Recognition of Ii-Key/MHC Class II Epitope Hybrids Derived from Proinsulin and GAD Hybrid by T Cells in Type 1 Diabetes

Authors M. Vadacca[1], M. G. Valorani[2,3], E. von Hofe[4], N. L. Kallinteris[4], R. Buzzetti[5], P. Pozzilli[1,2]

Affiliations Affiliation addresses are listed at the end of the article

Key words
- Ii-Key hybrid
- type 1 diabetes
- MHC class II

Abstract

In order to determine whether the Ii-Key technology can enhance the presentation of specific epitopes associated with type 1 diabetes, we have designed and synthesized a series of Ii-Key/proinsulin and GAD epitope hybrid peptides. Peptides of proinsulin and GAD shown to be recognized by CD4+ T cells of type 1 diabetes patients have been selected from the literature and modified with Ii-Key. A total of 23 Caucasian type 1 diabetes subjects and 17 normal subjects as controls were included in the study. Reactive T cells were identified using an IFN-γ ELISPOT assay. We selected 5 proinsulin and 5 GAD epitopes. Regarding the activity of the proinsulin Ii-Key hybrids, 3 out of 15 patients (20%) demonstrated a positive response to one or more Ii-Key hybrid peptides compared no responders in the control subjects. 2 out of 8 patients demonstrated a positive response to one or more Ii-Key/GAD65 hybrids. Proinsulin Ii-Key hybrids and peptides were recognized only by DR3/DR4 0302+ve diabetic patients. Control subjects showed no detectable response to stimulation with Ii-Key hybrids or peptides, neither for proinsulin nor GAD65. We have now shown that the use of Ii-Key-modified MHC class II epitopes, derived from proteins associated with insulin-secreting cells, can detect the presence of specifically activated CD4+ T helper cells with greater sensitivity than unmodified epitopes in the standard ELISPOT assay. The use of these technologies may be of use in identifying patients at the earliest stages of type 1 diabetes.

Introduction

Type 1 diabetes is the result of a T cell immune-mediated destruction of pancreatic β-cells in the islets of Langerhans [1]. In diabetic animal models and in humans, a correlation between disease onset and cytokine production, in particular interferon (INF-γ by T lymphocytes, has been observed stimulating the generation of inflammatory cellular immune responses [2]. Type 1 diabetes is also associated with the major histocompatibility complex (MHC) class II genes with an important role being played by the highly polymorphic HLA class II immunoregulatory molecules, DR and DQ [3,4]. Both these molecules are essential for the presentation of specific peptides to autoreactive T cells [5]. In type 1 diabetes the specific epitopes recognized by pathogenic T cells remain poorly defined and the specific autoreactive T cells are difficult to detect due to their low number in the peripheral blood, their inhibition by regulatory T cells and the inability to assay cells from the inflammatory lesion [6,7]. Moreover, because autoantigen reactive T cells are also detected in healthy individuals, the development of new techniques to identify β-cell-reactive T cells with high specificity in type 1 diabetes subjects remains a major goal in the management of this disease [8].

A novel technique to boost the potency of MHC class II-presented epitope peptides has become available by modifying a given epitope with Ii-Key to produce an Ii-Key/epitope hybrid peptide [9–12]. Such Ii-Key hybrid peptides contain an immunomodulatory segment (Ii-Key moiety) of the MHC class II-associated invariant chain (Ii protein), which loosens the epitope-binding groove of MHC class II molecules to permit the direct insertion of a tethered MHC class II epitope [13]. The region of the invariant chain from which Ii-Key is derived (Ii 77–92; LRMKLPKPPKPVS QMR) was synthesized to test for biological activity related to regulation of MHC class II antigenic peptide binding because its primary sequence suggested a regulatory structure signal (6 positive amino acids, no negative amino acids,

received 26.02.2011
accepted 23.03.2011

Bibliography
DOI http://dx.doi.org/10.1055/s-0031-1275705
Horm Metab Res 2011; 43: 1–7

ISSN 0018-5043

Correspondence
Dr. M. Vadacca, MD
University Campus Bio-Medico
Via Alvaro del Portillo 21
00128 Rome
Italy
Tel.: +39/062/254 11
Fax: +39/062/254 14 56
m.vadacca@unicampus.it

4 spaced prolines, and recurrent cationic-hydrophobic doublets reminiscent of protease-cleavage sites) [14]. Studies on the structure activity of 160 homologues revealed a shorter core sequence (LRMKLPK) with significantly greater activity than the original 16-amino acid peptide. The shortest active sequence is constituted by 4 amino acids (LRMK) [9,15]. In in vitro model systems, the enhancement in stimulation over the free epitope peptide can be up to >250 times when the N-terminus of the epitope is covalently linked through a chemical spacer to the C-terminus of the Ii-Key peptide forming an Ii-Key/antigenic epitope hybrid [10,11]. The mechanistic hypothesis has been that the Ii-Key moiety binds initially to an allosteric site just outside the MHC class II epitope binding groove inducing a conformational change for more accessible antigenic epitope charging [10]. In prior studies, linking Ii-Key to a HER-2/*neu* MHC class II epitope induced much greater IFN-γ release from peripheral blood mononuclear cells (PBMC) of breast cancer patients than did the comparable HER-2/*neu* epitope-only peptide [16]. A number of other MHC class II epitope peptides similarly have demonstrated significantly enhanced potency after linking to Ii-Key, both in vitro and in vivo (11).

In order to determine whether the Ii-Key technology can enhance the presentation of specific epitopes associated with T1D to more sensitively identify auto-reactive T cells, we have designed and synthesized a series of Ii-Key/proinsulin and GAD hybrid peptides.

Research Design and Methods

Design and synthesis of Ii-Key/MHC class II epitope hybrids

Proinsulin and GAD peptides previously shown to be recognized by CD4+ T cells of T1D patients were selected for these studies. Complete sequences of the human proinsulin and GAD65 proteins were obtained from Genbank. Putative DRB1*0301 and DRB1*0401 epitopes within the sequences of the experimentally characterized peptides were identified by application of the Rammensee SYFPEITH program (http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.html). In the case of relatively short peptides, the sequence for analysis by the program was extended by 5 amino acids at both the N- and C-termini. The sequence of the top-scoring epitopes predicted for DRB1*0301 and DRB1*0401 were plotted against the primary amino acid sequence of the experimentally studied peptides. Ii-Key hybrid peptides were designed to contain: a) constant LRMK-*ava*- (where *ava*=aminovaleric acid; 5-aminopentanoic acid), and b) peptide sequences with a constant C-terminus, but varying at the N-terminus by one amino acid among members of the set, in a nested deletion pattern. N-termini ends of the peptides were acetylated and C-termini ends amidated to block catabolism by amino- and carboxypeptidases, respectively. Within a homologous series, the longest and shortest hybrids were taken for initial synthesis plus the epitope-only peptide as a control. In addition, every other member of the series also was synthesized. All peptides were dissolved in sterile distilled water (5 mg/ml) and stored at −80 °C until used.

Subjects

A total of 23 Caucasian type 1 diabetes subjects (diagnosed according to the criteria of the American Diabetes Association) were studied (13 males aged 4–33 years; mean age 15.3±7 SD; 10 females aged 4–33 years; mean age 15.8±9 SD). A total of 17 normal subjects (11 males and 6 females) without diabetes or any other autoimmune disease were studied as controls. The clinical characteristics of study subjects are summarized in **Table 1, 2**. This study was approved by the Ethical Committee of University Campus Biomedico within the framework of the IMDIAB group mechanistic studies.

Table 1 Clinical characteristics and HLA genotype of type 1 diabetic patients and normal subjects tested for proinsulin-Ii/Key hybrids

Subjects	Sex	Age (years)	Duration of diabetes (months)	DRB1 genotype
Diabetic subjects				
1	M	21	22	DR3/DR3 0201
2	F	22	30	DR4/DR4 0302
3	M	21	29	DR3/DR4 0302
4	M	8	37	DR3/DR4 0302
5	M	22	28	DR3/DR4 0302
6	M	16	37	DR3/DR4 0302
7	F	13	34	DR3/DR4 0302
8	F	10	36	DR4/DR4 0302
9	F	7	26	DR3/DR4 0302
10	M	9	36	DR3/DR3 0201
11	M	12	38	DR3/DR4 0302
12	M	14	30	DR3/DR4 0302
13	M	12	37	DR4/DR4 0302
14	M	8	33	DR3/DR4 0302
15	F	11	34	DR3/DR3 0201
Normal subjects				
1	M	39	–	DR1/DR4
2	M	44	–	DR8/DR4
3	M	54	–	DR3/DR6
4	F	27	–	–
5	F	45	–	DR3/DR5
6	M	26	–	–
7	M	27	–	–
8	F	28	–	–
9	M	24	–	–
10	F	25	–	–

Table 2 Clinical characteristics of type 1 diabetic patients and normal subjects tested for GAD-Ii/Key hybrids

	Sex	Age (years)	Duration of diabetes (months)	DRB1 genotype
Diabetic subjects				
1	F	11	11	
2	M	14	12	
3	M	16	1	
4	F	4	11	
5	F	21	24	DR2/DR3
6	F	26	31	
7	F	33	4	DR8/DR4 0302
8	M	21	12	
Healthy subjects				
1	M	28	–	DR8/6/DR8/6
2	M	29	–	DR8/6/DR8/6
3	M	29	–	DR7/DR7
4	F	32	–	–
5	F	29	–	–
6	M	27	–	–
7	M	29	–	–

HLA typing

Blood samples were collected after fasting in the morning. Samples were stored at −20 °C until used for genomic extraction of DNA. Genomic DNA was extracted using QIAamp DNA Blood Kit (QIAGEN Genomics Inc., Bothell, WA). Typing for HLA-DRB1 and DQB1 loci was performed as previously described by Buzzetti et al. [17].

ELISPOT assay

Heparinized, freshly drawn blood samples from each subject were assayed within 6 h. PBMC were isolated by Ficoll Hypaque density gradient centrifugation (ICN Biomedicals Inc., USA).

ELISPOT assays were performed in triplicate using the BD Pharmingen kit for IFN-γ (BD™ ELISPOT Human INF-γ ELISPOT Set, BD Biosciences, San Diego, CA, USA) (Kallinteris melanoma, Nectaria). Briefly, 100 µl of purified anti-human INF-γ, diluted in sterile coating buffer (PBS, pH 7.2), was added to each well of the ELISPOT plate and allowed to coat overnight at 4 °C. On day +1, the coating antibody was discarded. Wells were washed with 200 µl/well blocking solution [cell culture medium (RPMI 1640) containing 10 % human AB serum and 1 % penicillin-streptomycin-glutamine (Gibco-BRL)] and the plate was incubated for 2 h at room temperature with blocking solution.

Irradiated autologous antigen presenting cells (APCs) were pulsed at a density of 10^6 APCs/ml with Ii-Key hybrids or epitope-only control peptides (50 µg/ml) in complete medium and dispensed in triplicate to the 96-well ELISPOT plate. Subsequently, PBMC suspensions (10^6 PBMC/ml, 100 µl/well) were added to each well followed by the addition of IL-12 (25 pg/ml final concentration). Positive control wells contained cell culture medium with PMA/Ionomycin (50 ng/ml and 1 µg/ml final concentration, respectively). ELISPOT plates were incubated at 37 °C in a humidified incubator at 5 % CO_2 for 96 h. On day +5, cell suspensions were aspirated and the wells washed twice with deionized (DI) water first and then with 200 µl/well PBS containing 0.05 % Tween-20. Subsequently, 100 µl per well of biotinylated anti-human INF-γ antibody was added. The plate was incubated for 2 h at room temperature. Wells were washed twice with 200 µl/well PBS. Streptavidin-HRP (100 µl/well) was dispensed into the wells. After 1 h at RT, 100 µl of final substrate solution (3-amino-9-ethylcarbazole) was added to each well and incubated until the appearance of red spots in the wells (20–30 min). The plate was dried and stored in the dark until it was analyzed with Eli. Analyse 4.0 software. The criteria for spot size, circularity, and color density were determined by comparing control and experimental wells. Partially overlapping spots were separated and noise signals caused by substrate precipitation and nonspecific antibody binding were eliminated. Relative IFN-γ production was estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot size x the number of spots. Counting results were validated by the human eye to judge whether all spots were counted accurately and artifacts excluded. The number of cytokine-producing cells in antigen stimulated wells was measured in relation to the negative control wells (medium alone or epitope-controls). The difference between spot counts in the antigen-stimulated culture vs. control wells revealed the number of antigen-specific T cells present in the experimental wells. All data were expressed as the mean of triplicate determinations for each antigen.

Statistical analysis

ELISPOT assays for IFN-γ production were performed in triplicate. Statistical analysis was performed using GraphPad statistical software, version 3.0.

Results are expressed as mean ± standard deviation or median plus/minus the range, where appropriate. Results were considered significant at $p < 0.05$. The number of cytokine-producing cells in antigen stimulated wells and in negative control wells (irrelevant peptide) was compared using the one-way ANOVA test. Kruskall-Wallis test was performed if data was not Gaussian distributed. Bonferroni or Dunns post-tests, in Gaussian or non-Gaussian distribution, respectively, were used where appropriate.

Results

Design and synthesis of Ii-Key/Proinsulin MHC class II epitope hybrids

On the basis of the reported data, we selected 5 proinsulin epitopes and 5 GAD epitopes (**Fig. 1, 2**). Peptides of proinsulin and GAD65 (**Table 3, 4**) were synthesized by Commonwealth Biotechnologies Inc., 601 Biotech Drive, Richmond, VA 23225, USA. All peptides were found to be >98 % pure by analytical HPLC and mass spectrometry (Commonwealth Biotechnologies, Richmond, VA, USA).

T cell response to Ii-Key/Proinsulin and Ii-Key/GAD hybrid and native peptides

Spontaneous production of INF-γ was present at similar, very low levels in both patients and controls (median number of spots 0, mean number 4.1, range 0–22/100 000 cells, p NS). All cases and control subjects showed a detectable and significant INF-γ response to stimulation with the polyclonal T cell stimulus PMA/Ionomycin, and the frequencies and magnitude of these responses were similar in both groups. INF-γ upon PMA/I stimulation was 428.1 ± 89 (mean ± SD) and 403 ± 99 for patients and controls, respectively (p NS).

Regarding the activity of the proinsulin Ii-Key hybrids, 3 out of 15 patients (20 %) demonstrated a positive response to one or more Ii-Key hybrid peptides compared with none of control subjects (**Fig. 3**). 2 out of 8 patients (25 %) demonstrated a positive response to one or more Ii-Key/GAD65 hybrids (**Fig. 4**). In all cases, the Ii-Key hybrids were more active than the epitope-only peptide. In fact, they induced up to 3 times more INF-γ responding cells than did the epitope-only peptide. The hybrid structure appears to induce a stronger signal as indicated by a

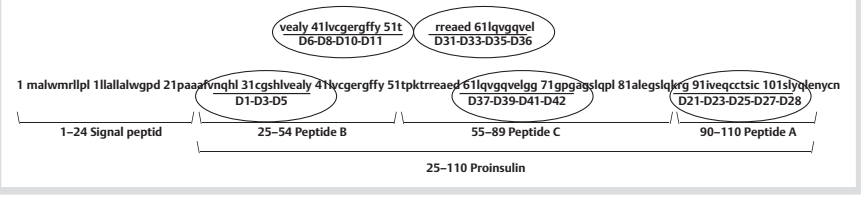

Fig. 1 Peptides of proinsulin recognized by CD4+ T cells of type 1 diabetes patients.

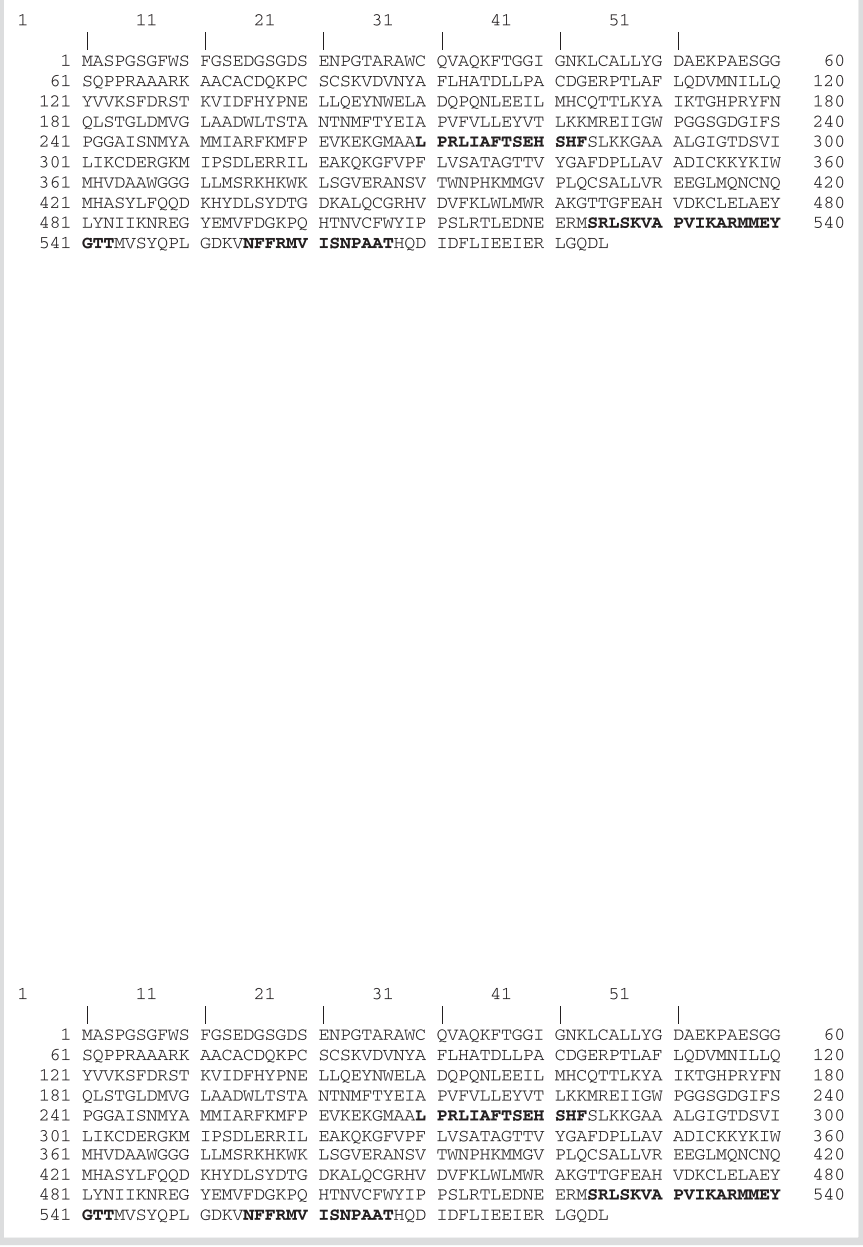

Fig. 2 Peptides of GAD65 recognized by CD4+ T cells of type 1 diabetes patients.

higher frequency of cytokine producing cells. All the other patients and all controls did not show any reactivity neither for epitope-only peptides nor for Ii-Key hybrids peptides.

The proinsulin Ii-Key hybrids were shown to be recognized only in DR3/DR4 0302+ve diabetic patients. Control subjects showed no detectable response to stimulation with Ii-Key hybrids or other peptides, neither for proinsulin nor for GAD65. 4 out of 10 control subjects tested using proinsulin Ii-Key hybrids were HLA-DR3 or DR4 positive and did not show any detectable response to stimulation with the Ii-Key hybrids or peptides.

Finally, we did not observe a significant relationship between the presence of GAD or insulin autoantibodies and INF-γ production, neither for insulin nor for GAD Ii-Key hybrids.

Discussion

The higher frequency of specific INF-γ producing T cells in subjects with type 1 diabetes compared to HLA-matched controls is very likely the result of T cells primed in vivo during the spontaneous autoimmune response [18]. The studies performed here were designed to better detect and understand the specific repertoire of reactive T cells. To enhance antigen presentation by resting APC in our test system, we used the Ii-Key modified epitope hybrids. This is a new strategy for augmenting the potency of MHC class-II restricted epitopes and results in a profound increase in stimulation of T cells both in vitro and in vivo [19]. It is also a powerful tool to efficiently expand CD4+ve T cells ex vivo. Data from the literature indicates that Ii-Key epitope hybrids are more potent and might be used for diagnostic purposes. In an initial preliminary study, HIV gag-hybrid peptides consistently elicited IFN-γ response [11]. In a study of HER2+ve breast cancer patients, an Ii-Key modified HER-2/*neu* epitope hybrid peptide greatly increased the detection of HER2 responsive T cells of the patients' PBMC compared to an unmodified peptide as measured by ELISPOT [15]. Further, in a Phase I clinical trial, immunization with the same Ii-Key/HER2 epitope hybrid led to an increase both in delayed-type hypersensitivity

Table 3 Design of Ii-Key/Proinsulin hybrids

D1	Ac-LRMK-*ava*-	NQHLCGSHLVEALY	-NH_2
D3	Ac-LRMK-*ava*-	HLCGSHLVEALY	-NH_2
NP5	Ac-	NQHLCGSHLVEALY	-NH_2
D6	Ac-LRMK-*ava*-	VEALYLVCGERGFFYT	-NH_2
D8	Ac-LRMK-*ava*-	ALYLVCGERGFFYT	-NH_2
D10	Ac-LRMK-*ava*-	YLVCGERGFFYT	-NH_2
NP11	Ac-	VEALYLVCGERGFFYT	-NH_2
D21	Ac-LRMK-*ava*-	GIVEQCCTSICSLYQ	-NH_2
D23	Ac-LRMK-*ava*-	VEQCCTSICSLYQ	-NH_2
D25	Ac-LRMK-*ava*-	QCCTSICSLYQ	-NH_2
D27	Ac-LRMK-*ava*-	CTSICSLYQ	-NH_2
NP28	Ac-	GIVEQCCTSICSLYQ	-NH_2
D31	Ac-LRMK-*ava*-	RREAEDLQVGGVEL	-NH_2
D33	Ac-LRMK-*ava*-	EAEDLQVGGVEL	-NH_2
D35	Ac-LRMK-*ava*-	EDLQVGGVEL	-NH_2
NP36	Ac-	RREAEDLQVGGVEL	-NH_2
D37	Ac-LRMK-*ava*-	LQVGGVELGGGPGA	-NH_2
D39	Ac-LRMK-*ava*-	VGGVELGGGPGA	-NH_2
D41	Ac-LRMK-*ava*-	GVELGGGPGA	-NH_2
NP42	Ac-	LQVGGVELGGGPGA	-NH_2

NP-5, -11, -28, -36, -42 are the native proinsulin peptides, whereas peptides D1 through D41 are the Ii-Key/Proinsulin hybrid peptides. The Ii-Key (LRMK) segment of the immunoregulatory Ii protein was linked through a simple polymethylene *ava* spacer to the different proinsulin epitopes by systematically deleting N-terminal amino acids

Table 4 Design of Ii-Key/GAD65 hybrids

G1P1	Ac-LRMK-*ava*-	LPRLIAFTSEHSHF	-NH_2
G1P2	Ac-LRMK-*ava*-	ALPRLIAFTSEHSHF	-NH_2
G1P3	Ac-LRMK-*ava*-	AALPRLIAFTSEHSHF	-NH_2
NPG1P4	Ac-	AALPRLIAFTSEHSHF	-NH_2
G2P1	Ac-LRMK-*ava*-	NFFRMVISNPAAT	-NH_2
G2P2	Ac-LRMK-*ava*-	VNFFRMVISNPAAT	-NH_2
G2P3	Ac-LRMK-*ava*-	KVNFFRMVISNPAAT	-NH_2
NP G2P4	Ac-	KVNFFRMVISNPAAT	-NH_2
G3P1	Ac-LRMK-*ava*-	SRLSKVAPVIKARMMEYGTT	-NH_2
NP G3P2	Ac-	SRLSKVAPVIKARMMEYGTT	-NH_2
G3aP1	Ac-LRMK-*ava*-	LSKVAPVIKARMM	-NH_2
G3aP2	Ac-LRMK-*ava*-	RLSKVAPVIKARMM	-NH_2
G3aP3	Ac-LRMK-*ava*-	SRLSKVAPVIKARMM	-NH_2
NP G3aP4	Ac-	SRLSKVAPVIKARMM	-NH_2
G3bP1	Ac-LRMK-*ava*-	PVIKARMMYGTT	-NH_2
G3bP2	Ac-LRMK-*ava*-	VIKARMMYGTT	-NH_2
G3bP3	Ac-LRMK-*ava*-	IKARMMYGTT	-NH_2
G3bP4	Ac-LRMK-*ava*-	KARMMYGTT	-NH_2
NP G3bP5	Ac-	PVIKARMMYGTT	-NH_2

NP-G1P4, -G2P4, -G3P2, -G3aP4, -G3bP5 are the native GAD65 peptides, whereas the other peptides are the Ii-Key/GAD65 hybrid peptides. The Ii-Key (LRMK) segment of the immunoregulatory Ii protein was linked through a simple polymethylene *ava* spacer to the different GAD65 epitopes by systematically deleting N-terminal amino acids

and in vitro proliferation of T cells after restimulation with the immunizing and epitope-only peptide [16]. Studies with HLA-DR4 transgenic mice demonstrated that Ii-Key/gp100 (46–58) MHC class II melanoma hybrid mediated a significantly greater frequency of IFN-γ memory recall T cell responder cells compared to those generated by the native peptide sequence [18]. In short, there are now many examples where Ii-Key/MHC class II epitope hybrid peptides induce stronger Th1 responses compared to epitope-only peptides both in vitro and in vivo [11,15].

Using PBMC from patients with type 1 diabetes, we show that the number of reactive INF-γ producing cells is higher than in controls where INF-γ secreting T cells were not detectable. Initial data show that some proinsulin Ii-Key hybrids, but not the epitope-only peptide, stimulate a reaction in some type 1 diabetes subjects. In order to increase the sensitivity of the test we decided to extend the assay to GAD65 Ii-Key hybrids. In different reports using an ELISPOT assay, GAD reactive T helper cells in PBMC from type 1 diabetic patients have been identified at a higher frequency than by other assays [6,7]. Several reports indicate GAD as the major antigen in type 1 diabetes especially in young adolescents and adults. Using GAD hybrids we found a positive response to Ii-Key hybrids in 2 out 8 patients (25%). Although the rationale behind the use of this technology relates to more selective and sensitive detection of lymphocyte subpopulations in peripheral blood, preliminary data presented here demonstrated that positive results can be obtained only in few patients. The data suggests that our test offers a specificity of 100%, but may lack sensitivity. We propose to combine the use the proinsulin and GAD65 Ii-Key MHC Class II-hybrids in order to increase the sensitivity of the assay compared to insulin and GAD65 Ii-Key MHC Class II-hybrid alone. In a study by Oling et al., it has been demonstrated that GAD65 and proinsulin tetramers binding CD4T cells are detectable more often in peripheral blood of type 1 diabetes subjects and at-risk subjects than normal subjects [20]. Peakman and colleagues [21] have identified a panel of naturally processed islet epitopes by direct elution from APC bearing HLA-DR4. They reported IFN-γ secreting cells in response to 6 IA-2 peptides and 3 proinsulin peptides using an ELISPOT assay. The lack of discrimination of responses among those peptides mimics our data. However, there did not appear to be strong differences in responses among: a) type 1 diabetes subjects with HLA-DR4, b) type 1 diabetes subjects without DR4, and c) normal subjects in those studies. In contrast, our test showed a very high specificity for type 1 diabetes subjects.

IFN-γ has biological effects on the insulin production of the β-cells. Given the important immunoregulatory role of IFN-γ for enhancing the cytolytic activity of natural killer cells, monocytes, and macrophages, GAD reactive CD4+ve T cells that secrete IFN-γ may play an important role in vivo by promoting the development of type 1 diabetes in genetically susceptible individuals. Further study with controls matched for HLA DR3 and DR4 and T1D associated HLA DQ genotypes could help us to better verify the specificity of this test.

It should be noted that our type 1 diabetes population included both adults and children. Type I diabetes is heterogeneous with respect to the age of onset and also with respect to disease progression in susceptible subjects. The majority of our patients are of adult onset-type, however, the clinical criteria for T1D (extensive weight loss, low body mass index, hyperketonuria, presence of autoantibody, primary insulin dependency) were fulfilled. It has been shown that at the time of T1D onset, the pancreas contains islets in all stages of destruction, and the destructive process continues until all beta cells are destroyed. Nevertheless, we studied patients who were not newly diagnosed but who had disease of short duration; the analysis of the T cell response profile indicates that even after a few years of overt disease, different patterns of T cell responses to proinsulin/GAD can still exist. The response can be confined to a single immunodominant epitope as identified in DRB1*0301/0401 patients, or it can be spread over other autoreactive peptides.

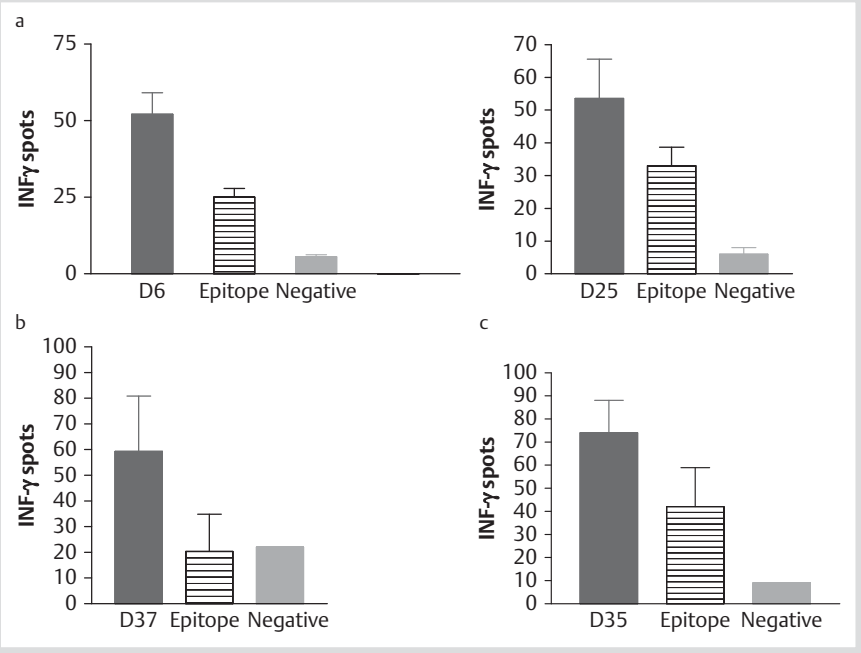

Fig. 3 Response to Proinsulin/Ii-Key/hybrids. **a**: Male, 22 years, HLA-DR3/DR4; **b**: Male, 8 years, DR3/DR4 0302; **c**: Female 32 years, DR3/DR4 0302.

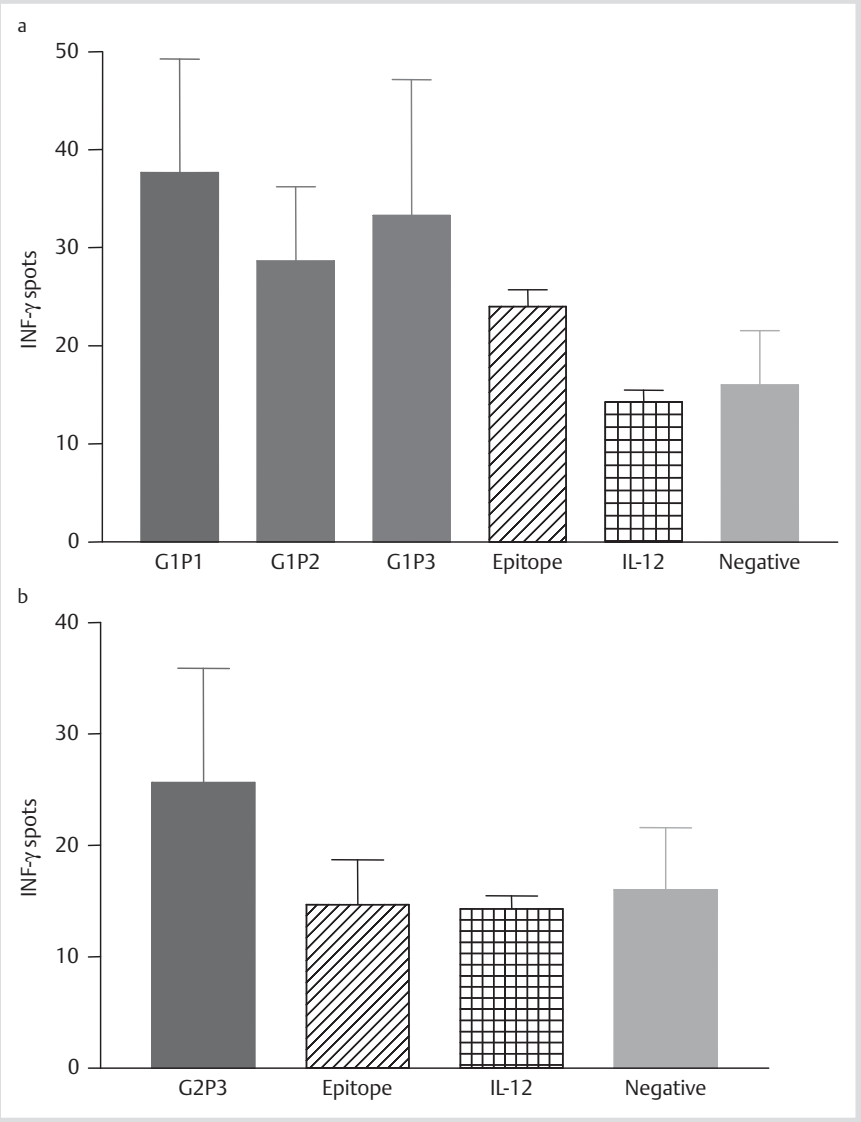

Fig. 4 Response to GAD/Ii-Key/hybrids. **a**: Female, 32 years; **b**: Male, 14 years.

Taken together, these data suggest that the T cell response profile can vary from an effector repertoire restricted to only a single epitope, to one with a rather broad autoimmune specificity based on recognition of multiple epitopes. Precise definition of the MHC/peptide specificity and functional properties of CD4+ve T cell populations are required for a complete mechanistic understanding of the disease process in type 1 diabetes.

The obvious diversity of T helper cell epitopes recognized by type 1 diabetes subjects makes it unlikely that an epitope specific immune intervention will be easily developed for type 1 diabetes. It is possible however, that the initial response is directed against particular determinants, as is the case for the NOD mouse model. Investigation of the T cell response in different disease stages may resolve this issue, and perhaps allow a specific immunotherapy to be initiated at a stage when sufficient numbers of βcells are still available to maintain metabolic homeostasis. Since extensive studies have confirmed the role of the ELISPOT assay to detect early antigen-directed activation of lymphocyte subpopulations in type 1 diabetes, we believe that the combined use of Ii-Key-hybrids in the ELISPOT assay might assure a more selective and sensitive cytokine detection in response to antigen stimulation. In conclusion, although this is a preliminary study, nonetheless the data are of interest.

Further studies are in progress including participation of more patients, in particular those with newly diagnosed T1D in childhood.

Conflict of Interest

This work was supported by an educational grant by Antigen Express-Generex Biotechnology. The peptides used in this study were designed in collaboration with Antigen Express, made, and donated by them. We independently planned and performed the experiments described in this paper. No other conflicts are reported but 2 authors (EVH and NKL) are Generex employees.

Affiliations

[1] Department of Endocrinology and Diabetes, University Campus Bio-Medico, Rome, Italy
[2] Bart's & the London School of Medicine & Dentistry, Centre of Diabetes, Institute of Cell and Molecular Science, Queen Mary University of London, UK
[3] Immunology Research Department, Livio Patrizi Foundation, Research Group BIOS, Rome, Italy
[4] Antigen Express Inc., Worcester, MA, USA
[5] Department of Clinical Science, University Sapienza, Rome, Italy

References

1 *Ott PA, Herzog BA, Quast S.* Islet-cell antigen-reactive T cells show different expansion rates and Th1/Th2 differentiation in type 1 diabetic patients and healthy controls. Clin Immunology 2005; 115: 102–114
2 *Karlsson Faresjo MGE, Ludvigsson J.* Diminished Th1-like response to autoantigens in children with a high risk of developing type 1 diabetes. Scan J Immunol 2005; 61: 173–179
3 *Galgani A, Petrone A, Spoletini M, Hodge A, Del Buono ML, Locatelli M, Buzzetti R, Diabfin Study Group.* HLA class II typing in newborns reveals a low frequency of the DRB1*04 allele and a high frequency of DRB1*11 allele in three regions of Continental Italy. Hum Immunology 2004; 65: 366–372
4 *Barrat BJ, Payne F, Lowe CE, Hermann R, Healy BC, Harold D, Concannon P, Gharani N, McCharty MI, Olavesen MG, McConmark R, Guja C, Ionescu-Tirgoviste C, Undlien DE, Ronningen KS, Gillespie KM, Tuomilheto-Wolf E, Tuomilehto J, Bennett ST, Clayton DG, Cordell HJ, Todd JA.* Remapping the Insulin Gene/IDDM2 Locus in type 1 Diabetes. Diabetes 2004; 53: 1884–1889
5 *Aribi M.* Candidate genes implicated in type 1 diabetes susceptibility. Curr Diabetes Rev 2008; 4: 110–121
6 *Meierhoff G, Ott PA, Lehmann PV.* Cytokine detection by ELISPOT: relevance for immunological studies in type 1 diabetes. Diabetes Metab Res Rev 2002; 18: 367–380
7 *Schloot NC.* Comparison of cytokine ELISPOT assay formats for the detection of islet antigen autoreactive T cells. Report of the Third Immunology of Diabetes Society T-cell Workshop. J Autoimmun 2003; 21: 365–376
8 *Endl J, Rosinger S, Schwarz B, Friedrich SO, Rothe G, Karges W, Scholsser M, Eiermann T, Schendel DJ, Boehm BO.* Co-expression of CD25 and Ox40 (CD134) receptors delineates autoreative T cells in type 1 diabetes mellitus. Diabetes 2006; 55: 50–60
9 *Xu M, Jackson R, Adams S, Humphreys RE.* Studies on activities of invariant chain peptides on releasing or exchanging of antigenic peptides at human leukocyte antigen-DR1. Arzneimittelforschung 1999; 49: 791–799
10 *Humphreys RE, Adams S, Koldzic G.* Increasing the potency of MHC class II-presented peptides by linkage to Ii-peptide. Vaccine 2000; 18: 2693–2697
11 *Kallinteris NL, Powell D, Blackwell CE, Kim M, Lu X, Wu S, Humphreys RE, Xu M, von Hofe E.* Ii-Key/MHC class II epitope peptides as helper T cell vaccines for cancer and infectious disease. Front Biosci 2006; 11: 46–58
12 *Kallinteris NL, Wu S, Lu X, von Hofe E, Humphreys RE, Xu M.* Linkage of Ii-Key segment to gp100 (46–58) epitope enhances the production of epitope-specific antibodies. Vaccine 2005; 23: 2336–2338
13 *Xu M, Li J, Gulfo JV, Von Hofe E, Humphreys RE.* MHC class II allosteric site drugs: new immunotherapeutics for malignant, infectious and autoimmune diseases. Scand J Immunol 2001; 54: 39–44
14 *Voutsas IF, Gritzapis AD, Mahaira LG, Salagianni M, von Hofe E, Kallinteris NL, Baxevanis CN.* Induction of potent CD4+ T cell-mediated antitumor responses by a helper HER-2/neu peptide linked to the Ii-Key moiety of the invariant chain. Int J Cancer 121: 2031–2041
15 *Kallinteris NL, Lu X, Blackwell CE, von Hofe E, Humphreys RE, Xu M.* Ii-Key/MHC class II epitope hybrids: a strategy that enhances MHC class II epitope loading to create more potent peptide vaccines. Expert Opin Biol Ther 2006; 6: 1311–1321
16 *Holmes JP, Benavides LC, Gates JD, Carmichael MG, Hueman MT, Mittendorf EA, Murray JL, Amin A, Craig D, von Hofe E, Ponniah S, Peoples GE.* Results of the first phase I clinical trial of the novel Ii-key hybrid preventive HER-2/neu peptide (AE37) vaccine. J Clin Oncol 2008; 26: 3426–3433
17 *Buzzetti R, Galgani A, Petrone A.* Genetic prediction of type 1 diabetes in a population with low frequency of HLA risk genotypes and low incidence of the disease (the DIABFIN study). Diabetes Metab Res Rev 2004; 20: 137–143
18 *Kotani R, Nagata M, Moriyama H.* Detection of GAD65-reactive T cells in type 1 diabetes by immunoglobulin-free ELISPOT assays. Diabetes Care 2002; 25: 1390–1397
19 *Kallinteris NL, Wu S, Lu X, Humphreys RE, von Hofe E, Xu M.* Enhanced CD4+ T-cell response in DR4-transgenic mice to a hybrid peptide linking the Ii-Key segment of the invariant chain to the melanoma gp100 (48-58) MHC class II epitope. J Immunother 2005; 28: 352–358
20 *Oling V, Marttila J, Ilonen J, Kwok WW, Nepom G, Knip M, Simell O, Reijonen H.* GAD65- and proinsulin-specific CD4+ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects. J Autoimmun 2005; 25: 235–243
21 *Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M.* Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. J Clin Invest 2004; 113: 451–463

Author,
Please check the Figure 2.
Thank you