Autoantibodies against Type I Interferons as an Additional Diagnostic Criterion for Autoimmune Polyendocrine Syndrome Type I

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Context: In autoimmune polyendocrinopathy syndrome type I (APS-I), mutations in the autoimmune regulator gene (*AIRE*) impair thymic self-tolerance induction in developing T cells. The ensuing autoimmunity particularly targets ectodermal and endocrine tissues, but chronic candidiasis usually comes first. We recently reported apparently APS-I-specific high-titer neutralizing autoantibodies against type I interferons in 100% of Finnish and Norwegian patients, mainly with two prevalent *AIRE* truncations.

Objectives: Because variability in clinical features and age at onset in APS-I frequently results in unusual presentations, we prospectively checked the diagnostic potential of anti-interferon antibodies in additional APS-I panels with other truncations or rare missense mutations and in disease controls with chronic mucocutaneous candidiasis (CMC) but without either common *AIRE* mutation.

Design: The study was designed to detect autoantibodies against interferon-α2 and interferon-ω in antiviral neutralization assays.

Setting and Patients: Patients included 14 British/Irish, 15 Sardinian, and 10 Southern Italian *AIRE*-mutant patients with APS-I; also 19 other patients with CMC, including four families with cosegregating thyroid autoimmunity.

Outcome: The diagnostic value of anti-interferon autoantibodies was assessed.

Results: We found antibodies against interferon-α2 and/or interferon-ω in all 39 APS-I patients *vs.* zero of 48 unaffected relatives and zero of 19 British/Irish CMC patients. Especially against interferon-ω, titers were nearly always high, regardless of the exact APS-I phenotype/duration or *AIRE* genotype, including 12 different *AIRE* length variants or 10 point substitutions overall (n = 174 total). Strikingly, in one family with few typical APS-I features, these antibodies cosegregated over three generations with autoimmune hypothyroidism plus a dominant-negative G228W AIRE substitution.

Conclusions: Otherwise restricted to patients with thymoma and/or myasthenia gravis, these precocious persistent antibodies show 98% or higher sensitivity and APS-I specificity and are thus a simpler diagnostic option than detecting *AIRE* mutations. **(*J Clin Endocrinol Metab* 93: 4389–4397, 2008)**

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Abbreviations: AD, Addison's disease; AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; APS-I, autoimmune polyendocrine syndrome type I; AVINA, antiviral neutralization assay; CARD, caspase recruitment domain; CC, chronic *Candida* infections; CMC, chronic mucocutaneous candidiasis; hAT, autoimmune hypothyroidism; HP, hypoparathyroidism; IFN, interferon; MG, myasthenia gravis; OMIM, Online Mendelian Inheritance in Man; SLE, systemic lupus erythematosus.

Studies on monogenic autoimmune diseases have greatly increased our understanding of normal mechanisms of self-tolerance and of its breakdown (1–4). For example, the autoimmune polyendocrine syndrome type I [APS-I: Online Mendelian Inheritance in Man (OMIM) 240300] is caused by autosomal (mostly recessive) mutations in the autoimmune regulator gene (*AIRE*) (3, 4). This highly interactive transcriptional regulator (see Fig. 1) normally governs the expression in thymic medullary epithelial cells of peripheral tissue-restricted antigens such as insulin (5); these delete potentially autoaggressive nascent T cells (2, 6, 7) and apparently select specific regulatory T cells (8, 9).

Also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), APS-I is more prevalent in Finnish, Norwegian, Sardinian, and Iranian Jewish populations. Highly variable clinically, genetically, and serologically, it typically starts in the first or second decades with chronic cutaneous and/or mucosal candidiasis (CC), followed by autoimmune hypoparathyroidism (HP) and/ or adrenocortical failure [Addison's disease (AD)] (1). Probands with any two of this triad (or relatives with just one) meet current criteria for diagnosis of APS-I. Frequent additional targets may be ectodermal (skin, nails, and teeth), endodermal (stomach, liver, and intestines), gonadal, and sometimes other endocrine glands such as the thyroid or, especially in Finland (1), the pancreatic islets. The well known autoantibodies, often to intracellular enzymes, co-occur mainly with autoimmunity against specific target organs (1, 10–12) and are not general/early markers for APS-I (12).

Increasingly, patients with prodromal APS-I or with less typical presentations are also proving to have *AIRE* mutations (1, 13–15). However, their detection can be time consuming, costly, locally unavailable, or occasionally unsuccessful, even in apparently typical patients (1). Surprisingly, we find high-titer neutralizing autoantibodies against interferon (IFN)-α (usually all its 12 molecularly distinct subtypes), and especially the approximately 60% identical IFN-ω, in 100% of Finnish and Norwegian APS-I patients with (mostly truncating) mutations detected in both *AIRE* alleles and even in some where they have not (yet) been found (16).

These antibodies seem highly specific for APS-I. Otherwise, we find them only in patients with myasthenia gravis (MG) or thymoma (20–30%) or both together (~70%), but not in numerous other autoimmune, infectious, or neoplastic diseases (17) (see Table 3). Titers in APS-I are almost always high initially and persist for decades thereafter, so they obviously have potential diagnostic value. So far, however, we have systematically tested only Nordic patients with large truncations of the 545-amino-acid AIRE protein [*e.g.* R257X in Finland; p.C322 fsX372 (c.967/979del13) in Norway], although about 50 other mutations have now been reported in *AIRE* (3, 4, 7, 18–25). Pathogenic mutations are located along its length (Fig. 1), but they show few phenotypic correlations.

We have now tested 39 additional patients with different truncations (from the United Kingdom, Ireland, and central Sardinia) (18) or rare point substitutions (from Apulia in the heel of Southern Italy) (20) plus six from the unique family 1 (from central Italy) (22) who presented mainly with autoimmune hypothyroidism (hAT; see Fig. 2) and a dominant-negative G228W substitution (23).

Despite their early onset and high prevalence in APS-I, the chronic *Candida* infections remain unexplained. Chronic mucocutaneous candidiasis (CMC) can instead occur sporadically or in families (*i.e.* without *AIRE* mutations), but its diagnosis is purely clinical; no genetic or biochemical markers are currently available. However, in some families, it shows autosomal dominant (OMIM 11458) or recessive OMIM 212050) inheritance, sometimes cosegregating with thyroid autoimmunity (27). We have tested 19 such patients for anti-IFN antibodies, partly in case they have undetected mutations in *AIRE*, its partners, or targets and partly as additional disease controls. Here we show that, despite many differences in both clinical phenotypes and *AIRE* genotypes, the anti-IFN antibodies are remarkably consistent. They correlate almost perfectly with APS-I disease, even now that we have tested more than 30% of the approximately 500 patients reported worldwide (28), and about 40% of their mutations.

Patients and Methods

Patients

With informed consent and ethics committee approval, we tested the earliest available sera from four patient panels: 1) 15 APS-I patients from Sardinia (18) (disease durations $\geq$10 yr); 2) 10 APS-I patients from Apulia, Southern Italy (20) (durations ~10 yr); 3) six patients with atypical APECED from family 1 (durations up to 32 yr) (22) (also four unaffected relatives; see Fig. 2). Family 1 has recently been rescrutinized for parathyroid and adrenal function, gastric and islet cell autoantibodies and candidiasis, and ectodermal dystrophies. A fourth group included 33 patients from a United Kingdom/Ireland study on candidiasis (Ryan, K.R., and D. Lilic, submitted), including 14 with APECED plus *AIRE* mutations and 19 with sporadic or familial CMC with or without hAT. Because most of the unusual patients had presented years previously, early samples were seldom available. The healthy controls included 21 age- and sex-matched children undergoing general anesthesia for non-

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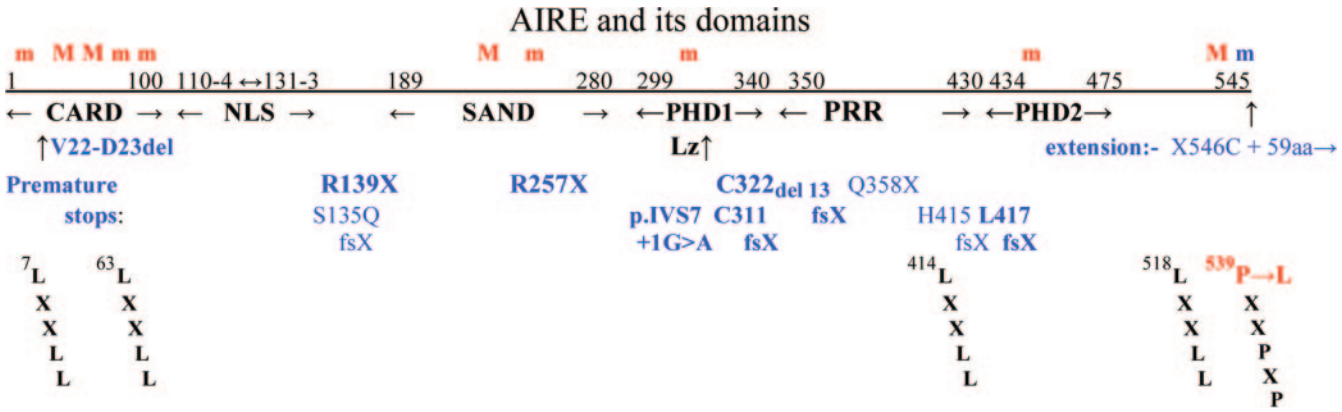

FIG. 1. The *AIRE* mutations tested and the domains and actions they affect. The point substitutions so far tested are shown in *red*: M and m, tested in dominant/homo- or heterozygous states, respectively. The length variants are in *blue*; premature stops often follow an intervening frameshift (fsX). LXXLL, Nuclear receptor-binding motifs; Lz, Leu zipper; NLS, nuclear localization signal; PHD, plant homeodomain (Zn finger-containing protein-protein interactive domains found in many transcriptional regulators); PRR, proline-rich region (found in many proteins that regulate transcription at the chromatin level); PXXPXP, important for transactivation; SAND, Sp100, AIRE, Nuc p41/75, DEAF domain, potentially DNA-binding. CARD (26) overlaps the homogeneously staining region and is thought to be crucial for AIRE homodimerization. Additional references on AIRE domains are given at the end of the supplemental data.

infectious conditions (*e.g.* for circumcisions or hernias) and 11 laboratory adults.

Antibody assays

The ELISA and antiviral neutralization (AVINA) assays have been described (16, 17). In brief, for AVINA, dilutions of patient (or control) sera were preincubated for 2 h with diluted IFN-α2 (Hoffmann-La Roche, Basel, Switzerland) or IFN-ω (Bender and Co., Vienna, Austria) (10 U/ml). Human glioblastoma cells (line 2D9) (16) were added; after an additional 24 h, they were challenged with encephalomyocarditis virus for 24 h, washed, stained with 0.05% amido blue black, fixed, and washed before absorbance was read at 620 nm. Apart from the Apulians, all sera were tested blind of their APS-I or relative status.

Results

Overall, the results in the present samples are remarkably clear (summarized in Table 1 and supplemental Fig. A, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). We found neutralizing antibodies against IFN-ω in all 39 APS-I patients with mutations in both *AIRE* alleles and in all six patients in family 1 (see Fig. 2). Titers were at least 1:4500 in all 31 Italians and at least 1:600 in the 13 from the United Kingdom/Ireland; most were much higher (Table 2), up to $1{:}4 \times 10^6$ (16). They were also high against IFN-α2 (≥1:3200) in 30 of the 31 Italians (with one exception aged 82 in family 1) and in all 14 patients from the United Kingdom/Ireland (≥7000).

We detected no antibodies against either IFN-α2 or IFN-ω (>1:60 background) in 48 unaffected Italian relatives (Table 2 and supplemental Fig. A), in 68 new disease controls with either CMC (supplemental Table A) or AD alone, or in numerous previous disease or healthy controls, numbering about 800 overall (Table 3).

Antibodies against IFN-ω were readily detected in the APS-I patients, 1) whatever their exact *AIRE* mutations, from position 8 to the stop codon (Table 1) or their (hetero)zygosity (supplemental Table B), and 2) in all the patients without the full APS-I triad (Tables 2 and 3), indeed, regardless of their exact clinical features or durations (up to 32 yr in patient III-1 in family 1; details in supplemental Tables C and D).

Evidently, the anti-IFN antibodies are not an effect of any one disease component or mutation. In our previous studies (15, 16, 25), they were also present from the earliest sampling time onward; the single exceptional patient later became very strongly positive (16). In every informative case, titers have remained consistently high for up to about 30 yr (16). Moreover, 11 of 11 Finnish patients diagnosed clinically were antibody positive when first tested (16) and subsequently proved to have mutations in both *AIRE* alleles (not shown). Thus, these antibodies seem highly disease-specific and must have significant diagnostic value (see below and Table 3).

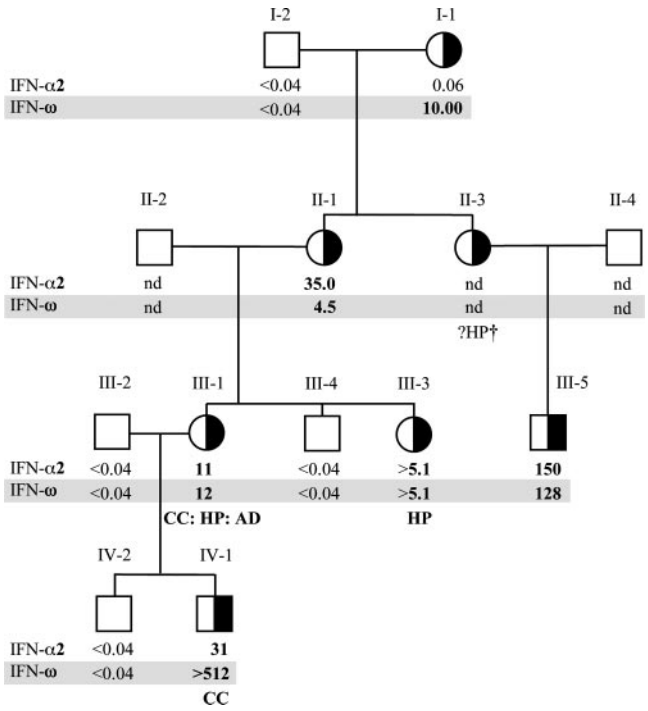

FIG. 2. Family 1 pedigree. III-1 is the proband. *Half-black circles and squares* indicate females or males with AIRE G228W mutation plus hAT (plus anti-thyroid peroxidase antibodies), which was the only manifestation in three of them. The *numbers below* indicate anti-IFN titers ($\times 10^{-3}$). †, This aunt had severe hypocalcemia in childhood and died suddenly at age 40 (22).

TABLE 1. Summary of anti-IFN antibodies and *AIRE* mutations in APS-I patients

AIRE mutations	Ref.	n	Neutralizing antibodies against IFN-α2	Neutralizing antibodies against IFN-ω
Truncations/extensions				
R139X hom		12	≥+ or –	≥++ or +
R139X/not detected		1	+++	+++
R257X hom	15, 16	57	54 ≥ +; 3[a] –	56 ≥ +; 1[a] –
C322fsX372 hom	15	10	+++ to –	100% ≥ +
p.IVS7 + 1G→A,[b] hom	15	2	+++ or –	≥++
C311fsX376 hom		1	+++	+++
R257X/H415fsX422	15	2	+++	+++
L417fsX422 hom	15	1	++	++
R257X/X546C + 59 aa	15	4	+++	+++ to ++
Totals			8/151[c] negative	1/151[c] negative initially
Missense mutations				
R8C + S135Q[d]		1	–	++
R8C + L97P		1	+++	+++
W78R hom		3	+++	+++
W78R + V22-D23del		1	+++	+++
W78R + P252L		1	+++	+++
W78R + Q358X		3	+++	+++
Y85C hom	29	1	++ (ELISA)	ND
R92W + truncations	25	2	+++ to ++	+++ to ++
G228W + wt family 1		5	+++ to +	+++ to +
Grandmother		1	–	++
C311Y + R257X		2	+ or –	++
C446G + R257X		1	–	++
P539L hom		1	++	++
Totals			5/23[c] negative	0/22[c] negative

–, ≤1:60; +, >1:120; ++, >1:10,000; +++, >1:50,000. aa, Amino acids; hom, homozygous; ND, not done; wt, wild type.

[a] One initially negative patient was strongly positive against both IFNs in both subsequent bleeds (16).

[b] Also called c.879 + 1G→A.

[c] Totals include additional compound heterozygotes omitted here for clarity.

[d] Also called c.402delC.

Do anti-IFN antibodies show any correlations with clinical features?

With autoimmune features of APS-I

Notably, antibodies against IFN-ω were clearly present in all the less typical patients, even in the 20 of 44 with only one or two of the APS-I triad (Tables 2 and 3 and supplemental Tables C and D). Indeed, titers did not correlate clearly with the numbers or durations of APS-I features.

Both the anti-IFN antibodies and the main clinical features in the newly tested APS-I patients appear broadly similar to those in Nordic series reported previously (supplemental Table D). However, hAT may be less frequent in the latter. It was reported in only about 10% of Norwegians (15) and only about 30% of Finns with APS-I (usually as a late sign) (1), whereas it was noted here in four of the 10 Southern Italians. It was also the one disorder common to all the six patients tested in family 1, only one of whom developed AD, after a 25-yr delay, and eventually, therefore, the full APS-I triad (Table 2 and Fig. 2). Nevertheless, all six had antibodies against IFN-ω (and five also against IFN-α2); these showed no clear differences in the three with CC and/or HP and the three without.

With candidiasis

All of our 14 new British and Irish APS-I patients with CC plus R257X and/or p.C322fsX372 mutations were strongly positive for anti-IFN antibodies (Table 2). Remarkably, so were all the 13 rare APS-I patients either without CC at their last assessment (n = 11) or whose CC began only in adulthood (n = 2) (supplemental Table A). They include five of the present Italian patients (four from family 1; Fig. 2) and several others reported recently (15, 25). Although titers were strongly positive against IFN-ω in all of them, they were low or negative against IFN-α2 in five of the 13 but high in the other eight (supplemental Table A).

Do anti-IFN antibodies show any correlations with *AIRE* mutations?

AIRE truncations/extensions

In fact, we found antibodies against IFN-α2 and IFN-ω in all the 34 newly tested patients with three different AIRE truncations (Table 2 and supplemental Table B). These included the Sardinians with the short R139X, even one with only one mutation detected (supplemental Table B). More surprisingly, titers were also high in the patients with the V22-D23 mini-deletion (lower Table 2) or the

TABLE 2. Anti-IFN antibodies in newly tested APS-I patients with truncations or missense mutations in both *AIRE* alleles

AIRE mutation	APS-I triad/total patients tested	Neutralizing antibodies[a]			
		Anti-IFN-α2		Anti-IFN-ω	
		No. positive/total	Titer × 10^{-3}	No. positive/total	Titer × 10^{-3}
Length variants					
Sardinia					
R139X hom	7/12	12/12	>512–14	12/12	>512–14
R139X/C322fsX372	1/2	2/2	500–5	2/2	256–180
Unaffected relatives[b]		0/33	All <0.04	0/33	All <0.04
Apulia					
C311fsX376 hom	0/1	1/1	>256	1/1	200
Unaffected relatives[b]		0/1	<0.04	0/1	<0.04
UK and Ireland					
R257X and/or C322fsX372	8/14	14/14[c]	>512–7	14/14[c]	>512–0.6
Totals	16/29	29/29		29/29	
Missense *AIRE* mutations					
Apulia					
W78R hom	2/3	3/3	>256–64	3/3	135–25
W78R/P252	1/1	1/1	128	1/1	110
W78R/V22-D23del	0/1	1/1	256	1/1	110
W78R/Q358X	3/3	3/3	>256–70	3/3	256–70
P539L hom	1/1	1/1	50	1/1	12
Unaffected relatives[b]		0/10	All <0.04	0/10	All <0.04
Rome family 1					
G228W/wt	1/6	5/6	331–0.06	6/6	1024–4.5
Unaffected relatives		0/4	All <0.04	0/4	All <0.04
Totals	8/15	14/15		15/15	
Grand totals	24/44	43/44		44/44	

p.C322fsX372 is also called c.967–979del 13. wt, Wild type.

[a] The titers of anti-IFN antibodies are listed as the highest to lowest in each group.

[b] The unaffected relatives from Sardinia were all heterozygous for *AIRE* mutations; one Apulian was V22_D23del/wt heterozygous, and three were Q358X/wt (one with non-autoimmune nodular hyperthyroidism).

[c] One 7-yr-old boy had similarly high titers despite having only CC (onset age 2 yr) of the triad.

stop codon mutation/extension (supplemental Table D). They were similar in nearly all the recently reported Nordic and U.S. cases, again regardless of their APS-I durations (supplemental Table D).

Point substitutions

Titers were also high against both IFN-α2 and IFN-ω in all nine newly tested Southern Italian patients with missense mutations (Table 2). In stark contrast, they were never above background in any of the 48 heterozygous relatives tested here (Tables 2 and 3).

This precise cooccurrence with APS-I disease is especially striking in the (nonconsanguinous) family 1 (Fig. 2 and Table 2), whose hAT is coinherited with the AIRE G228W mutation but without the APS-I triad in six of the seven cases (22). The anti-IFN antibodies show similar (dominant) cosegregation with AIRE G228W over three generations, being clearly negative in their four available unaffected relatives.

These findings are further supported by recent reports of high-titer anti-IFN antibodies in: 1) two Slovaks with one R92W plus one truncating *AIRE* mutation (25) but without CC (supplemental Tables A and D), 2) one Y85C homozygote (with the full APS-I triad) who was Iranian in origin (29), and 3) five Finnish or Norwegian patients with four other point substitutions (supplemental Table D).

Diagnostic specificity and sensitivity

Table 3 summarizes our entire experience. We have now found anti-IFN antibodies in all 174 APS-I patients tested, including at least 47 without the full triad, two with only a single *AIRE* mutation detected, and another two with none found initially who later proved to be R257X homozygous (supplemental Table B). Notably, we found no anti-IFN antibodies in three final patients, each with just two of the APS-I triad and no *AIRE* mutations detected; there are other serious doubts about their APS-I diagnoses (supplemental Table B) (15, 16, 25).

In stark contrast, these antibodies were found in none of 58 unaffected relatives in total, none of 84 single disease controls for APS-I, none of 119 healthy subjects, and only one of 733 additional controls with other infectious, autoimmune, or neoplastic diseases (Table 3). Even that one has systemic lupus erythematosus (SLE), where IFN-α is heavily implicated (31).

Therefore, apart from their well known prevalence in patients with MG and/or thymoma (16, 17), anti-IFN-ω antibodies seem

TABLE 3. Neutralizing antibodies against IFN-α2 and IFN-ω as diagnostic markers for APS-I; comparisons with disease and healthy controls

Donor group	Patient numbers tested			
	This paper	Previously (Ref.)	Anti-IFN-α2	Anti-IFN-ω
APS-I				
APS-I total	45	123 (15, 16, 25)	166/174	173/174[a]
APS-I but without full APS-I triad	20	≥27 (15, 16, 25)	≥43/47	47/47
APS-I with <2 *AIRE* mutations detected initially	1	6 (15, 16, 25)	4/7	4/7
Controls				
Healthy controls	49 (supplemental Table A)	70 (17)	0/119	0/119
Unaffected APS-I relatives	48 (Table 2)	10 (16)	0/58	0/58
Subtotals			0/177	0/177
Disease controls				
APS-2	27	9 (16, 25)	0/36	0/36
AD alone	49	11 (16, 25)	0/60	0/60
HP alone		2 (16)	0/2	0/2
CMC alone ± hAT	19 (Supplemental Table A)	3 (16)	0/22	0/22
IDDM		(17)	0/29	0/29
Thyroid autoimmunity		(17)	0/25	0/25
Pemphigoid		(17)	0/67	0/67
Di George syndrome	48		0/48	0/48
Neurological		(17)	0/124	0/124
Neoplastic		(17)	0/70	0/70
Postviral		(17)	0/94	ND
Rheumatological				
OA, giant cell arteritis, polymyalgia rheumatica, RA		(30)	0/70	0/70
SLE			1/50[b]	0/50
Subtotals			1/695	0/601
Grand totals			1/874	0/780
Sensitivity			95.4%, 166/174[a]	99.4%, 173/174[a]
Specificity			99.9%, 873/874	100%, 780/780
Predictive values				
Positive			99.4%, 166/167	100%,173/173
Negative			99.1%, 873/881	99.9%, 780/781

IDDM, Isulin-dependent diabetes mellitus; ND, not done; OA, osteoarthritis; RA, rheumatoid arthritis.

[a] In Ref. 16, just one *AIRE*-mutant patient (E) initially tested negative against both IFN-α2 and IFN-ω, but she was strongly positive in both subsequent samples (for unknown reasons). Because the present paper focuses on early diagnosis, we have classed her as negative because of her first sample.

[b] This patient had neutralizing titers (1/3,500 to 1/10,000) against IFN-α2 in her first and later samples (over 13 yr). One other SLE patient had low titers against IFN-α8 only (<1/1000) and was consistently negative against IFN-α2 over about 10 yr. We never detected neutralizing antibodies against IFN-ω in any sample from either patient.

tightly restricted to APS-I, where their sensitivity, specificity, and predictive values exceed 98% (Table 3).

Technical caveats

Testing against IFN-ω and IFN-α2

In the 174 APS-I cases, we noted eight exceptions where titers against IFN-α2 were scarcely above background in the first sample (Tables 2 and 3), although they rose subsequently in at least two (16). Therefore, we favor also testing against IFN-ω, which nearly always revealed substantial titers. The lowest noted here were 1:1500 *vs.* IFN-ω and 1:200 *vs.* IFN-α2 or 1:600 and 1:7000 (Table 2 and supplemental Fig. A). Overall, these two antibodies correlated only modestly in the present samples ($r = 0.41$).

Binding vs. *neutralizing assays*

We have also tested 143 of the 174 APS-I samples by ELISA against IFN-α2 and 58 by RIA against *in vitro* translated IFN-ω (43). In general, sera with high (or low) titers in one assay have high (or low) titers in the other. However, a few exceptional sera with moderate neutralizing titers (~1:45,000) (especially from MG patients) have given only weak ELISA signals (~20% saturating). These latter overlap with the values in occasional control or postviral samples (Meager, A., unpublished) (17).

With their consistently low backgrounds (≤1:120), neutralization assays highlight the autoantibodies in APS-I or MG/thymoma patients more starkly than binding assays. Alternative neutralization tests based on IFN-inducible proteins, *e.g.* MxA, are now available for these autoantibodies (32, 33); some of them are simpler than AVINA. Such tests are sensitive, robust, and straightforward and should be readily applicable in routine diagnostic laboratories. They are also quicker, simpler, and cheaper and may yield fewer false negatives (supplemental Table B) than defining mutations in *AIRE*, among which many laboratories screen only for the commoner ones.

Discussion

We summarize here an almost perfect correlation between autoantibodies to IFN-ω and APS-I-type disease in a grand total of 174 patients with 12 different *AIRE* length variants and 10 point substitutions scattered from position 8 to the stop codon. The correlation applies equally to patients from different Italian regions (with particularly heterogeneous mutations), Ireland and United Kingdom (this report), Finland (16), Norway (15), Slovakia (25), and the United States (29, 34). Together, these represent around one third of all the cases, and nearly 40% of the mutations, so far reported worldwide (28).

Anti-IFN antibodies as diagnostic markers

We found neutralizing antibodies against IFN-ω in all the 174 APS-I patients we have tested, whether they had the full APS-I triad or not and whatever their clinical phenotypes or *AIRE* genotypes. We also found antibodies against IFN-α2 in 166 of them. Not only were they clearly positive at or near diagnosis (with only one exception) (16), even at age 3.3 yr, but also in every subsequent sample (up to 32 yr later).

Clearly, therefore, these antibodies are highly specific for APS-I. They are also much more omnipresent than any others so far reported (10–12). These appear later, may be more transitory, correlate more with the particular organs targeted, and so have lower overall sensitivities (10–12).

We propose that the AVINA, a sensitive biological assay for neutralizing anti-IFN antibodies, be considered a front-line diagnostic test for APS-I. These antibodies can be detected much more simply, quickly, and cheaply than *AIRE* mutations (especially in regions where these are more heterogeneous than in Finland) and apparently with fewer false negatives. Their greatest practical value will probably be in identifying prodromal or atypical cases (1, 13–15), which may pose significant clinical challenges. Especially in children, *e.g.* presenting with isolated CC, early diagnosis can alert physicians to the potential for fatal HP demanding lifesaving monitoring and treatment.

We therefore propose that patients with just one of the APS-I triad could be assigned a provisional diagnosis of APS-I if they test positive for anti-IFN antibodies, subject to subsequent clinical evolution and/or identification of *AIRE* mutations.

Thus, the initial criteria might be any two of: 1) CC, 2) HP, 3) AD, or 4) *AIRE* mutations or anti-IFN antibodies (Fig. 3).

Additionally, we recommend testing for these antibodies in young patients with other atypical presentations of APS-I such as unexplained hepatitis, keratopathy, periodic rash with fever, chronic diarrhea, severe obstipation, or pernicious anemia.

If such a definition is formally accepted, then one should remember that these neutralizing antibodies are also common in patients with late-onset MG (after age 40) and/or thymomas. These disorders seem unlikely to be confused because: 1) they are rare in childhood/adolescence, when APS-I usually presents, and 2) endocrine autoimmunity is rare in both, and so is CMC. Otherwise, we have detected anti-IFN antibodies in only one of more than 600 disease controls, with SLE (17). Together with 161 healthy controls, these give a sensitivity and specificity of more than 98% and equally high positive and negative predictive values (Table 3).

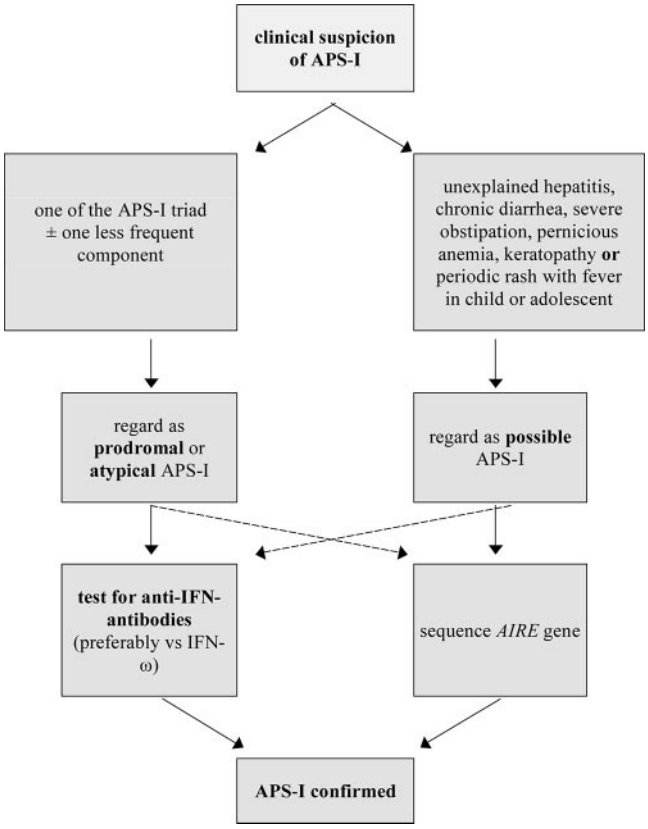

FIG. 3. Diagnostic options for atypical or prodromal APS-I.

There are two methodological caveats. Occasional APS-I patients have high-titer neutralizing antibodies against IFN-ω but not IFN-α2 (or rarely vice versa), so optimal diagnostic tests should include both types. Second, we strongly favor neutralization assays, because of their consistently low backgrounds and uniquely sensitive detection/quantification of functionally relevant neutralizing antibodies that recognize correctly folded, active IFNs. By contrast, although they may be invaluable for newly cloned autoantigens that are not easily purified in bulk, ELISAs and RIAs can also detect antibodies specific for unfolded targets (12). ELISAs are also much more error prone; because of their variable backgrounds and lower signal to noise ratios, they can detect low levels of low-affinity binding (but nonneutralizing) antibodies, *e.g.* after intercurrent infections (17).

Anti-IFN antibodies and *Candida* infections

Chronic infection is surprisingly uncommon in APS-I, despite the high levels of neutralizing anti-type I IFN antibodies. These are clearly not a general feature in CMC patients without *AIRE* mutations, whether sporadic or familial with or without hAT (supplemental Table A). Indeed, the absence of these antibodies in the present United Kingdom/Irish families with CMC argues strongly against such atypical presentations of APS-I as those in family 1.

In general, the anti-IFN antibodies in APS-I cannot be a mere side effect of *Candida* infections. They preceded the CC in every informative case (16). Titers were also high against IFN-ω in all

the 13 unusual APS-I patients who had not yet developed CC when tested, even by ages 28–82, as in family 1 (supplemental Table A). By contrast, antibodies to IFN-α were almost negative in some of the rare patients who developed CC late (in their 20s; supplemental Table A), a possible hint that, when present, they predispose to CC. Perhaps more likely, the antibodies and the CC may be parallel results of some shared defects upstream.

In patients with or without *AIRE* mutations, the immune defects selectively predisposing to *Candida* infections are unknown. Dysregulated cytokine production in response to *Candida* now seems likelier (35) than the disordered effector T cell function invoked initially (36) but seldom confirmed subsequently, either *in vivo* or *in vitro* (37). Although others argue that this dysregulation is an effect of the chronic *Candida* infections rather than their cause (2), the anti-IFN antibodies might be involved indirectly, possibly by inhibiting dendritic cell maturation and activation (44).

Genotype/phenotype/serotype correlations

Because the clinical picture in the present 15 Sardinian patients was broadly similar to that in Finns and Norwegians (A. Meloni, to be published), it seems largely unaffected by environmental factors or genetic background. However, there could be biases in Italy toward thyroid autoimmunity and possibly against insulin-dependent diabetes mellitus type 1 relative to Nordic APS-I , even though this Sardinian population is especially susceptible to sporadic type 1 diabetes.

Not only does the present study confirm the inferred heritability of this autoantibody response (16), but it also extends our previous findings to patients with both additional point substitutions and shorter truncations in the AIRE sequence, again regardless of the varied clinical pictures. These antibodies co-occurred with all the disease-causing *AIRE* mutations we tested, whatever their diverse biochemical effects, even the P252L that is the wild type in chimpanzees (38).

Figure 1 summarizes the recognized AIRE domains. Together with its LXXLL motifs and its two plant homeodomains, the recently discovered caspase recruitment domain (CARD) region at the N terminus highlights the great potential interactivity of AIRE, *e.g.* with cAMP response element binding protein-binding protein (CBP), a global *trans*-activator (26). The latter interactions may be impaired by W78R and Y85C, and likely by R92W, which are predicted to protrude from the CARD surface. Mutant W78R, G228W, and R257X products are clearly synthesized in transfected cells and retain the characteristic cytoplasmic fibrillar staining pattern; the former two are also found in nuclear dots, normally a correlate of *trans*-activation by AIRE (7, 19, 20, 26, 28; also supplemental Refs. b–d).

Moreover, G228W has clear dominant-negative, although quantitative, effects on coexpressed wild-type AIRE both *in vitro* (23) and *in vivo* in transgenic mice (39). The perfect cosegregation of these antibodies with disease manifestations and *AIRE* mutations in family 1 members argues strongly that their disorders truly are part of the APS-I spectrum and not merely coincidental. Wild-type AIRE is known to inhibit expression of some of its targets while enhancing that of others (39, 40). Possibly, the contrasting rarity of AD and 100% prevalence of hAT in family 1 might reflect such opposing effects of mutant AIRE on thymic expression of key thyroid or adrenocortical targets and/or on the resulting selection of effector or regulatory T cells. Alternatively, variant promoter sites in target autoantigen genes (as noted in MG patients) (41) might differ quantitatively in their sensitivity to residual wild-type AIRE.

Such Mendelian (dominant or recessive) inheritance of a spontaneous autoantibody response is extraordinary, especially in outbred humans (16). Its almost 100% penetrance in APS-I patients, whatever their exact mutation or clinical picture, and its early/persistent positivity suggest to us that it is a marker of some common/very early event in pathogenesis before the various outcomes diverge; these striking clues are discussed elsewhere (42).

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