *(170,171)*, Takayasu's disease *(172)*, and Crohn's disease *(173)* have all presented with pituitary manifestations.

Tuberculosis of the sella turcica *(174)* was not uncommon in the early 1900's and features in Sheehan's series of cases. It is also still a consideration in regions that have a high prevalence of tuberculosis, especially as HIV/AIDS is contributing to its resurgence. A tuberculin test and antigen polymerase chain reaction (PCR) of CSF are informative *(170)*. Alternatively, new sensitive and specific tests of lymphocyte interferon-gamma responses to tuberculous antigens are increasingly being used. Syphilis was also more common last century but is a consideration in patients with other manifestations who are *treponema pallidum* hemagglutination positive.

Pituitary Autoantibodies

Pituitary autoantibodies and their relation to lymphocytic hypophysitis have recently been reviewed *(175)*. The other autoimmune endocrinopathies, such as Hashimoto's

thyroiditis, Addison's disease, type 1 diabetes mellitus, and Graves' disease, have been traditionally considered as organ-specific processes. Their respective target autoantigens are tissue-specific or cell-specific enzymes *(7,176,177)*, hormones *(178)*, or receptors *(179)*. Yet, islet cell antibodies (ICA) detected by IF in patients with type 1 diabetes recognize not only insulin-secreting beta cells but multiple pituitary cells *(180)* as well as islet alpha cells, delta cells, and those making pancreatic polypeptide *(181)*. This ICA reactivity is not completely preabsorbed by glutamic acid decarboxylase (GAD) 65 and islet antigen (IA) 2, suggesting that there are other relevant islet cell autoantigens *(182)*. Patients with Graves' disease can also have autoantibodies that cross-react with the pituitary *(183)*, and those with Hashimoto's thyroiditis can develop an encephalopathy that is related to their thyroid autoantibody status and not some concurrent neurological condition *(184,185)*. One interpretation has been that pituitary autoantibody reactivity is therefore non-specific, but this is partly contradicted by recent data on autoantibodies to type 2 iodothyronine deiodinase (D2) *(186)*. This enzyme is expressed in both the pituitary and the thyroid.

The pituitary contains at least five different hormone-secreting cell types. If there are cell-specific or enzyme-specific targets, then pituitary autoantibodies in a patient with lymphocytic hypophysitis and isolated ACTH deficiency are probably going to be different to those from a patient with isolated TSH deficiency or panhypopituitarism. A number of techniques have been used to look for pituitary autoantibodies as summarized in Table 1. Some methods, such as IF, identify the target cell type and sub-cellular localization but not the target protein. Others, such as IB, identify the molecular weight of the target protein but not the cell of origin. Finding a pituitary-specific autoantigen is not so simple, as the enzymes in the pituitary are also present in the hypothalamus and neuroendocrine tissues including the placenta—for example, neuron-specific enolase (NSE) *(187)*, prohormone convertase (PC) *(188)*, and the family of carboxypeptidases (CPs) *(189)*. Using a candidate autoantigen approach, Tanaka et al. looked at the expression profile of active genes in the human pituitary gland and found two pituitary gland-specific factor (PGSF) 1a and PGSF2. These factors have been recognized by sera from patients with rheumatoid arthritis, which intuitively would exclude them as specific autoantigens. However, there is preliminary evidence that rheumatoid patients may have subtle pituitary dysfunction *(190)*.

Complement Consumption Assays

Pituitary autoantibodies were first sought using a complement consumption assay and crude autopsy pituitary gland homogenate *(191)*. This type of assay works on the basis of the interaction of antigen-antibody complexes with complement and is particularly neither sensitive nor specific. The results in 128 normal peripartum women linked the development of positive antibody status 5–7 days postpartum (seen in 18%) with symptoms suggestive of pituitary dysfunction 6–12 months later, but hormonal data were lacking.

Indirect IF Assays

The first study was by Goudie *(192)*, who was unable to find positive anti-pituitary reactivity. In 1969, Nerup et al. *(193)* also unsuccessfully attempted to demon-

Table 1
Techniques and Substrates Used for the Detection of Pituitary Autoantibodies

Technique	*Pituitary substrate*		*References*
Complement consumption	Human	Autopsy material	Engelberth and Jezkova, 1965 *(191)*
Indirect immunofluorescence	Human	Autopsy material	Bottazzo et al., 1975 *(55)*
			Pouplard et al., 1985 *(202)*
		Fresh material from surgery	Bottazzo et al., 1975 *(55)*
			Mirakian et al., 1982 *(180)*
			Gluck et al., 1993 *(193)*
		Fetal glands	Scherbaum et al., 1987 *(197)*
			Gluck and Scherbaum, 1990 *(200)*
			Gluck et al., 1993
	Primate	Cymologous monkey	Gluck and Scherbaum, 1990 *(200)*
		Rhesus monkey	Maghnie et al., 1994 *(196)*
			Maghnie et al., 1995 *(197)*
		Baboon	Gluck and Scherbaum, 1990 *(200)*
			De Bellis et al., 2003 *(198)*
			De Bellis et al., 2005 *(201)*
	Non-primate	Rat	Bottazzo et al., 1975 *(55)*
			Pouplard et al., 1980 *(200)*
			Hansen et al., 1989 *(183)*
			Møller et al., 1985 *(201)*
			Sugiura et al., 1986 *(203)*
			Kobayashi et al., 1988 *(203)*
			Gluck and Scherbaum, 1990 *(200)*
			Kajita et al., 1991 *(82)*
			Fetissov et al., 2002 *(223)*
		Bovine	Bottazzo et al., 1975 *(55)*
			Gluck and Scherbaum, 1990 *(200)*
		Guinea pig	Pouplard, 1982 *(196)*
			Pouplard et al., 1985 *(202)*
		Porcine	Hansen et al., 1989 *(183)*
			Gluck and Scherbaum, 1990 *(200)*
		Sheep	Gluck and Scherbaum, 1990 *(200)*
	Cell lines	Murine AtT_{20} and Rat GH_3	Sugiura et al., 1987 *(84)*
			Komatsu et al., 1988 *(204)*
			Kajita et al., 1991 *(82)*
Immunoblotting	Human		Crock et al., 1993 *(205)*
			Crock, 1998 *(40)*
			Strömberg et al., 1998 *(208)*
			Nishiki et al., 2001 *(206)*

			Takao et al., 2001 *(210)* Goswami et al., 2002 *(118)* O'Dwyer et al., 2002 *(207)* O'Dwyer et al., 2002 *(187)* Bensing et al., 2004 *(81)* Bensing et al., 2005 *(37)*
	Primate	Rhesus monkey	Crock et al., 1993 *(205)*
	Non-primate	Rat	Yabe et al., 1995 *(212)* Yabe et al., 1998 *(213)* Kikuchi et al., 2000 *(215)* Nishino et al., 2001 *(214)*
		Porcine	Kobayashi et al., 1997 *(212)* Kobayashi et al., 1998 *(211)*
Enzyme-linked immunosorbent assay	Human	Adenoma cells	Keda et al., 2002 *(216)*
	Non-primate	Rat	Yabe et al., 1998 *(213)* Kikuchi et al., 2000 *(215)* Nishino et al., 2001 *(214)* Keda et al., 2002 *(216)*
		Porcine	Kobayashi et al., 1998 *(211)*
In vitro transcription translation and immunoprecipitation of pituitary proteins	Human		Tanaka et al., 2002 *(219)* Tanaka et al., 2003 *(190)* Tanaka et al., 2003 *(220)* Tatsumi et al., 2003 *(188)*

strate pituitary autoantibodies in 16 patients with idiopathic hypopituitarism (and 232 controls) using IF on fresh human surgical pituitary tissue from breast cancer sufferers as well as monkey and rabbit pituitaries. None of the 16 had other autoimmune conditions except one with thyroglobulin antibodies. In 1975, Bottazzo et al. *(55)* first described autoantibodies to pituitary prolactin-secreting cells using indirect IF in 287 patients with endocrine autoimmunity, but none had clinical hypopituitarism.

The IF assay is still widely used, but it has rarely identified pituitary autoantibodies in patients with biopsy-proven or suspected lymphocytic hypophysitis *(27,194)*. This method recognizes the conformational structure of antigens, their sub-cellular localization, and the pituitary cell type targeted. In general, the titer of pituitary autoantibodies found by IF is low. The choice of pituitary substrate is problematic in terms of species specificity issues, ethical issues, and limited supply.

Human Tissues as Substrate. Bottazzo et al. *(55)* used a four-layer double-fluorochrome method on fresh, human pituitaries from women whom had undergone hypophysectomy for breast cancer. These glands did not have entirely normal

histology, as they had prolactin cell and GH cell hypertrophy and a reduced number of basophils. Prior treatment with stilbestrol and prednisone may have accounted for these changes. Sera from 10 patients with autoimmune polyendocrinopathy and 9 patients with a single endocrine autoimmune disease gave a diffuse, finely granular cytoplasmic IF on pituitary cells. None had hypopituitarism. Conversely, none of the 13 patients with idiopathic panhypopituitarism gave positive results.

Subsequently, using undiluted serum, antibodies to "multiple pituitary cell types" were shown in patients with newly diagnosed diabetes and their high-risk first-degree relatives *(180)*. There was a striking correlation between pituitary cell antibodies and positive ICA. The authors speculated that this may have indicated a viral trigger for diabetes that simultaneously involved the pituitary. The idea is supported by the work of Onodera et al. *(195)* discussed in "Animal Models."

Pouplard *(196)* had shown in 1982 that immunoglobulins from normal sera bind through Fc receptors to the surface of corticotrophs but not those in fetal pituitary glands. Thus, pituitary antibodies against corticotrophs need to be interpreted with caution. Scherbaum et al. *(197)* have shown that pituitary autoantibodies to corticotrophs in Cushing's disease patients are associated with an unfavorable outcome after microsurgical resection. They have not published results on patients with lymphocytic hypophysitis.

Other cell types targeted have included thyrotrophs, gonadotrophs in patients with cryptorchidism and their mothers *(198)*, and somatotrophs *(199)*. Again, no patients with hypophysitis were studied.

Non-Human Tissues as Substrate. Although fresh human tissue would be ideal, the ethical issues of using fetal glands and the limited supply of surgical tissue make this untenable. Non-human tissues raise the problems of species specificity because of heterophile antibodies as outlined by the study from Gluck and Scherbaum *(200)*.

Bottazzo's original publications concluded that baboon pituitary was the optimal non-human tissue substrate. Recently, De Bellis and colleagues *(201)* have revisited IF using young baboon pituitary glands in patients with idiopathic GHD and autoimmune endocrine diseases. Specific staining of somatotrophs alone was typical of isolated GHD; however, more diffuse staining of other cells was seen in patients with GHD and other autoimmune diseases.

A range of other pituitary tissues has been used including guinea pig *(202)*, rat *(203)*, porcine *(183)*, and a murine AtT20 cell line *(84)*. Positive reactivity has been seen in patients with cryptorchidism *(202)*, isolated ACTH deficiency *(203)*, the empty sella syndrome *(204)*, and Graves' disease *(183)*.

Immunoblotting Assays

The IB (or Western) assay was developed to overcome the problems with IF *(205)*. The preparation of whole pituitary glands by homogenization means that the immunoreactivity detected by patient sera is to proteins of a particular molecular size rather than a specific pituitary cell type. In addition, the initial centrifugation step gives a pellet containing nuclei and mitochondria. If this fraction contains any potential autoantigens, they will be discarded at this step. Proteins from the membrane or cytosolic fractions

are then denatured, separated electrophoretically by size and transferred to a membrane. The assay method is outlined in Fig. 5. In contrast to IF, patient sera react with linear epitopes rather than a three-dimensional structure.

In the original IB article *(205)*, both membrane and cytosolic pituitary fractions were probed with sera from pediatric patients with GHD and normal pediatric control sera. Autoantibodies were identified to a 45-kDa pituitary-specific membrane protein in 1 of 19 patients with idiopathic GHD and the empty sella syndrome. One other patient with idiopathic GHD and 1 of 14 patients with secondary GHD had autoantibodies to a 43-kDa membrane protein, found in both pituitary and brain. None of 27 control subjects had these autoantibodies. In light of recent IF data in an adult cohort of Italian patients with GHD *(201)*, it will be of great interest to identify the protein(s) seen by IF and to see whether they have the same molecular weights as those in our IB study. Nishiki et al. *(206)* also identified pituitary-specific antibodies to 43-kDa, 49-kDa, or 68-kDa membrane proteins in 5 of 13 patients with lymphocytic hypophysitis, 1 of 12 patients with infundibuloneurohypophysitis, but none of 4 patients with isolated ACTH deficiency. These proteins are of great interest but have yet to be further characterized. No other membrane studies have been published.

Subsequent IB studies using pituitary cytosolic preparations in a series of 10 patients with biopsy-proven hypophysitis and 22 patients with suspected disease showed autoantibodies to a 49-kDa protein in 70% patients with biopsy-proven hypophysitis, 50% with suspected disease, and 9.8% normal controls. A number of other autoantigens were identified, particularly a 40-kDa protein. Titers as high as >1:1000 were seen in contrast to IF studies that have consistently used undiluted sera or dilutions up to 1:8. Species specificity experiments using IB demonstrated that the 49-kDa protein was conserved across species *(40)* but also demonstrated the extent of tissue cross-reactivity that can confound IF results.

The 49-kDa protein was purified using column chromatography, sequenced, and identified as alpha-enolase *(207)*. Enolase has three isoforms, one of which is found

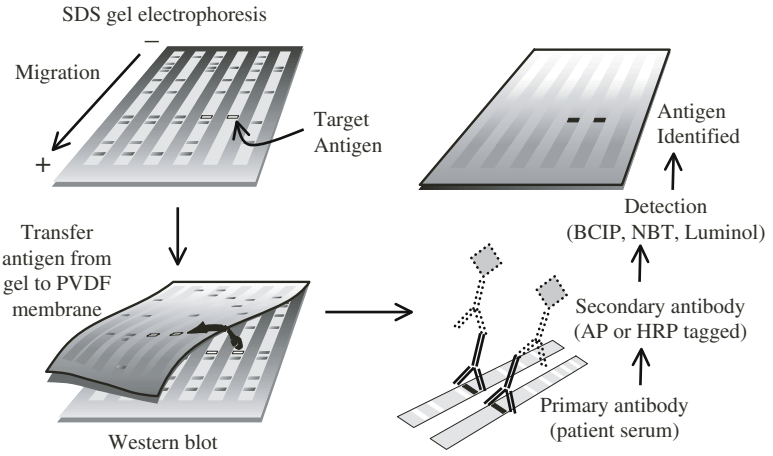

Fig. 5. Schematic representation of the immunoblotting (IB) assay for pituitary autoantibodies.

in neuroendocrine tissues (NSE). A study using two-dimensional gel electrophoresis showed that serum from a peripartum woman with lymphocytic hypophysitis recognized NSE in both the placenta and the pituitary *(187)*. It was hypothesized that the sharing of placental and pituitary antigens may explain the association of lymphocytic hypophysitis with pregnancy. The study by Goswami et al. *(118)* of Indian women with true Sheehan's syndrome who developed hypopituitarism up to 8 years later also hints at this link.

Anti-enolase antibodies have been found in a wide range of patients with classical non-organ-specific autoimmunity *(40)* but also in up to 20% of patients with pituitary adenoma and 5–10% of control subjects using IB. Using another method (in vitro transcription translation [ITT]; *see* In Vitro Transcription Translation or Immunoprecipitate of Pituitary Proteins), the incidence of these antibodies in tumor patients was even higher (46%), suggesting that they may not be a reliable discriminator of an autoimmune condition.

Isolated ACTH deficiency has been reported in association with autoimmune diseases *(83,208)*, including lymphocytic hypophysitis *(38,209)*. In a large Polish series of patients with ACTH deficiency (isolated in 61 of 65), 51% (33 of 65) had another autoimmune disease and 85% (55 of 65) had positive thyroid autoantibodies. IB identified a novel 36-kDa pituitary cytosolic autoantigen in 12 patients (18.5%) compared with 2 of 57 healthy controls (3.5%, $p < 0.021$) *(37)*. Patients with autoantibodies to the 36-kDa protein had a higher frequency of thyroglobulin autoantibodies than the patients whose sera were not immunoreactive. This target autoantigen has not been further characterized as yet. A Japanese study of nine patients with isolated ACTH deficiency demonstrated that seven (77.8%) had autoantibodies to a 22-kDa human pituitary cytosolic protein, subsequently identified as GH *(210)*. The same study found these autoantibodies in 11 of 15 (73%) patients with lymphocytic hypophysitis. GHD was found on testing in 9 of 11 patients with autoantibodies. GH reactivity was lost by preabsorption with pancreatic antigens *(211)*, a finding that mirrors Bottazzo's earlier observations in patients with diabetes and ICA cross-reactivity with the pituitary.

The empty sella syndrome almost certainly has a heterogenous etiology with one component being end-stage hypophysitis. Bensing et al. *(81)* studied a group of 30 patients with empty sella syndrome, 15 of whom had type 2 diabetes or impaired glucose tolerance and a body phenotype of central obesity. They did not have evidence of high-titer pituitary autoantibodies compared with controls, but only four patients (13%) had pituitary dysfunction. Therefore, it appears that patients with an empty sella syndrome and normal pituitary function are very unlikely to have had hypophysitis.

Enzyme-Linked Immunosorbent Assay

The first group to investigate an enzyme-linked immunosorbent assay (ELISA) was Yabe et al. *(211–213)*. Rat or porcine pituitary antigens from tissue preparations were used. A typical ELISA procedure was developed using the cytosolic fraction of homogenized rat pituitary glands, coated onto the ELISA plate at alkaline pH. Serum reactivity to bound antigens was detected using a peroxidase-conjugated second antibody and a colored substrate. Measurement of absorbance at an appropriate wavelength related to the concentration of pituitary autoantibodies present. This approach presents a cocktail of potential autoantigens and preserves the three-dimensional structure of the antigens. However, the advantage may be nullified if a low level of target autoantigen

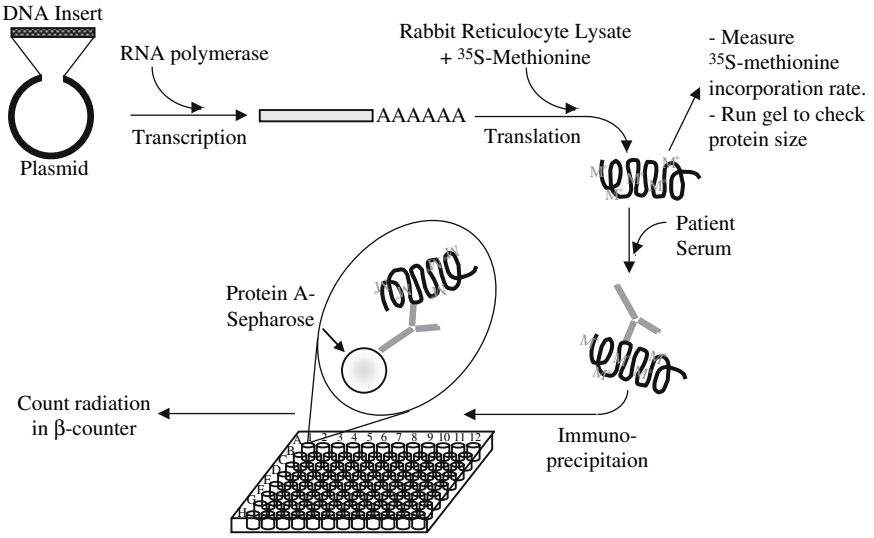

Fig. 6. Schematic representation of the immunoprecipitation (in vitro transcription translation [ITT]) assay for pituitary autoantibodies.

(cytosolic or membrane) is masked by other proteins or if there is significant species cross-reactivity. Pituitary autoantibodies were detected by this method in patients with non-insulin-dependent diabetes mellitus *(212)* and autoimmune thyroiditis *(214)* as well as various pituitary disorders *(215)*. 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 *(211)*.

Keda et al. *(216)* modified the ELISA by using human pituitary adenoma cells to develop a cellular variant. Serum from patients with idiopathic hyperprolactinemia or idiopathic-isolated GHD had autoantibodies more frequently to prolactin-secreting cells and GH-secreting cells, respectively, than patients with other forms of pituitary diseases.

Evaluation of these ELISA techniques using sera from biopsy-proven lymphocytic hypophysitis patients has yet to be performed.

In Vitro Transcription Translation and Immunoprecipitation of Pituitary Proteins

The latest assay uses rabbit reticulocyte lysate to produce recombinant pituitary proteins in vitro. Methionine residues on these proteins are labeled with ^{35}S, and the proteins then mixed with patient sera and protein-A sepharose in an immunoprecipitation step *(217)*. The method is outlined schematically in Fig. 6. A number of potential pituitary autoantigens have been tested in this system. Tanaka et al. *(218)* tested two novel PGSFs, PGSF1a and PGSF2, isolated from a hypothalamic cDNA expression library. Other candidates studied included enolase; GH; the prohormone-processing enzymes, PC, PC1/3, and PC2; CPE; and PC2-regulatory protein, 7B2 *(188)*. None of these enzymes is pituitary specific.

Positive antibody indices to PGSF1a were found in 1 of 3 (33%) patients with biopsy-proven hypophysitis and 2 of 10 (20%) patients with isolated ACTH deficiency *(219)*. Reactivity to PGSF2 was seen in 2 of 14 (14%) patients with suspected hypophysitis

or infundibuloneurohypophysitis and 3 of 14 (21%) patients with hypopituitarism. Anti-GH antibodies were detected in 2 of 8 (25%) patients with hypophysitis (1 of whom was biopsy proven), 2 of 14 (14%) with hypopituitarism, and 2 of 31 (6.5%) with other autoimmune diseases. None of the antibody indices was above 2, which implies very low titer reactivity. Patients with pituitary adenomata did not show any reactivity to either PGSF1a or PGSF2 *(219)*, but 5 of 11 (45%) patients had antibodies against PC1/3 *(188)* and 6 of 11 (55%) to 7B2 compared with 2 of 14 (14%) patients with lymphocytic hypophysitis for both antigens *(188)*. PGSF1a antibodies have also been detected in 20 of 26 (77%) rheumatoid arthritis patients *(190)*. Before these latest results are dismissed as non-specific, it will be important to exclude subtle pituitary hormone dysfunction in these patients.

Enolase was tested as an autoantigen in the ITT assay by Tanaka et al. *(220)*. They demonstrated positive autoantibodies in 7 of 17 (41%) patients with lymphocytic hypophysitis, 6 of 30 (20%) with non-functioning pituitary macroadenoma, 4 of 17 (23.5%) with other autoimmune diseases, and 2 of 46 (4.3%) healthy controls. These results are similar to those reported with IB except for the high prevalence in patients with pituitary adenomata. In the ITT system, enolase antibodies appear quite non-specific.

The ITT assay has the potential to test multiple target autoantigens in high-throughput assays that are less labor intensive than IF or IB. However, expression of proteins in this system does not always guarantee that they are in their native conformation required for binding of patient sera *(221)*.

Autoantibodies to Pituitary Hormones

There are limited studies showing that pituitary hormones can be targets, analogous to insulin as a major target autoantigen in type 1 diabetes. In 1993, Mau et al. *(222)* demonstrated by IB, anti-ACTH and anti-GH antibodies in two of six patients with empty sella syndrome and anti-ACTH and anti-TSH antibodies in three of five patients with pituitary tumors. Six normal controls were negative. Positive antibodies did not correlate with hormonal function *(222)*. A significant subset of sera from patients with anorexia nervosa and bulimia nervosa contains antibodies against MSH and/or ACTH *(223)*. IB studies from Kikuchi et al. *(215)* and Takao et al. *(210)* identified a 22-kD protein as a target autoantigen, subsequently shown to be GH.

Animal Models

Experimental induction of cellular or humoral autoimmunity by sensitizing animals with autologous pituitary antigens is one of the major criteria for organ-specific autoimmunity. In 1967, Levine *(224)* successfully induced "allergic adenohypophysitis" by injecting rat pituitary tissue homogenate emulsified in complete Freund's adjuvant into 14 rats. Six animals (43%) developed focal or diffuse mononuclear cell infiltration of the pituitary within 2–3 weeks of a single injection. The addition of pertussis toxin as a second adjuvant increased disease incidence to 75%. The hypophysitis was more severe in a sub-group of pregnant and postpartum rats, analogous to the human condition. Subsequent experiments *(225)* showed guinea pig pituitary extracts were the most successful inducer of disease in the rat model, whereas human and bovine tissues were poor inducers. Similar experiments in rabbits gave similar results *(226)*. In 2001, Watanabe et al. *(227)* revisited Levine's experiment in female Lewis rats. Although no

severe lymphocytic infiltration of the adenohypophysis was seen, they identified GH, TSH, and luteinizing hormone (LH) as major autoantigens. These findings accord with the data from Japanese clinical studies *(210)*.

One of the most interesting experiments in this field was by Beck and Melvin in 1970 *(228)* who induced autoimmune hypophysitis in a female rhesus monkey using repeated exposure to human placental extracts and chorionic gonadotrophins over 3 years. No evidence was presented to show that the lympho-plasmacytic infiltrate had affected pituitary function. The pituitary and placenta share the expression of many molecules, including such autoantigens as NSE *(187)*. Whether this is relevant to the close association of lymphocytic hypophysitis and pregnancy or to Sheehan's syndrome and delayed hypopituitarism with pituitary antibodies *(118)* is intriguing but unclear.

Virally Induced Hypophysitis

Four animal models of virally induced pituitary autoimmunity have been reported. In the first model, mice infected with reovirus type 1 developed autoantibodies against anterior pituitary, islets of Langerhans' and gastric mucosa, as well as to hormones such as insulin and GH *(195)*. This model is consistent with IF data in newly diagnosed type 1 diabetes patients *(180)*. Yoon et al. *(229)* injected male golden Syrian hamsters with rubella virus E1 and E2 glycoproteins and detected pituitary cell autoantibodies by IF within 3 weeks in 95% of animals. All animals had diffuse inflammatory infiltrates in their pituitary glands, but by 8 weeks, only 20% of animals still had autoantibodies. Neonatal thymectomy almost completely prevented the disease, implying that it was T-cell mediated, but it could not be transferred by autoantibodies. T-cell transfer experiments were not conducted.

The other models were part of recent studies into gene therapy for pituitary disease. Adenovirus-mediated gene transfer studies in sheep, using direct stereotaxic injection, showed evidence of a severe inflammatory reaction with lymphocytic infiltration, venulitis, and periglandular fibrosis *(230)*. Expression of influenza nucleoprotein as a transgene under the control of the human GH locus-control region localized this virus to secretory vesicles in pituitary somatotrophs. Activation of monoclonal CD8 T cells specific to the viral protein resulted in spontaneous autoimmune hypophysitis of the pituitary gland targeting somatotrophs. In turn, this resulted in significantly reduced GH levels in adult mice and a dwarf phenotype *(231)*. In a follow-up study, these authors showed that antigen dose, T-cell precursor frequency, the degree of lymphopenia, and the context of target antigen expression are all important modulators of disease expression *(232)*. These studies highlight the potential problems with the use of therapeutic strategies based on vaccination against soluble pituitary proteins. However, they also support the theory that viral infections can trigger hypophysitis and that cases of viral meningoencephalitis preceding hypophysitis are not just coincidental occurrences but pathogenetic.

Treatment

Management of a patient with lymphocytic hypophysitis is dictated by the rapidity of onset, by the severity of symptoms and signs, and by the certainty of the clinical diagnosis. Even when hypophysitis is suspected preoperatively, it is not always possible to avoid surgery. The indications for surgical intervention include visual compromise

that cannot be rapidly improved with medical therapy, recurrent mass effects despite immunosuppression, and cases where the diagnosis of a pituitary adenoma or other tumor cannot be excluded *(14)*.

There is now a body of literature on the use of steroids as immunosuppressive agents in lymphocytic hypophysitis *(12,18)*. Prednisolone was first tried successfully in 1980 by Mayfield et al. *(27)*. There has been only one prospective trial of high-dose methylprednisolone therapy (120 mg/day for 2 weeks, then tapering doses over 1 month) *(68)*, where four of nine patients had some improvement in hormonal function and seven had a reduction in size of the mass on MRI scan. Methylprednisolone has been tried up to lymphocytotoxic doses of 1 g *(65,233)*. Dexamethasone has also been used *(11,234)* but can cause severe Cushingoid features *(142)*. Prolonged steroid use can lead to bilateral necrosis of the head of the femur *(59)*, among many other toxicities. Alternative treatments have been used in individual cases including low-dose stereotactic radiotherapy *(164,235)*, methotrexate *(22,60)*, and azathioprine *(64)*.

Conservative management is more likely to be successful in those cases that present in a sub-acute fashion, such as with hypopituitarism and a resolving pituitary mass. As spontaneous remission has been reported *(49,105)*, many endocrinologists and neurosurgeons now advocate a conservative approach, with or without a trial of immunosuppression. However, it should be stressed that a response to immunosuppressive (rather than replacement) doses of steroids does not necessarily confirm a diagnosis of lymphocytic hypophysitis. A number of conditions can be steroid responsive including dysgerminoma, neurosarcoidosis, Wegener's granulomatosis, and Langerhans' cell histiocytosis.

There is clearly a role for surgical intervention in some patients; however, aggressive resection of inflammatory tissue almost always results in permanent hypopituitarism *(14)*. There are rare cases where recurrence of the inflammatory mass has required a second surgery *(21,22,74,135,235,236)*. In these, surgery was very effective at relieving symptoms, particularly headache and visual field defects.

FUTURE DIRECTIONS

Future directions for lymphocytic hypophysitis research hinge on finding the relevant target autoantigens and identifying reliable autoantibody markers for the disease. Hopefully, these will give us new insights into the underlying autoimmune trigger(s) and mechanisms. New therapeutic approaches with "biologicals" are targeting these mechanisms in other diseases, such as rituximab therapy for thyroid-associated ophthalmopathy *(237)* and CD3 antibody therapy in new-onset type 1 diabetes *(238)*. It is therefore critical to establish the immunopathogenesis of lymphocytic hypophysitis to tailor any future immunotherapies.

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