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Autoantibodies to Steroidogenic Enzymes in Autoimmune Polyglandular Syndrome, Addison's Disease, and Premature Ovarian Failure

SHU CHEN, JOANNA SAWICKA, CORRADO BETTERLE, MIKE POWELL, LOUISE PRENTICE, MARINA VOLPATO, BERNARD REES SMITH, AND JADWIGA FURMANIAK

FIRS Laboratories, RSR Ltd. (S.C., J.S., M.P., L.P., B.R.S., J.F.), Llanishen, Cardiff, Wales, United Kingdom CF4 5DU; and Istituto di Semeiotica Medica, University of Padua (C.B., M.V.), Padua, Italy; the Department of Medicine, University of Wales College of Medicine (B.R.S., J.F.), Heath Park, Cardiff, Wales, United Kingdom CI4 4XN

ABSTRACT

Autoantibodies to steroidogenic enzymes, steroid 17α-hydroxylase (17α-OH), cytochrome P450 side-chain cleavage enzyme (P450scc), and steroid 21-hydroxylase (21-OH), were measured using specific and sensitive immunoprecipitation assays (IPAs) in patients with various forms of autoimmune adrenal disease. Autoantibodies to 17α-OH were detected in 6 of 11 (55%) patients with autoimmune polyglandular syndrome (APS) type I, 8 of 24 (33%) patients with APS type II, 11 of 56 (20%) patients with adrenal cortex antibody (ACA; measured by immunofluorescence)-positive patients without Addison's disease, and only 3 of 64 (5%) patients with Addison's disease. Autoantibodies to P450scc were found at a prevalence similar to those to 17α-OH: in 5 of 11 (45%) APS type I patients, 10 of 24 (42%) APS type II patients, 11 of 56 (20%) ACA-positive patients without Addison's disease, and only 6 of 64 (9%) patients of the Addison disease group. Autoantibodies to 21-OH were found in a majority of patients with APS type I (7 of 11; 64%), APS type II (23 of 24; 96%), Addison's disease (41 of 64; 64%), and ACA-positive patients without Addison's disease (48 of 56; 86%). All sera that were positive for 17α-OH or P450scc were also positive for 21-OH autoantibodies, except in 1 case. There was good agreement between the presence of ACA measured by immunofluorescence and 21-OH antibodies measured by IPA in all patient groups studied, and this indicates that 21-OH is a major autoantigen in adrenal autoimmune disease regardless of whether the disease presents as isolated Addison's disease or APS type I or type II. Autoantibodies to 17α-OH and P450scc appeared to be the major components of the steroid-producing cell antibodies measured by immunofluorescence. No autoantibodies to 21-OH, 17α-OH, or P450scc were detected in 17 sera from patients with premature ovarian failure without evidence of adrenal autoimmunity (as judged by immunofluorescence studies), except for 1 serum in which low levels of 17α-OH antibodies were found. Overall, our studies indicate that ^{35}S-labeled 17α-OH, P450scc, and 21-OH can be used successfully in IPAs for their respective autoantibodies. Assays such as these may well be valuable in the immunological assessment of patients at risk for or suspected of adrenal autoimmunity. (*J Clin Endocrinol Metab* **81:** 1871–1876, 1996)

RECENT STUDIES have shown that steroid 21-hydroxylase (21-OH) is a major autoantigen in autoimmune Addison's disease (1–4). In addition, autoantibodies to two other steroidogenic enzymes have been described in patients with adrenal and gonadal autoimmunity; in particular, steroid 17α-hydroxylase (17α-OH) and the P450 side-chain cleavage enzyme (P450scc) (5, 6). However, there are some discrepancies between reports on the distribution of autoantibodies to 21-OH, 17α-OH, and P450scc among patients with Addison's disease and patients with autoimmune polyglandular syndrome types I and II (4, 6–9). We now describe new assays for autoantibodies to 17α-OH and P450scc based on ^{35}S-labeled autoantigens produced in an *in vitro* transcription/translation (TnT) system. These assays have been used to study autoimmunity to steroidogenic enzymes in patients with adrenal and gonadal autoimmunity.

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Address all correspondence and requests for reprints to: Dr. J. Furmaniak, FIRS Laboratories, RSR Ltd., Parc Ty Glas, Llanishen, Cardiff, Wales, United Kingdom CF4 5DU.

Materials and Methods

Sera were obtained from 1) 11 patients with APS type I (8 females and 3 males; mean age, 26 yr; range, 13–45 yr), 4 of whom had premature ovarian failure (POF); 2) 24 patients with APS type II (20 females and 4 males; mean age, 43 yr; range, 16–83 yr), 5 with POF; 3) 56 patients who were ACA positive without Addison's disease (51 females and 5 males; mean age, 36; range, 5–71 yr), 3 with POF; 4) 64 patients with Addison's disease (39 females and 25 males; mean age, 32 yr; range, 6–62 yr); and 5) 17 patients with isolated POF (mean age, 26 yr; range, 16–39 yr).

Sera were also obtained from 17 patients with autoimmune thyroid disease (AITD; 7 Graves' disease and 10 Hashimoto's thyroiditis; 14 females and 3 males; mean age, 47 yr; range, 30–85 yr), 19 patients with insulin-dependent diabetes mellitus (IDDM; 12 females and 7 males; mean age, 24 yr; range, 9–50 yr), 10 patients with myasthenia gravis (8 females and 2 males; mean age, 45 yr; range, 42–63 yr), and 28 sex- and age-matched healthy blood donors. Sera from patients with AITD were highly positive for thyroglobulin and/or thyroid peroxidase autoantibodies, and sera from Graves' patients were positive for TSH receptor autoantibodies. All IDDM patients were positive for autoantibodies to glutamic acid decarboxylase (GAD_{65}), and patients with myasthenia gravis were all positive for acetylcholine receptor autoantibodies.

The autoantibodies specified in the above patient groups were measured with RIA kits available from RSR (Cardiff, UK). Disease diagnosis was based on clinical, immunological, and biochemical grounds.

Immunofluorescence (IF) studies

ACA were detected by a classical indirect IF technique, using thin cryostatic sections of normal bovine adrenal gland as the source of antigen and fluorescein isothiocyanate-conjugated goat antihuman IgG, as previously described (10, 11). Sera were tested undiluted, and if positive, ACA titers were determined by retesting sera in serial 2-fold dilutions until reaching the end point.

Steroid cell autoantibodies (StCA) were tested using cryostat sections of normal human ovary and testis by an indirect complement fixation test, as described previously (12).

Production of ^{35}S-labeled autoantigens and immunoprecipitation assay (IPA)

^{35}S-labeled 17α-OH, P450scc, and 21-OH were prepared using an *in vitro* transcription/translation reaction, as described previously (4, 13), and analyzed by SDS-PAGE (4). The IPAs were carried out using the method of Colls *et al.* (4), with 20 μL serum for the 17α-OH and P450scc autoantibody assay.

In the case of 17α-OH and P450scc autoantibodies, the results were expressed as arbitrarily defined units (see *Results*) and 21-OH autoantibody levels as an index (4).

Rabbit antibodies (Ab) specific for 21-OH (from FIRS Laboratories, RSR Ltd.), 17α-OH (from Dr. M. Waterman, Nashville, TN), and P450scc (from Dr. Bon-Chu Chung, Taipei, Taiwan) were used in some experiments.

Results

Preliminary studies with ^{35}S-labeled 17α-OH preparations and a selection of sera from patients with adrenal autoimmunity showed that some sera could precipitate up to 35% of the ^{35}S-labeled material. In the case of 28 individual healthy blood donors, a mean (±SD) value of 3.7 ± 0.5% binding was observed, with a range of 2.7–5.1%. One positive serum was selected to prepare a standard curve based on arbitrary units. One unit per mL (serum dilution, 1:1024) immunoprecipitated about 7% of the [^{35}S]17α-OH preparations. About 12% of the labeled preparation was precipitated by 64 U/mL (serum dilution, 1:16). Studies with the 28 healthy blood donors gave a mean (±SD) of 0.045 ± 0.12 U/mL, and this suggested a lower detection limit (mean ± 3 SD) of approximately 1 U/mL. Using this criterion, 17α-OH Ab could be detected in 6 of 11 (55%) patients with APS type I, 8 of 24 (33%) patients with APS type II, 3 of 64 (5%) patients with Addison's disease, 11 of 56 (20%) ACA-positive patients without Addison's disease, and 1 of 17 (6%) patients with isolated POF (Table 1). Individual values for 17α-OH in the different patient groups are shown in Fig. 1A.

Studies similar to those with 17α-OH were carried out using ^{35}S-labeled P450scc. Some sera from patients with autoimmune adrenal disease bound more than 50% of the ^{35}S-labeled P450scc preparations. With 20 individual healthy blood donor sera, a mean ± SD of 4.7 ± 0.77% binding was observed, with a range of 3.5–6.2%. One positive serum was used to prepare a P450scc autoantibody standard curve based on arbitrary units in a manner similar to the studies with 17α-OH Ab. One unit per mL (serum dilution, 1:64) immunoprecipitated about 9% of the [^{35}S]P450scc preparations, and 32 U/mL (serum dilution, 1:2) immunoprecipitated about 18%. Studies with the 20 individual healthy blood donor sera gave a mean ± SD of 0.17 ± 0.1 U/mL, suggesting a lower detection limit of about 1 U/mL.

As summarized in Table 1, autoantibodies to P450scc were detected by IPA in sera from 5 of 11 (45%) APS type I patients, 10 of 24 (42%) APS type II patients, 6 of 64 (9%) Addison's disease patients, and 11 of 56 (20%) ACA-positive patients without Addison's disease. None of the isolated 17 POF sera was positive for P450scc autoantibodies. Individual serum results for the different patient groups are shown in Fig. 1B.

TABLE 1. Autoantibodies in different patient groups

Group	ACA	21-OH	StCA	17α-OH	P450scc
APS I	8/11 (73)	7/11 (64)	5/11 (45)	6/11 (55)	5/11 (45)
APS II[a]	21/24 (87)	23/24 (96)	8/22 (36)	8/24 (33)	10/24 (42)
ACA(+)[b]	56/56 (100)	48/56 (86)	12/56 (21)	11/56 (20)	11/56 (20)
Addison[c]	14/17 (82)	41/64 (64)	1/17 (6)	3/64 (5)	6/64 (9)
Isolated POF	0/17	0/17	0/17	1/17 (6)	0/17

Percentages are given in *parentheses*.
[a] StCA data were only available for 22 of 24 APS II sera.
[b] ACA-positive patients without Addison's disease.
[c] Immunofluorescence data (ACA and StCA) were only available for 17 out of the 64 Addison sera studied. Of the 17 sera, 13 (76%) were positive for 21-OH autoantibodies.

Table 1 and Fig. 1C show the results of 21-OH autoantibody measurements in the different patient groups. These Ab were detected in 7 of 11 (64%) patients with APS type I, 23 of 24 (96%) patients with APS type II, 41 of 64 (64%) patients with Addison's disease, 48 of 56 (86%) patients who were ACA positive without Addison's disease, and 0 of 17 patients with POF.

There was good agreement between ACA results by IF and 21-OH Ab measured by immunoprecipitation assay in the 108 patients with APS types I and II, Addison's disease patients, and the group of ACA-positive patients without Addison's disease (Table 2). Of the 108 sera, 88 were positive for both ACA and 21-OH Ab, 7 were negative for both ACA and 21-OH Ab, 10 were positive for ACA but negative by IPA, and only 3 sera were positive by IPA but negative for ACA by IF (Table 2).

In terms of disease in young patients, 4 patients with APS type I, 1 with APS type II, 3 from the group of ACA positive patients without Addison's disease, and 5 from the isolated Addison's disease group (13 patients in total) were 16 yr old or younger. Of these 13 patients, 11 (85%) were positive for 21-OH Ab, 3 (23%) for 17α-OH Ab, and 2 (15%) for P450scc Ab.

There was good agreement between positive StCA by IF and 17α-OH Ab and/or P450scc Ab detected by immunoprecipitation assay (Table 3). In a study of 26 StCA-positive sera from patients with APS types I and II or Addison's disease or ACA-positive patients without Addison's disease, 24 were positive for either 17α-OH or P450scc autoantibodies (16 sera were positive for autoantibodies to 17α-OH, 17 sera were positive for P450scc autoantibodies, and 9 sera were positive for both 17α-OH and P450scc Ab; Table 3). All StCA-positive sera were also positive for ACA by IF and for 21-OH Ab by IPA, except 1 serum (no. 2 in Table 3) that was borderline positive by IPA (2.2 U/mL), but the presence of 21-OH Ab could be demonstrated in this serum by Western blotting (4).

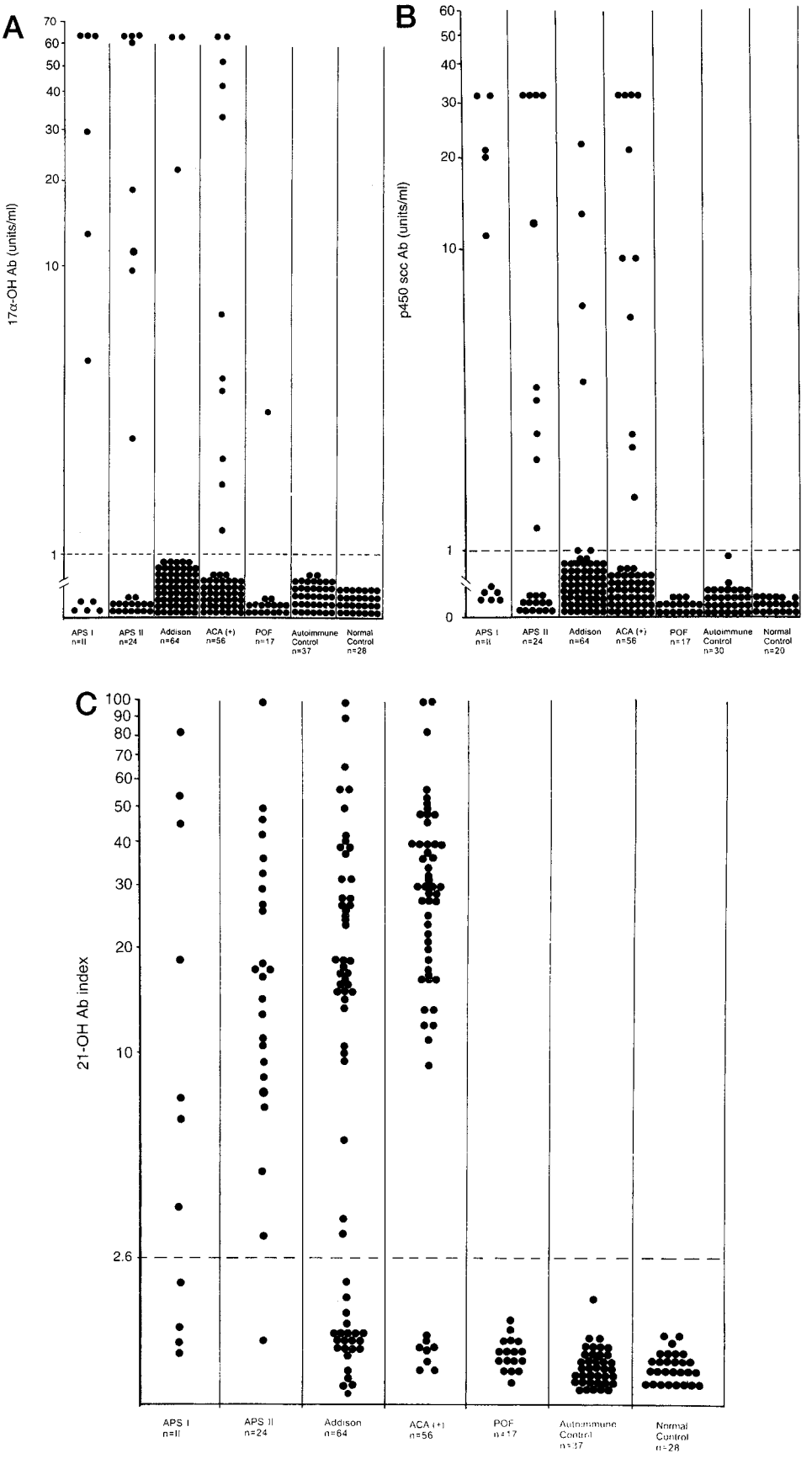

FIG. 1. Autoantibodies to 17α-OH, P450scc, and 21-OH measured by IPA in different patient groups. ACA(+), Sera from patients positive for adrenal cortex Ab (ACA) but without Addison's disease. Normal control, Sera from healthy blood donors. POF, Patients with POF without evidence of adrenal autoantibodies. *Broken horizontal lines* show the limit of detection. Full details of the different patient groups are given in the text. A, Autoantibodies to 17α-OH. The autoimmune control group consisted of sera from 10 patients with IDDM, 10 with myasthenia gravis, and 17 with AITD. B, Autoantibodies to P450scc. The autoimmune control group consisted of sera from 10 patients with IDDM, 10 with myasthenia gravis, and 10 with AITD. C, Autoantibodies to 21-OH. The autoimmune control group is the same as that in A.

TABLE 2. ACA determined by IF and 21-OH Ab determined by IPA in 108 sera from patients with APS types I and II, Addison's disease patients and ACA-positive patients without Addison's disease

	ACA IF (+)	ACA IF (−)
21-OH IPA (+)	88	3
21-OH IPA (−)	10	7

None of the control sera from patients with AITD, IDDM, or myasthenia gravis was positive for 21-OH, 17α-OH, or P450scc autoantibodies (Fig. 1).

Analysis of TnT produced ^{35}S-labeled 21-OH, 17α-OH, and P450scc by SDS-PAGE followed by autoradiography showed that these three antigens (which ran as 55-kDa bands) were major ^{35}S-labeled components of their respective reaction mixtures (data not shown).

Rabbit Ab to 21-OH, 17α-OH, and P450scc (diluted 1:100) precipitated about 50% of their respective ^{35}S-labeled antigen (data not shown). The individual rabbit antisera did not cross-react with each other, *i.e.* 21-OH antibody did not react with 17α-OH or P450scc *etc.*

Additional specificity data are given in Table 3, where it can be seen that some sera positive for 21-OH Ab were negative for 17α-OH Ab and/or P450scc Ab. Also, some 17α-OH-positive sera were negative for P450scc Ab and *vice versa*. Furthermore, in the 17α-OH IPA, the addition of increasing amounts of unlabeled 17α-OH (produced in the TnT reaction) resulted in a dose-dependent inhibition of binding of [^{35}S]17α-OH to 17α-OH autoantibodies. Similarly, the addition of unlabeled P450scc (also produced in the TnT reaction) inhibited in a dose-dependent manner the binding of [^{35}S]P450scc to autoantibodies in the IPA (data not shown).

Discussion

Our studies indicate that 17α-OH Ab occur relatively frequently in patients with APS types I and II and ACA-positive patients without Addison's disease (55%, 33%, and 20%, respectively), whereas they occur infrequently in Addison's disease (5%). Only 1 of 17 (6%) patients with POF had detectable serum 17α-OH autoantibodies, and this was in a relatively low concentration. These results can be compared with observations from other laboratories using methodologies different from our own. For example, our finding of a relatively high prevalence of 17α-OH autoantibodies in APS type I is in agreement with some studies (5, 8, 14), but other reports suggest a lower prevalence or an absence of these autoantibodies in APS type I sera (6, 7, 9).

In our IPA studies, 17α-OH autoantibodies were detected in 8 of 24 APS type II patients, and this can be compared with frequencies of 1 of 9 reported by Uibo *et al.* (8) and 0 of 15 by Song *et al.* (7). In the case of isolated Addison's disease, our finding that 17α-OH Ab occur only occasionally is in agreement with most other reports (7–9).

Using the IPA, autoantibodies to P450scc were found predominantly in patients with APS types I and II (45% and 42%, respectively). In addition, 20% of ACA-positive patients without Addison's disease were P450scc antibody positive. The occurrence of P450scc Ab in isolated Addison's disease was low (9%), and no isolated POF sera were P450scc positive. Our data can be compared with those of Uibo *et al.* (8), who reported over 50% positivity for P450scc Ab in patients with APS type I, but did not find reactivity to P450scc in any of the 9 APS type II sera and in any of the 8 sera from patients with idiopathic Addison's disease. In contrast, Winqvist *et al.* (9) detected P450scc Ab in all 7 APS type I sera studied and in nearly 70% of sera from patients with Addison's disease. However, Song *et al.* (7) did not find reactivity to P450scc in APS type I (0 of 5), APS type II (0 of 15), or Addison disease (0 of 8) sera. Our findings that P450scc Ab occur frequently in APS type I are in agreement with those of Uibo *et al.* (8) and Winqvist *et al.* (9). Winqvist *et al.*'s report (9) of a high prevalence (69%) of P450scc Ab in Addison sera as judged by Western blot analysis is not confirmed by our IPA data.

In the current IPA study, 21-OH Ab were found in most patients with APS types I and II, Addison's disease patients, and ACA-positive patients without Addison's disease; this is in agreement with our earlier report carried out on smaller patient groups (4). 21-OH Ab in APS type I were also detected in 15 of 36 sera by Uibo *et al.* (8) and in 4 of 5 sera by Song *et al.* (7). However, Winqvist *et al.* (9) were unable to detect autoantibodies to 21-OH in the group of APS type I sera (n = 7) they studied. Our finding of a high prevalence of 21-OH Ab in APS type II (96%) is similar to the results reported by Song *et al.* (7) (93%) and Uibo *et al.* (8) (78%).

Autoantibody levels can vary over the course of autoimmune disease (10, 11); therefore, discrepancies in the prevalence of 21-OH, 17α-OH, and P450scc Ab in different studies could be related to the duration of the disease. In the present study, sera were obtained from patients with different disease durations (from newly diagnosed up to several years after diagnosis); therefore, the prevalences reflect the overall occurrence. Analysis of autoantibodies to steroidogenic enzymes in patients at different stages of autoimmune adrenal disease is currently under way.

Some of the discrepancies between different reports on the occurrence of autoantibodies to 17α-OH, P450scc, and 21-OH mentioned above could be related to the different autoantigen preparations and detection methods used. In the case of 21-OH, formation of 21-OH autoantibody-binding sites appears to be dependent on cooperation between the central and the C-terminal parts of the 21-OH molecule (13, 15, 16). Consequently, fragments of 21-OH expressed in bacteria and used in Western blotting studies may not react well with 21-OH autoantibodies in some sera. A similar situation may well occur in the case of 17α-OH and P450scc antibody-binding sites, as most epitopes reactive with endocrine autoantibodies appear to be conformational (17, 18). In view of these potential problems, IPAs based on ^{35}S-labeled 17α-OH, P450scc, and 21-OH produced in the *in vitro* transcription/translation system seem to have major advantages over assays based on bacterially produced fragments. Similar assays using ^{35}S-labeled GAD_{65} have been shown to be very sensitive and specific for measuring GAD_{65} Ab (19, 20).

There was good agreement between the presence of 21-OH Ab measured by IPA and the presence of ACA measured by IF in all of the patient groups studied. Furthermore, 21-OH Ab were found in 11 of 13 ACA-positive sera from patients 16 yr old or younger. Consequently, 21-OH is a major au-

TABLE 3. Autoantibodies in StCA-positive patients

Patient no.	Diagnosis	ACA titer	21-OH index	17α-OH (U/mL)	p450scc (U/mL)	17αOH or P450scc	17α-OH and P450 scc
1	APS I	64	7.5	29.6	21	+	+
2	APS I	32	2.2	64	11.1	+	+
3	APS I	>320	82.2	Negative	>32	+	−
4	APS I	320	3.6	64	>32	+	+
5	APS I	80	54.7	>64	20	+	+
6	APS II	256	7.0	Negative	Negative	−	−
7	APS II	>320	10.5	Negative	3.5	+	−
8	APS II	>320	48.6	2.5	Negative	+	−
9	APS II	20	17.4	62.3	Negative	+	−
10	APS II	80	103.4	Negative	>32	+	−
11	APS II	80	41.8	>64	>32	+	+
12	APS II	40	12.7	>64	Negative	+	−
13	APS II	10	7.9	11.6	11.2	+	+
14	ACA (+)	512	36.5	Negative	9.1	+	−
15	ACA (+)	512	82.2	Negative	>32	+	−
16	ACA (+)	4	16.1	Negative	>32	+	−
17	ACA (+)	256	51.2	>64	Negative	+	−
18	ACA (+)	16	45.9	52.6	5.9	+	+
19	ACA (+)	64	11.9	Negative	Negative	−	−
20	ACA (+)	256	20.9	>64	>32	+	+
21	ACA (+)	320	102.2	33.8	Negative	+	−
22	ACA (+)	80	18.2	1.7	>32	+	+
23	ACA (+)	256	48	Negative	21	+	−
24	ACA (+)	640	36.5	Negative	2.4	+	−
25	ACA (+)	320	39.6	1.2	Negative	+	−
26	Addison	40	26.1	>64	Negative	+	−
Total		26(+)	26(+)	16(+)	17(+)	24(+)	9(+)

toantigen in adrenal autoimmune disease regardless of patient age or whether the disease presents as isolated Addison's disease or as APS type I or APS type II.

In the current study we have had the opportunity to study autoantibodies to the three steroidogenic enzymes (21-OH, 17α-OH, and P450scc) in a group of patients (n = 56) who were ACA positive by IF but did not have Addison's disease, although they had a range of other autoimmune diseases, including AITD, IDDM, myasthenia gravis, and vitiligo. 21-OH Ab were found in 86% of these patients, 17α-OH in 20%, and P450scc Ab in 20%. Previous studies (10) indicate that about half of the patients in this group are likely to develop autoimmune adrenal disease.

By definition, StCA detected by IF react with adrenal cortex tissue sections and other steroid hormone-producing tissues (internal theca of the follicles in the ovary, Leydig cells in the testis, and syncytiotrophoblast in the placenta) (21). Of the three steroidogenic enzymes that appear to be the autoantigens in autoimmune adrenal disease, only 21-OH is adrenal specific, 17α-OH is expressed in adrenals and gonads, and P450scc is present in adrenals, gonads, and placenta (8, 14, 21). Our studies showed good agreement between StCA measured by IF and 17α-OH Ab and/or P450scc Ab measured by IPA. This good agreement suggests that autoantibodies to 17α-OH and/or P450scc are major components of StCA activity. However, it is possible that some StCA contain autoantibodies to as yet unidentified autoantigens (9).

IF studies have indicated that StCA are usually associated with the presence of ACA (12, 21), and this is confirmed by the data shown in Table 3, where all 26 StCA-positive sera were also positive for ACA. In this group of sera, 21-OH Ab were always present when 17α-OH Ab or P450scc Ab were present. We found one APS I serum low positive (4.6 U/mL) for 17α-OH Ab but negative for 21-OH Ab and P450scc Ab (data not shown); however, overall, our results indicate that the autoantibody responses to 17α-OH and P450scc do not usually occur in the absence of an autoantibody response to 21-OH.

The 17 sera we studied from patients with isolated POF (POF in the absence of adrenal cortex autoantibodies by IF) did not contain detectable 21-OH Abs or P450scc Abs, and only 1 of the 17 showed low levels of 17α-OH Abs. Similarly, 21-OH, 17α-OH, and P450scc Ab were not found in an analysis of 7 POF sera reported by Wheatcroft *et al.* (22). Both of these observations are in agreement with IF studies which show that StCA are not usually found in patients with isolated POF (12, 21, 23, 24). Among our patients with evidence of adrenal autoimmunity by IF and/or IPA, 12 had POF. All of the 12 were 21-OH Ab positive, and 11 of 12 were 17α-OH Ab and/or P450scc Ab positive. These observations are in agreement with IF studies, which show that these types of patients are usually ACA and StCA positive (12, 23–25).

Overall, our studies indicate that 17α-OH, P450scc, and 21-OH labeled with ^{35}S in the *in vitro* transcription/translation reaction can be used successfully in IPAs for their respective autoantibodies. Measurement of these adrenal autoantibodies should be helpful in the differential diagnosis of adrenal insufficiency, particularly in view of the changing spectrum of this condition (26, 27–29). Our current studies with IPAs for adrenal and gonadal autoantibodies indicate that autoantibodies to 21-OH are almost always present when 17α-OH and/or P450scc autoantibodies can be detected, and this suggests that measurement of 21-OH Ab

should be the first step in immunological assessment of patients at risk for or suspected of adrenal autoimmunity. Assays for 17α-OH Ab and P450scc Ab could then be carried out in patients positive for 21-OH Abs to assess the extent of adrenal and gonadal autoimmunity.

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