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Identification of Tyrosine Hydroxylase as an Autoantigen in Autoimmune Polyendocrine Syndrome Type I

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Patients with the autosomal recessively inherited autoimmune polyendocrine syndrome type I (APS I) have autoantibodies directed against several endocrine and nonendocrine organs. In this study a new autoantigen related to this syndrome, tyrosine hydroxylase, was identified in sera from patients with alopecia areata through immunoscreening of a scalp cDNA library. Immunoreactivity against *in vitro* expressed tyrosine hydroxylase was found in 41 (44%) of the 94 APS I patients studied and this reactivity correlated with the presence of alopecia areata ($P = 0.02$). These findings further stress the importance of enzymes involved in neurotransmitter biosynthesis as important immune targets in APS I.

Key Words: **alopecia areata; catecholamine biosynthesis; autoantibodies; tyrosine 3-monooxygenase.**

Autoimmune Polyendocrine Syndrome type I (APS I), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a recessively inherited disease caused by mutations in a recently identified gene on human chromosome 21 (1) and on mouse chromosome 10 (2). The gene, named AIRE for autoimmune regulator, encodes a novel protein with the characteristics of a transcription factor (3, 4).

Mucocutaneous candidiasis, hypoparathyroidism and adrenocortical insufficiency comprise the classical triad of manifestations in APS I (5) and the clinical diagnosis requires the presence of at least two of these three manifestations. Other autoimmune manifestations such as autoimmune chronic active hepatitis, gonadal failure, malabsorption, and insulin-dependent diabetes mellitus (IDDM) are often present in these patients. Ectodermal conditions such as vitiligo, alopecia, nail and enamel dystrophy are also frequent (1, 6). Patients with APS I display high titer autoantibodies against structures in the affected organs, which often are tissue-specific key enzymes (7–13), a reactivity for which APS I has been instrumental in identifying autoantigens of importance in more common autoimmune diseases (14).

Alopecia areata is characterized by a sudden patchy hair-loss, usually on the scalp, leaving circumscribed, bald areas for various lengths of time. Regrowth often occurs, but in the more severe cases of alopecia, the hair loss remains permanent throughout life and may affect the entire scalp (alopecia totalis) or even the entire body (alopecia universalis). Isolated alopecia areata is probably a heterogeneous disorder, with both genetic and environmental factors contributing (15, 16) to its development. Association of alopecia areata has been reported with other autoimmune diseases, e.g., thyroiditis or pernicious anemia (16, 17), suggesting an autoimmune etiology. It is supported by high prevalence of certain MHC haplotypes in alopecia areata (18) and the presence of autoantibodies against hair follicles in sera from patients with alopecia areata (19, 20).

Although alopecia is a common feature in APS I, affecting 29 to 37% of the patients (6), little is known about the immunoreactivity against hair follicles in these patients. With immunohistochemical staining we have previously shown that patients with APS I have autoantibodies directed against the anagen hair follicle and that this reactivity is associated with alopecia totalis (21). The present study was undertaken in an attempt to further elucidate the autoantibody targets of dermal structures. Using immunoscreening, we were able to identify tyrosine hydroxylase (TH), the

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rate-limiting enzyme in catecholamine biosynthesis, as an autoantigen associated with alopecia areata in APS I. Interestingly, TH is a member of the same family of tetrahydropteridine-dependent enzymes as tryptophan hydroxylase, recently identified as an important intestinal autoantigen (13).

MATERIALS AND METHODS

Sera. Sera were obtained from 94 APS I patients of Swedish (n = 10), Italian (n = 13), Norwegian (n = 9) and Finnish (n = 62) origin. Clinical characteristics of the Swedish patients (12, 21, 22) the Italian (6), Norwegian (13) and the Finnish patients (1) have previously been described. There were 46 men and 48 women in the study: 4/6 in the Swedish, 8/5 in the Italian, 3/6 in the Norwegian and 31/31 in the Finnish groups. Alopecia (areata or totalis) was present in 3 out of 10 (30%) Swedish, 4 out of 13 (31%) Italian, 2 out of 9 (22%) Norwegian and 20 out of 62 (32%) Finnish APS I patients. Twenty (43%) of the men and 9 (19%) of the women had alopecia. Seven patients with alopecia areata not related to APS I were included from the Department of Dermatology at our hospital. Sera from 35 Swedish and 30 Finnish healthy blood donors were used as controls. Sera from 224 patients with newly diagnosed IDDM and 20 patients with Addison's disease were also tested. A polyclonal rabbit antiserum against human TH (isoform I) was used as a positive control (23).

Enzymes. The expression and purification of human TH isoforms 1–4 from BL 21 DE3 pLysS *E. coli* has been described elsewhere (24, 25). Bovine TH was purified from adrenal medulla as previously described (26). The protein concentration was determined using an extinction coefficient of 1.04 cm^{-1} for 1 mg/ml human TH at 280 nm, as reported for bovine TH (26).

Construction of cDNA library. Preparation of 5 μg of poly(A)$^+$ mRNA from 50 mg of human scalp including intact hair follicles was performed with Dynabeads mRNA DIRECT kit (Dynal A. S., Oslo, Norway) and used for cDNA-library synthesis (λZAP-EXPRESS cDNA Synthesis Kit, Stratagene, CA) according to the protocol supplied by the manufacturer. Fractions containing cDNA larger than 500 base pairs were pooled and ligated into λZAP-EXPRESS vector arms. The library, containing 3.0×10^6 cDNA clones, was then amplified once. Scalp tissues were taken in connection with neurosurgical interventions and with the approval of the local ethics committee.

Isolation of the tyrosine hydroxylase clone. Immunoscreening of the cDNA-library with one patient serum diluted 1:3000 was performed (10). A phagemid vector pBK-CMV was excised *in vivo* from the λZAP clones in the *E. coli* strain XLOLR with helper-phage Exassist according to the Stratagene protocol. The sequence was analyzed with AmpliTaq DNA Polymerase, FS, dye Terminator and dye Primer kits (Perkin Elmer, ABI, Foster City, CA) and λZAP vector specific primers (Scandinavian Gene Synthesis, Köping, Sweden and Perkin Elmer) on a 373A DNA Sequencer (Applied Biosystems, Foster City, CA). The full-length clone was sequenced by primer walking with unlabeled, internal specific primers (KEBO, Solna, Sweden). The DNA sequence was then compared to international databases using BLAST (NCBI).

Subcloning into pSP64-poly(A) vector. Polymerase chain reaction, with Expand High Fidelity PCR System (Boehringer Mannheim, GmbH, Germany), was used for subcloning of the cDNA-insert from the pBK-CMV phagemid into a pSP64 vector (Promega, Madison, WI) designed for *in vitro* transcription and translation. Upper and lower primers (KEBO) were designed to anneal to the cDNA at the first ATG and stop codons respectively and also containing 15 5′-flanking bases constituting *Hin*dIII and *Bam*HI restriction sites, respectively. The PCR-product and pSP64 vector were digested with the appropriate restriction enzymes (Life Technologies, Inc., MD) and after ligation into the pSP64-poly(A) vector the sequence was analyzed as described above to ensure the correct sequence.

In vitro transcription and translation (ITT) and immunoprecipitation. Plasmids were purified with Qiagen miniprep kit (Qiagen GmbH, Hilden, Germany). *In vitro* transcription and translation (ITT) of the purified plasmids was performed using the TNT SP6-coupled reticulocyte lysate system (Promega). The correct size of the radioactive product was analyzed on a SDS-PAGE minigel (BioRad, Richmond, CA) according to standard protocols. The [^{35}S]-radiolabeled TH protein was used for immunoprecipitation with patient sera in a 96-well plate assay as described elsewhere (27). The results were expressed as a TH index ((cpm sample − cpm negative control)/(cpm positive control − cpm negative control) × 100). Each sample was analyzed in triplicates. One APS I patient with a high titre of anti-TH antibodies was used as a positive control and one of the blood donors was used as a negative control in each microwell plate. The upper normal limit of TH antibody index was set to 27, which was the mean value for blood donors plus 3 SD.

Western blot. Seventy-five ng of TH protein was used in each lane. SDS-PAGE (28) and Western blots (29) were performed as described. Sera from the 10 Swedish APS I patients, 10 blood donor and the polyclonal rabbit anti-TH1 antibody were all diluted 1:300.

Measurement of TH enzyme activity. The enzyme (TH1) was incubated for 2 min at 30°C, in the presence of 0.5 mg ml^{-1} catalase, 100 μM $Fe(II)SO_4$, 250 μM (6R)-tetrahydrobiopterin, 1 mM dithiothreitol, 25 μM L-[3,5-^{3}H]tyrosine and 40 mM NaHepes, pH 7.0, using the ^3H_2O release assay (30). The enzyme activity was measured in triplicates in the absence of serum or in the presence of serum diluted 100-fold in a final assay volume of 100 μl.

Statistics. Fisher's exact test was used to compare the frequencies of reactivity against tyrosine hydroxylase with the various disease components.

RESULTS

Identification of TH as an autoantigen. Ten positive clones were identified when immunoscreening of the λZAP-scalp library was performed using a serum from one of the Swedish APS I patients with alopecia areata. All positive clones were subcloned into the pSP64-polyA vector. The corresponding radioactive proteins were generated by *in vitro* transcription and translation and used for immunoprecipitation with a panel of ten APS I sera. The majority of the clones were recognized solely by the individual serum used for the screening, but one clone was identified by 5 of the panel of 10 APS I sera. The DNA-sequence of this purified clone was identical to human tyrosine hydroxylase isoform 2.

Immunoprecipitation of the recombinant TH with patient sera. When the cDNA was subcloned into pSP64 polyA vector and used for ITT, the incorporation-rate of [^{35}S]-methionine was about 2.7% of total radioactivity. Analysis on a SDS-PAGE gel showed a band with the expected size of approximately 60 kDa (data not shown). Forty-one out of 94 (44%) patient sera showed reactivity against TH (Table 1); 5 of the 10 (50%) Swedish, 6 of the 13 (46%) Italian, 28 of the 62 (45%) Finnish and 2 of the 9 (22%) Norwegian patients. There was a significant correlation between autoantibodies against TH and the presence of alopecia areata ($p = 0.02$) since

TABLE 1

Immunoreactivity against *In Vitro* Expressed Tyrosine Hydroxylase Isoform 2 (TH) and Correlation with Alopecia in the Four Groups and the Total of 94 Patients with APS I

Country	Alopecia/Total number	TH positive with alopecia	TH positive without alopecia	*P* value[a]
All	29/94 (31%)	18/29 (62%)	23/65 (35%)	0.02
Sweden	3/10 (30%)	3/3 (100%)	2/7 (29%)	0.17
Italy	4/13 (31%)	3/4 (75%)	3/9 (33%)	0.27
Finland	20/62 (32%)	12/20 (60%)	16/42 (38%)	0.17
Norway	2/9 (22%)	0/2	2/7 (29%)	>0.99

[a] Calculated by use of Fisher's test.

18 of the 29 (62%) patients with alopecia had autoantibodies against TH compared with only 23 of 65 (35%) of the patients without alopecia. No correlation with other clinical manifestations of APS I was found (Table 2). Vitiligo was present in 21 of the 94 (22%) APS I patients. Although 12 of these 21 (57%) patients showed TH reactivity, no significant correlation with vitiligo was found. Only 1 of 224 sera from patients with type I diabetes mellitus displayed a significant reactivity against tyrosine hydroxylase. No reactivity against TH was found in sera from any of 7 patients with alopecia areata unrelated to APS I, 20 patients with Addison's disease or 65 blood donors.

Western blot. Sera from 10 Swedish APS I patients and 10 healthy blood donors were tested in Western blot against each of the four isoforms of human TH. Only the 5 sera that immunoprecipitated the *in vitro* expressed TH2 protein showed reactivity against TH in Western blot (Fig. 1) and equal reactivity was seen against all four isoforms of tyrosine hydroxylase (data not shown).

Inhibition of tyrosine hydroxylase by serum from APS I patients. Sera from 4 of the 5 Swedish APS I patients with positive immunoreactivity against TH were tested. The enzyme activity (TH1) was significantly reduced by 40% by three of these sera whereas sera from the fourth patient and 10 healthy blood donors reduced the activity only by about 20% (Fig. 2). No linear relationship existed, however, between the extent of enzyme inhibition and the titre of TH autoantibodies. At a 100-fold dilution of control serum, the TH activity was typically about 80% of the activity without serum. We have previously reported a similar nonspecific inhibition of both side chain cleavage enzyme (29) and tryptophan hydroxylase (13) activity by control serum. This effect could be due to the presence of free amino acids or other substances in the serum. In comparison, the inhibition by protein A-Sepharose purified IgG from blood donors was always less than 10% at a final concentration of 10 μg/ml and the difference between patients and blood donors then became larger (data not shown). Further characterization of the enzyme inhibition (patient #2 in Fig. 2) demonstrated a non-competitive type of enzyme inhibition (vs. cofactor or amino acid substrate) that was comparable for all four isoforms of human TH, as well as bovine TH (data not shown).

DISCUSSION

In the present study we describe the identification of tyrosine hydroxylase (TH), as an autoantigen in APS I.

TABLE 2

Immunoreactivity against *In Vitro* Expressed Tyrosine Hydroxylase Isoform 2 (TH) and Correlation with Clinical Manifestations in 94 Patients with APS I

Clinical disorder	Number with disorder	TH positive with disorder	TH negative with disorder	*P* value[a]
Alopecia areata	29/94 (31%)	18/29 (62%)	23/65 (35%)	0.02
Hypoparathyroidism	78/94 (83%)	34/78 (44%)	7/16 (44%)	>0.99
Adrenal insufficiency	78/94 (83%)	37/78 (47%)	4/16 (25%)	0.17
Intestinal dysfunction	22/94 (23%)	10/22 (45%)	31/72 (43%)	>0.99
Insulin-dependent diabetes mellitus	12/94 (13%)	5/12 (42%)	36/82 (44%)	>0.99
Chronic active hepatitis	17/94 (18%)	8/17 (47%)	33/77 (43%)	0.80
Gonadal failure	31/94 (33%)	16/31 (52%)	25/63 (40%)	0.40
Pernicious anaemia	15/94 (16%)	6/15 (40%)	35/79 (44%)	>0.99
Vitiligo	12/94 (22%)	12/21 (57%)	29/73 (40%)	0.21

[a] Calculated by use of Fisher's test.

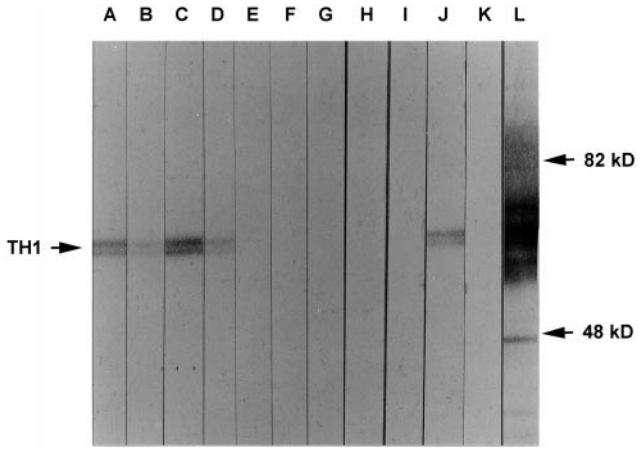

FIG. 1. Western blot of human TH1 expressed in *E. coli*, with APS I patient and control sera incubated at a dilution of 1:300. Lane A-J: sera from 10 Swedish APS I patients; lane K: serum from a healthy blood donor; lane L: polyclonal rabbit anti-TH1 serum. Arrows to the right indicate the mobilities of prestained molecular weight standards.

TH is a member of the closely related group of tetrahydropteridine-dependent amino acid hydroxylases, which also includes tryptophan hydroxylase (TPH) and phenylalanine hydroxylase. We have recently shown that TPH, the rate limiting enzyme in the synthesis of serotonin, is an intestinal autoantigen in APS I patients (13). Interestingly, sera from patients with APS I also react with aromatic-L-amino-acid decarboxylase (AADC) (10), an enzyme involved in both the monoaminergic and serotonergic biosynthetic pathways.

TH catalyzes the conversion of tyrosine to L-dopa, the precursor of the catecholamines dopamine, norepinephrine and epinephrine. TH exists as four alternatively spliced isoforms (TH1-TH4) (31). The amino acid sequence is identical in all four isoforms at the catalytic C-terminal part but differs in the regulatory N-terminal end. The clone isolated in the immunoscreening was found to correspond to isoform 2. All five APS I sera reacting with this clone in the *in vitro* transcription and translation (ITT) assay recognized all four isoforms in Western blots, indicating common epitopes. Among the isoforms, TH1 and TH2 are the most widely distributed forms, being mainly expressed in the central nervous system, but also in the sympathetic nervous system including the adrenal medulla (32). In rats and mice TH is also at significant levels expressed in the β-cells of the pancreas (33). No correlation was, however, found between insulin-dependent diabetes mellitus and TH reactivity among APS I patients. Only 1 out of the 224 sera obtained from patients with newly diagnosed, isolated type I diabetes showed TH reactivity in the ITT assay. In the skin, undifferentiated keratinocytes of the hair follicles and epidermal layers are likely to be a source for TH mRNA. TH activity has been detected in human skin keratinocytes *in vitro* by means of measuring ^{3}H-labelled L-tyrosine metabolism (34). Human hair follicles and cultured keratinocytes have also been shown, using RT-PCR technique to be a source of TH (35, 36). A role for TH in keratinocytes has been implicated where upregulation of TH activity and subsequent elevated levels of norepinephine and epinephrine induce β2-adrenergic receptors, which, via calcium influx, initiate keratinocyte differentiation (37). However, we were not able to find significant staining of the keratinocytes in hair follicles or epidermis in acetone fixed cryo preserved sections or citrate buffer treated paraffin fixed sections of human scalp by use of APS I patient sera or specific anti-TH antibodies. An explanation for this could be that the expression of TH in the keratinocytes is below the detection level of this methodology or that epitopes recognized by patient sera are hidden within the three-dimensional protein structure, only being exposed when TH is denatured in Western blot. Staining of nerve fibers around hair follicles and arrector pili muscles were, however, seen with the specific anti-TH antibodies (data not shown) and peripheral autonomic fibers are known to contain TH. In murine skin, hair cycle-dependent fluctuations of sensory and autonomic nerve fiber density have been demonstrated and a dramatic increase in the number of TH containing nerve fibers was seen in the early anagen phase during regrowth following depilation (38). The correlation between TH-autoantibodies and alopecia may thus be due to an autoimmune attack on nerve-endings close to the hair follicles, perhaps of importance for their maturation.

When comparing immunoreactivity against *in vitro* expressed TH with various clinical manifestations found in the 94 APS I patients, a correlation was found only with alopecia areata ($p = 0.02$) (Table 2). Among our APS I patients alopecia areata was twice as com-

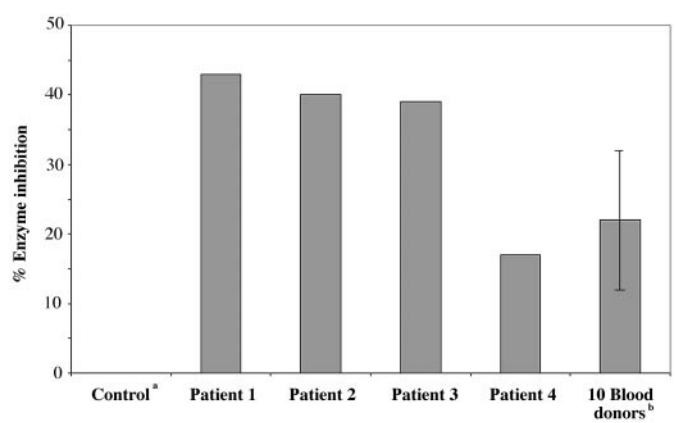

FIG. 2. TH enzyme inhibition with 4 APS I patient sera and 10 blood donors. [a]Incubation as described under Materials and Methods. [b]Blood donors are presented with highest and lowest individual values indicated.

mon in men (43%) than in women (19%) ($p = 0.01$). The frequency of TH reactivity did not, however, differ between sexes and can not explain this observation, perhaps suggesting that there are other antigens yet to be identified in the hair follicle or that males are more sensitive to develop alopecia areata by other mechanisms.

The results of the enzyme inhibition assay implies that the APS I patients have a heterogeneous population of anti-TH antibodies capable of inhibiting the enzyme activity, but to differing degrees. No linear relationship between the extent of enzyme inhibition and the anti-TH titer was found and no enzyme inhibition was found in the serum of one patient with anti-TH antibodies as measured in the ITT assay. Interestingly, while many APS I sera in a previous study displayed strong inhibitory antibodies against TPH (13), the inhibition of TH (Fig. 2) activity was less pronounced. This was somewhat surprising considering the extensive sequence homology and structural similarity of the tetrahydropteridine-dependent hydroxylases. The antibodies were not species-specific, as all isoforms of human TH, as well as bovine TH were significantly inhibited by APS I patient sera.

Tyrosine hydroxylase belongs to a group of highly homologous tetrahydropteridine-dependent enzymes together with tryptophan hydroxylase and phenylalanine hydroxylases for which the three-dimensional structures have recently been determined (39). Autoantibodies against TH and TPH may recognize both crossreactive and unique epitopes that are located on the surfaces of these proteins. The exact structures of these epitopes can therefore not be determined correctly unless the intact three-dimensional molecule structure is preserved. The existence of autoantibodies against TH and TPH, respectively, and our knowledge of the three-dimensional structure will allow "intelligent" construction of chimeric proteins where changes introduced would not perturb the gross over-all three-dimensional structure, giving a unique possibility to more closely study interactions between autoantibodies and antigens.

To our knowledge, this is the first demonstration of autoantibodies directed against TH. The finding of TH as an autoantigen further strengthens the observation that many autoantigens in APS I are key enzymes and also points to the biosynthetic pathway of neurotransmitters as an important target. Possibly, these enzymes share properties that make them potent triggers of the immune system. Alternatively, the presentation of these enzymes in the thymus may be incomplete, since they are expressed rather late during fetal development or due to a pathogenetic event caused by defective expression of the AIRE gene in the thymus. Although this study indicates a role of TH in the pathogenesis of alopecia in APS I, further studies are needed in order to evaluate the exact role of the TH immunoreactivity in the etiology of alopecia areata either isolated or as part of APS I. The identification of TH together with our previous identification of tryptophan hydroxylase, two members of the highly homologous family of tetrahydropteridine-dependent hydroxylases, may also help to elucidate important structural properties of conformational epitopes recognized by the immune system.

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