International Immunology, Vol. 11, No. 6, pp. 951–956

A shared TCR CDR3 sequence in NOD mouse autoimmune diabetes

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Keywords: CDR3, insulin-dependent diabetes mellitus, TCR

Abstract

T cells involved in autoimmune diseases have been characterized by the genetic elements used to construct their autoimmune TCR. In the present study, we sequenced the α and β chains of the TCR expressed by a $CD4^+$ T cell clone, C9, functional in NOD mouse diabetes. Clone C9 can adoptively transfer diabetes or, when attenuated, C9 can be used to vaccinate NOD mice against diabetes. Clone C9 recognizes a peptide epitope (p277) of the 60 kDa heat shock protein (hsp60) molecule. We now report that the C9 TCR β chain features a CDR3 peptide sequence that is prevalent among NOD mice. This CDR3 element is detectable by 2 weeks of age in the thymus, and later in the spleen and in the autoimmune insulitis. Thus, a TCR CDR3β sequence appears to be a common idiotope associated with mouse diabetes.

Introduction

Mice of the NOD strain spontaneously develop autoimmune insulitis at ~1 month of age which progresses several months later to overt insulin-dependent diabetes mellitus (IDDM) (1). We have discovered that regulation of the autoimmune IDDM process can be influenced by T cell immunity to the p277 peptide corresponding to position 437–460 of the human 60 kDa heat shock protein (hsp60) sequence (2). Overt IDDM in NOD mice is preceded by spontaneous T cell activity to peptide p277. T cell clones reactive to p277, such as clone C9, can adoptively transfer hyperglycemia and early insulitis, and vaccination with the p277 peptide itself can prevent (2) or even reverse IDDM (3). Treatment with peptide p277 is marked by down-regulation of anti-p277 T cell proliferation and by a switch in the cytokine profile of the T cell response to p277 from a T_h1 to a T_h2 phenotype (4). Active immunization to p277 conjugated to an immunogenic foreign carrier molecule can also induce transient diabetes (5). Thus, T cell immunity to p277 appears to be functionally involved in NOD diabetes.

We have also found that the C9 clone, when attenuated, can be used to vaccinate NOD mice against IDDM (2). Since the regulatory effects of T cell vaccination have been related to the TCR of the vaccinating T cells (6,7), the present investigation was done to characterize the TCR of C9 and to investigate its distribution among various T cell populations.

Methods

Animals

NOD/Lt (NOD) mice were raised in the animal facilities of the Weizmann Institute of Science. The breeding nucleus was the gift of Dr E. H. Leiter.

Antigens

Ovalbumin (OVA) was purchased from Sigma (St Louis, MO). Recombinant human hsp60 was kindly provided by Dr Ruurd van der Zee (University of Utrecht). The synthetic peptide p277 VLGGGCALLRCIPALDSLTPANED (2) was synthesized by standard Fmoc chemistry, purified on HPLC by reverse-phase chromatography using a CM12 column (Merck, Darmstadt, Germany). The sequence was confirmed by amino acid analysis.

T cell lines

The NOD T cell lines and clones, obtained from pre-diabetic NOD spleens, were selected *in vitro* by their response to recombinant hsp60, as described (2). A NOD anti-OVA T cell clone was generously provided by Dr Anne Cooke (Cambridge, UK). We have shown that hyperglycemia and insulitis can be adoptively transferred by C9 and other anti-p277 T cell clones of NOD mice (2).

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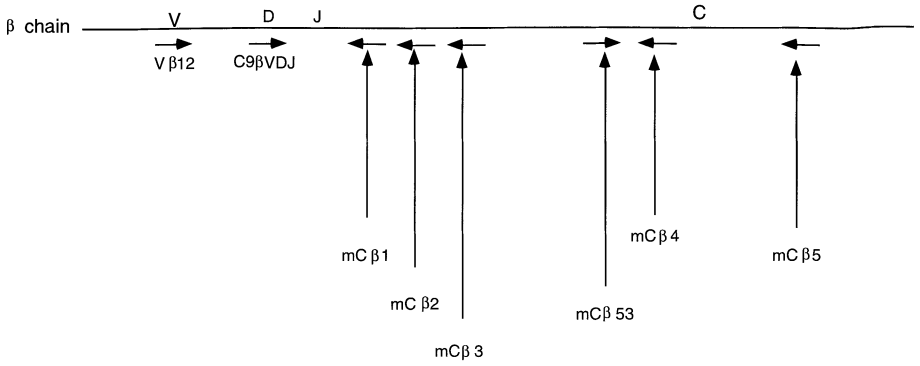
Transmitting editor: L. Steinman

Received 6 July 1998, *accepted* 1 March 1999



Designation	Sequence
C9βVDJ (CDR3β)	5' - TTAGGGGGTAACCAAGAC -3'
mCβ1	5' - GGAGACCTTGGGTGGAGTCAC -3'
mCβ2	5' - CTGCTTCTGATGGCTCAAA -3'
mCβ3	5' - GCCAAGCACACGAGGGTAGCC -3'
mCβ4	5' - ACTTGGCHGCGGAAGTGG -3'
mCβ5	5' - TCTCATAGAGGATGGTGGTGGCAGACA -3'
mCβ53	5' - CTGCTACCTTCTGGCACAATCC -3'

Fig. 1. Nucleic acid sequences that were used and their relative localizations on the β chain of the TCR. The arrows along the line of the β chain indicate the direction of PCR priming.

RNA and cDNA preparations

Cell lines. Following a 3 day activation by incubation with p277 as described (2), T cell blasts were collected and separated from cell debris and accessory cells on a Ficoll gradient, washed in PBS, and cultured for 2 more days in medium containing 10% T cell growth factor as described (2) to ensure that the cultures contained only living T cells. The T cells were then collected, washed in PBS and snap frozen in liquid nitrogen. Each sample was homogenized and total RNA extracted by the RNAzol kit (Cinna/Biotecx, Friendswood TX). Approximately 5 μg of total RNA was transcribed into first-strand cDNA in a 20 μl reaction of the cDNA cycle kit (Invitrogen, San Diego, CA) using oligo(dT) as a primer.

Spleen and thymus. Spleens or thymuses were removed from NOD mice and were immediately frozen in liquid nitrogen. RNA and cDNA were prepared as above.

Pancreatic islets. Pancreatic islets were prepared from NOD mice by the method of Gotoh *et al.* (8). Briefly, collagenase was injected into the bile duct, the duct was then ligated and the pancreas excised. Collagenase digestion was carried out in a shaking water bath at 37°C for 30–45 min and the tissue was then washed extensively in PBS. The islets were hand-picked from the digest using a dissecting microscope and frozen in liquid nitrogen. RNA and cDNA were prepared as described above.

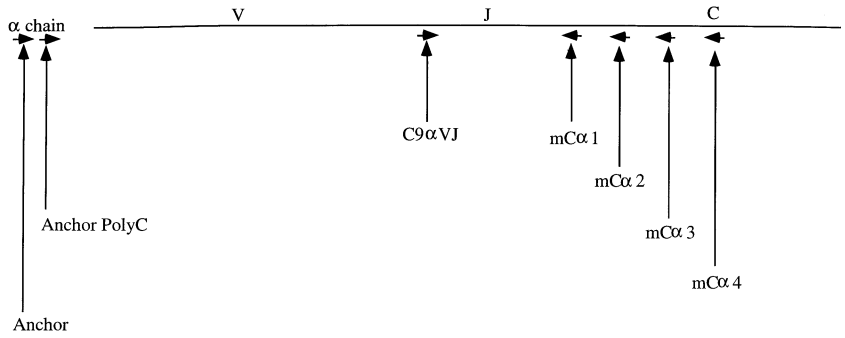
PCR amplification

Figure 1 is a schematic presentation of the TCR β chain with the relative positions and sequences of the various nucleic acid oligonucleotides used as primers in PCR reactions or as probes for hybridizations. The C region sequences were synthesized in the Department of Molecular Biology and Genetic Engineering, Hadassah Hospital, Mount Scopus, Jerusalem, Israel.

V_β panel. Aliquots of 3 μl of the cDNA preparation were put into a reaction mixture containing PCR buffer, 1 mM dNTP mixture, 1 μM of $mC_\beta3$ primer (see Fig. 1) and 20 U/ml Taq polymerase (USB, Cleveland, OH). This mixture was distributed into 19 reactions tubes, each containing a different V_β-specific oligonucleotide primer, obtained from Operon Technologies (Alameda, CA). The final concentration of the primer was 1 μM. The amplification was carried out in a DNA thermocycler (Perkin Elmer, Norwalk, CT) for 30 cycles. The cycle profile was as follows: denaturation at 94°C for 60s, annealing at 55°C for 60 s and elongation at 72°C for 60 s. Then 8 μl of each amplification reaction was subjected to electrophoresis on a 2% agarose gel (FMC, Rockland, ME), stained with ethidium bromide (Sigma, St Louis, MO) and visualized with UV light. In-gel hybridization (9) was performed for 1 h with 0.5 pmol/ml of the ^{32}P-labeled oligonucleotide probe $mC_\beta1$. The amplified products were sequenced directly according to Casanova *et al.* (10) with the Sequenase version II kit (USB).

C9 VDJ_β-specific oligonucleotide. Aliquots of 1 μl of each cDNA sample were put into the reaction mixture containing the C9 VDJ_β (CDR3β) oligonucleotide and the $mC_\beta3$ oligonucleotide as primers and analyzed as above. A higher specificity was achieved by raising the annealing temperature to 65°C. A ^{32}P-labeled C9 VDJ_β oligonucleotide was used as a probe in in-gel hybridizations with a V_β panel amplified by PCR as described (10).

C9 VDJ_β (CDR3β) PCR in individual mice. The TCR β chain was detected in the cDNA of spleen cells from individual NOD mice by a PCR reaction using the primers $mC_\beta53$ and



Designation	Sequence
C9αVJ (CDR3α)	5' - ATGAGAGGGCCTAATTAC -3'
mCα1	5' -GCAGGTTCTGGGTTCTGGATG -3'
mCα2	5' -AGGATCTTTTAACTGGTACAC -3'
mCα3	5' -GTCGGTGAACAGGCAGAGGT -3'
mCα4	5' -CGGACAATTGATTTGGGAGTC -3'
Anchor	5' -GLATGCGCGCGGCCGCGGAAGGCC -3'
Anchor PolyC	5' -GLHTGCGCGCGBCCGCGGAGGCCCCCCCCCCCCCC -3'

Fig. 2. Nucleic acid sequences that were used and their relative localizations on the α chain of the TCR. The arrows along the line of the α chain indicate the direction of PCR priming.

$mC_\beta 5$, and hybridization with the oligonucleotide $mC_\beta 4$. The presence of the C9-specific VDJ_β sequence (CDR3β) was detected in the same cDNA preparation by a PCR reaction using the C9 VDJ_β and $mC_\beta 3$ primers and hybridization with the biotinylated $mC_\beta 2$ primer. The Phototope detection kit (NEB New England, Boston, MA), was used to detect hybridization.

C9 V_α amplification. Figure 2 is a schematic presentation of the TCR α chain with the relative positions and sequences of the various oligonucleotides used.

Aliquots of 5 μl of the C9 cDNA first-strand preparation were tailed with dGTP using terminal transferase (TdT) under the conditions recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany) and 100 mM of dGTP in a total volume of 50 μl for 30 min. Then 10 μl of the tailing reaction was put in an anchored PCR reaction according to Acha-Orbea *et al.* (11). The first PCR round was done using Anchor Poly C and $mC_\alpha 4$; the second and third rounds were done using Anchor and $mC_\alpha 3$ and $mC_\alpha 2$ respectively. The amplification product was detected with the oligonucleotide probe $mC_\alpha 1$ cloned into pBluescript SK(+) (Stratagene) and sequenced according to the user manual of the Sequenase version II kit.

C9 VJ_α (CDR3α)-specific nucleotide. The oligonucleotide C9 VJ_α was used as a specific primer together with the $mC_\alpha 2$ primer for PCR amplification of different lines (Fig. 2), as described for the C9 VDJ_β amplification.

Results

The TCR nucleotide sequence of C9

To determine the nucleotide sequence of the α and β chains of the C9 TCR, cDNA was prepared from the mRNA extracted from the C9 clone. PCR amplification was performed using a C_β constant region oligonucleotide primer and each of 19 V_β-specific oligonucleotide primers.

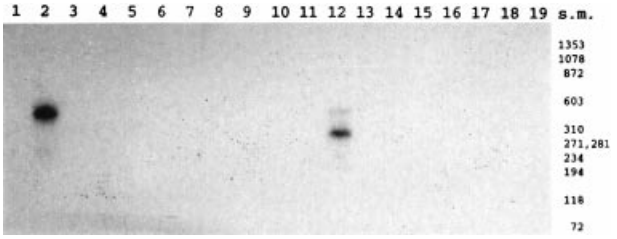


Fig. 3. Determination by PCR of TCR V_β gene usage in the C9 clone. Lanes 1–19: 19 different V_β amplification reactions with the $mC_\beta 3$ oligonucleotide primer (see Fig. 1). Lane s.m.: ϕx/*Hae*III size marker. The gel was hybridized with an internal $mC_\beta 1$ oligonucleotide probe and therefore the size marker is printed. The two positive bands for $V_\beta 2$ and $V_\beta 12$ indicate the existence of these segments in the C9 clone.

Of the 19 different V_β amplification reactions, only $V_\beta 2$ and $V_\beta 12$ PCR amplification products were visible (Fig. 3). After direct sequencing of both PCR products, the $V_\beta 2$ was found to be non-productive (out of reading frame). In contrast, the $V_\beta 12$ sequence was in-frame and thus productive (Table 1)

The greater number and variability of the α chain V regions made a similar panel amplification approach unworkable. Therefore, to sequence the α chain, we used an anchored PCR with dGTP tailed cDNA employing nested constant region oligonucleotide primers. The VJ_α (CDR3α) sequence of C9 was found to be MRGPNW (12); the V_α was BMB (13) and the J_α was 16.

TCR usage in other NOD T cell lines

We studied other T cell lines derived from the same pool of NOD lymphocytes that generated the C9 clone. The C7, N1, N26 and other clones that recognize the p277 peptide of human hsp60 were diabetogenic and could vaccinate NOD mice against developing diabetes (2). We also studied the N4 clone that reacts to *Mycobacterium tuberculosis* hsp65 but not to human hsp60, and is neither diabetogenic nor

Table 1. VDJ_β (CDR3β) sequences of T cell clones associated with IDDM

Mouse	Clone	Vβ	VDJ region sequence											Jβ
			V			N and D					J			
NOD	C9	12	A gcc	S agc	S agt	L tta	G ggg	G ggt			N aac	Q caa	D- gac	2.5
NOD	N4	11	A gca	S aga	S agc	L tta	**W** **t**gg	**T** g**ac**			N aac	Q cca	D- gac	2.5
NOD	4-1-E.2	12	A gcc	S agc		**R** ag**a**	L **ct**g	G gg**a**			N aac	Q caa	D- gac	2.5
Human	K2.12	8.1	A gcc	S agc	S agt	S agt	D gac	R agg	L tta	G ggc	N aat	Q cag	P ccc	1.5

The different V_β and J_β segments are indicated. The amino acid and nucleotide sequence differences from the C9 VDJ_β rearrangement are marked by bold letters. The sequence of NOD clone 4-1-E.2 is taken from Nakano *et al.* (14) and that of human clone K2.12 is taken from Duranovic-Bellow *et al.* (15).

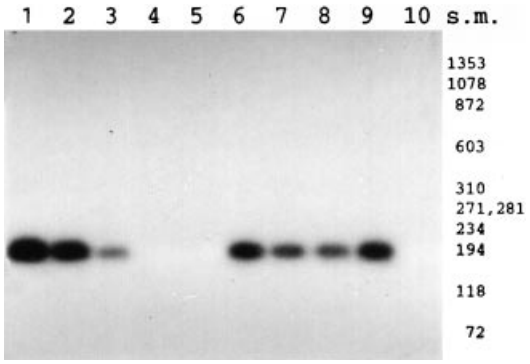


Fig. 4. The presence of sequences homologous to the C9 CDR3β rearrangement in different T cell lines. The assay was determined by PCR using the C9 CDR3β oligonucleotide primer. Lanes 1, 2, 6, 7, 8 and 9 are the results obtained from different lines specific for peptide p277. Lanes 4, 5 and 10 are from anti-OVA lines. Lane 3 is from clone N4.

vaccinates against diabetes (2), and an unrelated anti-OVA line.

Since the CDR3 regions of the α and the β TCR chains can each contribute to an idiotope, we prepared specific oligonucleotide probes based on the CDR3α and the CDR3β regions of the C9 clone. Using an α constant region and the CDR3α oligonucleotide primers, we obtained PCR amplification only for the anti-p277 T cell line. The β chain constant region and the CDR3β oligonucleotide primers resulted in PCR amplification for all the anti-p277 T cell lines. However, a faint amplification band could be detected for the N4 line (Fig. 4, lane 3). Anti-OVA lines were negative (Fig 4, lanes 4, 5 and 10).

For further analysis, we used the panel of 19 V_β-specific oligonucleotides and the β chain constant region oligonucleotide primers for PCR amplification. In all the NOD anti-p277 T cell lines we found, among others, a $V_\beta12$ amplification product. This was sequenced and found identical to that of C9. Amplification of cDNA derived from the N4 cell line produced, among others, a $V_\beta11$ PCR product. Sequencing of the product revealed a sequence in the CDR3β region of LWTNQD that was different from the LGGNQD sequence present in the C9 CDR3β. This difference in two amino acids could be attributed to a difference in 3 bp (Table 1). Note that the CDR3β sequences of C9 and N4 were the rearrangement products of two different V_β genes. The 3 bp difference in the CDR3β nucleotide sequence of C9 and N4 resulted in noticeably different PCR amplifications (Fig 4), demonstrating the usefulness of the PCR primer as a means for screening for the C9 idiotope and related sequences.

Table 1 includes the β chain CDR3 sequence of an NOD clone, 4-1-E.2, published by Nakano *et al.* (14). This clone was reported to transfer insulitis into diabetes-free 1-E^+ transgenic NOD mice. However, its specific antigen was not reported. Note that 4-1-E.2, like C9, expresses the $V_\beta12$ and $J_\beta2.5$ genes, and has a CDR3β region sequence similar in part to that of C9: C9-LGGNQD; 4-1-E.2-LGNQD.

Table 1 also includes the published sequence of the CDR3β chain of a human T cell clone reactive to β cells (15). The human clone K2.12 appears to express a CDR3 sequence with the LGNQ motif that differs by the lack of a G from the LGGNQ amino acid sequence of C9.

Distribution of the C9 CDR3β and CDR3α sequences in NOD tissues

In our NOD colony, insulitis is first evident around the age of 1 month. The intensity of the insulitis progresses and the insulin-producing β cells are destroyed. The female mice begin to develop overt hyperglycemia at ~3 months of age, and by 6 months of age essentially all the female mice are clinically ill and die unless treated with insulin. Male mice manifest a lag in the progression of insulitis and only ~50% develop overt IDDM. To see whether the presence of the C9 CDR3β rearrangement could be correlated with the progression of the autoimmune disease, we prepared cDNA from spleens and thymuses of 0.5-, 1-, 2- and 3-month-old NOD mice, pooling the organs of five mice in each age group. Islets were studied from 1- and 2-month-old NOD mice. The cDNA samples were amplified by PCR using the CDR3β or the CDR3α primers and constant region primers; $mC_\beta3$ and $mC_\alpha2$. All the CDR3α-primed PCR reactions were negative; but the CDR3β-primed PCR produced amplifications in both

Table 2. Tissue distribution of C9 VDJ_β (CDR3β) and C9 VJ_α (CDR3α) in NOD mice of various ages

Age (months)	C9 CDR3 probe	Female			Male		
		thymus	spleen	islets	thymus	spleen	islets
0.5	VJα	–	–	ND[a]	–	–	ND
	VDJβ	+	–	ND	+	–	ND
1	VJα	–	–	–	–	–	–
	VDJβ	+	+	+	+	+	–
2	VJα	–	–	–	–	–	–
	VDJβ	+	+	+	+	+	+
3	VJα	–	–	ND	–	–	ND
	VDJβ	+	+	ND	+	+	ND

Thymuses, spleens and islets of female and male NOD mice were tested for the presence of the C9 VDJ_β and C9 VJ_α-like sequences in the CDR3 regions of the TCR by PCR amplification with specific oligonucleotide primers.
[a]ND, not determined.

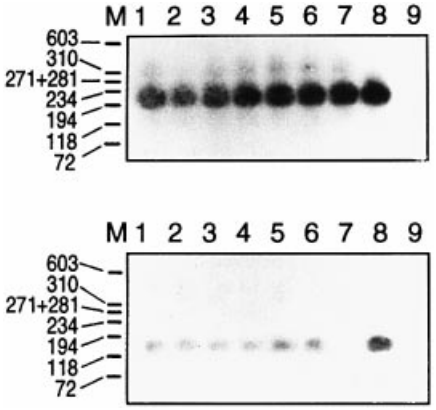


Fig. 5. The presence of C9 CDR3β-like rearrangements in individual NOD mice. The PCR reaction was used on cDNA from the spleen cells of individual 3-month-old female and male NOD mice (lanes 1–6). Lane 7 is a control anti-OVA T cell clone and lane 8 is the C9 clone itself. The water control is lane 9. (A) C_β products. (B) C9 CDR3β-like products.

female and male NOD mice. The results are summarized in Table 2. The thymus preparations were positive at all ages in both males and females. In the spleens, the C9 rearrangement was evident from the age of 1 month in the females and from 2 months in the males. The CDR3β was also detected in inflamed islets. The progression of the C9 CDR3β-specific signal from the thymus to the spleen and islets seems to correlate with the progression of the disease process.

Individual NOD mouse spleens are positive for the C9 CDR3β sequence

To confirm the prevalence of the C9 CDR3β motif in individual NOD mice, we prepared cDNA from each of the spleen cells of three male and three female mice; the mice were 3 months old. PCR reactions were used to detect a C_β product (Fig. 5A) and the C9 CDR3β product (Fig. 5B). It can be seen that each of the six NOD mice was positive for a C_β product (Fig. 5A, lanes 1–6) as were the OVA (Fig. 3A, lane 7) and C9 clone (Fig. 5A, lane 8). The C9 CDR3β probe was positive for each of the individual NOD mice (Fig. 5B, lanes 1–6) and for the C9 clone (Fig. 5B, lane 8). The OVA line was negative for the C9 CDR3β sequence (Fig. 5B, lane 7). Thus, individual NOD do express CDR3β sequences similar enough to the C9 CDR3β clone to be detected using the PCR reaction.

Discussion

In this study, the sequences of the mRNA coding for the α and β chains of the TCR of the diabetogenic NOD clone C9 were determined, and CDR3α and CDR3β probes were used to detect TCR sequences related to those of C9 in various T cell populations. We found that a CDR3β segment similar to that of C9 seems to be shared among individual NOD mice. It was detected by PCR in the thymus, spleen and islet infiltrates of different NOD mice as well as in the anti-p277 T cell clones and lines. In contrast, the C9 CDR3α sequence was undetectable in T cells sampled from the thymuses, spleens or islet infiltrates.

The prevalence of any CDR3β motif among different clones is unexpected. The CDR3 segments of the α and β chains of the TCR (VJ_α and VDJ_β) are produced by apparently random recombination of the 3′ ends of the V_α and V_β genes with the 5′ ends of J_α and DJ_β genes respectively. The randomness of CDR3 sequences is further enhanced by N insertions, nucleotides not directed by genetic templates (16). Could the apparent prevalence of the C9 CDR3β be an artifact of the PCR technique?

The specificity of the C9 CDR3β primer was indicated by hybridization of this oligonucleotide with the products of β chain PCR amplification using V_β and C_β primers. The reliability of the PCR primed with the C9 CDR3β oligonucleotide was confirmed by examining the TCR sequences of the targets involved: the N4 clone, which has a 3 bp difference (2 amino acids) from the C9 CDR3β primer, gave only a faint PCR product. Thus we may conclude that our C9 CDR3β was a reasonable probe to detect sequences similar to the C9 sequence.

Chance is also not a likely explanation for the results. Although two or three identical amino acids may arise by chance in functionally diverse CDR3 sequences, the shared LG(G/A)NQD sequence noted here is likely to be rare. Indeed

a computer search of the 488 mouse TCR sequences and the 1015 human TCR sequences present in the GenPep library (up-dated through July 1997) yielded no sequences identical to either LGGNQD or LGANQD, and only one sequence with one mismatch: I in place of either G or A, detected in a lymphoma cell (17).

Our detection of the C9 CDR3β-like segment in different NOD mice suggests that this motif might be associated with IDDM. Indeed, an independently isolated NOD clone, designated 4-1-E.2, was reported (14), like C9, to use $V_\beta12$ and $J_\beta2.5$, and had a CDR3β region (SRLGNQDTQY) remarkably similar to that of C9 which differs in the entire CDR3β region by an S in place of the R and an additional G: (SSLGGNQDTQY; the differences are underlined). Clone 4-1-E.2 was shown to adoptively transfer insulitis in non-diabetic 1-E^+ transgenic NOD mice (14). The 4-1-E.2 clone was found to proliferate in the presence of islets, but there was no report of the antigen recognized by this clone. Interestingly, Durinovic-Bello *et al*. reported the isolation of a T cell clone (K2.12) from a diabetic human with a TCR CDR3β motif of SRLGNQ (15). It remains to be seen if clones 4-1-E.2 and K2.12 can recognize hsp60 peptide p277.

Although we lack information about the ontogeny of T cells with similar CDR3 region sequences, it seems that anti-C9 anti-idiotypic T cells can regulate the activity of diabetogenic C9-like clones. T cell vaccination with C9 leads to down-regulation of anti-p277 reactivity and prevents the development of diabetes in NOD mice (2). Moreover, the accompanying paper shows that resistance to the development of diabetes could be obtained by active immunization of NOD mice to the C9 CDR3β peptide or by adoptive transfer of T cell lines specific for the C9 CDR3β peptide (18). These findings indicate that the C9 CDR3β sequence is not only detectable generally in NOD mice, the peptide expressed by this sequence is functionally meaningful to the mice. Indeed, the present findings may serve as a concrete example of the general concept of the immunological homunculus—the set of dominant self antigens such as hsp60 around which are organized naturally autoimmune T cells (19). Not only are the homunculus antigens highly conserved among individuals, but some of the T cells selected to recognize these antigens may bear similar TCR regions. The accompanying paper shows that the expression of NOD diabetes may depend on a balance between idiotypic and anti-idiotype T cell reactivity (18).

Acknowledgements

This study was done with the support of grants provided the Minerva Foundation, the Tauro Trust, and Peptor, Inc. D. E. was supported by a post-doctoral fellowship awarded by the Juvenile Diabetes Foundation International. I. R. C. is the incumbent of the Mauerberger Chair in Immunology and the Director of the Robert Koch-Minerva Center for Research in Autoimmune Disease. We thank Ms Doris Ohayon for helping prepare the manuscript.

Abbreviations

hsp60	60 kDa heat shock protein
IDDM	insulin-dependent diabetes mellitus
OVA	ovalbumin

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