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Molecular Mimicry in Type 1 Diabetes Mellitus Revisited: T-Cell Clones to GAD65 Peptides with Sequence Homology to Coxsackie or Proinsulin Peptides do not Crossreact with Homologous Counterpart

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ABSTRACT: Type 1 diabetes mellitus is a T-cell mediated autoimmune disease in which the insulin-producing pancreatic beta cells are selectively destroyed. Molecular mimicry and T-cell crossreactivity to β-cell autoantigens and environmental agents with sequence similarities have been a proposed mechanism underlying the pathogenesis of type 1 diabetes, but actual crossreactivity has not yet been demonstrated. We isolated and investigated T cells reactive to GAD65 peptides and homologous peptides of the Coxsackie virus protein P2C and proinsulin from recent onset type 1 diabetes patients, and tested their fine specificity and cytokine production profile. Six T-cell lines specific for GAD65 peptides (amino acids 491-530) with homology to proinsulin (B20-C14) were isolated from six newly diagnosed patients with type 1 diabetes, but none of the stable T-cell lines crossreacted to the homologous proinsulin peptides. Similarly, none of four T-cell lines reactive to GAD65 peptides (amino acids 247-280) with sequence homology to Coxsackie P2C (amino acids 30-50) crossreacted to the homologous viral peptide. Two T-cell lines corecognized a GAD65 peptide and a Coxsackie P2C peptide. However, the antigen-specific T-cell clones from these T-cell lines were reacting either with the GAD65 peptide or the Coxsackie P2C peptide using different restriction elements without cross-reacting to the homologous peptide. Our data demonstrate that homologous peptides previously proposed to serve as targets for crossreactivity indeed are immunogenic. Yet, T-cell clones did not crossreact with linear sequence homologies, despite strong T-cell responses to individual peptides. *Human Immunology 62, 299–309 (2001).* © American Society for Histocompatibility and Immunogenetics, 2001. Published by Elsevier Science Inc.

KEYWORDS: Molecular mimicry; autoimmune disease; Coxsackie virus; GAD65; proinsulin

INTRODUCTION

Type 1 diabetes mellitus is the result of a genetically associated immune-mediated process in which the insulin-producing pancreatic β-cells are thought to be destroyed by autoreactive T cells [1]. The majority of patients with type 1 diabetes mellitus develop humoral and cellular immune responses to defined islet autoantigens such as GAD65 (glutamic acid decarboxylase) [2–4], insulin [5, 6], proinsulin [7], and tyrosine phosphatase like proteins IA-2/IA-2β [8–10]. In animal models, T cells reactive to GAD65, insulin, and proinsulin have been shown to transfer insulitis and autoimmune diabetes [11–13]. NOD mice develop T-cell reactivity to GAD65 and GAD65 peptides sharing sequence homology with the viral protein P2C early in the disease process [14]. Prophylactic treatment of NOD mice with GAD or insulin prevents the development of autoimmune diabetes [14, 15].

It is widely anticipated that microbial antigens could be etiologic agents that initiate an (auto)immune T-cell response crossreactive with host proteins, a mechanism termed molecular mimicry [16–19]. Indeed, pathogenic T cells reactive to virus protein have been shown to

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TABLE 1 Age (years), duration of diabetes (days), HLA type, and gender of the patients with type 1 diabetes mellitus

Patient	Age	Duration of diabetes	HLA	Gender
ED	11.3	2	DR4/7, DQ2/8	F
RG	14.1	12	DR4/5(12), DQ7/8	M
BM	5.4	1	DR1/3, DQ5/2	M
YB	12.0	14	DR3/4, DQ2/8	M
JH	16.4	10	DR3/9, DQ2/9	M
KG	8.4	12	DR1/4, DQ5/8	M
FB	12.4	14	DR3/3, DQ2/2	M
AZ	9.2	1	DR3/4, DQ2/8	F
RP	13.2	5	DR3/4, DQ2/8	F
PN	14.4	13	DR13/8, DQ6/4	M
MG	8.1	1	DR4/4, DQ8/8	M

crossreact with homologous peptide from an autoantigen in human multiple sclerosis [20, 21] and in animal models of herpes stromal keratitis [22], encephalomyelitis [23], uveitis [24], and autoimmune diabetes [25]. A role of molecular mimicry between GAD65 and Coxsackie virus protein P2C in the pathogenesis of type 1 diabetes mellitus has been suggested [17, 19, 26, 27]. Corecognition of GAD65 and its homologous viral peptide of Coxsackie P2C has been reported on bulk culture level [4]. Likewise, the β-cell specific autoantigen proinsulin has a region of similarity with GAD65 suggestive of a potential T-cell epitope mimicry between these two islet antigens [28] and reactive T cells have been described on the bulk culture level [7]. However, bulk cultures consist of multiple T cells and an observed crossreactivity to more than one antigen or peptide might simply reflect T-cell proliferation of several T cells with different specificity [29].

We have generated autoreactive T-cell lines and clones from newly diagnosed patients with type 1 diabetes mellitus to elucidate T-cell crossreactivity. Antigen-specific T cells were generated with GAD65 peptides and their molecular mimicry peptides of Coxsackie P2C and homologous peptides of proinsulin. The ability to crossreact with the homologous peptide was analyzed studying antigen-specific T-cell proliferation, restriction elements, and cytokine production.

MATERIAL AND METHODS

Patients

After informed consent 30-ml peripheral blood was drawn from 11 newly diagnosed patients with type 1 diabetes mellitus who had been on insulin therapy for less than 2 weeks (Table 1). Peripheral blood mononuclear cells (PBMC) were used for the determination of T-cell autoreactivity.

Antigens

A panel of peptides spanning the homologous amino acid sequence (Figure 1) of human GAD65 (GAD65), human proinsulin, and Coxsackie virus protein P2C (with both isoforms described at aminoacid at position 48) was synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) [30]. Longer peptides were chosen for GAD65 peptides to test contribution of flanking sequences to immunogenicity. Due to synthesis difficulties and low yields, shortened peptides of GAD65 peptides 491-510 and 501-520 were tested in follow up studies (GAD 65 494-510 and 504-20). Peptides were tested as pools of either GAD65/proinsulin peptides or GAD65/Coxsackie P2C peptides for the initiation of all T-cell lines and the investigation of primary responses. The designation of peptides in the text refers to single peptides depicting the first and last position of the peptide (Figure 1) or to overlapping peptides (peptide pools), indicating the first and last amino acid position of the pool stretching the designated region. The baculovirus expressed 65 kDa isoform of human islet glutamic acid decarboxylas was prepared as described [31].

Generation of T-Cell Lines and Clones

PBMC were isolated by Ficoll centrifugation and resuspended in culture medium (Iscove's modified Dulbecco Medium [Gibco BRL, Paisely, Scotland] containing 10% autologous human serum). For the generation of T-cell lines, 1.5×10^5 freshly isolated PBMC were incubated in culture medium containing pools of peptides from the GAD65 and proinsulin homology region or pools of GAD65 and Coxsackie P2C region at a final concentration of 3 μg/ml per peptide.

The cells were incubated for six days in round bottomed 96 well plates (Costar, Cambridge, MA, USA) and then expanded in culture medium containing 10% Lymphocult T (Biotest, Dreieich, Germany) as a source of IL-2. At day 14, 2×10^5 T cells were restimulated with 1% PHA (Murex Biotech LTD, Darford, England) and 1×10^6 feeder cells (PBMC irradiated with 30 Gray) and after additional 3 days expanded by adding 10% Lymphocult T. T-cell cultures were repeatedly split and after a further 5- to 10-day incubation, frozen, and tested or cloned by limiting dilution at a cell concentration of 0.3 cells/well. Cloning was performed as described [32, 33]. In brief, T-cell lines were first restimulated with specific peptides and expanded by addition of IL-2. Consecutive cloning was performed using 1% PHA and feeder cells. Positive clones were expanded in additional rounds of stimulation with peptide-pulsed HLA-matched antigen-presenting cells (APC) or 1% PHA. Antigen specificity

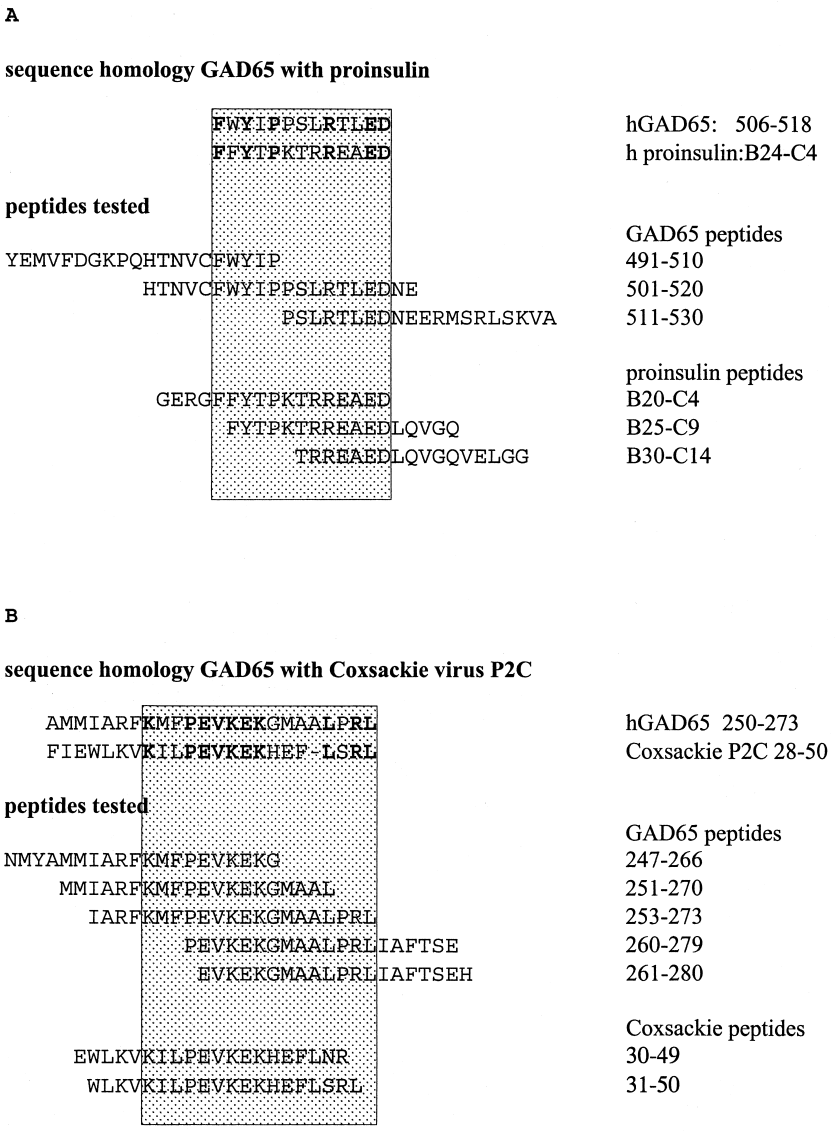

FIGURE 1 Amino acid sequence of synthesised peptides of human GAD65 peptides and the homology peptides of proinsulin (A) and Coxsackie P2C peptides (B).

of T-cell lines and clones was tested by incubating 10^4 T cells with 5×10^4 irradiated (20 Gray) autologous or HLA matched PBMC in the presence of antigen. For the determination of restriction elements or cytokine secretion, APC matched for the HLA-restriction element were used. Cells were cultured in triplicates in medium alone, or with a concentration range of 1- to 10-μg/ml peptides, 10 μg/ml GAD65, or IL-2 (Lymphocult) 10%. Blocking monoclonal antibodies to HLA-DR (B8.11.2, IgG2b) and HLA-DQ (SPV-L3, IgG2a) were used in inhibition studies at 10 μg/ml [32]. Cultures were set up in a final volume of 150-μl culture medium per well in a flat bottom 96-well microculture plates (Greiner, Nürtingen, Germany). After 3 days of incubation, 50 μl RPMI 1640 (Dutch modification; Gibco BRL) containing 0.5 μCi [^{3}H]thymidine (NEN Dupont, Boston, MA, USA) per well was added, and incubation was continued for 16 h. Cultures were then harvested onto glass fiber-filters and [^{3}H]thymidine incorporation was measured by liquid scintillation counting. The results are expressed as stimulation index (SI; mean cpm in presence of antigen divided by mean cpm without antigen).

T-cell lines were all tested and characterized after the initial stimulation with antigen that was followed by a second round of stimulation with PHA 1%. Before testing, T cells were frozen down and the same batch was used to perform all the experiments if not indicated otherwise. In case of reactivity to more than one peptide tested, cloning and expansion of T-cell clones was performed as described [31–33] and T-cell clones or sublines from one batch were investigated.

Flow cytometric analysis was performed using FITC

and PE labeled antibodies (Becton Dickinson, San Jose, CA, USA) specific for CD4 and CD8 as described [34]. Sequencing of T-cell receptor α and β was performed as described previously [35].

Cytokine Measurements

The T cells (2×10^4) and HLA-matched (5×10^4) APCs (irradiated 20 Gray) were incubated in medium supplemented with 10% heat inactivated FCS, with PMA 10 ng/ml (Sigma, Zwijndrecht, The Netherlands), and 1 mM ionomycin (Calbiochem, La Jolla, CA, USA), or with addition of GAD65 peptides, proinsulin peptides or Coxsackie P2C peptides at 2 μg/ml. For the determination of background cytokine release, irradiated APCs were incubated alone. Supernatants were harvested after 72-h incubation and tested for interferon-γ, IL-4, and IL-10 (Pelikine Compact Elisa Kit; CLB, Amsterdam, The Netherlands). Background cytokine release was below detection levels if not indicated otherwise. Parallel to the cytokine secretion assay, T cells from the same batch were tested in a proliferation assay to assure that reactivity described above was given.

Lymphocyte Stimulation Assay

To evaluate primary T-cell responses to molecular mimicry antigens, PBMC were tested in a primary lymphocyte stimulation assay as described previously [36]. The assays were performed in parallel to the generation of T-cell lines using the same antigen-concentrations with the protocol used for the T-cell lines. The results are expressed as SI of median proliferation (cpm) of antigen-specific proliferation divided by median proliferation (cpm) in presence of medium (background) [10]. A SI of >3 was considered positive.

RESULTS

T Cells Specific for GAD65/Proinsulin Peptides

T-cell lines specific for GAD65/proinsulin peptides were obtained from 6/11 (54.5%) of the patients and consisted predominantly of $CD4^+$ T cells as determined by FACS analysis (data not shown). Four T-cell lines, generated with a pool of GAD65 peptides and proinsulin peptides recognized GAD65 peptides only (Figure 2A). These T-cell lines proliferated specifically in presence of the peptides GAD65 491-510 (YB, JH, KG), GAD65 501-520 (ED), and GAD65 511-530 (YB) (Table 2). The restriction element was evaluated using partly matched APC and blocking antibodies for HLA-DR, -DQ, and -DP. T-cell responses towards these peptides were restricted by DR4 (ED), DR3 (YB, JH), and DQ5 (KG).

Two T-cell lines (RG and BM) proliferated specifically in presence of GAD65 peptides as well as to proinsulin peptides in the initial test (Figure 2A). However, when the same APC and the same batch of T cells were retested, the antigen-specific proliferative response towards the proinsulin peptides was lost.

The T-cell line and a clone derived of BM proliferated to GAD65 501-520 in a DR1 restricted fashion (Table 2). T-cell line RG recognized GAD65 501-520 in a DR4 restricted manner (Table 2A), and also responded to GAD65 511-530 with APCs matched for HLA-DR5-DQ7 (data not shown). No T-cell crossreactivity to the homologous proinsulin-peptide of these stable T-cell lines was found. The data indicate that stable T-cell lines isolated and specific for GAD65 peptide do not crossreact with the homologous peptide of proinsulin.

Only the GAD65 peptide reactive T-cell line JH proliferated in presence of whole GAD65 (SI 35.9, background proliferation 152 cpm).

T Cells Specific for GAD65/Coxsackie P2C Peptides

T-cell lines specific for GAD65/P2C peptides were obtained from 4/10 (40%) type 1 diabetes patients (Figure 2B) and consisted predominantly of CD4 positive T cells as determined by flow cytometry (data not shown). None of the T-cell lines proliferated in presence of whole protein GAD65. Two of the T-cell lines (RG and JH) proliferated to GAD65 peptides only and were restricted by HLA-DR: T-cell line RG recognized GAD65 peptide 251-270 in an HLA-DR5 [12] restricted fashion; T-cell line JH recognized GAD65 247-266 (Table 3) via HLA-DR3 and the overlapping peptide GAD65 251-270 (data not shown). The other two T-cell lines proliferated to GAD65 peptides as well as to P2C peptides (BM, YB) (Figure 2B). To evaluate whether the measured T-cell response was the combined result of T-cells with different antigen specificity or the result of a single crossreactive T cell, the T-cell lines were restimulated and cloned by limiting dilution.

Eleven clones were retrieved from BM (cloning efficiency $11/288 = 3.8\%$) of which one clone (BM.5) proliferated specifically to GAD65 247-266 (DR3 restricted) (Table 3) but not to the Coxsackie P2C peptide with sequence homology. A second clone (BM.10) recognized the Coxsackie P2C peptide 31-50 (DPB1*0401 restricted) (Table 3) without crossreactivity to the homologues GAD65 peptide. These results demonstrate that the observed corecognition of the original T-cell line is the result of at least two separate T-cell clones.

Seventy-six clones were obtained from YB (cloning efficacy 38.5%), of which 38 clones (50%) proliferated in presence of the Coxsackie PC-2 peptide only and not to the homologous GAD65 peptide. One T-cell clone (YB.35) recognized Coxsackie P2C peptide 261-280 as well as GAD65 261-280, but was subsequently shown to consist of two subclones. The minimal epitope of the

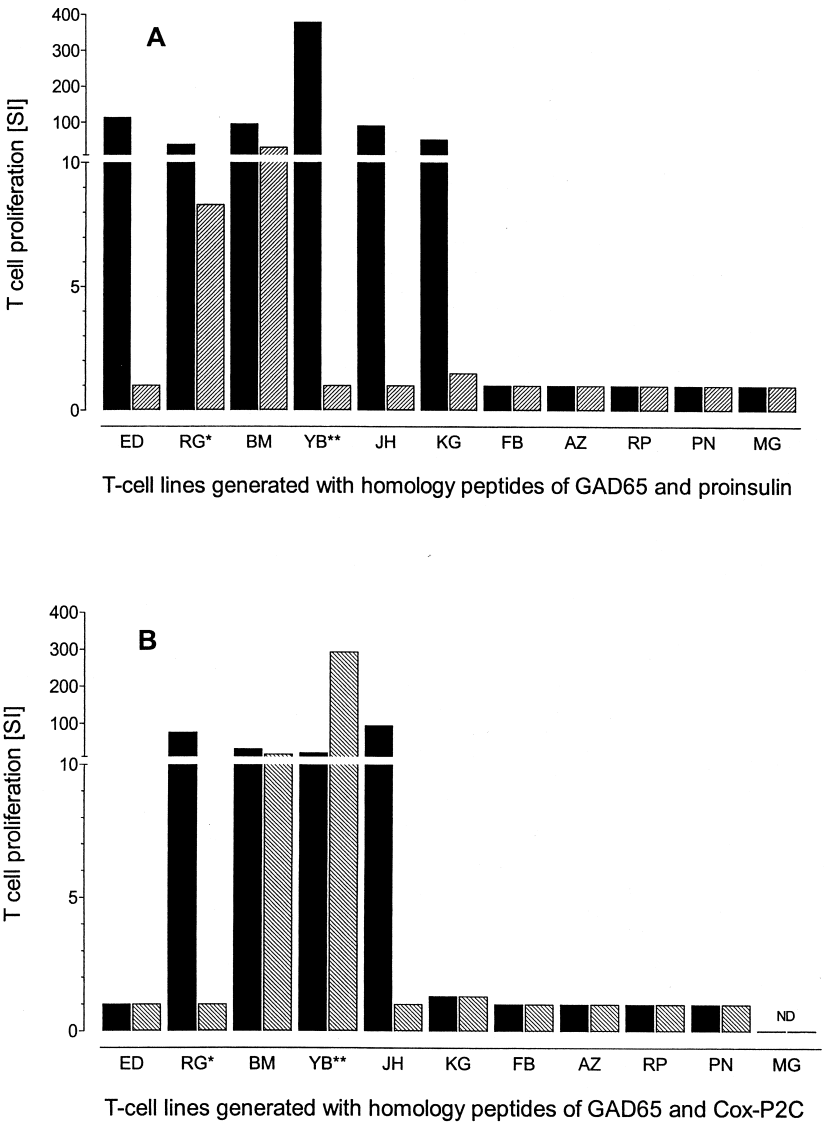

FIGURE 2 Antigen-specific proliferation (SI) of T-cell lines generated from newly diagnosed patients with type 1 diabetes. T-cell lines were tested after the initial incubation (antigen incubation followed by expansion with PHA). Autologous APC were used if not indicated otherwise. (A) Reactivity of T-cell lines generated with GAD65 peptides 491-530 and homologous proinsulin peptides B20-C14. Black bars represent reactivity towards GAD65 peptides, hatched bars represent reactivity towards proinsulin peptides. *APC: HLA-DR5(11/12), DQ7/7, **APC: HLA-DR 3/3, DQ2/2. (B) Reactivity of T-cell lines generated with GAD65 peptides 247-280 and homologues Coxsackie P2C peptides 30-50. Black bars represent reactivity towards GAD65 peptides, hatched bars represent reactivity towards Coxsackie P2C peptides. *APC: HLA-DR5 (11/12), DQ7/7, **APC: HLA-DR1/4, DQ5/8.

Coxsackie P2C peptide sequence recognized by YB.35 was mapped to Coxsackie P2C 30-41(EWLKVKILPEVK). This region displays linear sequence homology with GAD65 252-263. However, the GAD65 peptide recognized by YB.35 spans GAD65 261-280 (EVKEKGMAALPRLIAFTSEH), which overlaps with the Coxsackie P2C sequence by three amino acids only (Figure 1). T-cell recognition of the Coxsackie P2C peptide response was restricted by HLA-DQ8, whereas the GAD65 peptide response was restricted by HLA-DR4. The different restriction elements used by YB.35 together with recognition of almost nonoverlapping peptides of GAD65 and Coxsackie P2C suggested that YB.35 still consisted of different subsets of T cells. This was confirmed by T-cell receptor (TCR) analysis and FACs staining, which revealed at least two different T-cell populations in YB.35 (data not shown). Upon subcloning of YB.35, 24 T-cell clones were obtained of which three clones recognized the GAD65 261-280 but failed to crossreact to the Coxsackie P2C peptide. These results indicate that the crossreactivity to the Coxsackie P2C peptides and GAD65 peptides result from the occurrence of at least two different T-cell populations.

Cytokine Production Profile

Cytokine release of the antigen-specific T cells was determined after antigen-specific stimulation with the recognized peptide and the peptide with sequence homology (Table 4). For these experiments appropriate heterologous HLA-matched APCs were used. In parallel, T cells were stimulated with PMA and ionomycin. Upon removal of supernatant for cytokine measurements, T cells were pulsed with radioactive thymidine to assess proliferation and only those experiments were analyzed

TABLE 2 Reactivity of T-cell lines/clones generated with GAD65 peptides 491–530 and homologous proinsulin peptides B20–C14

Patient	HLA DR	HLA DQ	APC DR	APC DQ	Recognized peptide	Background proliferation	Stimulation index (SI) Antigen	+αDR	+αDQ	+αDP	Restriction element	Crossreactivity
YB	3,4	2,8	3,3	2,2	GAD 491–510	138 ± 57	269.4	1.9	156.9	150.8	DR3	—
JH	3,9	2,9	3,7	2,9	GAD 491–510	151 ± 41	95.6	2.8	111.0	73.3	DR3	—
KG	1,4	5,8	1,1	5,5	GAD 491–510	81 ± 46	94.3	39.9	1.1	28.2	DQ5	—
BM*	1,3	5,2	1,1	5,5	GAD 501–520	88 ± 92	308.0	9.5	219.9	n.d.	DR1	—
ED	4,7	2,8	4,7	7,9	GAD 501–520	131 ± 61	66.1	0.9	48.1	45.4	DR4[1]	—
RG	4,5	7,8	3,4	2,8	GAD 501–520	57 ± 10	65.4	0.7	93.6	75.1	DR4	—
YB	3,4	2,8	3,3	2,2	GAD 511–530	69 ± 10	13.4	1.3	16.4	15.0	DR3	—

Peptide specific proliferation (SE) of stable T-cell lines, blocking studies with anti-HLA antibodies and determination of restriction elements using partly matched, heterologous APC. Background proliferation is given in mean cpm ± SD. * T-cell clone.

[1] HLA-DR4 restriction element confirmed by PBMC panel studies (data not shown).

in which proliferation was reproduced as in previous experiments (data not shown).

The T-cell lines/and clones were of Th1/Th0-like phenotype, because they produced IFN-γ and IL-10 but low levels of IL-4 upon stimulation with either stimulus (Table 4). The majority of T-cell lines secreted cytokines in presence of one specific peptide but not with the homologous peptide. However, T-cell clone BM.10 secreted IFN-γ upon stimulation with GAD65 247-280 as well as in presence of the homologous sequence of Coxsackie P2C 31-50. Proliferation of BM.10 was only observed in presence of Coxsackie P2C 31-50 (Table 3) and not with the GAD65 of sequence homology (data not shown).

Association of Primary T-Cell Response and Reactivity of T-Cell Lines

The patients in the present study were investigated with regard to primary responses (LST) and on the levels of T-cell lines for reactivity homology peptides derived from GAD65, proinsulin, and Coxsackie P2C. One patient (KG) was not tested for primary responses. Positive primary responses to GAD65/proinsulin peptides or GAD65/Coxsackie P2C peptides were measured in 50% (5/10) patients (Table 5). Reactivity to the antigens at the stage of T-cell lines was detected in 6/11 (54.5%) patients tested. No correlation between initial ("primary") responses and reactivity of T-cell lines was detected.

DISCUSSION

Molecular mimicry between the nonstructural protein 2C of Coxsackievirus B4 and the autoantigen GAD65 has been a proposed mechanism underlying the activation of autoantigenic T cells in type 1 diabetes mellitus [17, 19]. We investigated stable T-cell lines that specifically recognized Coxsackie-virus P2C peptides and/or GAD65 peptides with sequence homology. Similar to previous studies in which T-cell reactivity to these antigens was observed in newly diagnosed patients with type 1 diabetes mellitus and their first degree relatives on the bulk culture level, we obtained such T cells from 40% (4/10) of the patients investigated [4, 37]. Our results

TABLE 3 Reactivity of T-cell lines/clones generated with GAD65 peptides 247–280 and homologous Coxsackie P2C peptides 30–50

Patient	HLA DR	HLA DQ	APC DR	APC DQ	Recognized peptide	Background proliferation	Stimulation index (SI) Antigen	+αDR	+αDQ	+αDP	Restriction element	Crossreactivity
BM.5*	1,3	5,2	3,4	2,8	GAD 247–266	21 ± 8	1896.1	1.9	1929.8	n.d.	DR3	—
JH	3,9	2,9	3,7	2,9	GAD 247–266	174 ± 266	118.3	0.9	43.6	148.1	DR3	—
RG	4,5(12)	7,8	11,12	7,7	GAD 251–270	63 ± 56	177.2	1.24	155.0	201.9	DR12	—
YB.35†	3,4	2,8	4,8	8,8	GAD 261–280	46 ± 2	210.3	250.6	1.7	n.d.	DQ8	—[3]
BM.10*	1,3	5,2[1]	3,4	2,8[2]	Cox 31–50	39 ± 37	234.6	190.0	173.9	4.1	DPw4	—
YB.35†	3,4	2,8	3,4	2,8	Cox 30–49, 31–50	150 ± 280	8.7	0.7	13.4	12.5	DR4[4]	—[3]

Peptide specific proliferation (SI) of stable T-cell lines, blocking studies with anti-HLA antibodies and determination of restriction elements using partly matched, heterologous APC. Background proliferation is given in mean cpm ± SD. * T-cell clone,† T-cell subline.

[1] DPB1* 1301, 0401, [2] DPB1*0201,0401. [3] Both experiments were performed with the same subline of YB.35. Additional subcloning and T-cell receptor sequencing revealed the existence of multiple T-cells and lack of cross reactivity on the clonal level of YB (data not shown).

[4] HLA-DR4 restriction element confirmed by PBMC panel studies (data not shown).

TABLE 4 Cytokine production profile (IFNγ, IL-4, and IL-10 [pg/ml]) of antigen-specific T-cells.

T-cell line		PMA + ionomycin			Specific peptide		
Patient	Antigen	IFNγ	IL-4	IL-10	IFNγ	IL-4	IL-10
GAD65/proinsulin peptide							
RG	GAD 501–520	17,310	3.2	108.5	116.5	—	—
	proins B20–C14				—	—	—
BM	GAD 504–520	>3,600	6.3	—	519.9	—	—
	proins B20–C14				95.6[1]	—	—
YB	GAD 491–510	270.4	—	—	>32,000	2.3	339.9
	GAD 511–530				1,439	—	12.9
	proins B20–C14				90.1[1]	—	—
KG	GAD 491–510	3,225	2.9	—	387.9	—	—
	proins B20–C14				—	—	—
GAD65/Coxsackie P2C peptide							
RG	GAD 251–270	5,500	3.9	14	306	—	22.6
	Coxsackie 30–50				n.o.	n.o.	n.o.
BM.5*	GAD 247–266	>32,400	298.3	—	3,445	68.1	58.2
	Coxsackie 31–50				—	—	—
BM.10*	GAD 247–280	>32,400	87.9	21.2	76.7	—	—
	Coxsackie 31–50				131.7	—	—
YB.35**	GAD 261–280	>10,600	24.4	97.2	131	—	—
	Coxsackie 30–50				4,590	4.6	60.3

Detection levels were 15 pg/ml for IFNγ, 1.2 pg/ml for IL-4, and 5 pg/ml for IL-10.
* T-cell clone; † T-cell subline; [1] Values were similar to background levels; n.o. = not obtained.

demonstrate that GAD65 peptides with homology to Coxsackie P2C protein indeed are immunogenic. However, none of the isolated T cells crossreacted to the peptide with linear sequence homology. Our observations suggest that previously published data of T-cell responses to homologous peptides in bulk culture are unlikely to be due to crossreactive T cells [4].

TABLE 5 Association of initial "primary" T-cell response (LST) and antigen-reactivity of T-cell lines to GAD65 peptide 491–530, proinsulin peptides (B20–C14), GAD65 peptide 247–280, and Coxsackie P2C peptides 30–50

	GAD/Proinsulin		GAD/Coxsackie	
Patient	LST	Line	LST	Line
ED	**6.8**	+	1.2	—
RG	0.4	+	0.6	+
BM	1.0	+	**3.0**	+
YB	0.5	+	0.8	+
JH	0.4	+	**4.5**	+
KG	nt	+	nt	—
FB	2.1	—	1.5	—
AZ	**6.9**	—	**4.7**	—
RP	1.7	—	2.3	—
PN	1.8	—	1.4	—
MG	1.7	—	**4.0**	nt
Positive response	2/10 (20%)	6/11 (55%)	4/10 (40%)	4/11 (36%)

Abbreviations: nt = not tested; + = specific; — = nonspecific, as depicted in Figure 2.

Several caveats in the experimental design of this study must be acknowledged. The T-cell lines obtained in this study could be a result of *in vitro* priming and may recognize peptides that are not relevant or naturally processed *in vivo*. Although this possibility cannot be excluded for all T-cell lines, one T-cell line (JH) generated with recombinant peptides also responded to whole protein GAD65. Whether the other T-cell lines respond to cryptic epitopes only or respond to whole GAD65 at a different concentration than tested was not resolved in this study. Another limitation of the study could be the generation of T-cell lines using peptide pools rather than single peptides that may have biased the T-cell response towards GAD65 peptides that override the response to Coxsackie P2C or proinsulin peptides because of different HLA binding affinities or different precursor frequencies. However, this strategy was driven by the original claims that peptides identical to those tested in this study provide evidence for crossreactivity [4]. Validation of this interesting observation formed the basis of our extended studies. Interestingly, two T-cell lines recognized GAD65 peptides as well as Coxsackie P2C peptides, but this corecognition was the combined result of different T-cell clones displaying reactivity to either GAD65 peptides or Coxsackie P2C peptides and using different restriction elements.

We were concerned with the limited degree of T-cell

crossreactivity between sysnthetic peptides and naturally processed peptides of autoantigens or viral protein, although this not a novel finding [38]. In studies in hen egg white lysozyme (HEL) transgenic mice, T cells raised upon immunization with HEL peptides did not crossreact with whole HEL, while T cells of HEL immunized mice crossreacted with peptides thereof [38]. This was explained by differences in epitope concentration on the APC or the weak interactions that exogenous synthetic peptides may have to allow negative selection in the thymus compared with naturally processed peptides. However, it is important to note that T cells reactive with synthetic "cryptic" peptides can be relevant to disease pathogenesis. First, our findings prove that T cells reactive with synthetic autoantigenic peptide circulate in. They may have escaped negative selection and be triggered by encountering peptides presented under unique circumstances such as viral infection or inflammatory leasions (insulitis) that modify the their threshold of activation. Intriguingly, Wen and colleagues [39] recently described a T-cell clone of HLA-DQ8 transgenic mice recognizing the same GAD65 epitope as some of our human T-cell clones, which were unable to respond to naturally processed GAD65. Yet, this T-cell clone was able to induce insulitis upon adoptive transfer to HLA-DQ8 transgenic mice, but not nontransgenic mice, suggesting that this type of T-cell reactivity to "cryptic" epitopes may be relevant to the pathogenesis of autoimmune diabetes.

We observed that initial ("primary" *in vitro*) responses in PBMC and secondary responses (T-cell lines and clones) to the peptides did not correlate. This could result from low precursor frequencies of peptide epitope specific T cells in peripheral blood and/or regulation of the primary response. We believe that *in vitro* priming is an unlikely explanation for this finding due to the short duration of the T-cell expansion.

The data indicate that crossreactivity on the (clonal) T-cell level towards GAD65 and the regions of sequence homology of Coxsackie P2C protein is not evident at disease manifestation of type 1 diabetes in our study. However, the method used may not necessarily be able to detect all crossreactive T cells. The T cells isolated produced Th1/Th0-like cytokines, which have been associated with destructive insulitis and type 1 diabetes mellitus [40–42]. Interestingly, one T-cell clone secreted IFNγ upon stimulation with GAD65 peptide as well as with Coxsackie P2C peptide, but proliferated in presence of the Coxsackie P2C peptide only. This indicates that crossreactivity can be measured by different parameters and careful evaluation of crossreactivity is necessary to fully address different qualities of the response.

T-cell studies in humans and animal models have suggested that linear sequence similarity of GAD65 and proinsulin may play a role in the pathogenesis of autoimmune diabetes. Proinsulin-reactive T cells in animal models are capable of transferring insulitis into healthy recipients, and proinsulin-peptide and GAD65-peptide reactive cells have been detected in human first degree relatives of patients with type 1 diabetes on the bulk culture level [7, 13, 28]. We obtained stable T-cell lines with reactivity to the region of sequence homology of proinsulin and GAD65 from 54.5% (6/11) of newly diagnosed patients. However, the stable T-cell lines recognize GAD65 peptides only and do not crossreact to proinsulin-peptides of sequence homology.

The restriction element utilized by the T cells was predominantly DR3 and DR4, both haplotypes predisposing to diabetes. Interestingly, HLA-binding affinities of the peptides investigated correspond well with their antigenicity [43–45]. GAD65 peptides 491-530 recognized high to intermediate binding affinity to HLA-DR1, DR3 and DR4, whereas binding of proinsulin-peptides (B20-C14) was rather weak [43]. The peptides of GAD65 with linear sequence homology to Coxsackie P2C, however, have similar binding characteristics [46] and stable T-cell lines and clones could be established. It is not clear whether the lack of proinsulin-reactive T cells is a result of negative selection in the thymus, too low precursor frequency in the peripheral blood, or a consequence of immune regulation or peripheral tolerance.

Database screening has been commonly used to search for molecular mimicry between unrelated proteins. Because of the large size of protein data bases, it is fairly easy to find several amino acid matches between unrelated proteins [16, 18, 28, 47, 48]. Sequence homology of the diabetes associated autoantigen GAD65 with Coxsackie P2C and proinsulin has been suggested to play an important role in initiating and perpetuating autoimmunity in diabetes [4, 17, 19, 26, 28, 49]. However, proof that crossreactivity on the T-cell level actually occurs in patients with type 1 diabetes is lacking. Recent murine studies suggest that Coxsackie virus can induce diabetes by bystander damage rather than molecular mimicry [50]. Our results indicate that it is rather unlikely that crossreactivity of the GAD65, Coxsackie P2C, and proinsulin is a common phenomenon at diabetes onset. Nevertheless, molecular mimicry is not unlikely to occur, since T-cell clones can equally well recognize two different peptides that share only very few amino acids but resemble each other in structure [21, 51, 52]. The identification of such amino-acid patterns representing mimicry epitopes obtained from dedicated peptide libraries are likely to reveal new peptides of sequence similarity to proteins not detected by searching for linear sequence homology.

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