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T cell reactivity to DR*0401- and DQ*0302-binding peptides of the putative autoantigen IA-2 in type 1 diabetes

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Summary: Type 1 diabetes is thought to be an autoimmune disease mediated by T lymphocytes recognizing critical islet cell antigens. Recently, the tyrosine phosphatase like protein IA-2 was suggested as a putative autoantigen in type 1 diabetes since autoantibodies are detected in sera of diabetic patients and prediabetic subjects. Similarly, T cell responses of peripheral blood lymphocytes of type 1 diabetic patients to this protein have been described. Only very few data is available about immunodominant epitopes of IA-2 recognized by T cells. We have studied T cell responses in type 1 diabetic patients and age and partly HLA matched controls to IA-2 peptides designed to bind HLA risk alleles of IDDM as DR*0401 and DQ*0302. Both diabetic patients and controls responded to IA-2ic and some of the peptides. Three peptides of the C-terminal region of IA-2 were recognised by T cells of a fraction of diabetic patients but at least two of these peptides triggered also T cell responses in DR*0401/DQ*0302-matched controls. Most peptides bound to different HLA alleles ("promiscous binders"). The identification of autoantigenic epitopes may offer clues to related sequences e.g. of viral origin what relates to models of diabetes pathogenesis ("molecular mimicry"). Secondly, the design of antigen- or even epitope-specific immune intervention strategies aiming at tolerization of disease specific T cells in type 1 diabetes may profit from the knowledge of immunodominant T cell epitopes of a putative autoantigen.

Introduction

Type 1 diabetes is an autoimmune disease probably mediated by T lymphocytes recognizing critical islet cell antigens (Bach, 1994). Some of the putative autoantigens in type 1 diabetes are identified as glutamic acid decarboxylase (GAD; Baekkeskov et al., 1990) and the tyrosine phosphatase-like protein IA-2 (Lan et al., 1994). The latter antigen has a strong homology to another putative autoantigen in type 1 diabetes, islet cell antigen 512 (ICA 512; Rabin et al., 1994), and is identical to the previously described tryptic fragment "40kD autoantigen" (Christie et al., 1992; Payton et al., 1995). Autoantibodies to these antigens have been detected in sera of patients before (Seissler et al., 1996; Christie et al., 1992; Verge et al., 1996; Mayrhofer et al., 1996) and at diagnosis of type 1 diabetes (Seissler et al., 1993; Wiest-Ladenburger et al., 1997; Morgenthaler et al., 1997). T cell responses to GAD 65 (Atkinson et al.,1992) or IA-2 (Durinovic-Bello et al., 1996) have been detected in peripheral blood lymphocytes (PBL's) of patients with type 1 diabetes but occasionally also in PBL's of healthy controls. Therefore, the T cell responses to putative autoantigens between healthy and diseased subjects may differ by different epitope recognition (Lohmann et al., 1994; Atkinson et al., 1994), activation state (Zhang et al., 1992), homing (Paronen et al., 1997) or other features.

In type 1 diabetes, different epitope recognition of GAD 65 between prediabetic individuals or patients with diabetes and healthy controls has been described (Lohmann et al., 1994; Atkinson et al., 1994). The aim of the present study was to define epitope specific IA-2 T cell recognition in HLA matched diabetic patients and controls. Therefore, we studied IA-2 autoantibodies and the response of T cells to peptides of the intracytoplasmic domain of IA-2 designed for binding motivs to the disease related HLA alleles DR*0401 and DQ*0302 (Nepom and Erlich, 1991).

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Table 1 Characterisation of type 1 diabetes patients and healthy controls, autoantibodies and T cell responses to IA-2ic peptides

No.	Age	HLA	IA2-Ab	Recognised IA2 peptides
D1	46	DR4,7/DQ201,302	–	608–620 (2.7), 797–809 (4.7), 933–945 (6.8)
D2	21	DR1,4/DQ101,302	–	933–945 (2.1)
D3	17	DR4,13/DQ6,8	–	854–866 (9.4)
D4	29	DR4,5/DQ301,302	+	797–809 (3.8), 933–945 (4.3)
D5	37	DR4,11/DQ301,302	–	854–866 (3.4)
D6	56	DR1,4/DQ501,302	–	933–945 (2.0)
D7	27	DR4/ DQ302	–	–
D8	19	DR1,4/DQ501,302	–	797–809 (4.4), 933–945 (2.2)
D9	22	DR1,4/DQ501,302	+	933–945 (2.2)
D10	27	DR1,4/DQ501,302	–	803–815 (2.3), 933–945 (2.6)
D11	24	DR4,7/DQ201,302	+	933–945 (3.0)
D12	29	DR4,8/DQ4,8	+	797–809 (2.7), 933–945 (2.1)
C1	35	DR4,11/DQ301,302	–	762–774 (2.8), 797–809 (7.0), 803–815 (2.6), 925–937 (2.4), 933–945 (5.4)
C2	27	DR4/DQ302, 301	–	933–945 (3.0)
C3	26	DR4/DQ301,302	–	95–107 (2.8), 925–937 (3.3)
C4	24	DR4,8/DQ302,401	–	933–945 (3.9)
C5	36	DR1,4/DQ501,302	–	–
C6	25	DR1,4/DQ501,302	–	797–809 (2.7), 933–945 (2.5)
C7	33	DR4,7/DQ301,302	–	–

Age, HLA class II characterisation, IA2-Ab status and T cell responses to IA2-peptides (SI in brackets) for 12 type 1 diabetes patients and 7 healthy controls

This approach allowed the detection of some potentially immunodominant T cell epitopes restricted by these HLA alleles.

Material and methods

Patients

Twelve HLA DR*0401 or DR*0404 and DQ*0302-positive type 1 diabetes patients less than 2 years from diagnosis and 7 HLA DR4/DQ302-positive healthy controls of comparable age and without family history of diabetes were included (Table 1). All patients were metabolically well controlled (HBA^{1c} < 7.5%) and without secondary diabetic complications. We studied only adult diabetic patients for ethical reasons since relatively large amounts of blood were needed for the T cell assays. The study was approved by the Ethical Committee at the University of Leipzig. HLA typing of IDDM patients and controls was performed by the single-step allele specific polymerase chain reaction.

Peptide Synthesis

Peptides of IA2 were selected by motifs predicting specific binding to either DR*0401 (Hammer et al., 1993; Sette et al., 1993; Max et al., 1994) or DQ*0302 (Kwok et al., 1996a and 1996b). They were synthesized using Fmoc chemistry on an automated peptide synthesizer, AMS 422 (SMPS 350, Zinsser Analytic, Frankfurt, Germany), and purified by reversed-phase HPLC. The final purity was over 90% as estimated by HPLC and mass spectrometric analyses. The sequences of peptides are shown in Table 2.

Table 2 Sequences of IA2 peptides

A) IA2 peptides predicted as DR*0401 binder	
95–107	LSWHDDLTQYVIS
546–560	GLQILQTGVGQREE
711–725	KEWQALCAYQAEPNT
797–809	MVWESGCTVIVML
854–866	FYLKNVQTQETRT
873–885	LSWPAEGTPASTR
B) IA2 peptides predicted as DQ*0302 binder	
608–620	DKERLAALGPEGA
741–753	YDHARIKLKVESS
762–774	SDYINASPIIEHD
803–815	TVIVMLTPLVEDG
832–844	YHVYEVNLVSEHI
925–937	VLNRMAKGVKEID
933–945	VKEIDIAATLEHV
955–967	KDQFEFALTAVAE

Sequences of the IA2-peptides tested for induction of T cell proliferation, single letter codes for amino acids are shown (for the complete sequence of IA2 see e.g. Lan et al., 1994)

Detection of autoantibodies to IA-2

Autoantibodies to IA-2 were detected in a radioligand assay using in vitro synthesised IA-2ic as described in detail elsewhere (Seissler et al., 1996; Morgenthaler et al., 1997). Briefly, IA-2ic was transcribed and translated in a single tube using the TnT coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-labelled methionine (Amersham Ltd, Amersham,

UK) following the manufacturer's instructions. 10,000 cpm of in vitro synthsised and ^{35}S-labelled protein were incubated with 5 µl serum on 96 well plates in duplicate followed by overnight incubation under agitation. Then 20 µl Protein A Sepharose (50% v/v in buffer A) were added for 2 h, followed by the transfer of the samples into 96-well filtration plates (Multiscreen BV 1.2 µm, Millipore). Plates were extensively washed with buffer and dried. After addition of 20 µl liquid scintillator (Microscint 20, Canberra Packard, Dreieich, Germany) to each well, bound radioactivity was counted in a microplate scintillation counter (Canberra Packard).

HLA binding measurements

For measuring binding/competition to HLA DR* 0401 and DQ*0302 we used our in vitro binding/ competition assay based upon gel filtration as described earlier (Kropshofer et al., 1992; Max et al., 1994). Briefly, solubilized HLA isolates (0.2 µM) purified by affinity chromatography from the LCL BSM (HLA DR*0401/ DQ*0302) were incubated for 40 hours with N-terminally fluorescently (AMCA-) labeled reference binding peptides (0.15 µM; for DR* 0401: influenza hemagglutinin; HA 306-318: PKYVKQNTLKLAT, for DQ*0302: CD20 249-262: EEDIEIIPIQEEEE) and with various concentrations of unlabeled competitor peptides in a 150 mM sodium phosphate buffer containing 15% (v/v) acetonitrile and 0.1% (w/v) Zwittergent 3-12. All samples were subsequently analysed using high performance size exclusion chromatography. The UV absorbance at 214 nm and fluorescence at 350/450 nm were detected simultaneously. The competition curves of the unlabeled peptides were determined by comparing the corresponding F/UV-values of each sample with an uncompeted reference sample.

Proliferation assays

The peripheral blood mononuclear cells (PBMC's) of the probands were separated by Ficoll-Hypaque (Lymphoprep, Biochrom KG, Berlin, Germany) density gradient centrifugation. After washing the cells twice in RPMI 1640 supplemented with Hepes/ Glutamine (Gibco, Paisley, UK)/2.5% FCS, the pellet was diluted in RPMI/ 10% autologous serum. Penicillin and streptomycin were used in all culture media.The proliferation assays were performed in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) with 2×10^5 cells per well in a final volume of 200 µl/well. Tetanus toxoid (TT) and phytohaemagglutinin (PHA) were used as positive controls. Twelve wells for each peptide, 6 wells for TT and PHA and 24 wells without antigen (negative control) were plated. Cultures were incubated at 37 °C, in 5% CO_2 atmosphere for 5 days. During the last 6 hours 0.5 µCi (^{3}H)-Thymidine (Amersham, Little Chalfont, UK) per well was added before harvesting and scintillation counting using a betaplate counter (Canberra Packard, Dreieich, Germany). Positive responses to antigen were defined by a stimulation index (SI) > 2 for peptides. Background proliferations were between 600 and 2000 cpm. Furthermore, significant responses to antigen had to exceed 1.000 cpm and at least 4/12 wells with peptide had to exceed mean + 3SD of negative controls.

$$SI = \frac{\text{Counts with antigen}}{\text{Counts without antigen}}$$

Differences between IDDM patients and controls were tested by Fisher's exact- and Student's t-test and regarded as significant by $p < 0.05$.

Results

Autoantibody responses to IA-2ic

IA-2ic autoantibodies were detected in the sera of 4/12 diabetic patients and none of the 7 healthy controls (Table 1; differences not significant, n.s.).

T cell responses to selected peptides of IA-2

A) Peptides selected for binding to DR*0401

We observed significant T cell responses to peptide 797–809 in 5/12 diabetic patients and 3/7 controls (n.s.) and to peptide 854–866 in 2/12 diabetic patients but none of the controls (n.s., Table 1). PBL's of one control and one patient responded to peptide 95–107. This region lies outside the intracytoplasmic domain of IA-2.

B) Peptides selected for binding to DQ*0302

PBL's of 9/12 diabetic patients and 4/7 controls showed significant responses to peptide 933–945 (Table 1, n.s.). Two controls but no diabetic patient responded to peptide 925–937. We observed responses to peptides 608–620, 741–753, 803–815, 762–774 and 832–844 in single controls and/or single diabetic patients.

HLA binding of selected IA-2 peptides

A) Binding to DR*0401

Peptide 854–866 exhibited relatively high affinity binding to DR*0401 (IC50 estimated 50µM) as expected by our prediction (Fig. 1A). Against our prediction, peptide 797–809 showed only negligible binding to DR*0401. Peptide 933–945, predicted as a binder to DQ*0302, bound DR*0401 with medium affinity (IC50 estimated 150 µM). Binding to DR*0401 was also observed for other peptides tested in this study as 711–725 or 873–885 (data not

shown) but these peptides induced no T cell responses.

B) Binding to DQ*0302

Peptide 933–945 was predicted as a binder for DQ*0302 and showed medium affinity binding to this allele (IC50 estimated 500 μM, Fig. 1B). Surprisingly, peptide 797–809 that was predicted to bind DR*0401 and bound this allele only marginally as outlined above, exhibited relatively good binding to DQ*0302 (IC50 estimated 140 μM). Peptide 854–866, predicted for DR*0401 binding and indeed binding this allele, showed already with 10 μM > 30% competition for DQ*0302 but could not be measured with higher concentration because of poor solubility (Fig. 1B). At least peptide 933–945 and likely also peptide 854-866 should be considered as "promiscous" HLA binders (Hammer et al., 1993) to both DR*0401 and DQ*0302 alleles. Again, peptides bound DQ*0302 with relatively high affinity (e.g. 762–774, 803–815, data not shown) but induced T cell responses only in PBL's of single patients and controls.

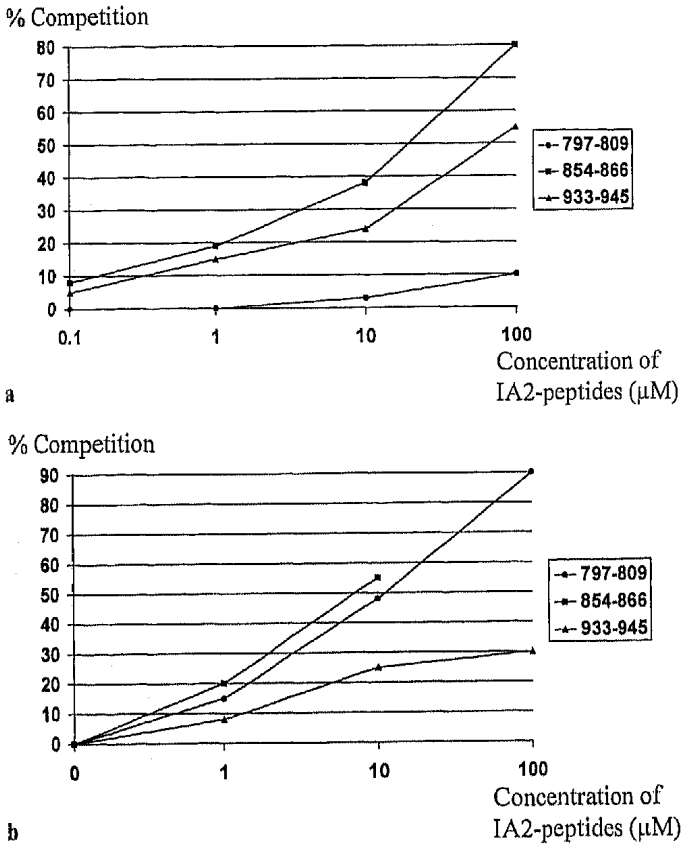

Fig. 1 HLA competition assay of selected IA2 peptides to DR*0401 (Fig. 1A) and DQ*0302 (Fig. 1B)

Discussion

The tyrosine phosphatase-like protein IA-2 has been identified as an important autoantigen in juvenile type 1 diabetes. Autoantibodies to IA-2 combined with those to GAD 65 are the most powerful markers to predict type 1 diabetes at least in relatives of diabetic patients (Seissler et al., 1996; Verge et al., 1996). Moreover, IA-2 autoantibodies are very specific for type 1 diabetes (Wiest-Ladenburger et al., 1997; Morgenthaler et al., 1997). In contrast to GAD 65, not much is known about T cell reactivity to IA-2. T cell responses by PBL's of IDDM patients to IA-2ic were reported but this finding was not exclusively specific for IDDM patients (Durinovic-Bello et al., 1996; Ellis et al., 1998). Our study confirms these reports but does not find statistical differences in T cell responses to IA-2ic peptides between IDDM patients and HLA matched controls.

Our approach predicting putative T cell epitopes by binding motifs for IDDM related HLA alleles as DR*0401 and DQ*0302 was indeed able to define at least three T cell epitopes of IA-2ic. These peptides were recognised by PBL's of more than a single diabetic patient and often also controls. In particular, peptides 933–945 and 797–809 were immunodominantly recognised in the context of DR*0401/DQ*0302 since PBL's of 9/12 diabetic patients and 4/7 controls or 5/12 diabetic patients and 3/7 controls did respond to these peptides, respectively. These T cell responses were HLA rather than disease specific in that responses did not differ between patients and HLA matched controls. Responses to peptide 854-866 were observed only in diabetic patients but seem to be relatively rare since this response was seen only in two out of twelve diabetic patients. Therefore, we found no common type 1 diabetes specific epitope of IA-2 by the approach applied. It may be that no such epitope exists or it may be missed because it is binding other HLA alleles or it is a poor HLA binder. The latter possibility is supported by our finding that a T cell epitope of GAD in human IDDM and NOD mice was an extremely poor binder of DQ*0302 but still induced significant DQ*0302 restricted T cell proliferation and cytokine production (Boyton, Lohmann et al., 1998). Moreover, we have only tested patients with established diabetes where already a spreading of the immune response to many T cell epitopes has to be expected (Lehmann et al., 1993). Longitudinal studies including prediabetic subjects are necessary to identify early, possibly more focussed T cell responses to IA-2 epitopes.

Recently, a first study has been published about epitope specific T cell recognition of IA-2 in healthy controls and diabetic patients (Honeyman et al., 1998).Our study differed from the work of Honeyman et al. in that latter has i) investigated subjects at high risk for type 1 diabetes, ii) only accepted subjects homozygeous for DR4/DQ*0302 or DR3/DQ*0201 or heterozygeous for these both alleles, iii) used a different strategy of peptide selection. Nevertheless, the results are largely consistent since two of the 3 IA2-peptides we have described as immunodominant

(797–809, 854–866) are closely matched by peptides described as T cell epitopes in the study of Honeyman et al. (793–808, 847–862). On the other side, some of the T cell epitopes in the latter study (e.g. 805–820) are partly matched by our peptides (803–815) but were found to be immunogenic only in single patients or controls.

We have also measured HLA binding of the peptides showing significant T cell responses. Surprisingly, some peptides did not only bind the expected HLA allele but also the second allele tested. E.g. peptide 797-809 predicted as DR*0401 binder showed only weak binding for DR*0401 but turned out to be the best DQ*0302 binder. Therefore, we were not able to detect the HLA restriction of these peptide specific T cell responses because of the promiscuity of HLA binding features (Hammer et al., 1993). Similar findings have been described for GAD 65 peptides (Lohmann et al., 1996). We are currently trying to dissect the HLA restriction of these IA2 peptides by the establishment of peptide specific T cell clones. However, this approach does not exclude different HLA restriction in different clones specific for the same T cell epitope.

Our findings may be of relevance for the understanding of the pathogenesis of IDDM. It is known now for several human autoimmune diseases that potentially autoreactive cells do exist in the peripheral T cell repertoire without being harmful for the subject (Zhang et al., 1992). These cells ignore the self target because i) they are not activated (Zhang et al., 1992), ii) the epitopes that they recognise are not generated naturally processed ("cryptic epitopes", Sercarz et al., 1993), iii) they cannot home to the target organ (Paronen et al., 1997). One possible explanation how such ignorant T cells may be activated belongs to the concept of "molecular mimicry". Indeed, it was shown for T cell clones specific for peptides of the putative multiple sclerosis autoantigen, myelin basic protein, that these clones recognised several viral peptides (Wucherpfennig and Strominger, 1995). We have also checked our immunodominant IA-2 peptides for homologies to viral sequences. Some sequences fit the predicted HLA binding motifs and will be investigated for potential T cell crossreactivity. Furthermore, future prevention trials for IDDM aiming on tolerization of antigen specific T cells (Weiner et al., 1993) may profit from the knowledge of dominant epitopes of autoantigens e.g. by designing the tolerogen cocktail or by monitoring these trials (Zhang et al., 1996).

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