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Lecture

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

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Patients and methods

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

Immunoblotting assay

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

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

Analysis

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

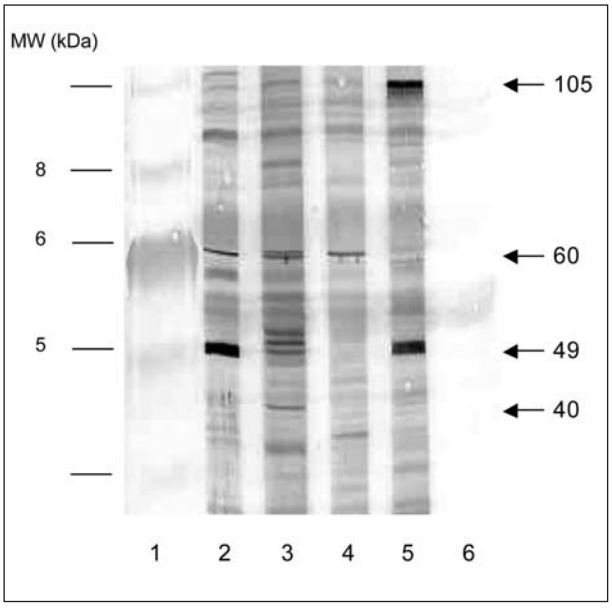

Figure 1

Results

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

Autoantibodies to human pituitary enolase were detected in 25 of the 67 APECED patients in the earliest (i.e. initial) sample of their series, compared to 5 of 68 controls (chi-square, 1df = 17.11, p<0.001; odds ratio (OR)=7.32). The youngest age at which this reactivity was seen was 5.6 years. Fourteen APECED patients had negative early samples but seroconversion was detected subsequently, as early as 10.3 years of age and as late as 53.2 years. With the exception of two patients, once autoantibodies were demonstrated in a patient, all subsequent samples in that patient remained positive (Figure 1). These latter two patients had only two samples each, at intervals of 6.9 and 15.3 years. Thus in total, 39 of the 67 APECED patients (58%) were positive for pituitary enolase autoantibodies (Table 1).

In an earlier study, 24 of our patients were examined for antibodies against candidal enolase. Nineteen of them were found to have a high titre of antibodies in their serum to recombinant candidal enolase expressed from *Candida albicans* (18). Studying the same sera we now found 12 of these 19 patients positive for human enolase antibodies, and seven negative (Table 2). While all these seven were positive for candidal enolase antibodies, only one of them had a high titre. Of the total of eight patients with such high titre candidal enolase antibodies, the other seven were also positive for autoantibodies to human enolase. Conversely, human enolase autoantibodies were detected in all the five of the 19 patients that were negative for the candidal enolase antibodies, or had only low titres.

Autoantibodies against other pituitary cytosolic proteins

Amongst the 67 APECED patients, 19 had no serum reactivity against human pituitary cytosolic proteins. Some of the sera identified several other pituitary target autoantigens, with 18 patients displaying reactivity to more than one pituitary cytosolic autoantigen. Although the molecular weights of these autoantigens are known, they have yet to be characterized further.

Two patients had antibodies directed against a 105 kDa pituitary cytosolic protein, and eight patients to a 60 kDa cytosolic protein (Table 1 and Figure 2). The youngest age at which reactivity against the 105

Table 1. Endocrine and non-endocrine components of APECED and serum pituitary autoantibodies

Age, years	0-5	5-10	10-15	15-20	20-30	30-40	40-50	Over 50	All
No. of endocrine components per patient (percentages)									
1 or more	33.8	77.9	92.1	95.6	98.5	100	100	100	100
2 or more	4.4	27.9	50.0	66.2	82.4	91.2	94.1	94.1	73.5
3 or more	1.5	5.8	23.5	38.2	45.6	50.0	54.4	55.9	57.4
None	66.2	22.1	8.8	4.4	1.5	0	0	0	0
Newly-Diagnosed Endocrine components									
Hypoparathyroidism	21	22	9	4	1	0	1	0	58 85.3%
Adrenal failure	5	21	17	4	5	1	1	0	54 79.4%
Diabetes mellitus	1	0	2	3	2	2	2	1	13 19.1%
Hypothyroidism	0	0	0	4	3	2	1	0	10 14.7%
Ovarian failure (of 36 females)	0	0	11	4	3	2	0	0	20 55.6%
Testicular failure (of 32 males)	0	0	1	1	0	2	0	0	4 12.5%
Growth hormone deficiency									3 4.4%
Newly-Diagnosed Non-endocrine components									
Alopecia	1	7	8	3	2	1	0	0	22 32.4%
Vitiligo	2	1	5	2	2	2	0	0	1 20.6%
Keratopathy	6	8	5	0	0	0	0	0	19 27.9%
Hepatitis	2	3	4	1	0	0	0	0	10 14.7%
Intestinal malabsorption	3	1	2	2	5	1	1	0	15 22.1%
Nephritis	0	0	0	1	1	1	0	0	3 4.4%
Iriti s	0	0	0	1	1	0	0	0	2 2.9%
Vasculitis	1	1	0	0	0	0	0	0	2 2.9%
Serum pituitary autoantibodies detected against human pituitary cytosolic proteins									
No. Patients tested in age group	2	13	24	30	38	24	10	3	67
Anti-human pituitary enolase	0	4	9	12	22	11	4	2	36
No. Patients positive	0	30.8	37.5	40.0	57.9	45.8	40.0	66.7	58
Percentages (%)									
40 kDa autoantigen (%)	0	7.7	4.2	6.7	10.5	12.5	40.0	33.3	14.7
45 kDa autoantigen (%)	0	7.7	8.3	10.0	18.4	12.5	10.0	33.3	16.2
60 kDa autoantigen (%)	0	0	8.3	10.0	7.9	8.3	20.0	33.3	11.8
105 kDa autoantigen (%)	0	0	4.2	0	5.3	4.2	0	0	2.9

kDa autoantigen was detected was 12.1 years, and for the 60 kDa pituitary cytosolic autoantigen, 13.7 years.

Autoantibodies to 40 kDa (Figure 2) and 45 kDa cytosolic proteins were detected in sera from 10 and

Table 2. Correlation of antibodies against two Enolases in the Finnish APECED cohort

Patient No	Age	Candida Enolase Antibodies	Human Pituitary Enolase Antibodies
1	16.5	-	-
	25.4	+	+
2	49.7	++	+
	51.6	++	+
	58.3	+++	+
3	21.1	+	+
4	27.1	+	-
	37.3	+	+
5	25.8	+	+
	29.4	+	+
6	34.3	+++	-
	44.2	++	-
7	41.7	-	-
	48.9	-	+
8	16.5	+	-
	30.7	+	-
9	23.6	-	-
	31.3	+	-
10	25.6	++	+
	35.4	+++	+
11	28.3	+	-
	41.5	-	-
12	24.0	-	+
	24.7	-	+
	34.7	-	+
13	12.3	-	+
	27.5	-	+
14	19.3	++	-
	24.3	+	+
	30.0	+	+
	32.6	++	+
15	23.4	-	+
	30.3	-	+
16	18.7	++	-
	33.9	+	+
17	57.4	+	-
18	22.9	+	-
19	17.8	++	+
20	32.2	+	-
21	9.0	-	+
22	8.8	++	+
23	12.5	+++	-
	15.7	+	-
	24.7	+	+
24	30.0	-	-
	31.9	-	+
	35.2	+	+
	42.7	-	+

11 patients, respectively. The youngest ages at detection were 8.6 and 5.5 years, respectively. Four control

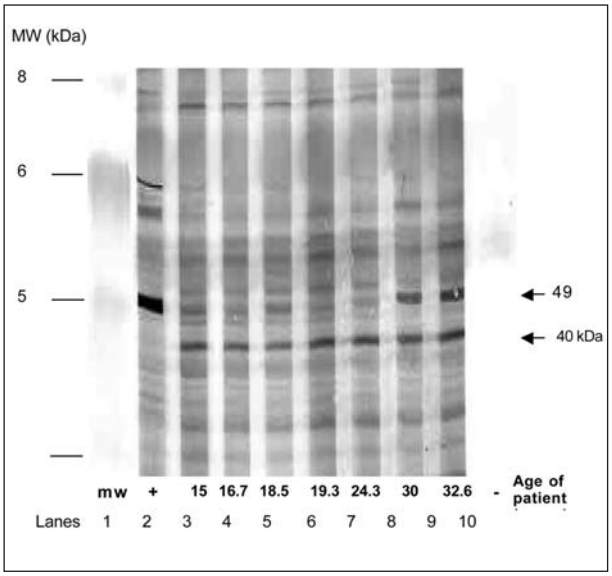

Figure 2

subjects had low titre antibodies directed against the 40 kDa cytosolic pituitary protein. No anti-45 kDa antibodies were found in control subjects.

Discussion

The detection of a range of pituitary autoantibodies in the sera of many APECED patients indicates that the pituitary can be part of the multi-organ involvement in this disease. The most remarkable feature of this study is the length of follow-up of the cohort over 26 years by one clinician. This has enabled us to demonstrate seroconversion in consecutive samples and the surprising stability of positive pituitary autoantibody status once achieved. Circulating organ-specific antibodies in APECED are highly predictive of adrenal and ovarian failure, but not of insulin dependent diabetes nor hypothyroidism (2). Although pituitary reactivity was relatively common in our series, hypopituitarism was very uncommon. The implication is that these autoantibodies are rarely pathogenic and may be epiphenomena. Immunoblotting is known to detect multiple bands of autoantibody reactivity, but some may be low titre "natural antibodies" and others "true" autoreactivity. We have characterized the 49 kDa cytosolic protein (discussed below) but

until the other relevant target autoantigens are further identified, it would be speculative to comment on their significance.

The predominant immunoreactivity was against a 49 kDa cytosolic protein. We have reported the association of 49 kDa pituitary cytosolic autoantibodies in 70% of cases of biopsy proven lymphocytic hypophysitis, to a lesser extent in other endocrinopathies (17), and in 28% patients with idiopathic hypopituitarism and 28% of their relatives (19). We have since identified the 49 kDa protein as enolase (20), a ubiquitous glycolytic enzyme that is highly conserved in evolution, with yeast enolase sharing 65% homology with human enolase (21). In the human, enolase exists as a homodimer or heterodimer of three isoforms, α-, β- and γ-enolase. We have recently shown by two-dimensional gel electrophoresis that autoimmune sera from patients with lymphocytic hypophysitis recognise both αα-and γγ-enolase in the human pituitary (22). γγ-enolase is neuron and neuroendocrine cell specific but shares 80% homology with the ubiquitous αα-enolase. It is likely that there is significant cross reactivity in patient sera between these forms and also potentially candidal enolase.

A number of the APECED patients in the current study were also involved in a research study looking at their antibody responses to *Candida albicans* infections, which are almost universal in this syndrome. Patient sera were used to immunoscreen a candidal cDNA expression library (18). Four cDNA clones were identified and found to be enolase, heat shock protein 90, pyruvate kinase and alcohol dehydrogenase. The reactivity to these antigens was studied by immunoprecipitation assays with in vitro-transcribed and translated proteins. Several cDNA clones encoding enolase were expressed in *E. coli* and studied by immunoblotting, with 84% of sera reacting with the recombinant candidal enolase. These results indicate that candidal enolase is a major antigen in APECED patients. It is possible that reactivity to human pituitary enolase is an epiphenomenon related to mucosal candidiasis, yet not all patients with candida antibodies had pituitary antibodies and vice versa.

However, the intriguing question is whether this cross-reactivity could represent a possible mechanism for autoimmunity in APECED patients through molecular mimicry. Some evidence already exists that antibodies directed against Candida can cross-react with mammalian tissues (23). Patients with thyroid, ovarian and adrenal, tissue-autoantibodies, exhibited significantly higher levels of Candida antibodies (60%), compared with tissue antibody negative sera (7.5%) and sera from healthy controls (10%). When sera containing high levels of Candida antibodies were pre-absorbed with tissue antigens, a 10-15% reduction in antibody titres was noted. Similarly, pre-absorption of thyroid antibody-positive sera with *C. Albicans* caused a reduction in thyroid antibody levels (23). The current study may offer a further demonstration of immunological cross reactivity between Candida and human endocrine tissues. Ultimately, further studies could indicate a possible pathogenic role of *Candida Albicans* in the development of autoimmune diseases in the genetically susceptible individuals affected by APECED.

The detection of pituitary autoantibodies, in patients with APECED suggests that the pituitary may be part of the multiorgan involvement and/or it shares antigens or cross-reactive epitopes with other target tissues in this disease. The one well characterised autoantigen so far is neuron specific enolase but it is not organ-specific, even if it is a marker of neuroendocrine autoimmunity. A possible link with candidal enolase is intriguing. The clinical significance of these autoantibodies in APECED remains to be determined as hypopituitarism is uncommon. Although pituitary autoimmune disease could develop over time in autoantibody-positive patients, the 26 year follow-up in our study makes this quite unlikely.

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