CLINICAL STUDY

Autoantibodies to human tryptophan hydroxylase and aromatic L-amino acid decarboxylase

Chiara Dal Pra^{1,2}, Shu Chen¹, Corrado Betterle³, Renato Zanchetta³, Vivienne McGrath¹, Jadwiga Furmaniak^{1,2} and Bernard Rees Smith^{1,2}

¹FIRS Laboratories, RSR Ltd, Parc Ty Glas, Llanishen, Cardiff CF14 5DU, UK, ²Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK and ³Department of Medical and Surgical Sciences, University of Padua, Via Ospedale, Civile 105, 35128 Padua, Italy

(Correspondence should be addressed to J Furmaniak; Email: firs@rsrltd.eclipse.co.uk)

(C Dal Pra is now at Department of Medical and Surgical Sciences, University of Padua, Via Ospedale, Civile 105, 35128 Padua, Italy)

Abstract

Objective: To assess the prevalence of autoantibodies (Abs) to tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase (AADC) in patients with different autoimmune diseases and to analyse their respective epitopes.

Design: TPH and AADC Abs were measured in an immunoprecipitation assay using ³⁵S-labelled full-length and fragments of TPH and AADC.

Methods: Patients with different autoimmune adrenal diseases ($n = 84$), non-adrenal autoimmune diseases ($n = 37$), idiopathic vitiligo ($n = 8$) and 56 healthy blood donors were studied.

Results: Fourteen of twenty-three (61%) of patients with autoimmune polyglandular syndrome (APS) type I and 1/34 (3%) of patients with isolated Addison's disease (AD) were positive for TPH Abs. None of the patients with APS type II ($n = 27$), coeliac disease ($n = 10$), autoimmune thyroid disease (AITD) ($n = 11$), type 1 diabetes mellitus (DM) ($n = 16$) or idiopathic vitiligo ($n = 8$) was positive for TPH Abs. AADC Abs were detected in 12/23 (52%) patients with APS type I, in 1/29 (3%) patients with APS type II and 1/34 (3%) patients with isolated AD. None of the patients with coeliac disease, type 1 DM, AITD or idiopathic vitiligo was positive for AADC Abs. TPH Abs were found to interact with the C-terminal amino acids (aa) 308–423, central aa 164–205 and N-terminal aa 1–105 of the TPH molecule. AADC Ab binding epitopes were within the C-terminal aa 382–483, the central aa 243–381 and the N-terminal aa 1–167.

Conclusions: Our study suggests that TPH Abs and AADC Abs react with several different epitopes and that different epitopes are recognized by different sera. The prevalence of TPH Abs and AADC Abs in patients with APS type I in our study is in agreement with previous reports. TPH Abs and AADC Abs were found very rarely in patients with other forms of autoimmune adrenal disease and were not detected in patients with non-adrenal autoimmune diseases.

European Journal of Endocrinology 150 313–321

Introduction

Autoimmune adrenal disease can present as isolated Addison's disease (AD) or as a component of autoimmune polyendocrine syndrome (APS) type I, type II or type IV (1). In addition, some patients may have serological markers of adrenal autoimmunity in the absence of clinical disease and these cases represent potential or subclinical AD (1). APS type I is an autosomal recessive disease and various mutations of the autoimmune regulator (AIRE) gene on chromosome 21q22.3 appear to be associated with this syndrome (2, 3). APS type I presents with at least two out of the three main component diseases of APS type I, i.e. AD, hypoparathyroidism and chronic mucocutaneous

candidiasis. In addition, patients with APS type I often develop one or more of the following diseases: hypogonadotropic hypogonadism, autoimmune thyroid disease (AITD), type 1 diabetes mellitus (DM), alopecia, vitiligo, chronic hepatitis, chronic atrophic gastritis, pernicious anaemia, malabsorption, keratoconjunctivitis, neoplasias and nail dystrophy. Consequently, various organ-specific and/or organ-non-specific autoantibodies (Abs) are detected in sera from patients with APS type I (1). Recently, the relationship between Abs to tryptophan hydroxylase (TPH) with intestinal dysfunction and Abs to aromatic L-amino acid decarboxylase (AADC) with autoimmune hepatitis and vitiligo in patients with APS type I have been reported (4–7). However, the prevalence of TPH Abs and AADC Abs in the patients

with other forms of AD has not been studied in detail. Consequently we have studied the prevalence of TPH Abs and AADC Abs in the patients with different forms of autoimmune AD and assessed which epitope regions on TPH and AADC are recognised by Abs.

Materials and methods

Patients

Serum samples were obtained from 23 patients with APS type I (13 females and 10 males; mean age 25 years; range 3–64 years), 29 patients with APS type II (23 females and 6 males; mean age 39 years; range 19–64 years) and 34 patients with isolated AD (18 females and 16 males; mean age 35 years; range 6–78 years). Sera from patients with non-adrenal autoimmune diseases were also used in the study; 16 sera from patients with type 1 DM all positive for glutamic acid decarboxylase (GAD) Abs (12 females and 4 males; mean age 16 years; range 3–73 years), 11 sera from patients with AITD all positive for thyroid peroxidase Abs and/or thyroglobulin Abs and/or thyrotrophin receptor Abs (10 females and 1 male; mean age 40 years; range 23–61 years) and 10 sera positive for tissue transglutaminase (tTG) Abs from patients with coeliac disease (7 females and 3 males; mean age 40 years; range 15–78 years). In addition, sera from 8 patients with idiopathic vitiligo and 56 healthy blood donors were used in the study. Patients gave informed consent for the study and healthy blood donor sera were purchased from Golden West Biologicals, Vista, CA, USA.

Disease diagnosis in each case was based on typical clinical, immunological and biochemical grounds (1). Patients with APS type I and type II were classified using the criteria for APS as described elsewhere (1). Adrenal cortex Abs (ACA) were detected by the classical indirect immunofluorescence technique using thin cryostatic sections of normal human adrenal glands, and steroid-producing cell Abs (StCA) were detected by a complement-fixing indirect immunofluorescence technique using normal human testis as described previously (8, 9). Autoantibodies to steroid 21-hydroxylase (21-OH) were measured using a diagnostic kit based on ¹²⁵I-labelled recombinant human 21-OH (RSR Ltd,

Cardiff, UK) (10). Autoantibodies to steroid 17 α -hydroxylase (17 α -OH) and to p450 cytochrome side chain cleavage enzyme (p450_{scc}) were detected using an immunoprecipitation assay (IPA) based on ³⁵S-labelled human autoantigens produced in an *in vitro* TnT rabbit reticulocyte system (Promega UK Ltd, Southampton, Hants, UK) as described previously (11). All other autoantibodies were detected in patient sera using diagnostic kits from RSR Ltd. The disease specificity and sensitivity of the above assays have been described in detail previously (1, 10–17).

Preparation of plasmids containing TPH and AADC sequences

The cDNAs that encoded TPH and AADC (cloned into the psp64-polyA vector) (5, 6) were kindly provided by Dr O Kämpe (Uppsala, Sweden).

Full-length TPH cDNA was subcloned into the BamHI site of pTZ18F as described previously (18) and full-length AADC cDNA was subcloned into the XbaI/BamHI site of the same plasmid. Various deletions of the TPH cDNA (from the C terminus) and various C-terminal deletions of the AADC cDNA were generated using naturally occurring restriction enzyme sites as shown in Table 1.

Analysis of reactivity of TPH Abs or AADC Abs with full-length and modified TPH or AADC proteins

Full-length and modified ³⁵S-labelled TPH or AADC proteins (Table 1) were produced in the *in vitro* TnT system (Promega) incorporating ³⁵S-methionine (Amersham Biosciences, Chalfont St Giles, Bucks, UK) as described before (19). Proteins synthesised from different constructs were analysed on 12% SDS-PAGE (20) followed by autoradiography using Fuji medical X-ray film (Genetic Research Instrumentation Ltd, Braintree, Essex, UK).

Binding of TPH Abs or AADC Abs to ³⁵S-labelled full-length and modified respective proteins were assessed using IPAs carried out in multiwell filter plates (Millipore (UK) Ltd, Watford, UK) as described previously (11, 19). Briefly, 50 μ l serum diluted 1:10 in assay buffer (200 mM NaCl, 150 mM Tris, pH 8.3,

Table 1 Summary of modifications of TPH and AADC proteins.

TPH			AADC		
Restriction sites used	Amino acids deleted	Mol. wt (kDa) of expressed protein	Restriction sites used	Amino acids deleted	Mol. wt (kDa) of expressed protein
None	None	51	None	None	54
PstI	424–444	48.7	BbrpI	382–483	42
BstXI	308–444	35.8	BluI	243–483	26.5
EcoRV	206–444	24	PstI	168–483	18
EcoRI	165–444	18.9			
StyI	106–444	12			

10 mg/ml bovine serum albumin, 0.5 mg/ml NaN_3 and 1% Tween 20) was incubated in duplicate with 50 μl ^{35}S -labelled protein diluted in assay buffer (30 000 dpm) for 2 h at room temperature followed by 1 h of incubation at room temperature with 50 μl Protein A-Sepharose (diluted 1:10 in assay buffer; Amersham Biosciences). The mixtures were then washed by filtration and the ^{35}S -labelled immune complexes bound to Protein A-Sepharose were counted in a scintillation β counter.

In the case of TPH Ab and AADC Ab IPAs, the Ab-positive reference was a serum from a APS type I patient with high levels of TPH Abs or AADC Abs. The negative reference was serum from an individual healthy blood donor. Results are expressed as antibody index as described (19) using the formula:

$$\left[\frac{(\text{dpm in the presence of test serum} - \text{d.p.m. in the presence of negative control})}{(\text{dpm in the presence of positive control} - \text{d.p.m. in the presence of negative control})} \right] \times 100$$

Autoantibody binding to each modified protein was assessed in two separate experiments (each in duplicate). Results are expressed as % Ab binding to modified protein relative to the binding to full-length protein using the formula:

$$\left[\frac{(\text{binding of test sample to modified } ^{35}\text{S-TPH or } ^{35}\text{S-AADC})}{(\text{binding of test sample to full length } ^{35}\text{S-TPH (or } ^{35}\text{S-AADC)})} \right] \times 100$$

The difference between mean binding of test samples to the modified protein relative to the full-length protein was assessed using Wilcoxon's matched pairs test. In addition, the difference in mean binding of test samples to different modified proteins was compared using Wilcoxon's matched pairs test.

Results

Expression of full-length and modified TPH or AADC in the TnT system

Full-length and modified recombinant TPH or AADC proteins produced in the TnT system were analysed by SDS-PAGE and autoradiography (data not shown). In the case of TPH proteins, ^{35}S -labelled products of the expected molecular weights (from 51 kDa to 12 kDa) were obtained from the respective constructs (Table 1). In the case of AADC proteins, ^{35}S -labelled products of the expected molecular weights (between 54 kDa and 18 kDa) were also obtained (Table 1).

Immunoprecipitation assays

APS type I sera with high levels of TPH Abs immunoprecipitated up to about 35% of the ^{35}S -labelled full-length TPH preparation. Sera from healthy blood

donors precipitated $0.73 \pm 0.24\%$ of this ^{35}S -labelled TPH (mean $\pm$ s.d.; range 0.5–1.8%; $n = 56$). Interassay coefficient of variation (C.V.) for a sample with high levels of TPH Ab ($31.5 \pm 1.3\%$ of ^{35}S -TPH binding; mean $\pm$ s.d.) was 4.2% ($n = 5$) and for a sample with medium TPH Ab level ($19.1 \pm 0.9\%$ binding; mean $\pm$ s.d.) it was 4.8% ($n = 5$). The intra-assay C.V. for a sample with high levels of TPH Ab ($30.4 \pm 1.1\%$ binding; mean $\pm$ s.d.) was 3.6% ($n = 10$) and for a sample with medium levels of TPH Ab ($18.2 \pm 1.0\%$ binding; mean $\pm$ s.d.) it was 5.7% ($n = 10$). The limit of detection in the ^{35}S -labelled TPH IPA was estimated as an index value of 2.3 based on the mean + 3 s.d. of the TPH Ab indexes obtained from individual healthy blood donor sera ($n = 56$) (Fig. 1A). 14/23 (61%) of patients with APS type I were positive for TPH Abs (index values from 8.0 to 100), none of 27 APS type II patients tested was positive and 1/34 (3%) of patients with isolated AD was positive for TPH Abs (low index value of 4.0). None of 10 patients with coeliac disease, none of 16 patients with type 1 DM, none of 11 patients with AITD and none of 8 patients with idiopathic vitiligo was positive for TPH Abs (Fig. 1A).

APS type I sera with high levels of AADC Abs immunoprecipitated up to about 60% of the ^{35}S -labelled full-length AADC preparation. Sera from healthy blood donors precipitated $2.9 \pm 0.5\%$ of the ^{35}S -labelled AADC (mean $\pm$ s.d.; range 2.1–3.9%; $n = 56$). The inter-assay C.V. for a sample with high levels of AADC Abs ($53.0 \pm 5.2\%$ of ^{35}S -labelled AADC binding; mean $\pm$ s.d.) was 9.8% ($n = 5$) and a C.V. of 15.9% ($n = 5$) was found for a sample with medium level of Abs ($26.3 \pm 4.2\%$ binding; mean $\pm$ s.d.). The intra-assay C.V. for a sample with high level of Abs ($56.6 \pm 3.8\%$ binding; mean $\pm$ s.d.) was 6.7% ($n = 7$) and 4.3% ($n = 8$) for a sample with medium levels of Abs ($27.3 \pm 1.2\%$ binding; mean $\pm$ s.d.). The limit of detection in the ^{35}S -labelled AADC IPA was estimated as an index value of 2.7 based on the mean + 3 s.d. of the AADC antibody indexes obtained from 56 individual healthy blood donor sera (Fig. 1B). 12/23 (52%) of patients with APS type I were positive for AADC Abs (index value from 2.8 to 125), 1/29 (3%) APS type II patients was positive for AADC Abs (index of 10.1) and 1/34 (3%) of patients with isolated AD was positive for AADC Abs (index of 9.3). However, none of the 10 patients with coeliac disease, 16 patients with type 1 DM, 11 patients with AITD or 8 patients with idiopathic vitiligo was positive for AADC Abs (Fig. 1B).

Detailed clinical and autoantibody information on the patients with APS type I, APS type II and isolated AD who were found positive for TPH Abs and/or AADC Abs are shown in Table 2. Eleven patients (10 with APS type I and 1 with AD) were positive for both TPH Abs and AADC Abs, 4 patients (all with APS type I) were positive for TPH Abs only and 3 patients (2 with APS type I and 1 with APS type II) for AADC Abs only.

Figure 1 Autoantibodies to (A) TPH and (B) AADC by IPAs in different patient groups. The broken lines show the cut-off points for positivity. This was determined as the mean+3 s.d. of the index values obtained with the 56 healthy blood donors (HBD) sera.

Table 2 Clinical and Ab characteristics of patients with APS I, APS II and isolated AD positive for TPH and/or AADC Abs.

Patient no.	Age (years)	Sex	Disease	TPH Ab ¹ index	AADC Ab ² index	21-OH Ab ³ (units/ml)	ACA	17α-OH Ab ⁴ (units/ml)	P450scc Ab ⁵ (units/ml)	StCA
1	8	M	APS type I; AD, C, HPT, RhA	64.2	53	92	+	Neg	8.9	Neg
2	31	F	APS type I; AD, C, HPT, POF, A, HT	34.6	57	1.7	+	> 64	> 32	+
3	28	F	APS type I; AD, C, HPT, POF, A, AG, PA, Ch	57.7	5	Neg	+	> 64	4	+
4	47	F	APS type I; AD, C, HPT, POF, PA, Sj, Ca, PN	29.3	Neg	268.3	+	Neg	19.9	+
5	10	M	APS type I; AD, C, HPT, A, V, Ma	63.3	23	1.5	+	Neg	4	+
6	15	M	APS type I; AD, C, HPT, A, V, Ma, HT	100	97.1	Neg	Neg	Neg	Neg	Neg
7	36	F	APS type I; AD, C, HPT, CD, POF, As	20.3	Neg	32.3	+	> 64	20	+
8	5	M	APS type I; AD, C, HPT, A, HT, Ma	83.2	5	51	+	Neg	> 32	+
9	9	M	APS type I; C, HPT, Ma, A, DM	74	Neg	61.3	+	6	Neg	+
10	33	M	APS type I; AD, C, HPT, V, Ma	83.8	76	2.5	+	> 64	Neg	+
11	12	F	APS type I; AD, C, HPT, HT, Sj	63.4	109	44.5	+	12.9	Neg	+
12	5	F	APS type I; AD, HPT	65.4	124.6	6.4	+	Neg	Neg	Neg
13	20	F	APS type I; AD, HPT, C	8.0	3.4	7.5	+	29.6	21	+
14	20	F	APS type I; AD, HPT	32.2	Neg	20.1	+	> 64	> 32	+
15	47	F	APS type I; AD, HPT, C, A, Ca, Sj, TS	Neg	100	Neg	Neg	Neg	Neg	Neg
16	8	F	APS type I; AD, HPT	Neg	2.8	36.5	+	Neg	4.6	+
17	32	M	AD, A	4.0	9.3	1710	+	Neg	Neg	Neg
18	44	F	APS type II; AD, GD	Neg	10.1	45.8	+	Neg	2.4	Neg

¹Index values >2.3 are positive; ²index values >2.7 are positive; ³values >1.0 units/ml are positive; ⁴values >1.0 units/ml are positive; ⁵values >1.0 units/ml are positive.

A, alopecia; AG, atrophic gastritis; AS, asplasia; C, candidiasis; Ca, cancer; Ch, cholelithiasis; CD, coeliac disease; GD, Graves' disease; HPT, hypoparathyroidism; HT, hepatitis; Ma, malabsorption; PA, pernicious anaemia; PN, peripheral neuropathy; POF, premature ovarian failure; RhA, rheumatoid arthritis; Sj, Sjogren's syndrome; TS, Turner's syndrome; V, vitiligo; Neg, negative.

In addition, 19/23 (83%) APS type I patients were positive for 21-OH Abs and 15/23 (65%) were positive for 17 α -OH Abs and/or p450scc Abs (Table 2). Furthermore, 5/23 (22%) were positive for tTG Abs and 9/23 (40%) were positive for GAD Abs (data not shown).

Antibody binding to modified TPH

Binding of TPH Abs from 11 different sera to full-length and modified TPH proteins is shown in Table 3. When the last C-terminal 21 amino acids were deleted (i.e. aa 1–423) there was little or no effect on mean TPH Ab binding relative to full-length TPH ($P = 0.168$). In contrast, more extensive C-terminal deletions reduced TPH Ab binding in all sera studied. In particular, TPH truncation at aa 307 (i.e. aa 1–307 expressed) resulted in reduction in TPH Ab binding relative to full-length (mean $48 \pm 28\%$; range 4–71%; $P < 0.01$). In the case of TPH with aa 1–205 expressed, the mean binding of TPH Abs was $52 \pm 31\%$ (range 4–79%) relative to full-length ($P < 0.01$). More extensive C-terminal deletions (i.e. aa expressed 1–164) resulted

in further changes in TPH Ab binding with mean binding relative to full-length of $37 \pm 22\%$ (range 3–68%) ($P < 0.01$). Deletion of a further 59 amino acids (aa 1–105 expressed) did not cause further changes in TPH Ab binding (mean binding $38 \pm 21\%$; range 3–63%; $P < 0.01$ relative to full-length) (Table 3). Sera ($n = 2$) from patients with APS type I that were negative for TPH Abs in IPA using the full-length ³⁵S-labelled TPH did not immunoprecipitate any of the modified ³⁵S-labelled TPH proteins (data not shown).

In terms of the effect of different TPH amino acid deletions on TPH Ab binding relative to each other, truncation at aa 307 had a marked effect compared with truncation at aa 423 (aa 1–423 expressed vs aa 1–307 expressed $P < 0.01$) and a further deletion of 87 amino acids had an effect compared with truncation at aa 307 (aa 1–307 expressed vs aa 1–205 expressed $P < 0.01$). When TPH with aa 1–164 expressed was studied, the TPH Ab binding was reduced even further (aa 1–205 expressed vs aa 1–164 expressed $P = 0.01$) and this effect was not changed when a further 59 amino acids were deleted (aa 1–164 expressed vs aa 1–105 expressed $P = 0.720$) (Table 3).

Table 3 Binding of TPH Ab in 11 APS type I sera to intact and modified TPH.

		Binding of serum to modified ³⁵ S-TPH Binding of serum to full-length ³⁵ S-TPH × 100					
Serum no.	Relative TPH Ab level in IPA using full-length ³⁵ S-TPH ¹	Full length (1–444)	C-terminal deletions				
			1–423 expressed	1–307 expressed	1–205 expressed	1–164 expressed	1–105 expressed
1	100	100	100	63	69	49	47
2	68	100	87	4	4	3	3
3	78	100	111	68	75	68	63
4	63	100	91	6	5	5	8
5	86	100	91	72	79	52	58
6	114	100	95	71	75	52	47
7	49	100	99	30	33	26	35
8	97	100	105	66	72	48	52
9	13	100	80	14	16	10	14
10	117	100	101	71	79	49	44
11	107	100	96	65	69	50	49
Mean		100	96	48	52	37	38
S.D.		0	9	28	31	22	21
P ²			P = 0.168	P < 0.01	P < 0.01	P < 0.01	P < 0.01
P ³						P = 0.720	
P ³					P = 0.01		
P ³				P < 0.01			
P ³			P < 0.01				

¹ 23–39% of ³⁵S-full-length TPH bound to serum 1 in these experiments and the results shown for sera 2 to 11 are relative to serum 1 binding; healthy blood donor sera bound 0.7–1.8% of ³⁵S-full-length TPH.

² P values shown are for the difference between TPH Ab binding to full-length and modified ³⁵S-TPH.

³ P values shown are for the difference between TPH Ab binding to differently modified ³⁵S-TPH.

Autoantibody binding to modified AADC

AADC Ab binding to full-length and modified AADC proteins in 10 patients studied is shown in Table 4. When the C-terminal 102 amino acids were deleted (aa 1–381 expressed) there was no effect on mean

AADC Ab binding relative to full-length (mean 84 ± 27%; range 30–110; P = 0.176). However, the effect on individual sera varied, in the case of three sera the ability to bind AADC with the C-terminal 102 amino acids missing was reduced (range 30–55% relative to full-length) whereas in the case

Table 4 Binding of AADC Ab in ten APS type I sera to intact and modified AADC.

Serum no.	Relative AADC Ab level in IPA using full-length ³⁵ S-AADC ¹	Binding of serum to modified ³⁵ S-AADC Binding of serum to full-length ³⁵ S-AADC × 100			
		C-terminal deletions			
		Full length (1–483)	1–381 expressed	1–242 expressed	1–167 expressed
1	100	100	100	61	9
2	99	100	104	74	20
3	65	100	30	4	10
4	96	100	104	36	20
5	66	100	55	4	6
6	77	100	58	4	14
7	104	100	100	50	23
8	80	100	100	13	5
9	72	100	110	38	6
10	14	100	83	13	30
Mean		100	84	30	14
S.D.		0	27	26	8
P ²			P = 0.176	P < 0.01	P < 0.01
P ³				P = 0.139	
P ³			P < 0.01		

¹ 45–46% of ³⁵S-full-length AADC bound to serum 1 in these experiments and the results shown for sera 2 to 10 are relative to serum 1 binding; healthy blood donor sera bound 2.5–2.6% of ³⁵S-full-length AADC.

² P values shown are for the difference between AADC Ab binding to full-length and modified ³⁵S-AADC.

³ P values shown are for the difference between AADC Ab binding to differently modified ³⁵S-AADC.

of seven sera the binding was affected little or not at all (range 83–110%). In contrast, truncation at aa 242 (aa 1–242 expressed) had a marked effect on mean AADC Ab binding ($30 \pm 26\%$; range 4–74%; $P < 0.01$ compared with full-length). Further C-terminal amino acid deletions (i.e. aa 1–167 expressed) had a clear effect on AADC Ab binding in all sera studied with a mean binding relative to full-length of $14 \pm 8\%$; range 5–30%; $P < 0.01$. Sera ($n = 2$) from patients with APS type I that were negative for AADC Abs in IPA using the full-length ^{35}S -labelled AADC did not immunoprecipitate any of the modified ^{35}S -labelled AADC proteins (data not shown).

In terms of the effect of different modifications in relation to each other, truncation at aa 242 had a marked effect on AADC Ab binding compared with truncation at aa 381 (aa 1–382 expressed vs aa 1–242 expressed $P < 0.01$) but a further deletion (aa 1–167 expressed) had no effect on AADC Ab binding compared with truncation at aa 242 (aa 1–242 expressed vs aa 1–167 expressed $P = 0.139$).

Discussion

In our study, TPH Abs and AADC Abs were found in a majority of patients with APS type I (61 and 52% respectively). In contrast, only 1/29 patients (3%) with APS type II was positive for AADC Abs and 1/34 patients (3%) with isolated AD was positive for both TPH and AADC Abs. Previous studies have reported that 38/80 (48%) of APS type I patients were positive for TPH Abs (6) and 35/69 (51%) of APS type I patients positive for AADC Abs (5).

The prevalence of malabsorption among the patients with APS type I has been reported to vary from 6 to 22% (1). In our study, 5 out of 23 (22%) APS type I patients presented with malabsorption and all 5 patients were positive for TPH Abs (Table 2). However, 9/18 (50%) APS type I patients without malabsorption were also positive for TPH Abs. This can be compared with a previous study in which 17/19 (89%) APS type I patients with gastrointestinal symptoms were positive for TPH Abs as well as 21/61 (34%) APS type I patients without gastrointestinal symptoms (6).

Vitiligo and chronic active hepatitis in APS type I have been reported to occur in 8–25% and 5–31% of patients respectively (1). Of the APS type I patients with vitiligo and/or chronic active hepatitis, 6/8 (75%) were positive for AADC Abs in our study. However, 6/15 (40%) APS type I patients without vitiligo and/or chronic active hepatitis were also positive for AADC Abs. Husebye *et al.* (5) have reported that 11/12 (92%) APS type I patients with hepatitis were positive for AADC Abs; however, 24/57 (42%) of their patients without hepatitis were also positive for AADC Abs. In the same study, 12/15 (80%) APS type I patients with vitiligo had AADC Abs compared with 23/54 (43%) of APS type I patients without vitiligo

(5). None of the 8 patients with idiopathic vitiligo we have studied was positive for AADC Abs.

Our studies and those of others (6) have shown that TPH Abs are detected in a higher proportion of patients with malabsorption but are also present in those without malabsorption. In addition, the prevalence of AADC Abs is higher in patients with vitiligo and/or hepatitis but also a considerable proportion (about 40%) of patients without these diseases are AADC Ab positive (5). These observations suggest that the disease specificity of TPH Abs for malabsorption in APS type I and disease specificity of AADC Abs for vitiligo and/or hepatitis requires further investigation.

In our study, a large proportion of APS type I patients were positive for Abs to 21-OH (83%), 17 α -OH and/or p450sc (65%) (Table 2). Furthermore, 40% of patients had detectable GAD Abs but did not have type 1 DM. In addition, some patients ($n = 5$) were positive for IgA tTG Abs (22%) (16) but none had coeliac disease. Three of these patients had normal duodenal biopsy results while 2 other patients did not undergo the biopsy.

Thus, the majority of APS type I patients had more than four different autoantibodies. This is consistent with the well-known presence of multiple autoantibodies, sometimes in the absence of clinical disease, in sera from APS type I patients (1, 21–23).

Mutations in the AIRE gene are associated with APS type I (2, 3, 24) but no relationship between mutations in the AIRE gene and the ability to produce autoantibodies in patients without APS type I (for example Graves' disease or Hashimoto's thyroiditis) has been found (25). In our study, TPH Abs and AADC Abs were detected almost exclusively in patients with APS type I. The APS type II and AD patients who were positive for these autoantibodies may have had a mutation in the AIRE gene and represented an unidentified or atypical APS type I (24, 26, 27). However, we were unable to carry out analysis of the AIRE gene in these two patients.

TPH is a member of the bipterin-dependent hydroxylases family (1) together with tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH) and autoantibodies to TH and PAH have been described in patients with APS type I (28). In addition, autoantibodies to histidine decarboxylase, a histamine-synthesising enzyme that is pyridoxal phosphate dependent have been reported very recently in patients with APS type I (29, 30). Other known targets of autoimmune response in patients with APS type I such as GAD and AADC are also pyridoxal phosphate-dependent enzymes. The reasons why the autoimmune responses in APS type I involve particularly the enzymes important for the biosynthesis of neurotransmitters is not clear at present (1, 28–30).

In our study, a series of modified TPH and AADC proteins labelled with ^{35}S was used to analyse autoantibody epitopes. At least three regions on the TPH

molecule were found to be involved in the interaction with TPH Abs: the C-terminal region aa 308–423, central region aa 164–205 and N-terminal region aa 1–105. The varying effects of different modifications of the TPH molecule on TPH Ab binding in different patients suggested that TPH Abs in patient sera recognise multiple epitopes. At least some of the TPH Ab binding sites were located or were dependent on the amino acids within the catalytic domain of TPH (aa 308–444) and an inhibiting effect of TPH Abs on TPH enzyme activity has been reported previously (28). In the case of two patients, however, the TPH Ab epitope(s) appeared to be located only within the C-terminal region (aa 292–423) (Table 3; sera nos 2 and 4).

In the case of AADC, the amino acids involved in the AADC Ab binding were located in different parts of the molecule including the C-terminal region of aa 382–483, the middle region (aa 243–381) and the N-terminal region (aa 1–167). AADC Abs in patient sera appeared to be directed to at least two different regions except for one serum (Table 4, no. 10) that appeared to react principally with an epitope(s) dependent on aa 242–381.

Husebye *et al.* (31) have reported that AADC Abs in patient sera can inhibit AADC activity *in vitro* and that different sera have different inhibiting effects, suggesting a heterogeneity of autoantibody binding sites and this is in agreement with the observations reported here. Furthermore, the relationship between the domains important for enzyme activity and domains involved in autoantibody binding has been demonstrated in the case of other autoantibodies reactive with the enzymes (32, 33).

Our experiments with modified TPH and AADC proteins showed that different stretches of amino acids covering almost the entire sequence of the molecules were important for autoantibody binding in the case of both antigens. Involvement of long and different stretches of amino acids in the autoantibody binding is a known feature of conformational binding sites (34). It is possible therefore, that the TPH Ab and AADC Ab binding sites are conformational; i.e. are formed by three dimensional folding of the molecule. Alternatively, the involvement of long stretches of amino acids in autoantibody binding may be related to epitope spreading processes (32, 35). Furthermore, the different binding pattern of TPH Abs and AADC Abs to modified respective antigens in different patient sera suggested heterogeneity of the autoantibodies in terms of the epitope recognition.

Overall, our study on the prevalence of TPH Abs and AADC Abs in sera from patients with APS type I is in agreement with previous reports (5, 6). In addition, we have shown that TPH Abs and AADC Abs are present infrequently in the sera from patients with adrenal autoimmune diseases other than APS type I. In the case of patients with these other forms of autoimmune

adrenal disease who were positive for TPH Abs or AADC Abs, testing for AIRE gene mutations may be helpful in improving the disease diagnosis (24, 26, 27).

Acknowledgements

We thank Dr Olle Kämpe of Uppsala University, Sweden for providing the cDNAs coding for TPH and AADC and Carol James for preparation of the manuscript. C D P was in receipt of a fellowship from RSR Ltd.

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Received 18 June 2003

Accepted 4 December 2003