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ORIGINAL ARTICLE

Serological proteome analysis reveals new specific biases in the IgM and IgG autoantibody repertoires in autoimmune polyendocrine syndrome type 1

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Abstract

Objective: Autoimmune polyendocrine syndrome type 1 (APS 1) is caused by mutations in the *AIRE* gene that induce intrathymicT-cell tolerance breakdown, which results in tissue-specific autoimmune diseases. *Design*: To evaluate the effect of a well-defined T-cell repertoire impairment on humoral self-reactive fingerprints, comparative serum self-IgG and self-IgM reactivities were analyzed using both one- and two-dimensional western blotting approaches against a broad spectrum of peripheral tissue antigens. *Methods*: Autoantibody patterns of APS 1 patients were compared with those of subjects affected by other autoimmune endocrinopathies (OAE) and healthy controls. *Results*: Using a Chi-square test, significant changes in the Ab repertoire were found when intergroup patterns were compared. A singular distortion of both serum self-IgG and self-IgM repertoires was noted in APS 1 patients. The molecular characterization of these antigenic targets was conducted using a proteomic approach. In this context, autoantibodies recognized more significantly either tissue-specific antigens, such as pancreatic amylase, pancreatic triacylglycerol lipase and pancreatic regenerating protein 1α, or widely distributed antigens, such as peroxiredoxin-2, heat shock cognate 71-kDa protein and aldose reductase. As expected, a well-defined self-reactive T-cell repertoire impairment, as described in APS 1 patients, affected the tissue-specific self-IgG repertoire. Interestingly, discriminant IgM reactivities targeting both tissue-specific and more widely expressed antigens were also specifically observed in APS 1 patients. Using recombinant targets, we observed that post translational modifications of these specific antigens impacted upon their recognition. *Conclusions*: The data suggest that T-cell-dependent but also T-cell-independent mechanisms are involved in the dynamic evolution of autoimmunity in APS 1.

Keywords

AIRE, APS 1, autoantibody repertoire, post translational modifications, T-dependent and T-independent mechanisms

History

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Introduction

Autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; OMIM ID: 601240; 240300), also known as ''Autoimmune Polyendocrine Syndrome type 1'' (APS 1), is a rare monogenic autosomal recessive disease associated with autoimmune regulator (*AIRE*) gene mutations [1,2]. The *AIRE* gene is located on chromosome 21(21q22.3) and encodes the Aire protein, which is expressed in thymic medullary epithelial cells (mTECs), but also in dendritic cells and monocytes [1] in spleen and lymph nodes [3]. Aire expresses many structural and functional characteristics common to transcriptional regulators [4]. Experimental models using Aire$^{-/-}$ animals have shown that Aire is involved in intrathymicT-cell-negative selection because it promotes ectopic expression of a subset of peripheral tissue-specific antigens (TS-Ags) by mTECs [5,6]. Loss of Aire-dependent thymic expression of a peripheral TS-Ag (such as mucin-6) results in autoimmune reactivity against this protein [7]. Aire is also expressed in extra-thymic Aire-expressing cells (eTACs), like myeloid and lymphoid cells [8], in lymph nodes and spleen, where it regulates a set of TS-Ags, suggesting that Aire expression has broad transcriptional consequences for TS-Ag presentation in the periphery. Interestingly, the genes regulated by AIRE in eTACs had no overlap with AIRE-regulated genes in the thymus, suggesting a complementary role in the maintenance of self-tolerance [3]. Aire$^{-/-}$ mice develop tissue-specific autoantibodies

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(autoAbs) and lymphocyte infiltrates in multiple organs [5, 6]. In humans, APS 1 is characterized by several tissue-specific autoimmune diseases [9] associated with organ-specific but also non-organ-specific autoAbs detected in the serum [10]. Antibodies against cytokines have also been reported [11,12]. APS 1 patients develop endocrine autoimmune diseases, such as adrenal insufficiency, hypoparathyroidism, hypogonadism, type 1 diabetes mellitus and Hashimoto thyroiditis, and non-endocrine autoimmune diseases, such as pernicious anemia, hepatitis, alopecia, vitiligo and candidiasis [9]. Thus, APS 1 represents a unique monogenic human model in which a well-demonstrated T-cell tolerance breakdown occurs that can result in several tissue-specific autoimmune diseases. Although numerous APS 1tissue-specific target antigens have been described, the global systemic self-antibody repertoire remains to be defined. We have previously performed such a global immunoproteomic approach in healthy subjects and in patients with different autoimmune diseases [13–15]. In healthy subjects, the human Ab repertoire is thought to be well conserved and restricted to a few self-antigens in homologous tissues [16]. Nevertheless, in each subject, singular patterns were found, possibly related to individual responses against exoantigens [13]. Interestingly, a distortion of serum self-IgG patterns in organ-specific autoimmune diseases that predominantly involve T cells, such as multiple sclerosis, has been demonstrated in our laboratory [13]. The intriguing aspect of these data was that discriminant reactivities were supported by widely distributed antigens [14]. To define more precisely the pathophysiological significance of these fingerprints, we evaluated, in the present study, the autoreactive antibody response in a pathology where well-defined molecular defects, related to tolerance induction processes, have been described. To evaluate T-cell-dependent and T-cell-independent involvement in APS 1, we compared self immunological patterns obtained with both IgG and IgM autoAbs and characterized the respective molecular targets recognized.

Subjects and methods

Patients

Sera from 48 patients were analyzed. The samples were obtained from 14 patients with APS 1 (group 1:9 males, 5 females, mean age = 33 ± 14 years), 17 patients with other autoimmune endocrinopathies (OAE) (group 2:6 males, 11 females, mean age = 47.6 ± 15.1 years) and 17 healthy controls (group 3:9 females, 8 males, mean age = 33.1 ± 9.2 years). APS 1 patients were clinically diagnosed according to Neufeld criteria [17] and confirmed by DNA sequencing as described previously [18]. OAE patients presented either single or multiple endocrinopathy (Table 1). Sera were collected with the subjects' written consent and the study was approved by the local ethics committee.

Anti-cytokine ELISA

Serum reactivities towards interleukin (IL)-22, IL-17A, IL-17F, IFN-omega and IFN-alpha2A (all from R and D Systems, Minneapolis, MN), were evaluated in both APS 1 and OAE patients using ELISAs, as previously reported [12], with either anti-human IgG or IgM alkaline phosphatase-conjugate (Sigma-Aldrich, Poole, UK) as the secondary antibody.

Tissues

Tissue samples were extracted from post-operative fragments with the patients' written consent or from post-mortem samples. Adrenal tissue was obtained from adrenalectomies performed during nephrectomies for kidney adenocarcinoma; none of the adrenal tissues had been invaded by tumors. Pancreas samples were obtained during postmortem dissections within 6 h of death and were performed with the approval of the local ethics committee.

Western blotting and related analytical procedures

One-dimensional electrophoresis (1-DE) or two-dimensional electrophoresis (2-DE) was performed as described in [14]. For immunostaining, the gels were blotted onto Hybond-P PVDF membranes (Amersham Pharmacia Biotech Europe GmbH, Saclay, France) using a semidry protocol (8 mA per cm^2) as in [14]. Dilution of secondary antibodies coupled to peroxidase was 1/5000 for anti-human Fcμ and 1/10 000 for anti-human Fcγ antibodies. Superimposition and alignment of antibody reactivity was performed using Diversity Database Fingerprint software (version 22; BioRad, Hercules, CA) for 1-DE and PDQuest software (BioRad, Hercules, CA) for 2-DE.

Two-dimensional electrophoresis (2-DE)

Tissue homogenization and 1-DE protein separation were done as previously described [11]. Briefly, 100 mg of each tissue was homogenized in a detergent solution (4% Triton X100, 1X anti-protease cocktail; Sigma, St Louis, MO, USA) and was ground using a grinding kit (GE Healthcare) before protein precipitation with a 2D cleanup kit (GE Healthcare). The supernatant was removed and the pellet was resuspended in 250 ml of sample buffer (8 M urea/2 M thiourea [Sigma], 4% CHAPS [Sigma]). Protein concentration was determined using the Bradford assay (BioRad, Hercules, CA). Proteins (500 mg per gel) were eluted into rehydration buffer (8 M urea/2 M thiourea [Sigma], 2% CHAPS [Sigma], DeStreak reagent [15 mg/ml, GE Healthcare] and ampholytes [1% IPG buffer, GE Healthcare]) before first separation according to their isoelectric points along a nonlinear immobilized pH-gradient (IPG) strip (pH 3–11 NL, 18 cm long) using an IPGphor III apparatus (GE Healthcare), as described elsewhere [14]. For the second dimension, equilibrated strips were loaded onto 8–18% SDS-polyacrylamide gels and electrophoresis was performed as in [19]. One preparative gel was stained with CBB G-250 (Sigma) and used for spot cutting and protein sequencing. The remaining gels were electroblotted onto ECL membranes (GE Healthcare).

In-gel digestion and MALDI-TOF/TOF MS analysis

Protein identification was performed using a Proteineer™ workflow from BrukerDaltonics (Bremen, Germany). Colloidal Coomassie blue-stained spots were excised from preparative 2D gels using a spot picker (PROTEINEER sp™)

Table 1. Main clinical characteristics of APS 1 and OAE patients. *AIRE* gene mutations are detailed in APS 1 patients.

Patient	Age (gender)	*AIRE* gene mutations	Clinical manifestations	Autoantibodies							
				TPO	TG	TBII	GAD	IA2	21 OHase	GPC	tTg
APS 1											
1	37 (F)	c.967_979del13/c.967_979del13	HPT, AI, DM, OI, PA, C, A, K				+	+	+	+	
2	28 (M)	c.1193delC/c.1193delC	HPT, AI, PA, C, M	+	+				+		
3[a]	42 (F)	c. 1097 C>T/c. 769 C>T	HPT, AI, OI, PA, C								
4	52 (M)	c.769C>T/c.967_979del13	HPT, AI, C, A, K	+							
5[b]	31 (F)	c.967_979del13/c.967_979del13	HPT, AI, OI, PA, C, A						+		
6[b]	26 (M)	c.967_979del13/c.967_979del13	C, A, K								
7	25 (M)	c.966_978del13/c.967_979del13	HPT, AI, C, A						+		
8	23 (M)	c.967_979del13/c.967_979del13	AI, DM, C, A,M				+				
9	51 (M)	c.769C>T/c.14-1-28G>C	HPT, AI, M, C				+		+		
10	15 (M)	c.967_979del13/c.967_979del13	AI, C, A, M				+				
11	9 (M)	c.769C>T/c.967_979del13	AI, C, A						+		
12	32 (M)	c.967_979del13/c.967_979del13	HPT, AI, T, A, K, C	+	+		+		+		
13	39 (F)	c.967_979del13/c.967_979del13	AI, OI, C		+						
14[a]	57 (F)	c. 1097 C>T/c. 769 C>T	HPT		+						
OAE											
15	25 (F)	ND	T, OI	+	+						
16	25 (F)	ND	DM, PA, M				+	+			+
17	60 (M)	ND	HPT, T, PA	+	+					+	
18	62 (F)	ND	T, OI, PA	+	+					+	
19	57 (F)	ND	DM, T, PA	+	+		+			+	
20	74 (F)	ND	AI, DM, T	+	+				+		
21	56 (F)	ND	DM, T, M	+	+		+	+			+
22	23 (F)	ND	T	+							
23	63 (M)	ND	AI, DM, T	+	+				+		
24	47 (F)	ND	AI, T	+	+	+			+		
25	43 (F)	ND	AI, DM, T	+	+	+					
26	54 (F)	ND	AI, T	+	+				+		
27	48 (M)	ND	DM, M								
28	35 (F)	ND	T	+	+	+					
29	61 (M)	ND	T, PA	+	+	+				+	
30[c]	39 (M)	ND	AI						+		
31[c]	37 (M)	ND	AI								

Clinical manifestations: HPT: Hypoparathyroidism; AI: Adrenal insufficiency; DM: Diabetes mellitus; T: Thyroiditis; OI: Ovarian insufficiency; PA: Pernicious anemia; M: Malabsorption; K: Keratitis; A: Alopecia; C: Candidiasis.

Specific antibodies: TPO: anti-thyroperoxidase; TG: anti-thyroglobulin; TBII: thyroid-binding inhibitory immunoglobulin; GAD: anti-glutamic acid decarboxylase 65 (GAD 65); IA2: anti-tyrosine phosphatase; 21 OHase: anti-steroid 21 hydroxylase ; GPC: anti-gastric parietal cells; tTg: anti-tissular transglutaminase.

M = male, F = female; ND = not done; [a]siblings, [b]siblings, [c]siblings.

and placed onto 96-well microtiter plates. In-gel digestion and sample preparation for MALDI-TOF/TOF analysis were performed according to the manufacturer's instructions using a digester/spotter robot (PROTEINEER dp™) and a digestion kit (DP 96 standard kit, BrukerDaltonics, Bremen, Germany). The MALDI target plate (AnchorChip™, BrukerDaltonics, Bremen, Germany) was covered with a cyanohydroxycinnamic acid (CHCA) matrix (0.3 mg/ml in acetone:ethanol, 3:6 v/v). Extracted peptides were applied directly onto the CHCA matrix thin layer. The molecular mass measurements were performed in automatic mode using FlexControl™ 22 software on an Ultraflex™ TOF/TOF instrument (BrukerDaltonics, Bremen, Germany), in the reflection mode for the MALDI-TOF peptide mass fingerprint (PMF) and in LIFT mode for the MALDI-TOF/TOF peptide fragmentation fingerprint (PFF). External calibration was performed using a peptide calibration standard kit (BrukerDaltonics, Bremen, Germany). Peak lists were generated from MS and MS/MS spectra using Flexanalysis™ 24 software (BrukerDaltonics, Bremen, Germany). Database searches using Mascot (Matrix Science Ltd., London, UK) and PMF datasets were performed via ProteinScape 13 (BrukerDaltonics, Bremen, Germany). Searches were conducted for monoisotopic peptide masses using the NCBI and Swiss-Prot protein databases and Mascot (www.matrix-science.com). Various parameters were used for database searches: mammal species, one missed cleavage, chemical partial modifications (oxidation of methionines, cysteines modified by carbamidomethylation) and a mass tolerance of 75 ppm and 0.5 Da for fragment ions. Criteria used to accept the identifications included the probability score and the number of matched peptides (minimum of six peptides).

Recombinant proteins

GST-tagged full length recombinant proteins were purchased from ABNOVA (Aachen, Germany): pancreatic amylase (AMY2A, AAH07060); pancreatic triacylglycerol lipase (PNLIP, AAH14309.1); pancreatic regenerating protein 1α (REG1A, AAH05350); aldose reductase (AKR1B1AAH00260); peroxiredoxin 2 (PRDX2, AAH00452.1); heat shock cognate 71-kDa protein (HSPA8, AAH16179). They were all produced in wheat

germ cell-free system. Five micrograms were loaded on SDS-PAGE mini-gels (Biorad, Hercules, CA), and were processed as described earlier in the western blotting procedure.

Statistical analysis

Data were expressed in binary mode (0 = absence of an antigenic band; 1 = presence of an antigenic band) to analyze IgG and IgM antibody patterns using the Chi-square test (a p value ''P''$< 5 \cdot 10^{-2}$ was judged as significant). This approach enabled us to select antigens indicative of qualitatively different immune recognition among the three groups, within groups 1 and 2 and within groups 1 and 3.

Results

Validation of serum and western blotting procedure

Anti-cytokine antibody reactivity (anti-IFN alpha2A, IFN-omega, anti-IFN-lambda1, anti-IL-17A anti-IL-17F, and anti-IL-22) was evaluated in APS 1 and OAE patients, in order to qualify the sera of the two groups (Supplemental Table 1). IgM anti-cytokine reactivity and IgG anti-IL-17A were never observed neither in OAE, nor in APS-1 patients. By contrast, IgG anti-IFN-alpha2A, anti-IFN-omega, anti-IL22 and anti-IL-17F were predominantly observed in APS 1 compared to OAE patients (90%, 80%, 70% versus 27%, 18% and 0%, respectively). Anti-IFN-lambda1 Ab (IgG or IgM) were never observed in APS 1 or OAE patients (data not shown).

To evaluate the preservation of relevant antigenic targets after the protein extraction procedure and to test the quality of pancreatic and adrenal tissues as selected targets in this present work, we first evaluated the ability of monoclonal IgG antibodies to detect representative antigenic markers of these tissues. As expected, glutamic acid decarboxylase 65 (GAD 65) and steroid 21 hydroxylase (21OHase) expression was respectively preserved in pancreatic or adrenal tissues. However, NACHT leucine-rich-repeat protein 5 (NALP 5) expression was not observed in these two tissues, in contrast to the parathyroid tissue (see Supplemental Figure 1).

To enlarge the spectrum of analysis of T-cell-independent and T-cell-dependent self-reactive Abs, the reactivity of the two isotypes IgM and IgG were respectively evaluated towards adrenal and pancreatic protein extracts. A similar analysis was preliminarily performed with sera collected in healthy subjects. As illustrated in Figure 1A, each isotype was able to recognize protein bands whose expression was shared by the two tissues (e.g. ~37 kDa for IgG; ~25 kDa for IgM; black arrows). In addition, each isotype recognized tissue-specific antigens (~50 kDa for IgG in adrenal tissue; ~63–65 kDa for IgM in pancreatic tissue; white arrows in Figure 1A and B). Moreover, a same tissue-specific antigenic band was recognized by the two isotypes (~20 kDa for adrenal tissue; ~60 kDA for pancreatic tissue; black arrows in Figure 1B).

Serum self-IgG and self-IgM reactivities restrictively observed in APS 1 patients

As shown in Figure 2, serum self-IgG and self-IgM responses against adrenal and/or pancreas protein extracts were

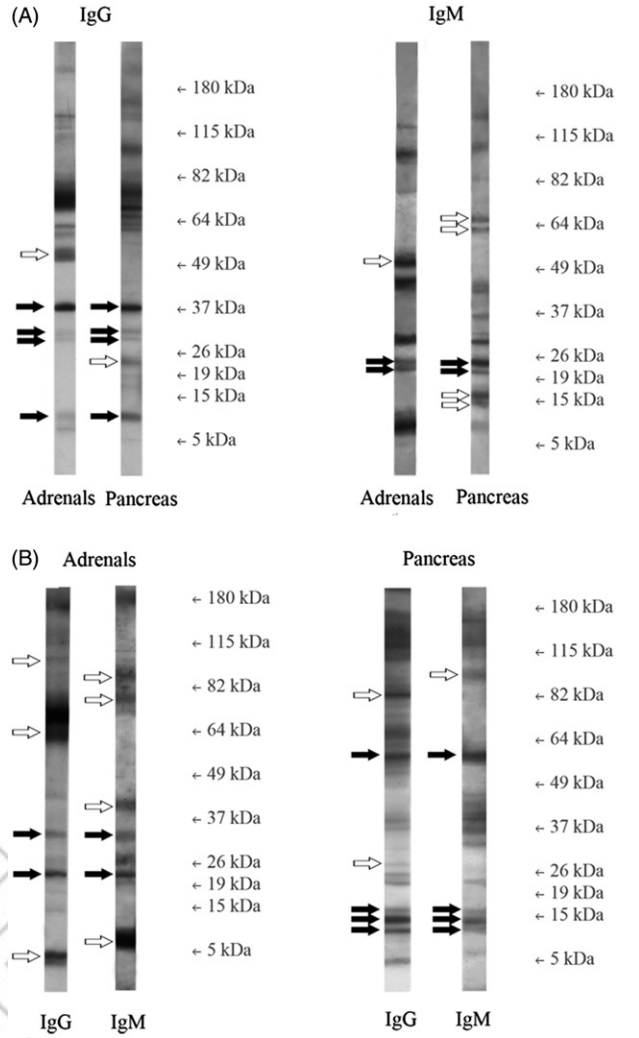

Figure 1. Detection of both IgG and IgM self reactivities against adrenal and pancreatic tissues with sera of healthy subjects. As illustrated with a representative serum collected in one healthy subject, western blotting analysis revealed detectable IgM and IgG autoreactivities against adrenal or pancreatic tissues. When we considered the antigenic targeting by IgG or IgM antibodies with regard to the tissue tested (A), we found that some IgG or IgM self reactivities were specific to a given tissue (white arrows). In contrast, other IgG or IgM Abs shared self reactivities for adrenal and pancreatic tissues (black arrows). When we considered the antigenic targeting with regard to the two Ab isotypes (B), we found that some IgG and IgM self reactivities were only observed in a given tissue (white arrows). In contrast, other IgG and IgM Abs shared self reactivities in adrenal or pancreatic tissues (black arrows).

quantitatively (numbers of bands) and qualitatively (molecular mass of recognized bands) heterogeneous within subjects, indicating that inter-individual variability occurs. Firstly, there were more antigenic bands in patients with APS 1 or OAE than in healthy controls. When we considered all the sera studied, serum self-IgM reactivity was quantitatively greater than self-IgG reactivity against both extracts, in both groups of patients. For pancreatic extracts, 82 different antigenic bands were identified in self-IgM patterns, while 56 bands were noted in self-IgG patterns ($p < 0.001$). Moreover, for adrenal extracts, 71 antigenic bands were identified in self-IgM patterns, while only 45 bands were noted for self-IgG patterns ($p < 0.001$). A similar difference was observed in all groups (APS 1, OAE and healthy subjects). In addition,

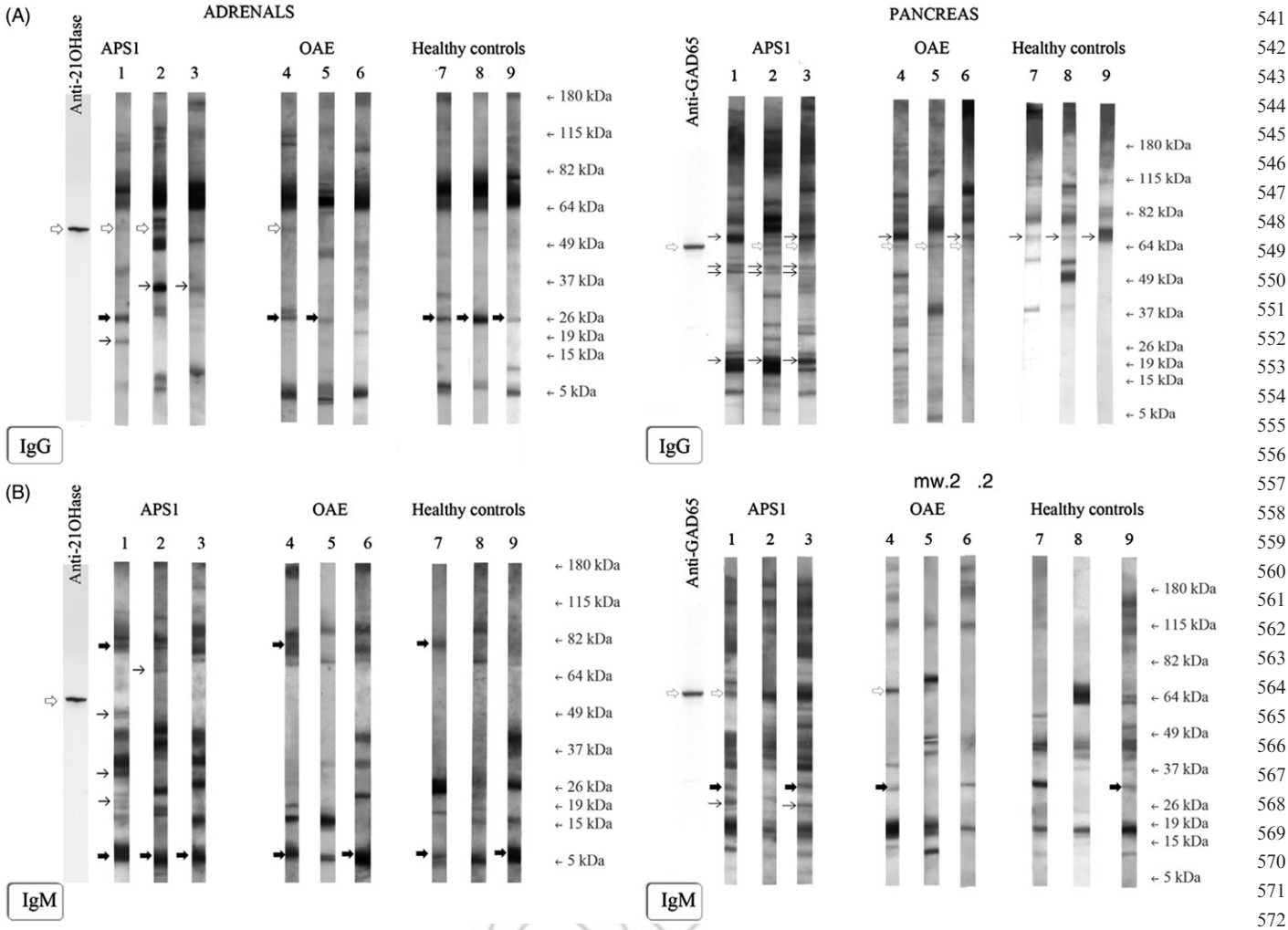

Figure 2. Comparison of IgG and IgM reactivities against adrenal and pancreatic tissues in APS 1, OAE and healthy controls. Serum self-IgG (A) and self-IgM (B) reactivities were respectively evaluated against adrenal and pancreatic tissues in APS 1, OAE patients and healthy subjects. With respect to the tissue tested, co-alignment with anti-21 OHase (adrenal) and anti-GAD 65 (pancreas) monoclonal antibodies was respectively found for some APS 1 and OAE patients (white arrows). Some IgG and IgM self reactivities were shared between APS 1, OAE and healthy controls (black arrows). In contrast, some IgG and/or IgM self reactivities were only detected in APS 1 patients (thin black arrows).

Table 2. Characterization of discriminant antigens by MS and MS/MS. Antigens preferentially recognized by APS 1 patients (χ^2 test results).

Name of antigenic band	Isotype concerned	UniProtKB/ Swiss-Prot references	Name	Theoretical mass (observed mass) (kDa)	Theoretical IP (observed IP)	MS Mascot score	MS Sequence coverage	MS/MS Mascot score	MS/MS Sequence coverage
Adrenal tissue									
Ad p66	IgM	P11142	Heat shock cognate 71-kDa protein	70.7 (66)	5.4 (5.3–5.5)	169	33.3	217.8	6.32
Ad p36	IgG	P15121	Aldose reductase	35.7 (36)	6.6 (6.1–6.9)	191	50.2	132.7	12.69
Ad p25	IgM/IgG	P32119	Peroxiredoxin-2	21.7 (25)	5.6 (5.6)	190	53.3	537.8	33.5
Pancreatic tissue									
Pc p55	IgG	P04746	Pancreatic α-amylase	57.7 (55)	6.7 (7.1)	201	45.6	338	2.77
Pc p53	IgM/IgG	P16233	Pancreatic triacylglycerol lipase	49.5 (53)	6.2 (7.0)	187	68.9	561.7	16.7
Pc p22	IgM	P05451	Pancreatic regenerating protein 1 alpha	18.7 (22)	5.6 (5.5)	120	54	365.5	30.7

some antigenic bands detected either in adrenal or in pancreatic tissues were common in all sera collected (black arrows in Figure 2A and B).

Marking of antigenic bands related to 21OHase for adrenal extracts and GAD65 for pancreatic extracts revealed a co-alignment of bands only detectable in some APS 1 and OAE patients and never detectable in healthy subjects (see Supplemental Table 2). Immune reactivity against 21OHase or GAD65 was only observed when patients presented adrenal or pancreatic autoimmune diseases. Compared to classical assays performed in routine to define specific Abs towards theses Ag, western blotting procedure is less sensitive. In spite

of the singularity found in each pattern, variabilities related to a specific group were observed. Thus, some antigenic bands were only detected on adrenal and/or on pancreatic extracts in APS 1 patients when IgG and/or IgM isotypes were evaluated. We then focused statistical analysis (i) on reactivities observed on adrenal tissue in APS 1 and OAE patients when adrenal insufficiency occurred in these two groups, and (ii) on reactivities observed on pancreatic tissue in APS 1 and OAE patients when pancreatic insufficiency occurred in these two groups. In these two situations, specific reactivities towards either adrenal or pancreatic tissues were specifically observed in APS 1 group.

The presence or absence of protein bands of reactivity was evaluated for each tissue. Computer-assisted alignment and additional statistical studies allowed us to localize singular IgG and/or IgM bands of reactivity detected in adrenals (p66, p36 and p25) and in pancreas (p55, p53 and p22) (thin black arrows in Figure 2A and B). For self-IgG patterns (see Supplemental Figure 2A), the Chi-square test identified two bands unique to adrenal tissue (Ad p36 and Ad p25) and two bands unique to pancreatic tissue (Pc p55 and Pc p53), which were significantly more often recognized by APS 1 patients, compared to OAE patients and healthy controls. For self-IgM patterns (see Supplemental Figure 2B), the Chi-square test identified two bands unique to adrenal tissue (Ad p66 and Ad p25) and two bands unique to pancreatic tissue (Pc p53 and Pc p22), which were significantly more often recognized by APS 1 patients compared to the other two groups. Thus two protein bands (Ad p25 and Pc p53) were recognized both by IgG and IgM.

Characterization of discriminant antigenic bands of reactivity

To further characterize the discriminant bands of reactivity, we used a serological proteomic approach. Identification of antigenic targets of such reactivities was first performed by comparing 1-D and 2-D immune patterns. Sera were used to identify antigenic candidates on a proteomic map obtained after 2-D electrophoresis performed for each tissue. Two-dimensional electrophoresis followed by immunoblotting revealed the presence of multiple antigenic spots for pancreatic (Figure 3) and adrenal protein extracts (Figure 4). Then, superimposition of antigenic spots and protein spots revealed by a standard colloidal Coomassie blue-stained two-dimensional gel electrophoresis enabled us to select proteins for further in-gel digestion and MALDI-TOF/TOF analysis on the basis of peptide mass matching [19].

This approach enabled us to identify some proteins as potent discriminant antigens for each tissue using the

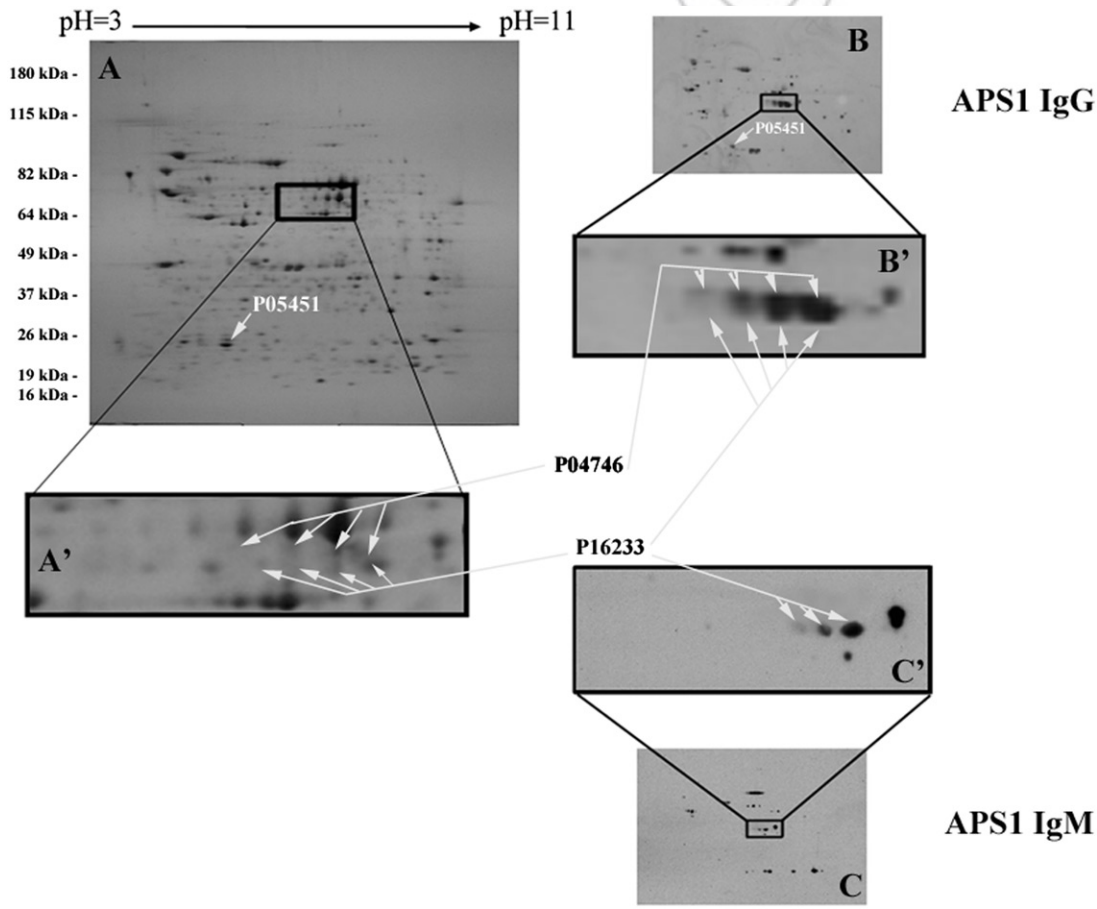

Figure 3. Immuno-proteomic characterization of pancreatic antigens recognized by IgG and IgM. Pancreatic protein extracts on colloidal Coomassie blue-stained two-dimensional electrophoresis gels (A) were compared with two-dimensional western blots (A′) of an APS 1 serum sample that was reactive against numerous bands recognized by either IgG (B and B′, magnification) or IgM (C and C′, magnification). Superimposition of the two two-dimensional patterns enabled the location of the antigenic proteins to be determined before mass spectrometry characterization. Discriminant antigenic bands were indicated with their corresponding Swiss-Prot references. Two-dimensional electrophoresis gel and two-dimensional western blot illustrations were magnified to distinguish P04746 (B′) and P16233 (C′).

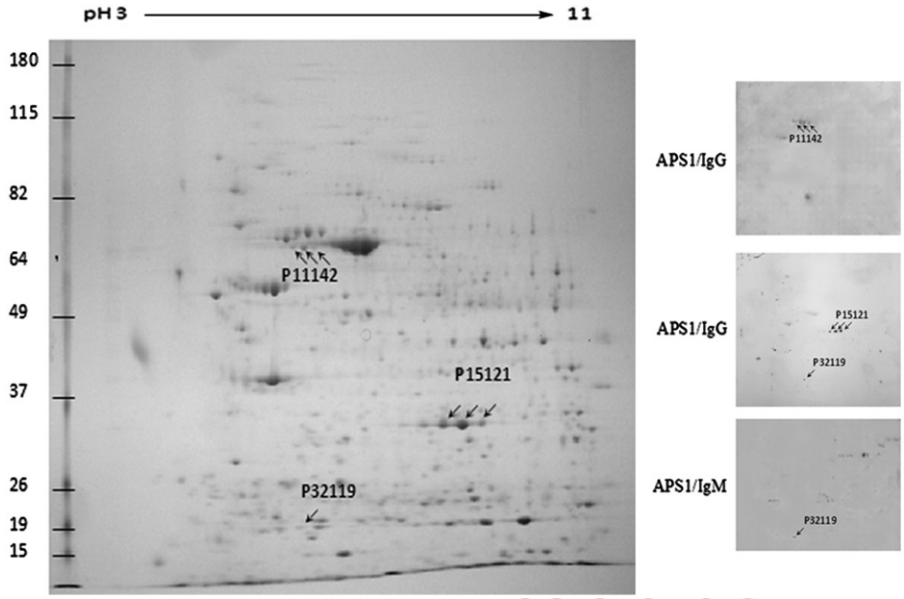

Figure 4. Immuno-proteomic characterization of adrenal antigens recognized by IgG and IgM. Adrenal protein extracts on colloidal Coomassie blue-stained two-dimensional electrophoresis gels (A) were compared with two-dimensional western blots of an APS 1 serum sample that was reactive against numerous bands recognized by IgG (at the top) or IgM (at the bottom) (B). Superimposition of the two two-dimensional patterns enabled the location of the antigenic proteins to be determined before mass spectrometry characterization. Discriminant antigenic bands were indicated with their corresponding Swiss-Prot references.

Table 3. Level of concordance of autoreactivity according to the origin of the antigens (tissue or recombinant) and to the isotype (IgG or IgM).

		Autoreactivity frequency		Concordance level	
Target	Isotype	In tissue	On recombinant protein	Presence of reactivity	Absence of reactivity
Adrenal tissue					
Heat shock cognate 71-kDa protein	IgM	50%	50%	60%	40%
Aldose reductase	IgG	40%	50%	100%	85%
Peroxiredoxine-2	IgG	50%	60%	100%	80%
	IgM	40%	40%	75%	85%
Pancreatic tissue					
Pancreatic alpha-amylase	IgG	100%	90%	90%	–
Pancreatic triacylglycerol lipase	IgG	100%	40%	40%	–
	IgM	50%	30%	30%	71%
Pancreatic regenerating protein 1 alpha	IgM	50%	0%	0%	100%

SWISS-PROT database (Table 2). The antigens identified in our study were either tissue-specific or ubiquitous proteins. Some antigens were targeted both by self-reactive IgM and IgG reactivities: one for adrenal tissue, and two for pancreatic tissue. As shown in (Figure 3 and Table 2), pancreatic (Pc) p55 was characterized as pancreatic α-amylase (P04746), Pc p53 as pancreatic triacylglycerol lipase (P16233), and Pc p22 as pancreatic regenerating protein 1α (P05451). Furthermore, adrenal (Ad) p66 was characterized as heat shock cognate 71-kDa protein (P11142), Ad p36 as aldose reductase (P15121) and Ad p25 as peroxiredoxin-2 (P32119) (Figure 4 and Table 2).

Evaluation of discriminant reactivities observed in APS 1 patients against targeted recombinant proteins

In order to strengthen the data observed on tissue-extracted proteins, we performed 1-D blotting with recombinant proteins identified by the 2-D approach. Both IgG and IgM reactivities were tested in all APS 1 sera against the six discriminant antigens.

For the IgG isotype, and except for lipase, the autoreactivity frequency was similar between tissue protein extracts and recombinant proteins (Table 3). Moreover, in terms of presence or absence of reactivity, concordance levels were higher than 80% when IgG reactivities were evaluated either on tissue extract or recombinant proteins. For the IgM isotype, whatever the frequencies observed, the concordance in terms of presence or absence of reactivity was lower than 75% (Table 3).

Discussion

In previous studies, specific serum autoAbs were usually investigated by techniques using purified self-Ags and/or relevant peptides from preselected targets [20]. We previously demonstrated the value of using a large panel of antigens derived from different tissue extracts to analyze the serum autoAb repertoire in organ-specific autoimmune disease [13,15]. Using this approach, we illustrated that the specific antibody response associated with a pathological condition could be rich and diverse and not only focalized on a restricted set of antigenic targets. Among the different reactivities, some of them could constitute a specific pathological signature of the disease. In this study, we aimed to analyze the diversity of the autoantibody repertoire in APS 1, not for diagnostic purposes, but to appreciate potential biases specifically associated with this condition. APS 1 is classically described as an Aire-mediated T-dependent disease. In this view, we performed a specific analysis of both the

IgG- and IgM-specific auto-antibody repertoires in APS 1 patients versus controls, to evaluate the specific distortion restricted to the IgG repertoire in this disease, whereas the IgM patterns were attempted not to be changed.

We have first evaluated anti-cytokine Ab reactivity in order to assess whether our patients are representative of both APS 1 and OAE patients reported elsewhere [12]. We chose then to analyze the autoAb repertoire on both adrenal and pancreatic protein extracts based on the large diversity of autoAb specificities observed in the sera of APS 1 patients and controls in these tissues. Other tissue protein extracts have been evaluated (gastric, ovarian, testis, liver, thyroid, parathyroid, skin) (not shown) and reveals less global reactivity or more homogeneous autoreactive patterns between groups of patients, as observed previously [10]. Moreover, adrenal and pancreatic tissue protein extracts contained specific antigens which have been described as being targeted by autoAb associated with clinical manifestations reported in APS 1 and OAE, such 21OHase and GAD 65. We have confirmed that, using monoclonal Abs with our western blotting procedure, we could reveal these specificities in these tissues when Abs were present in serum of individuals. As expected, we did not reveal NALP5 expression in these two tissues, whereas it was observed in the parathyroid protein extract. Thus, we could not detect anti-NALP5 autoAbs in this study.

We first evaluated the global richness of the immune repertoire. With regard to the IgM autoAb panels, we observed in all individuals a more diversified repertoire than with IgG, which could be related to the natural immune repertoire (‘*immunculus*’) that has been described as being largely composed of IgM autoAbs [21]. By contrast, we observed a more diversified IgG autoAb repertoire in patients suffering from autoimmune diseases, namely APS 1 and OAE, than in healthy subjects. This phenomenon could be related to at least two events. First, a specific defect of the educational process of the immune system leading to the persistence of autoreactive immune cells could contribute to the enlargement of this autoreactiveAb repertoire in patients. Secondly, the tissue damage associated with the autoimmune process could contribute to enlarging the panel of autoantigens that are expressed in altered tissues and presented to these immune cells. This phenomenon may itself contribute both to the preservation of autoimmune specificities and to the emergence of new autoreactiveAb specificities, generating a neo-repertoire. These two processes may also act by a summation effect.

In a second step, we analyzed the intra-individual variations of the autoreactive patterns between adrenal and pancreatic tissues. We observed that some IgM or IgG reactivities were co-aligned between the two tissues, suggesting that a cluster of widely distributed auto-antigens could be targeted by these reactivities. By contrast, some bands of reactivity were exclusively observed on either the adrenal or the pancreatic protein extracts, suggesting tissue-specific autoimmune targeting. We next studied the inter-individual variations of the autoreactive patterns independently on the adrenal or pancreatic tissues. Whereas we did not observe any difference in terms of number of bands of reactivity between APS 1 and OAE, we hypothesized that qualitative distortions could be specifically associated with the Aire-related pathological process in APS 1. This condition is described as a T-dependent autoimmune disorder which preferentially impacts the IgG autoAb repertoire. Surprisingly, in APS 1 patients compared to the two control groups, our approach demonstrated as much as specific distortions in IgM repertoire that in IgG repertoire. In our study, some IgM specificities were shared by different APS 1 patients, suggesting that the autoreactivities supported by this isotype are sustainable and not transitory reactivity brought to switch to the IgG class. Several studies have reported an extrathymic expression of Aire that influenced the T-cell repertoire [3,5]. Our data suggest that Aire expression deficiency in peripheral lymphoid organs could also impact the autoreactive IgM repertoire. Sustained IgM production by B cells has been associated with two different B-cell subpopulations in humans. It has been reported that during germinal center differentiation, follicular B cells could maturate into long-lasting IgM-expressing memory B cells through T-dependent mechanisms [22]. By contrast, T-independent mechanisms generate marginal zone B cells which produce IgM in response to non-peptidic epitopes [23,24]. Interestingly, numerous studies have focused on changes affecting B-cell homeostasis and T-cell-independent marginal zone (MZ) B-cell subsets in Aire$^{-/-}$ mice [25–28]. In addition, recent studies have underlined the fact that Aire can regulate T-cell-independent B-cell responses through B-cell-activating factor of the TNF family (BAFF) [28].

Using a serological proteomic approach, we did not observe any discriminant reactivities towards 21OHase or GAD specifically associated with APS 1 condition. These results could be related to the presence of common reactivities in the control group of patients with OAE, since both antibodies could be observed in both APS 1 and other polyendocrinopathies. By contrast, we noted that some reactivity were statistically more observed in the APS 1 group. They targeted tissue-specific antigens such as amylase, lipase and pancreatic regenerating protein 1 alpha. They also recognized three ubiquitous antigens: peroxyredoxine-2, heat shock cognate 71-kDa protein and aldose reductase. The discriminant recognition of amylase, lipase and pancreatic regenerating protein 1 alpha emphasizes pancreatic exocrine dysfunctions widely evoked either in APS 1 patients who could develop malabsorption caused by several mechanisms such as exocrine pancreatic insufficiency [29–35] or in experimental models such as NOD Aire-deficient mice [5,36].

The discriminant targeting of aldose reductase, a ubiquitous protein mainly expressed in adrenal glands [37], by IgG Abs in APS 1 patients, requires consideration. It has been shown that the expression of aldose reductase is regulated by Aire in mTECs in mice [5]. In addition, the Aire-dependent expression of other ubiquitous Ags has also been described in eTACs localized in lymph nodes and the spleen [3]. Multi-organ inflammation in Aire-deficient models is also known to be associated with the presence of serum autoAbs against proteins specifically produced by these organs. In our study, the discriminant targeting of some ubiquitous antigens, such as peroxyredoxine-2 and heat shock cognate 71-kDa protein, could be indicative of endogenous danger signals involving cellular oxidative stress. It can be compared to biomarkers

previously described in systemic autoimmune disorders [38,39].

In parallel with the combination of reactivities classically associated with APS 1 diagnosis, our data highlight some biomarkers that could be associated with a particular tissue alteration (exocrine pancreatic-specific antigens) or more general pathological processes associated with autoimmune diseases. We aimed to design an *in vitro* assay to evaluate the presence of these reactivities, using recombinant antigens. IgG reactivities towards amylase, aldose reductase and peroxyredoxine-2 were also observed in APS 1 patients when we used recombinant proteins as targets. By contrast, IgM reactivities against HSP71, REG-1A, and lipase were not concordantly observed between tissue extracts and recombinant proteins. When detected, such IgM reactivities were systematically observed at a lower frequency when we used recombinant proteins. Such a discrepancy between the immunoproteomic approach and an *in vitro* assay using recombinant targets has previously been observed [40]. To avoid the impact of folding on antigenic recognition, we chose to use the same one-dimensional electrophoresis experimental procedure. The denaturing conditions lead to the linearization of proteins, whatever their origin: tissue extracts or purified wheat germ recombinant proteins. Nevertheless, the presence of post transcriptional modifications (PTMs), such as glycosylation, on the targeted antigens could support these observations. Eukaryotic PTMs are not observed in the wheat germ expression system, so that specific modifications of native proteins are not present on the recombinant protein. Interestingly, we observed a major reactivity discrepancy when we focused on the IgM isotype. Once again, these observations could highlight the impact of the thymo-independent processes associated with the dynamic changes in the IgM repertoire in APS 1 patients.

Conventional immunoassays are usually performed with limited antigenic targets, the choice of which has been driven by a supposedly well-known physiopathogenic rationale. Advances in proteomic methodologies (*in vitro* gene expression, 2-DE and mass spectrometry) have allowed the emergence of broad spectrum analysis methods. These approaches have been developed to overcome the limits of conventional methods. Based on a ''without any *a priori*'' strategy, they offer a simultaneous analysis of a large spectrum of reactivities, which surpasses the physiopathogenic hypotheses and offers an integrative interpretation of results. When applied to the APS 1 condition, this immunoproteomic methodology not only reveals the expected IgG repertoire biases, it also identifies IgM repertoire distortions. The latter alterations could be partially associated with T-independent immunological events related to the impact of post-translational modifications of antigens. Our results highlight the fact that AIRE also impacts the presentation of thymo-independent antigens. It points out that autoimmune alterations observed in APS 1 are not only related to Aire-driven T-cell clonal deletion deficiency. At an individual level, this approach highlighted original antigenic targets, potentially associated with tissue injury and cellular dysfunctions related to the singular clinical evolution in each patient.

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Declaration of interest

Authors have no conflict of interest.

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Conceived and designed the experiments: SD, EP, DL, LP. Performed the experiments: EP, HK, AR, VLD, MB, SDB, PSW. Analyzed the data: SD, EP, DL. Contributed reagents/materials/analysis tools: EP, AR, JLW. Wrote the paper: SD, EP, DL, HK, JLW, LP.

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Supplementary material available online

Supplementary Tables I–II and Supplementary Figures 1–2

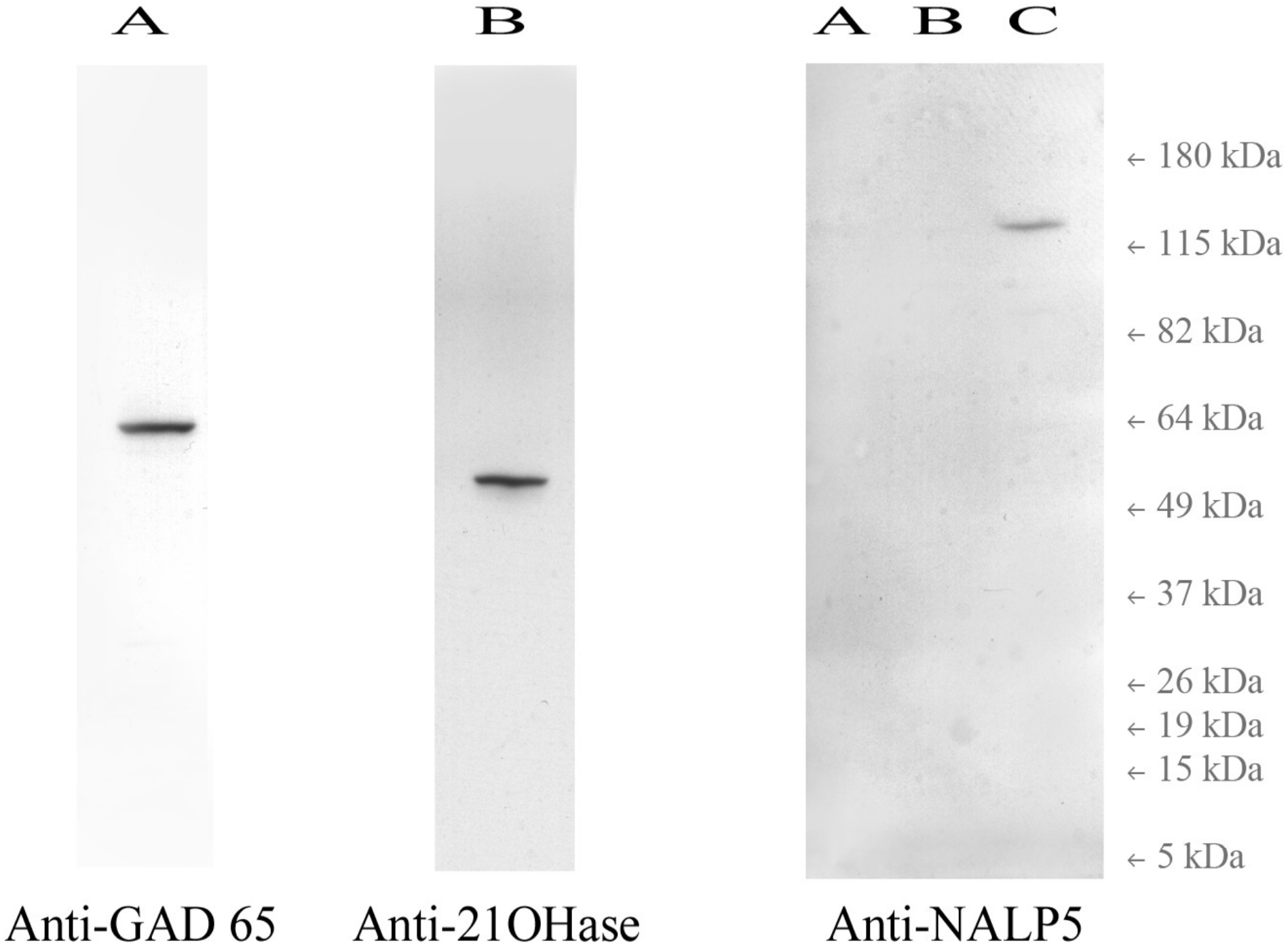
Supplemental table 1. Frequency of detection of IgG and IgM anti-IFN-alpha2A, anti-IFN –omega, anti-IL-22, anti-IL-17A and anti-IL-17F antibodies in APS 1 and OAE patients

	IFN-alpha2A		IFN –omega		IL-22		IL-17A		IL-17F	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
APS 1 patients	90%	0%	40%	0%	80%	0%	0%	0%	70%	0%
OAE patients	27%	0%	0%	0%	18%	0%	0%	0%	0%	0%

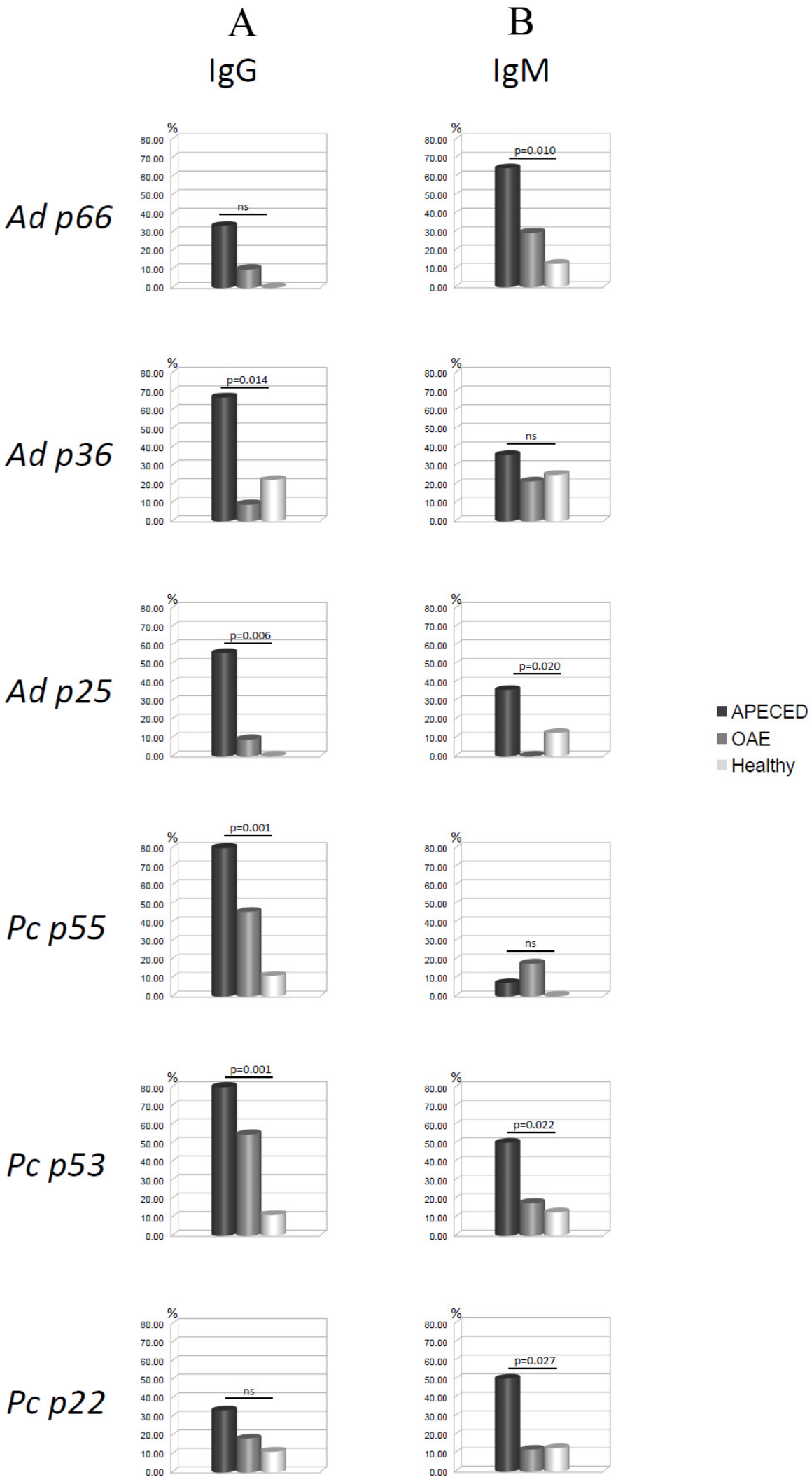
Supplemental table 2. Frequency of detection of a co-alignment with anti 21 OHase and anti GAD65 IgG monoclonal antibodies in APS 1 patients, OAE patients and healthy controls.

	GAD65		21OHase	
	IgG	IgM	IgG	IgM
APS 1 patients	33%	25%	60%	0%
OAE patients	40%	26%	46%	0%
Healthy controls	0%	0%	0%	0%

Supplemental Figure 1. Preservation of expected antigenic specificities on selected tissue targets used in western blotting. Monoclonal IgG antibodies against glutamic acid decarboxylase 65 (GAD65), steroid 21 hydroxylase (21 OHase) and NACHT leucine-rich repeat protein 5 (NALP 5) were tested against pancreatic (A), adrenal (B) and parathyroid tissues (C). As expected, expression of GAD65 and 21 OHase was found in pancreatic and adrenal tissues, respectively. However, NALP 5, detectable in parathyroid tissue, was undetectable in the two other tissues.


A
B
A B C
← 180 kDa
← 115 kDa
← 82 kDa
← 64 kDa
← 49 kDa
← 37 kDa
← 26 kDa
← 19 kDa
← 15 kDa
← 5 kDa
Anti-GAD 65
Anti-21OHase
Anti-NALP5

Supplemental Figure 2. Discriminant antibody reactivities detected in APS 1 patients. After Chi square analysis, the prevalence of discriminant self Ab reactivities against adrenal extracts (Ad p66, Ad p36 and Ad p25) and the prevalence of pancreatic protein extracts (Pc p55, Pc p53 and Pc p22) were respectively shown for IgG (A) and IgM (B) in APS 1 and OAE patients and in healthy subjects.


A
IgG
B
IgM
Ad p66
Ad p36
Ad p25
Pc p55
Pc p53
Pc p22
%
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ns
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ns
p=0.027
APECED
OAE
Healthy