CLINICAL STUDY

Autoantibodies against recombinant human steroidogenic enzymes 21-hydroxylase, side-chain cleavage and 17α-hydroxylase in Addison's disease and autoimmune polyendocrine syndrome type III

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Abstract

Objective: To evaluate the frequency of autoantibodies (Ab) against 21 hydroxylase (21OH), side-chain cleavage (SCC) and 17α-hydroxylase (17OH), in Addison's disease (AD) and autoimmune polyendocrine syndrome type III (APSIII).

Design and Methods: We used radiobinding assays and in vitro translated recombinant human 35S-21OH, 35S-SCC or 35S-17OH and studied serum samples from 29 AD (18 idiopathic, 11 granulomatous) and 18 APSIII (autoimmune thyroid disease plus type 1 diabetes mellitus, without AD) patients. Results were compared with those of adrenocortical autoantibodies obtained with indirect immunofluorescence (ACA-IIF).

Results: ACA-IIF were detected in 15/18 (83%) idiopathic and in 1/11 (9%) granulomatous AD subjects. 21OHAb were found in 14/18 (78%) idiopathic and in the same (9%) granulomatous AD subject. A significant positive correlation was shown between ACA-IIF and 21OHAb levels ($r^2 = 0.56$, $P < 0.02$). The concordance rate between the two assays was 83% (24/29) in AD patients. SCCAb were found in 5/18 (28%) idiopathic (4 of whom were also positive for 21OHAb) and in the same (9%) granulomatous AD subject. 17OHAb were found in only 2/18 (11%) idiopathic and none of the granulomatous AD patients. Two APSIII patients were positive for ACA-IIF, but only one was positive for 21OHAb and SCCAb. 17OHAb were found in another two APSIII patients.

Conclusions: Measurement of 21OHAb should be the first step in immune assessment of patients with AD and individuals at risk for adrenal autoimmunity, in addition to ACA-IIF. Due to their low prevalence in AD, measurement of SCCAb and 17OHAb should be indicated only for 21OHAb negative patients and/or for those with premature ovarian failure, regardless of ACA-IIF results.

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Introduction

Autoimmune destruction of the corticosteroid-producing cells is responsible for 70–90% of cases of Addison’s disease (AD) (1, 2). Adrenocortical autoantibodies, conventionally detected by indirect immunofluorescence (ACA-IIF), are sensitive markers of autoimmune AD and are found in 60 to 80% of patients with clinically idiopathic AD (2–4).

The steroidogenic enzyme 21-hydroxylase (21OH) is the major adrenal autoantigen associated with AD (5), but autoantibodies (Ab) against other P450 cytochromes (such as side-chain cleavage enzyme, SCC (6), and steroid 17α-hydroxylase, 17OH (7)) can also be detected in subjects with the autoimmune polyendocrine syndrome type I (APS1) or with AD associated with premature ovarian failure (8). Autoantibodies against 21OH (21OHAb), detected by a radiobinding assay, have a high diagnostic sensitivity and specificity for autoimmune AD (9–12).

Given the frequent association of AD with other autoimmune diseases (2, 13–15), the identification of subjects with an ongoing autoimmune AD is especially important in clinical management of endocrine autoimmune diseases such as Hashimoto’s thyroiditis, Graves’ disease, type 1 diabetes mellitus (IDDM) and premature ovarian failure (POF). 21OHAb can be found in 3% of Graves’ disease and in up to 2% of IDDM patients (11, 16). The presence of 21OHAb in adult patients with other autoimmune endocrinopathies
and absent overt hypoadrenalinism is considered a marker of low progression to AD (17). It was recently shown that the levels of 21OHBAb correlate with the degree of adrenal dysfunction in preclinical AD subjects, which suggests that 21OHBAb is the best immune marker of ongoing adrenal autoimmunity (18).

The aim of our study was to evaluate the frequency of 21OHBAb, SCCAb and 17OHBAb in AD and APS type III (APSIII) patients. We used radiobinding assays with in vitro translated recombinant human 13S-21OH, 13S-SCC or 13S-17OH, and compared the results of these assays with those obtained with the classical IIF technique (ACA-IIF).

**Subjects and methods**

Serum samples from 29 Brazilian patients with AD (15 males/14 females; median age: 53 years, range 20–79 years; median disease duration: 3 years, range 0.1–38 years), consecutively diagnosed at the Division of Endocrinology of the Universidade Federal de São Paulo were studied. Diagnosis of primary adrenocortical insufficiency was based on both clinical and laboratory findings (blunted 60 min cortisol response to an acute adrenocorticotropin (ACTH) stimulation: 21 ± 1.5 μg/dl, range 0.4–5.5 μg/dl, normal ≥ 18 μg/dl) (19). All patients were on steroid replacement therapy at the time of the study.

Patients were investigated for all possible causes of AD. Diagnosis of post-tuberculosis or post-paracoccidioidomycosis AD was based on anamnestic data, purified protein derivative (PPD) test, presence of antibodies against *Paracoccidioides brasiliensis* and on demonstration of adrenal calcification by computerized tomography (CT) (20, 21). The presence of other autoimmune diseases, as well as that of organ-specific autoantibodies, such as glutamic acid decarboxylase autoantibodies (GAD65Ab) or thyroid autoantibodies (thyreo peroxidase antibodies, TPOAb; thyroid microsomal antibodies, TMSA) was also investigated.

Prior to the evaluation of 21OHBAb, SCCAb and 17OHBAb, patients with primary adrenocortical insufficiency were assigned to two etiological groups, according to their clinical characteristics: (1) Clinically idiopathic: consisted of 18 patients without evidence of granulomatous or metastatic diseases. A total of 10/18 (56%) patients had one or more autoimmune associated endocrinopathies, characterizing an APS type II (APSII); the remaining eight (44%) had isolated AD. CT of the adrenal glands showed volume reduction (suggestive of atrophy) in 15 of these patients (Table 1). (2) Granulomatous: consisted of 11 patients with calcification on adrenal CT scanning due to tuberculosis (n = 4) or paracoccidioidomycosis (n = 3). Patients 4, 5, 8 and 10 had a positive PPD test. Patient 1 had antibodies against *Paracoccidioides brasiliensis* and patients 6 and 7 had been treated for paracoccidioidomycosis several years before the study. In the remaining four patients, the etiology of AD was attributable to a granulomatous disease on the basis of the finding of calcified adrenals on CT. Two patients were positive for thyroid autoantibodies, one for GAD65Ab and one had vitiligo (Table 1).

We also analyzed serum samples from 18 APSIII patients (15 females/3 males; median age: 37 years, range 20–68 years). Both IDDM and an autoimmune thyroid disease were present in all these subjects, but none had AD (22) (Table 2). None of the patients was receiving corticosteroid therapy at the time of the study.

Serum samples from 90 Brazilian healthy subjects (57 males/33 females; median age: 28 years, range 19–81 years) served as controls.

Written informed consent was obtained from all subjects after the study protocol had been approved by the Ethical Committee on Human Research of the University Hospital.

**Indirect immunofluorescence (ACA-IIF)**

ACA were detected by a classical IIF technique, using thin cryostatic sections of normal bovine adrenal glands as the source of antigen (23). ACA-IIF titers were determined by testing serial twofold dilutions of the serum until reaching the end point.

**21OHBAb, SCCAb and 17OHBAb radiobinding assays**

The full-length human cDNAs of 21OHa, SCC and 17OH were subcloned into the plasmid vector pcDNA II (Invitrogen, San Diego, CA, USA), under the control of SP6 promoter. The cDNA of human SCC (24) and of human 17OH (25) were a kind gift of Dr Walter Miller, Department of Pediatrics and Metabolic Research Unit, University of California, San Francisco, CA, USA. Recombinant human 13S-21OH, 13S-SCC and 13S-17OH were produced by in vitro coupled transcription and translation following procedures similar to those described for the production of 13S-GAD65 (26).

The presence and levels of antibodies to steroidogenic enzymes were determined in radiobinding assays similar to those for 21OHBAb (9) and GAD65Ab (26). In each antibody analysis, 15 000 c.p.m. of the trichloroacetic acid (TCA)-precipitable 13S-labeled enzyme were immunoprecipitated, in duplicate, with 2 μl serum (final serum dilution 1:25). After an overnight incubation at 4°C on a rotating platform, the immunoprecipitated proteins were transferred to filtration plates, and the antibody-bound 13S-labeled enzyme was separated from free antigen by addition of 50 μl 40% protein A-sepharose (Pharmacia, Uppsala, Sweden) and incubation for 45 min on a plate shaker. After several washes, the sepharose-bound radioactivity was finally determined in a liquid scintillation counter.
Table 1  Clinical characteristics of the 29 Brazilian AD patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease duration (years)</th>
<th>Sex/age (years)</th>
<th>AID</th>
<th>TMSA and/or TPOAb</th>
<th>GAD65Ab</th>
<th>ACA-IIF</th>
<th>21OHAb</th>
<th>SCCAb</th>
<th>17OHAb</th>
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<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>M/60</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
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<td>−0.020</td>
<td>−0.005</td>
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<td>0.9</td>
<td>M/52</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.020</td>
<td>0.006</td>
<td>−0.020</td>
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<tr>
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<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.060</td>
<td>0.030</td>
<td>0.050</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>F/34</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
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<td>−0.003</td>
<td>−0.006</td>
</tr>
<tr>
<td>5</td>
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<td>NEG</td>
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<td>0.040</td>
<td>0.010</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>M/62</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.130</td>
<td>0.030</td>
<td>0.010</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>M/61 V</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.090</td>
<td>0.110</td>
<td>0.005</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>M/69</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.001</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>M/60</td>
<td>NEG</td>
<td>NEG</td>
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<td>0.250</td>
<td>0.030</td>
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<td>10</td>
<td>28</td>
<td>M/68</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
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<td>−0.007</td>
<td>−0.040</td>
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<tr>
<td>11</td>
<td>38</td>
<td>M/79</td>
<td>NEG</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>0.002</td>
<td>−0.020</td>
<td>−0.050</td>
</tr>
</tbody>
</table>

**Granulomatous AD**

**Idiopathic AD**

Table 2  Clinical characteristics of the 18 APSIII patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age (years)</th>
<th>APS III</th>
<th>TPOAb</th>
<th>GAD65Ab</th>
<th>ACA-IIF</th>
<th>21OHAb</th>
<th>SCCAb</th>
<th>17OHAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/38</td>
<td>IDDM + H</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>0.010</td>
<td>0.005</td>
<td>0.050</td>
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<tr>
<td>2</td>
<td>F/30</td>
<td>IDDM + GD</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.020</td>
<td>−0.020</td>
<td>0.050</td>
</tr>
<tr>
<td>3</td>
<td>F/37</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>0.007</td>
<td>−0.020</td>
<td>−0.030</td>
</tr>
<tr>
<td>4</td>
<td>F/39</td>
<td>IDDM + H + POF</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.680</td>
<td>0.580</td>
<td>−0.140</td>
</tr>
<tr>
<td>5</td>
<td>M/28</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.120</td>
<td>−0.030</td>
<td>0.010</td>
</tr>
<tr>
<td>6</td>
<td>F/52</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>0.040</td>
<td>−0.010</td>
<td>0.040</td>
</tr>
<tr>
<td>7</td>
<td>F/31</td>
<td>IDDM + GD</td>
<td>NEG</td>
<td>POS</td>
<td>NEG</td>
<td>−0.002</td>
<td>0.004</td>
<td>−0.020</td>
</tr>
<tr>
<td>8</td>
<td>F/31</td>
<td>IDDM + GD + H + SMS</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.030</td>
<td>0.004</td>
<td>0.050</td>
</tr>
<tr>
<td>9</td>
<td>F/26</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.050</td>
<td>−0.007</td>
<td>0.220</td>
</tr>
<tr>
<td>10</td>
<td>F/34</td>
<td>IDDM + GD</td>
<td>NEG</td>
<td>POS</td>
<td>NEG</td>
<td>−0.030</td>
<td>−0.004</td>
<td>−0.005</td>
</tr>
<tr>
<td>11</td>
<td>F/57</td>
<td>IDDM + H + CG</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.040</td>
<td>−0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>12</td>
<td>F/60</td>
<td>IDDM + H + V</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.040</td>
<td>−0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>13</td>
<td>F/36</td>
<td>IDDM + GD + MG</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>0.030</td>
<td>−0.010</td>
<td>−0.030</td>
</tr>
<tr>
<td>14</td>
<td>F/20</td>
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<td>NEG</td>
<td>NEG</td>
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<td>0.003</td>
<td>0.290</td>
</tr>
<tr>
<td>15</td>
<td>M/68</td>
<td>IDDM + H + V + HP + H</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.030</td>
<td>0.008</td>
<td>−0.040</td>
</tr>
<tr>
<td>16</td>
<td>F/42</td>
<td>IDDM + GD + V</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>0.030</td>
<td>−0.009</td>
<td>0.080</td>
</tr>
<tr>
<td>17</td>
<td>F/36</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>−0.090</td>
<td>−0.070</td>
<td>−0.030</td>
</tr>
<tr>
<td>18</td>
<td>M/55</td>
<td>IDDM + GD</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>−0.100</td>
<td>−0.110</td>
<td>−0.180</td>
</tr>
</tbody>
</table>

AID: autoimmune disease clinically manifested (H: Hashimoto’s thyroiditis; GD: Graves’ disease; POF: premature ovarian failure; CG: chronic atrophic gastritis; IDDM: type 1 diabetes mellitus; V: vitiligo); POS: positive; NEG: negative; *: isolated idiopathic AD.

H = Hashimoto’s thyroiditis; GD = Graves’ disease; POF = premature ovarian failure; CG = chronic atrophic gastritis; IDDM = type 1 diabetes mellitus; V = vitiligo; SMS = stiff man syndrome; MG = myasthenia gravis; HG = hypergonadotropic hypogonadism; HP = hypoparathyroidism; POS = positive; NEG = negative.
To control for the interassay variation, antibody levels were expressed as relative indices (21OHAb index, SCCAb index and 17OHAb index) using a positive and two negative control sera in each assay. Each index was calculated as follows: (mean c.p.m. of the unknown sample – mean c.p.m. of two negative standards)/(mean c.p.m. of the positive standard – mean c.p.m. of the two negative standards). The upper level of normal of each assay was calculated using the mean + 3 S.D. of the results obtained with 90 Brazilian healthy control subjects.

**Other assays**

TMSA were detected by semiquantitative agglutination (Sera-Tek microsomal antibody test kit, Ames, IN, USA, normal <1/100). TPOAb were detected by a 125I-immunoradiometric assay kit (Bio-Line S.A., Brussels, Belgium, normal <100 U/ml). GAD$_{65}$Ab were determined by a radiobindng assay with in vitro translated recombinant human antigen (normal GAD65 index <0.035) (26). Steroid-cell autoantibodies (SICA-IIIF) were determined by indirect immunofluorescence using cryostatic sections of monkey ovary and testis (Bios GmbH, Grafelfing, Germany).

Antibodies against *Paracoccidioides brasiliensis* were detected by immunodiffusion (27). Delayed cellular hypersensitivity to tuberculosis (PPD test) was evaluated by the subcutaneous injection of 2 U tuberculin in the anterior face of the forearm.

**Statistical analysis**

Differences in frequencies of autoantibodies between the two AD patient groups, or between AD and APSIII patients, or between AD or APSIII patients and control subjects were tested by the Chi square method or the Fisher’s exact test, when appropriate. Because the autoantibody levels were not normally distributed among the patients, the relationship between ACA-IIIF titer or 21OHAb index and disease duration was analyzed by linear regression, after logarithmic transformation of antibody levels. Spearman’s rank correlation test was used to verify correlation between ACA-IIIF titer and 21OHAb index. The Mann-Whitney test was used to evaluate differences in autoantibody indices. A $P$ value less than 0.05 was considered significant.

**Results**

**ACA-IIIF in Addisonian patients and healthy controls**

ACA-IIIF were detected in 16/29 (55%) AD patients (15/18 (83%) from the idiopathic and 1/11 (9%) from the granulomatous group ($P < 0.001$)) and in none of the 90 healthy control subjects ($P < 0.001$) (Table 1, Fig. 1). No significant correlation was found between the presence or titers of ACA-IIIF and the duration of AD ($r = -0.39$, $P = $ not significant (NS)). Among patients with idiopathic AD, ACA-IIIF were detected in 8/10 (80%) with APSII and in 7/8 (88%) with isolated AD ($P = $ NS).

**21OHAb in Addisonian patients and healthy controls**

In the 21OHAb assay, immunoprecipitation was 30% with the positive standard serum and 6% and 4% with the two negative standard sera. The intra-assay and interassay coefficients of variation were 5% and 12% respectively for the positive, and 4%-13% and 4%-12% respectively for the negative standards.

The upper level of normal for the 21OHAb index was set at 0.14. 21OHAb were detected in 15/29 (52%) AD patients (14/18 (78%) from the idiopathic and 1/11 (9%) from the granulomatous group ($P < 0.001$)) and in none of the 90 control subjects (Table 1, Fig. 2a). The 21OHAb index was significantly higher in idiopathic (median 0.52, range: 0.04 to 1.18) than in granulomatous individuals (median 0.03; range: –0.06 to 0.50) ($P = 0.001$).

Although levels of 21OHAb tended to decrease with increasing disease duration ($r = -0.13$), the correlation between 21OHAb index and the duration of AD did not reach statistical significance. Among patients with idiopathic AD, 21OHAb were detected in 8/10 (80%) with APSII and in 6/8 (75%) with isolated AD ($P = $ NS). A positive and significant correlation was found between ACA-IIIF titer and 21OHAb index ($r^2 = 0.56$, $P < 0.02$). Concordance between results for both assays was 83% (24 out of 29). A total of 17/18 (94%) patients with clinically idiopathic AD was positive for either ACA-IIIF or 21OHAb.
SCCAb in Addisonian patients and healthy controls

In the SCCAb assay, immunoprecipitation was 36% with the positive standard serum and 5% and 4% with the two negative standard sera. The intra-assay and interassay coefficients of variation were 5% and 13% respectively for the positive, and 4–9% and 4–10% respectively for the negative standards.

The upper level of normal for the SCCAb index was set at 0.15. SCCAb were detected in 6/29 AD patients (21%) (5/18 (28%) from the idiopathic and 1/11 (9%) from the granulomatous group (P=NS)) and in 1/90 (1%) of the healthy control subjects (Table 1, Fig. 2b).

The SCCAb positive patient from the granulomatous group was also positive for ACA-IIF and 21OHAb. In the idiopathic group, all but one patient positive for SCCab were also positive for 21OHAb. The two patients with POF were positive for SCCAb, including the patient negative for 21OHAb and positive for ACA-IIF. Among patients with idiopathic AD, SCCAb were detected in 5/10 (50%) with APSII and in 0/8 (0%) with isolated AD (P=0.036).

17OHAb in Addisonian patients and in healthy controls

In the 17OHAb assay, immunoprecipitation was 25% with the positive standard serum and 6% and 5% with the two negative standard sera. The intra-assay and interassay coefficients of variation were 4% and 12% respectively for the positive, and 4–11% and 4–14% respectively for the negative standards.

The upper level of normal for the 17OHAb index was estimated at 0.10. 17OHAb were detected in 2/29 (7%) AD patients (both from the idiopathic group (11%)) and in none of the control subjects (Table 1, Fig. 2c). One of the patients (17OHAb index of 0.68) was also positive for ACA-IIF, 21OHAb and SCCAb, and had associated Hashimoto’s thyroiditis, positive GAD65Ab and irregular menses; the other (17OHAb index of 0.16) was negative for ACA-IIF, 21OHAb and SCCAb and had associated Graves’ disease (her menopause occurred at 53 years of age). Among patients with idiopathic AD, 17OHAb were detected in 2/10 (20%) with APSII and in 0/8 (0%) with isolated AD (P=NS).

StCA-IIF in Addisonian patients positive for SCCAb and/or 17OHAb and in the healthy control patient positive for SCCAb

StCA-IIF were detected in 3/6 (50%) idiopathic AD patients positive for SCCAb and/or 17OHAb (patients 12, 14 and 21). Among the idiopathic AD patients with POF, StCA-IIF was detected in only one (patient 21). The granulomatous AD patient positive for ACA-IIF, 21OHAb and SCCAb was negative for StCA-IIF. The healthy control subject positive for SCCAb was negative for StCA-IIF.

17OHAb in APS type III patients (Table 2 and Figs 1 and 2)

ACA-IIF, StCA-IIF, 21OHAb, SCCAb and 17OHAb in APS type III patients (Table 2 and Figs 1 and 2)

ACA-IIF were positive in 2/18 (11%) APSIII patients (P<0.001 vs idiopathic AD; P=0.03 vs controls). Patient 4 (ACA-IIF titer 1/1024) had IDDM, Hashimoto’s thyroiditis, POF and both GAD65Ab and
TPOAb. Patient 15 (ACA-IIF titer 1/16) had hypoparathyroidism, Hashimoto’s thyroiditis, hypergonadotropic hypogonadism, IDDM and vitiligo. He also had GAD_{65}Ab and TPOAb (Table 2).

The median 21OHAb, SCCAb and 17OHAb indices for APSIII patients were, respectively: 0.03 (range: −0.10 to 0.68), −0.01 (range: −0.11 to 0.58) and 0.003 (range: −0.18 to 0.29). The 21OHAb (P < 0.001) and SCCAb (P = 0.04) indices were significantly lower than those of the idiopathic AD patients. Among APSIII patients, 21OHAb and SCCAb were detected in only one (patient 4), whereas 17OHAb were detected in two (patients 9 and 14), none of them being positive for the other autoantibodies (Table 2).

Among the APSIII patients positive for SCCAb or 17OHAb, StCA-IIF were detected in patients 4 and 9.

**Association between SCCAb and/or 17OHAb positivity and gonadal insufficiency**

No association was observed between SCCAb and/or 17OHAb positivity and gonadal insufficiency in idiopathic AD patients. However, when APSIII patients were included in the statistical analysis, the presence of SCCab (but not that of 17OHAb) was significantly associated with gonadal insufficiency (P = 0.01). Only 4/36 (11%) patients presented with gonadal insufficiency: two AD women and two APSIII (one woman and one man), three of whom (75%) were positive for SCCab (and none for 17OHAb), as compared with 3/32 (9%) patients positive in the absence of gonadal failure. All three women with premature ovarian failure were SCCab positive.

**Discussion**

In the present study, we were able to detect 21OHAb, SCCab and 17OHAb in 78%, 28% and 11% respectively of idiopathic AD patients’ sera using radiobinding assays and recombining human steroidogenenic enzymes produced by in vitro coupled transcription and translation. Positivity for 21OHAb is similar to the prevalence of 64% to 86% reported by different laboratories (8–10) and confirms the key role of 21OH as the main autoantigen for development of adrenal autoimmune in isolated AD and in APSII (8, 28).

In our study, the concordance rate between ACA and 21OHAb in AD patients was 83%, with a significant positive correlation (r = 0.56) between the results of both assays. This figure is slightly lower than the 94% found by Betterle et al. (29). Positivity for only one of the two assays was as high as 28% (5 of 18) in autoimmune AD patients (3 for ACA-IIF and 2 for 21OHAb), stressing the need for performing both assays in order to increase diagnostic sensitivity.

Discrepancy between the ACA-IIF and 21OHAb tests may be due to different sensitivity and/or specificity of the assays. However, two of the three ACA-IIF positive-21OHAb negative patients were negative for both SCCab and 17OHAb, suggesting the presence of a surrogate antigen. Peterson et al. (30) observed no reactivity for 11β-hydroxylase, aromatase, 3β-hydroxysteroid dehydrogenase and adrenodoxin in the sera of 46 patients with APSI, strengthening the possibility of an as yet unidentified autoantigen involved in the autoimmune process. Recently, a 51 kDa antigen from the placenta and the granulosa cells has been shown to react with sera from the majority of AD patients positive for StCA-IIF (31). Although the ACA-IIF titers found in these patients were not in the lower range (1:16 and 1:32), the possibility of a false positive result cannot be excluded, since this is a semiquantitative method influenced by the presence of background staining, lipemia and quality of the adrenal cortex tissue (23). Among AD patients positive for SCCab and/or 17OHAb, discrepant results between StCA-IIF and SCCab and/or 17OHAb could also be due to the differences in the sensitivity and/or specificity of the assays.

In the two ACA-IIF negative-21OHAb positive AD patients, the use of radiolabeled antigen in the radio-binding assay allowed a more sensitive autoantibody detection system (30). On the other hand, the use of bovine instead of human adrenal tissue in the IIF may also explain the discrepant results seen in these patients. Differences in sequences of amino acids in the epitope regions of the human and bovine 21OH have been described (32), making it plausible that two populations of antibodies may coexist: one dependent on and one independent of the amino acids of human 21OH for binding (32, 33).

Since 94% of the idiopathic AD patients were positive for ACA-IIF and/or 21OHAb, their measurement should be the first step in the assessment of patients at risk for adrenal autoimmunity, which is in line with the observations reported by Laureti et al. (34). According to Chen et al., assays for SCCab and 17OHAb should then be carried out in ACA-IIF positive-21OHAb negative patients and/or to assess the extent of adrenal and gonadal involvement (8). However, one of our idiopathic AD patients was positive only for 17OHAb, probably resulting in her low levels of antibodies not being detected by ACA-IIF and StCA-IIF.

Differently from 21OH, which is restricted to the adrenal cortex, SCC and 17OH are shared between the gonads and the adrenal glands and can be associated with premature ovarian failure (35). In our study, half (2/4) of the SCCab positive AD women had premature ovarian failure. We also observed an association between SCCab positivity and premature ovarian failure when idiopathic AD and APSIII patients were analyzed altogether.

The specificity of ACA-IIF and 21OHAb for the diagnosis of autoimmune AD was 91%, since none of the healthy control subjects and only one granulomatous
AD patient was positive for both antigens (and also for SCCAb). One possible explanation for the presence of adrenal autoantibodies in one granulomatous AD patient is the presentation to the immune system of adrenal autoantigens resulting from inflammatory destruction of the adrenal cortex and subsequent autoantibody production in a genetically predisposed subject. Alternatively, one cannot rule out the possibility that a granulomatous adrenalitis supervened in a patient with an already ongoing autoimmune process (23).

Only one healthy control was SCCAb positive; this individual was SiCA-IIF negative. Autoantibodies against steroidogenic enzymes can be found in approximately 2% of normal subjects (9, 12). SCCAb seldom herald testicular failure in men, probably because the blood-testis barrier offers testicular protection (31). No relationship was observed between the presence of ACA-IIF or 21OHAb and APSII, which is in accordance with the results of a previous study (9). However, a significant relationship was found between the presence of SCCAb and APSII. Seissler et al. (36) have also found a higher prevalence of SCCAb (and also 17OHAb) in the sera of patients with APSII, as compared with patients with isolated AD, and suggested that the presence of these autoantibodies may point to the coexistence of or progression towards polyglandular autoimmune.

Detection of ACA-IIF and 21OHAb can accurately identify individuals at high risk for AD due to the long subclinical period marked only by the presence of circulating adrenal autoantibodies. This is particularly important in management of adult patients with endocrine autoimmune diseases often associated with AD, such as Hashimoto’s thyroiditis, Graves’ disease, IDDM and POF. In our study, ACA-IIF were detected in 2/18 (11%) patients with APSII, which is in accordance with the reported prevalence of 0.2% to 13.5% seen in autoimmune endocrinopathies (17, 37, 38).

One of our APSIII patients positive for ACA-IIF, 21OHAb, SiCA-IIF and SCCAb maintains, five years after the initial autoantibody detection, normal plasma levels of ACTH (14 pg/ml) and a normal cortisol response to ACTH stimulation (26.6 μg/dl); plasma renin activity is elevated (12.4 ng/ml/h) while she is on angiotensin converting enzyme (ACE) inhibitor therapy to reduce microalbuminuria. The other ACA-IIF positive APSIII patient initially had a subnormal cortisol (12.7 μg/dl) response to ACTH stimulation, which normalized (33 μg/dl) following treatment with prednisone. Although spontaneous remission of subclinical adrenocortical failure can occur in patients with low levels of ACA-IIF (18, 37), one cannot exclude the role of glucocorticoids in interrupting the autoimmune process (39, 40).

It has been proposed that ACA-IIF and/or 21OHAb positive patients without overt hypoadrenalism should have an ACTH test on a yearly basis to identify progression to adrenal failure (17); substitutive therapy should then be instituted at the first stage of impaired adrenal function in order to prevent acute adrenal crisis. In addition, treatment with glucocorticoids can downregulate the expression of autoantigens and prevent the destruction of unaffected adrenocortical cells, postponing the onset of clinical manifestation (39, 40).

In summary, in Brazilian AD patients of the present study, the autoimmune etiology was responsible for 66% of cases, and 21OH was shown to be the major autoantigen in isolated AD and in APSII. Thus, measurement of 21OHAb should be the first step in immune assessment of patients with AD and individuals at risk for adrenal autoimmunity, in addition to ACA-IIF. Due to their low prevalence in AD, measurement of SCCAb and 17OHAb should be indicated only for 21OHAb negative patients and/or for those with premature ovarian failure, especially but not exclusively in ACA-IIF positive patients.

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