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.

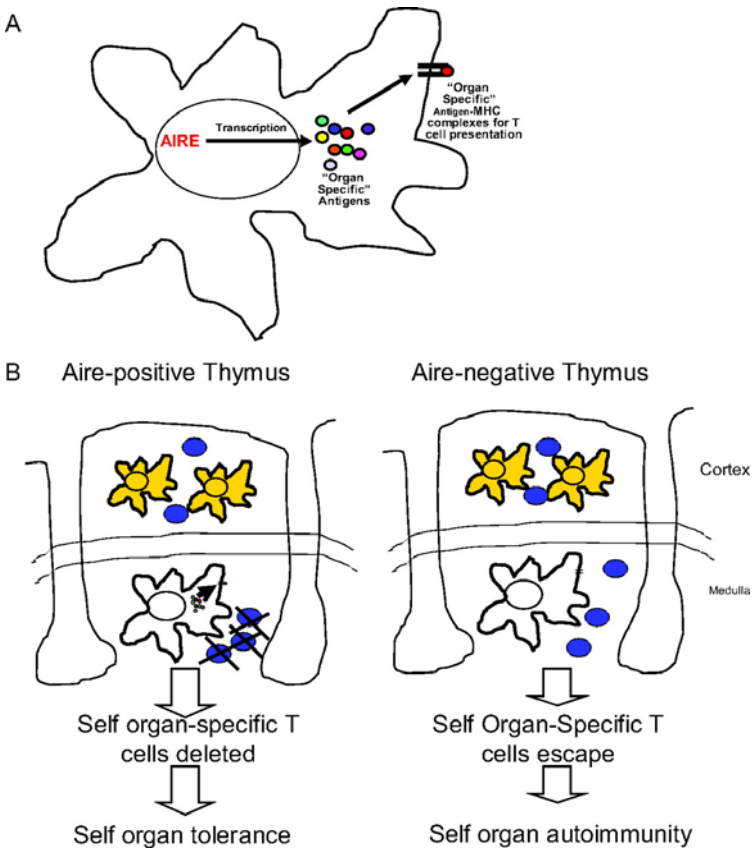

Figure 1. Model of the function of Aire in the thymus. **(A)** Aire appears to help mediate the transcription of many self-antigens in mTECs in the thymus. **(B)** Impact of Aire on T-cell selection. These self-antigens are then presented in the thymus to developing thymocytes (blue-colored cells) in 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)

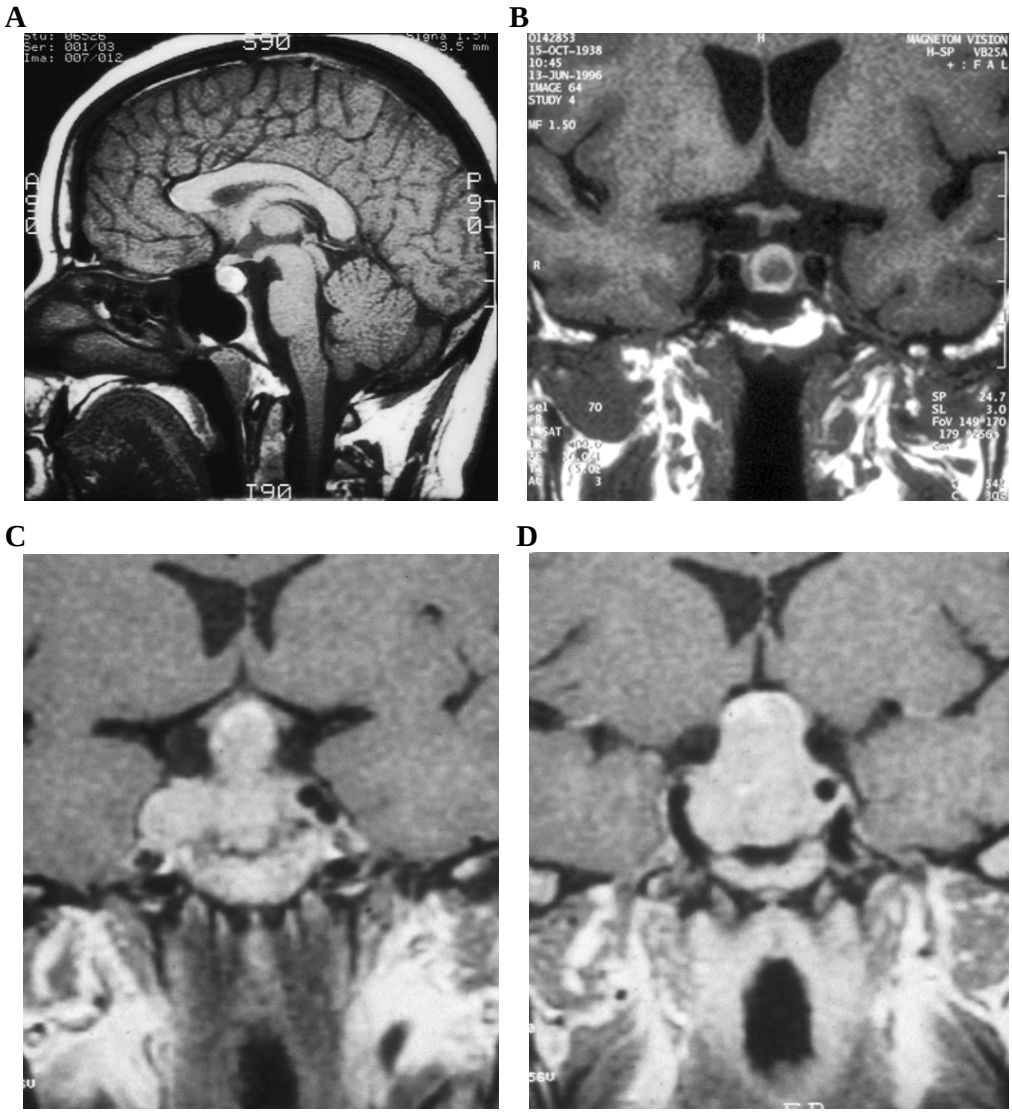

Figure 2. (A) Magnetic resonance imaging (MRI) scan of a classic case of lymphocytic hypophysitis in a 24-year-old woman who presented with symptoms of a pituitary tumor. Note the uniform enhancement with contrast with extension to the hypothalamus and **(B)** Coronal T1 weighted MRI scan of a 57-year-old 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.

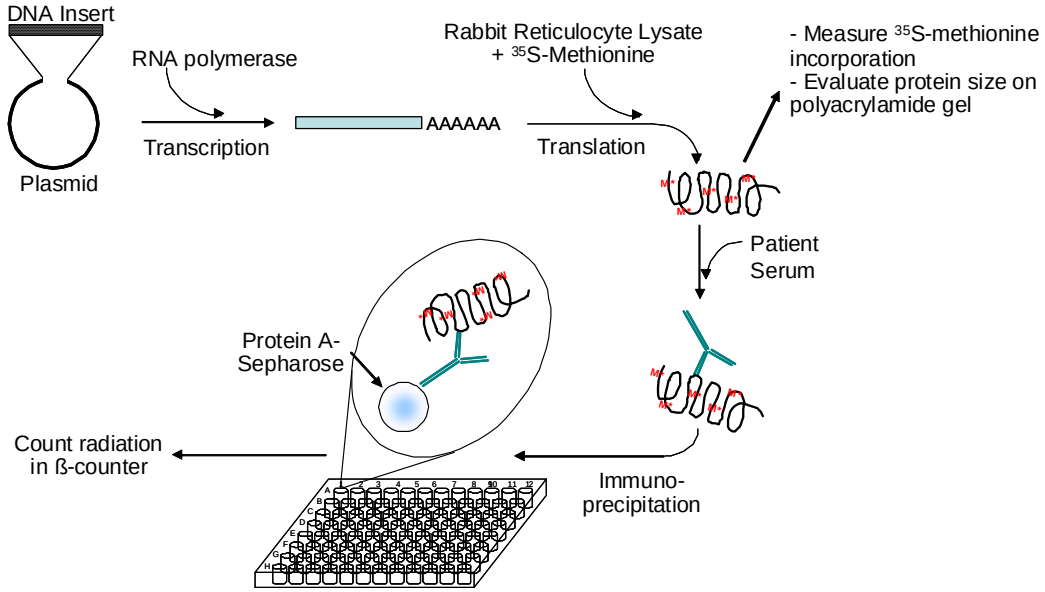

Figure 3: Schematic representation of the ITT assay (Crock *et al.* 2008) (213).

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).

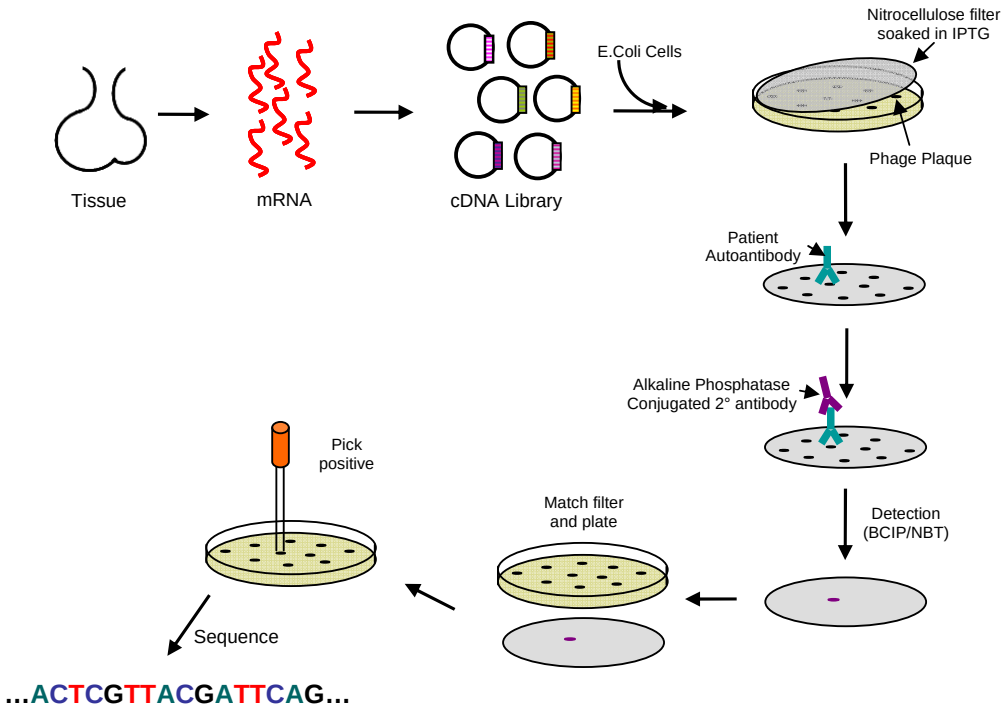

Figure 4: Schematic diagram of immunoscreening a cDNA expression library.

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.