



The spectrum of autoantibodies in IPEX syndrome is broad and includes anti-mitochondrial autoantibodies

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ABSTRACT

IPEX syndrome is a congenital disorder of immune regulation caused by mutations in the FOXP3 gene, which is required for the suppressive function of naturally arising CD4 + CD25 + regulatory T cells. In this case series we evaluated serum samples from 12 patients with IPEX syndrome for the presence of common autoantibodies associated with a broad range of autoimmune disorders. We note that 75% of patients (9/12) had 1 or more autoantibodies, an incidence far above the cumulative rate observed in the general population. The range of autoantibodies differed between patients and there was no predominant autoantibody or pattern of autoantibodies present in this cohort. Surprisingly, one patient had high-titer anti-mitochondrial antibodies (AMA) typically associated with primary biliary cirrhosis (PBC) although the patient had no signs of cholestasis. PBC is a well-characterized autoimmune disease that occurs primarily in women and includes the serological hallmarks of serum AMA and elevated IgM which were both present in this patient. PBC is virtually absent in children with the exception of one reported child with interleukin 2 receptor α (CD25) deficiency which is associated with an IPEX-like regulatory T cell dysfunction. Based on the present data and the available literature we suggest a direct role for CD4 + CD25 + regulatory T cells in restraining B cell autoantibody production and that defects in regulatory T cells may be crucial to the development of PBC.

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Keywords:

X chromosome
Autoimmune cholangitis
Autoantibodies
Pediatrics

1. Introduction

The immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare primary immune deficiency disease (PID) caused by mutations of the FOXP3 gene that maps to the centromeric region of the X chromosome. Unlike most PID in which increased susceptibility to infectious agents is the most prominent feature, patients with IPEX present most commonly with multi-organ autoimmunity. Symptoms typically become evident during infancy and include moderate to severe enteropathy, villous

atrophy, dermatitis (eczema and/or other skin manifestations), type I diabetes, thyroiditis and other autoimmune phenomena [1].

The genesis of immune dysregulation in IPEX is the absence of naturally arising CD4 + CD25 + FOXP3 + regulatory T cells (Tregs) [2], which are dependent on FOXP3 for their development and function [3]. Absence of the regulatory activity provided by this important T cell subset leads to unchecked effector T cell activation, which is thought to directly mediate much of the organ-specific autoimmunity observed in IPEX. In addition to cell-mediated autoimmunity, IPEX patients have evidence of humoral autoimmunity as well, demonstrated by the frequent onset of cytopenia and the presence of autoantibodies in some individuals with disease. These autoantibodies have been reported to target a variety of antigens expressed in the skin, gut, kidneys, pancreas, and on various hematopoietic cells [4]. Some of the reported autoantibodies appear to contribute to disease pathogenesis as evidenced by clinical

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Table 1
Genetics, clinical features, and serum autoantibodies in patients with the IPEX syndrome.

#	Mutation	Age	Plasma IgE(IU/ml)	Clinical manifestations	Serum autoantibodies
1	c.210 + 1G > A (Splicing) 1	3 years	3700	Enteropathy, skin disease, type 1 diabetes, thyroiditis, hepatitis, nephropathy (responder to hematopoietic stem cell transplantation)	ANA, h-tTG, RNP
2	c.210 + 1G > A (Splicing) 2	11 months	3210	Skin disease, nephropathy	h-tTG, Ribo-P
3	c.543C > T	2 months	1	Enteropathy	—
4	c.816 + 7G > C (Splicing)	17 years	842	Enteropathy, skin disease, type 1 diabetes, thyroiditis, nephropathy, recurrent infections	Chromatin
5	c.817G > T (p.A273S)	6 years	364	Enteropathy, skin disease, thyroiditis	ANA, Jo-1, Ribo-P, RNP, Scl-70, SS-A, SS-B
6	c.970T > C (p.F324L) 1 c.543C > T	5 years	3342	Enteropathy, skin disease	ANA
7	c.970T > C (p.F324L) 2 c.543C > T	6 years	34		h-tTG
8	c.1150 G > A (p.A384T)	11 years	2444	Enteropathy, skin disease	AMA
9	c.1157G > A (p.R386H)	4 months	NA	Enteropathy	ANA, Jo-1, Ribo-P, Scl-70, SS-A, SS-B
10	c.1169G > A (p.S390N)	5 years	2950	Enteropathy, skin disease, type 1 diabetes, recurrent infections	ANA, Centromere, SS-A
11	c.1190G > A (p.R397Q)	4 years	657	Enteropathy, skin disease, type 1 diabetes	Centromere
12	AAUAAA > AAUGAA (Poly A Signal)	Newborn	NA	Enteropathy, skin disease	—

responses to B cell depletion therapy in some patients [5] and unpublished data from T.R. Torgerson.

Despite the reports of tissue-specific autoantibodies in some patients with IPEX, there has been no systematic evaluation of either the prevalence or spectrum of antibodies to common autoantigens in this disorder. We have performed an extensive search for serum autoantibodies in a series of well-defined patients with IPEX syndrome and report that the prevalence of antibodies to common autoantigens is high in this cohort and that the spectrum of autoantibodies observed is quite broad. Interestingly, in addition to antibodies to common autoantigens, we observed antibodies to disease-specific autoantigens including anti-mitochondrial antibodies (AMA), which are typically associated with adult primary biliary cirrhosis [6–8].

2. Materials and methods

2.1. Patients

Serum samples from patients with a diagnosis of IPEX syndrome ($n = 12$), confirmed by sequencing of the *FOXP3* gene, were obtained from the Division of Immunology at the University of Washington and Seattle Children's Hospital, Seattle, WA, from the Department of Pediatrics, University of Florence, Meyer's Children's Hospital, Florence, Italy, and from the Department of Pediatrics, University of São Paulo, São Paulo, Brazil. The specific *FOXP3* mutations and clinical features of the enrolled patients are summarized in Table 1.

2.2. Autoantibody detection

Serum autoantibody screening was performed in a blinded fashion at a 1:100 dilution using a commercially available ELISA with microwell plates for gp210, Sp100, M2 EP IgG and IgA, Chromatin, h-tTG/DGP Screen, and Centromere (INOVA Diagnostics, Inc. San Diego, CA). Sera were also tested for autoantibodies to extractable nuclear antigens (ENA) using the QUANTA Plex TM SLE Profile 8 (INOVA Diagnostics, Inc. San Diego, CA, U.S.A.), which includes Sm, RNP, SS-A 60, SS-A 52, SS-B, Scl-70, Jo-1, Ribosomal P, and anti-IgG autoantigens. Serum ANA were evaluated by standard indirect immunofluorescence (IIF) on HEp-2 cells (Bion Enterprises Ltd., Chicago, IL). Briefly, reactivities were analyzed at serial dilutions in phosphate buffered saline (PBS) on human epithelial cell (HEp-2 cell) fixed slides in a moist chamber at room temperature for 30 min. After washing with PBS, slides were incubated with

fluorescent antibody conjugate (fluorescein isothiocyanate labeled goat anti-human immunoglobulin) in a moist chamber at a room temperature for 30 min. Immunofluorescent images were acquired using fixed exposure times and enhancement parameters using a 40X objective on a Axioskop50 fluorescence microscope (Carl Zeiss Inc, Thornwood, NY).

3. Results

3.1. Patient characteristics

Sera from 12 patients with IPEX syndrome (all males, ranging in age from newborn to 17 years old) were included in this study. The *FOXP3* mutation, IgE levels, and the clinical features of the enrolled cases are summarized in Table 1. Ten different *FOXP3* mutations, distributed throughout the gene and representing the majority of types of mutations observed in *FOXP3*, were included. Among the 12 patients, there were 2 sets of affected siblings. Of note, 92% (11/12) of the enrolled patients had enteropathy, and 75% (8/12) had typical skin manifestations. Thyroiditis and type 1 diabetes were found in 25% (3/12) and 33% (4/12) patients respectively. Only one of the patients had signs of hepatitis and two manifested nephropathy.

3.2. Serum autoantibodies detected

The autoantibodies identified in each patient are included in Table 1. Of note, 3/12 patients demonstrated celiac-disease-specific anti-tTG antibodies while 3/12 had positive anti-Ribo-P and 3/12 SS-A 52. Two/12 sera were reactive against RNP and centromere antigens. No serum reacted against gp210, Sp100, IgG, or Sm. Only 3/12 patients were negative for all tested autoantibodies. When tested for antinuclear antibodies (ANA) using indirect immunofluorescence on Hep2 cells, 5/12 serum samples manifested variable degrees of reactivity with a homogeneous pattern and these included the only IPEX case known to have signs of hepatitis (Patient #1).

Quite importantly, one serum sample manifested a positive pattern when tested for AMA of the IgA subtype while proving negative for IgG (Patient #8). This pattern is observed in patients with PBC and the clinical characteristics of this AMA-positive patient with IPEX syndrome are illustrated in Table 2. We note that plasma IgM and IgA levels were increased as well. Interestingly, this patient has not yet exhibited signs of cholestasis nor hepatocellular damage but has had an array of other severe complications associated with IPEX.

Table 2

Clinical, biochemical, and genetic characteristics of the AMA-IgA-positive patient.

FoxP3 mutation	c.1150 G > A (p.A384T)
Age	11 years 7 months
Clinical manifestations	Enteropathy Failure to Thrive Food Allergies Skin disease: Dermatitis Erythrodermic psoriasis Eczema Alopecia Endocrinopathy Eosinophilic meningitis EBV lymphoproliferative disease
Ongoing treatments	FK506, steroids, IVIG
Serum immunoglobulin	IgG: 1410 mg/dl IgM: 270 mg/dl IgA: 416 mg/dl IgE: 2444 IU/ml

4. Discussion

IPEX syndrome, caused by absence of functional Tregs, is the prototypic disorder of immune dysregulation caused by hemizygous mutations in the gene encoding the transcription factor FOXP3 [9]. Affected males typically present with systemic autoimmunity characterized by enteropathy, dermatitis, Type I diabetes, and thyroiditis, as well as other autoimmune processes including anemia, thrombocytopenia, neutropenia, hepatitis, and nephropathy [10]. In virtually all cases, IPEX is fatal unless treated with potent immunosuppressants [11], stem cell transplantation [12], and possibly B cell depletion. Some patients have recurrent bacterial, viral, or fungal infections related primarily to poor barrier function of the skin and gut epithelium and to the need for chronic immune suppression.

Various autoantibodies have been reported previously in IPEX including antibodies to a 75 kDa antigen expressed in gut and kidney [13], antibodies to skin antigens [5,14], and antibodies to organ-specific antigens (particularly pancreas and thyroid) [15]. No studies have systematically evaluated the prevalence of antibodies to autoantigens that are common in other autoimmune disorders in patients with IPEX. Our data demonstrated a high incidence of autoantibodies in this cohort of IPEX patients (75% of individuals). The number and type of autoantibodies detected did not seem to correlate with age or with particular mutations. In fact, autoantibody reactivity was different even among siblings with identical FOXP3 mutations. These data suggest a significant propensity of patients with IPEX to develop a broad spectrum of humoral autoimmunity and suggest that Tregs may exert a direct effect on B cells to limit autoantibody production and to maintain humoral immune tolerance. Several hypotheses have emerged about whether this occurs and how this effect may be mediated. However there is little concrete evidence to suggest how this may occur.

We were very interested to identify serum AMA IgA in one patient in this case series. AMA are the hallmark of PBC, an autoimmune cholestatic liver disease characterized by chronic inflammation [16] and the selective destruction of intrahepatic bile ducts mediated by autoreactive T cells [17,18]. PBC is most common among adult females [19] and is very rare in children [20] with solid genetic bases [21,22]. The subject in which we identified AMA-positive IgA antibodies has not yet manifested any sign of liver damage or cholestasis although there may be long latency between AMA appearance and disease onset [23]. We note that the serum AMA detected in this case was IgA and IgA-AMA have been repeatedly implicated indirect tissue injury based on their detection in the bile

of patients with PBC [24] and their capacity to induce cholangiocyte apoptosis [25,26], a putative key event in PBC autoimmunity [27–29]. It is also of interest that the patient plasma IgM levels were elevated, similar to what is observed in the majority of patients with PBC regardless of the AMA status or titer [30].

The identification of AMA autoantibodies in a patient with IPEX is particularly intriguing in light of our recent identification of positive AMA autoantibodies and a PBC-like syndrome in a child with CD25 (IL2R α) deficiency [31] and the similar scenario observed in murine models of CD25 deficiency [32,33]. Interestingly, although few patients with CD25 deficiency have been described, they have a clinical syndrome very similar to IPEX with diffuse, systemic autoimmunity including enteropathy, eczema, and type I diabetes [34,35]. Similarly, mice with CD25 deficiency exhibit similar autoimmune features to Foxp3-deficient mice [36]. Evaluation of the mechanism by which CD25 deficiency leads to a clinical phenotype similar to IPEX using murine models has revealed that CD25 expression and persistent IL-2 signaling are required to maintain high-level expression of Foxp3 in Tregs and are critical for maintaining homeostasis and competitive fitness of Tregs over time [36]. In the absence of IL-2 or CD25, Foxp3 is induced normally in Tregs but the cells are unable to maintain their long-term competitive function *in vivo* thereby making them unable to maintain immune homeostasis. The fact that AMA are rare in children but have been identified in two children with defects in Treg development or homeostasis suggests that regulatory T cells play a critical role in maintaining immune tolerance to this particular autoantigen and that investigation of Treg defects among other patients with AMA-positive PBC may prove fruitful. This is of particular interest based on previous data demonstrating quantitative defects in Tregs from patients with PBC [37] and the fascinating perspectives proposed in other autoimmune diseases [38]. Nevertheless, the etiopathogenesis of PBC should not overlook new potential mechanisms [39,40].

Finally, we are very pleased to recognize the contribution of Professor Harry Moutsopoulos to autoimmune disease. Professor Moutsopoulos's work has crossed many disciplines in immunology and has helped both children and adults with autoimmune disease. The recognition of Dr. Moutsopoulos's achievements in this dedicated issue is well deserved and part of a series of issues each year that recognize similar achievements [41–43].

Acknowledgements

Financial support provided by National Institutes of Health grant #DK39588.

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