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21-Hydroxylase epitopes are targeted by CD8 T cells in autoimmune Addison's disease

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ABSTRACT

In autoimmune adrenal deficiency, autoantibodies target the 21-hydroxylase (21OH) protein. However, it is presumed that autoreactive T cells, rather than antibodies, are the main effectors of adrenal gland destruction, but their identification is still lacking. We performed a T-cell epitope mapping study using 49 overlapping 20mer peptides covering the 21OH sequence in patients with isolated Addison's disease, Autoimmune Polyendocrine Syndrome 1 and 2. IFNγ ELISPOT responses against these peptides were stronger, broader and more prevalent among patients than in controls, whatever the disease presentation. Five peptides elicited T-cell responses in patients only (68% sensitivity, 100% specificity). Blocking experiments identified IFNγ–producing cells as CD8 T lymphocytes, with two peptides frequently recognized in HLA-B8+ patients and a third one targeted in HLA-B35+ subjects. In particular, the $21OH_{431–450}$ peptide was highly immunodominant, as it was recognized in more than 30% of patients, all carrying the HLA-B8 restriction element. This $21OH_{431–450}$ region contained an EPLARLEL octamer ($21OH_{431–438}$) predicted to bind to HLA-B8 with high affinity. Indeed, circulating EPLARLEL-specific CD8 T cells were detected at significant frequencies in HLA-B8+ patients but not in controls by HLA tetramer staining. This report enlightens disease-specific T-cell biomarkers and epitopes targeted in autoimmune adrenal deficiency.

1. Introduction

Autoimmune adrenal deficiency (Addison's disease, AD), which is the result of immune-mediated destruction of adrenal cortex, can occur either in isolation (IAD) or as one component of an autoimmune polyendocrine syndrome (APS 1 and APS 2) [1,2]. In these three clinical presentations, a key diagnostic tool is the presence of autoantibodies directed against the steroidogenic enzyme 21-hydroxylase (21OH) [3], which is selectively expressed in the adrenal cortex. These antibodies can also be present at a preclinical stage in patients at risk of adrenal failure (relatives of patients with AD or patients with other autoimmune diseases) in which they confer a cumulative risk of developing AD of 48% (100% in children) [4].

Although antibodies might be components of the disease pathogenesis [5], they are mainly considered as markers of a T cell-mediated destruction of the adrenal cortex. Histological studies of the adrenal tissue of deceased AD patients show a predominant mononuclear cell infiltrate [6,7]. IAD and APS2 are associated with certain HLA haplotypes or allelic combinations, in particular DR3-DQ2 and DR4-DQ8 [8,9] which are known to predispose to other T cell-mediated autoimmune diseases such as type 1 diabetes and celiac disease. Although less reported, association with the HLA A1-B8-DR3 ancestral haplotype has also been described in earlier studies [10,11]. APS 1 is caused by a mutation in the *AIRE* gene [12]. So far, only few reports suggest T cell reactivity in AD: Freeman et al. observed proliferation of PBMC in the presence of adrenal protein

Abbreviations: AD, Addison's disease; APS, Autoimmune polyendocrine syndrome; SFC, Spot forming cells; 21OH, 21-Hydroxylase.

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homogenate in patients with AD but not in controls [13]. More recently, proliferation and IFNγ secretion of T cells were reported in patients with AD in presence of the 21OH protein [14]. Additionally, defects in T cell functions were described in APS 2 patients: an impaired expression of caspase 3, involved in T cell apoptosis [15] and an impaired suppressive function of CD4+CD25+ regulatory T cells [16].

To date, the T cell antigen target(s) has however not been extensively studied. Research has been hampered by the lack of relevant experimental animal model. Whether 21OH is involved in the pathogenesis of autoimmune adrenal failure in humans remains elusive.

Because antibodies to 21OH are highly specific for AD, we focused on this protein as a candidate antigen that might trigger autoreactive T cells. Such a strategy was successfully used in type 1 diabetes, where identification of T cell autoantigen epitopes was guided by the known antigen specificities (insulin, GAD65 and IA-2) of islet autoantibodies [17]. We used the ELISPOT assay to quantify IFNγ-producing T cells from 16 patients with isolated AD, APS 1 and APS 2. This assay has been recognized as a sensitive method to track low frequency autoreactive T cells [18]. Using 20mer overlapping synthetic peptides covering the entire 21OH sequence, we identified CD8 T cell responses to 21OH epitopes in AD patients but not in controls. In particular, CD8 T cell response to one immunodominant HLA B8-restricted epitope was documented. These results document 21OH-specific T cell responses in AD patients, thus offering novel disease biomarkers and paving the way for further explorations into the pathogenic role of such T cells.

2. Materials and methods

2.1. Patients and controls

Patients were recruited from pediatric (CHU Sainte-Justine) and adult (Centre hospitalier de l'Université de Montréal) Endocrine Clinics. Local Ethics Committee approved the study and informed consent was obtained from each participant and/or participant's parents. Inclusion criteria for patients were a clinical diagnosis of primary adrenal deficiency [19] and presence of 21OH antibodies at diagnosis (>1 IU/ml). The 16 patients recruited (12 children <19years, 4 adults >19years) are described in Table 1. Control subjects were 9 diabetic children (C6, C7, C13, C9-11, C14, C16 and C17) who did not have 21OH antibodies and 8 healthy adult volunteers (C1-C5, C8, C12 and C15).

HLA class I (A, B) and HLA class II (DRB1) genotyping was determined by conventional PCR-sequence specific oligonucleotide hybridization technique (RSSO-Luminex, Ingen, France).

2.2. 21OH peptides

A bank of 49 peptides, 20 aa long and 10 aa overlap, spanning the entire 21OH sequence (source: Swiss-Prot P08686.1) was synthesized from PEPscreen Custom Peptide Librairies (Proimmune, Oxford, UK). 21OH peptides were named 1 to 49 according to the N- to C-terminal sequence order.

2.3. Elispot assay

Plates (Millipore S2EM004M99) were precoated overnight at 4 °C with anti-IFNγ mAb (Mabtech, Nacka Strand, Sweden 10 μg/ml). After a further 2 h saturation step using PBS 1% BSA (Sigma), cultures were performed in quadruplicate as following: fresh PBMCs were isolated by Ficoll gradient, enumerated using a haematological analyser SYSMEX XE-alpha (SYSMEX corporation, Kobe, Japan) and seeded in order to obtain 300 000 lymphocytes per well. PBMC were cultured in 100 μl total volume of serum-free culture medium (AIM-V, Invitrogen/Gibco, Carlsbad, CA) in presence of 21OH peptide(s). Final peptide concentration was 10 μg/ml. Control wells with no peptide had equal DMSO content (1%).

Tetanus toxoid (Aventis Pasteur, France) and CEF (Mabtech) containing viral CD8 T cell epitopes were used as positive controls. Polyclonal T cell stimulation was obtained with anti-CD3 mAb (OKT3, Janssen-Cilag, Australia). After a 40 h culture, cells were discarded and IFNγ secretion visualised using subsequently biotinylated anti-IFNγ mAb (Mabtech, 0.5 μg/ml) for 2 h, streptavidin-alkaline phosphatase (Mabtech,1/1000) for 1 h and the alkaline phosphatase Conjugate Substrate Kit (BIO-RAD). For blocking experiments, PBMCs were preincubated 20 min with anti-CD8 (25 μg/ml OKT8; eBioscience, UK) or anti-CD4 (10 μg/ml 13B8.2; Beckman Coulter, Fullerton, CA) mAbs or isotype controls. Spots were counted using a CTL Immunospot Analyzer (CTL, Cleveland, OH) and means of quadruplicate wells were calculated. The cut-off for a positive response was set at 3SD above the average basal reactivity (i.e., reactivity in the presence of DMSO diluent alone), as previously described in similar approaches. This cut-off was derived from a receiver-operator characteristics analysis of patients and controls data, comparing sensitivity and specificity of various cut-offs. To exclude values close to background, only those

Table 1
Patient characteristics.

Patient ID	Sex	Disease	Disease duration (years)	Age at time of study (years)	HLA-A	HLA-B	HLA-DR
P1	F	IAD	2	10	01–02	08–40	03–01
P2	F	IAD	1	15	23–02	08–4901	03–11
P3	M	APS 2 (thyroiditis)	19	50	nd	nd	nd
P4	M	IAD	0	12	11–68	08–27	03–04
P5	F	APS 1	5	20	03–3004	35–1517	1102–13
P6[a]	M	APS 1	4	9	2501–3002	35–44	04–1302
P7[a]	M	APS 1	9	14	3002–02	35–51	1302–15
P8	M	IAD	3	13	01–02	08–07	03–15
P9	M	APS 2 (diabetes)	4	18	23–2608	08–4901	03–0405
P10	F	IAD	1	15	03–24	08–4403	03–12
P11	M	IAD	2	14	03–02	07–51	0407–1501
P12	M	IAD	0	23	24–31	27–4001	03–04
P13	F	IAD	1	18	24–02	07–44	15-
P14	F	APS 2 (thyroiditis)	8	22	03–25	07–67	04–15
P15	F	IAD	0	10	03–02	35–51	3–16
P16	M	IAD	7	14	01–02	08–27	03–04

[a] Brothers