FLEXIBILITY OF THE T CELL REPERTOIRE

Self Tolerance Causes a Shift of T Cell Receptor Gene Usage in Response to Insulin

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The ligand of TCR-α/β consists of an antigenic peptide associated with self MHC molecules (reviewed in reference 1). The rules governing the association of the two components of the ligand have recently become increasingly clear (2–4). However, the molecular basis of ligand recognition by the TCR is still poorly understood. In the absence of information on the crystal structure of the TCR, one possible approach to this problem is to correlate primary structural features of the receptor with its fine specificity. Using this approach it has been shown that T cells responding to the same antigen often exhibit a limited heterogeneity of rearranged V and J gene segments (5–18). The use of certain gene segments, as well as of junctional sequences, tends to correlate with the specificity of the receptor, although a simple assignment of certain segments to peptide recognition and others to MHC recognition has not been possible so far. Obviously, these results do not imply that one particular gene segment can only be used in response to one particular antigen, and indeed, several examples demonstrate the use of the same gene segment in different responses (19, 20). An important aspect of these studies is that unwanted immune responses, such as those leading to autoimmune disease, could be selectively inhibited, if the responses use a small number of TCR gene segments (14–16, 18), and the gene segment usage remains constant in different individuals.

Among the immune responses studied so far, the response to bovine insulin (BI)[1] appeared to be an exception, in that no correlation between fine specificity and TCR gene segment usage could be established on the basis of seven characterized BI-specific TCRs (21, 22). To assess the heterogeneity and possible genetically controlled differences of receptor expression in the anti-BI response, we have studied a large number of BI-specific class II MHC-restricted T cell clones from mouse strains of C57/BL, BALB, 129, and DBA backgrounds. Our results demonstrate a predominant, although not exclusive, use of Vβ6 in response to insulin in C57/BL, BALB, and 129

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[1] *Abbreviations used in this paper:* Ano, nonoxidized bovine insulin A chain; Aox, oxidized bovine insulin A chain; BI, bovine insulin; Box, oxidized bovine insulin B chain; EI, equine insulin; HEL, hen egg white lysozyme; OI, ovine insulin; PCR, polymerase chain reaction; PE, phycoerythrin; PI, porcine insulin; PPD, purified protein derivative.

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mice. In contrast, in DBA mice where the great majority of T cells expressing Vβ6 and Vβ8.1 are deleted by self tolerance (23, 24), the BI-specific clones express, preferentially, Vβ8.2 and Vβ8.3, instead of Vβ6. This shift of TCR usage is not accompanied by a change of either responsiveness or fine specificity.

Materials and Methods

Mice. 8-wk-old C57BL/6 (B6), BALB.B (B.B), 129/J (129), B10.D2 (D2) (Olac, Bicester, UK), (C57BL/6 × DBA/2)F_1 (B6D2F_1) (Iffa Credo, L'Arbresle, France), and B6.C-H-2^{bm12} (bm12) (Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland) were used.

Antigens and Immunizations. BI, equine insulin (EI), ovine insulin (OI) and porcine insulin (PI), as well as oxidized BI A chain (Aox) and B chain (Box), and hen egg white lysozyme (HEL) were purchased from Sigma Chemical Co. (Brunschwig AG, Basel, Switzerland). Rat insulin was from Novo Industrie (Pharma Schweiz AG, Zürich, Switzerland). The "nonoxidized" BI A chain (Ano) was a generous gift from Dr. E. Rüde (Institut für Immunologie, Johannes Gutenberg Univ., Mainz, FRG). Ano corresponds to S-sulfonated A chain (A-SSO_3), where the oxidation of S in Cys residues is reversible, in contrast to Aox (A-SO_3), where this is irreversible. In terms of antigenicity, Ano is equivalent to disulfide-bonded A chains (25). Purified protein derivative (PPD) of tuberculin was from the Statens Seruminstitut (Copenhagen, Denmark). Mice were injected at the tailbase with 100 μg antigen in CFA (Strain H37Ra; Difco Laboratories Inc., Detroit, MI).

Establishment, Cloning, and Assay of T Cell Lines. 9 d after immunization, single cell suspensions were prepared from the inguinal and paraaortic lymph nodes, and the cells were cultured at 2 × 10^6/ml in RPMI 1640 supplemented with 0.5% mouse serum, glutamin, 2-ME, antibiotics, and 100 μg/ml of the priming antigen. Antigen-specific T cell proliferation was measured by [^{3}H]thymidine incorporation after 3 d of culture. Cell lines (5 × 10^5 cells/ml) were maintained by weekly restimulation with antigen, in culture medium with 10% FCS (otherwise as above), and 2.5 × 10^6 syngeneic irradiated (3,000 rad) spleen cells as feeders. The lines were cloned early (3 d) and late (2–3 mo) after the onset of culture by limit dilution (50, 5, 1, and 0.3 cell/well, respectively), in Terasaki plates with 2 × 10^4/well of feeder cells, in culture medium containing antigen and human rIL-2 (10 ng/ml; Sandoz Research Institute, Vienna, Austria). Clones were picked from wells with 1 and 0.3 cells, respectively, and expanded in antigen plus IL-2-containing medium. To test fine specificity, 2 × 10^4/well of cloned T cells were cultured in 96-well flat-bottomed microtiter plates with 5 × 10^5 syngeneic irradiated spleen cells (unless stated otherwise) with or without different concentrations of antigens, and [^{3}H]thymidine incorporation was measured 3 d later. Clones were assigned to belong to a particular clonotype after at least two separate tests with concordant results. The typed clones were expanded in IL-2 medium without feeder cells for 1–2 wk, and 6–10 × 10^6 cell aliquots were frozen in a dry, pelleted form for DNA and RNA preparation.

Flow Cytometry. Cells were resuspended at 4 × 10^6/ml in PBS supplemented with 2% FCS and 0.1% sodium azide. 50-μl aliquots of cell suspension were incubated with 50 μl of mAbs (used as hybridoma culture supernatants) in V-bottomed 96-well plates on ice for 45 min. The following mAbs were used: F23.1 (anti-Vβ8.1, 8.2, 8.3; reference 26), F23.2 (anti-Vβ8.2; reference 24), KJ16 (anti-Vβ8.1, 8.2; reference 27), and 44-22-1 (anti-Vβ6; reference 28). The cells were then washed and incubated with FITC-labeled (Fab')$_2$ of goat anti-mouse IgG (1:20 final dilution; Tago Inc., Burlingame, CA) for 30 min. In some experiments, the cells were stained with phycoerythrin (PE) -labeled L3T4 mAb, or with PE-labeled anti-Thy-1.2 (both from Becton Dickinson & Co., Mountain View, CA). After washing, the cells were resuspended in 1 ml PBS supplemented as above, and analyzed for surface fluorescence using a FACScan. Dot plots relating log fluorescence intensity (one or two color) to cell number were based on the analysis of 2,000 cells per sample.

Southern Blot Analysis. DNA was prepared from T cell clones (29) digested with Eco RI, Hind III, and Bam HI, and blotted onto nylon membrane filters (30). The DNA was then hybridized with ^{32}P random-labeled probes in 1.5 × SSPE, 1% SDS, 0.5% Blotto, 10% dextran sulfate, and 0.5% mg/ml salmon sperm DNA. Filters were washed twice in 3× SSC,

0.1% SDS, and twice in 0.3× SSC, 0.1% SDS at 65°C. The blots were autoradiographed by exposure at −70°C with intensifying screens. Blots were stripped by boiling in 0.1% SDS, 0.1× SSC for 30 min, and probed several times.

DNA Probes Specific for TCR α and β Gene Segments. Vβ probes (Vβ1 through Vβ16) were kindly provided by Dr. D. Loh (Washington University, St. Louis, MO). Vβ17 was a gift of Drs. J. Kappler and P. Marrack (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Dβ1 and Dβ2 probes were kindly provided by Dr. R. Haars (University of Ulm, FRG). The probe for Cβ is a 300-bp insert of a β chain cDNA clone (31). The Jβ1 probe is a 400-bp Bam HI–Eco RI fragment kindly provided by Dr. Karjalainen (Basel Institute for Immunology, Basel, Switzerland). The Jβ2 probe represents a 1-kb Cla I–Hind III fragment isolated from the clone pVBDFLβ I-9 (32). Vα1, Vα2, Vα4, Vα5, Vα6, Vα7, Vα8, Vα9, and Vα11 probes were generously provided by Dr. L. Hood (California Institute of Technology, Pasadena, CA). Vα3 and Vα13 probes were a gift of Dr. E. Palmer (National Jewish Center for Immunology and Respiratory Medicine). Probe Vα12 is a 200-bp Rsa I–Eco RI fragment isolated from the α chain cDNA clone pTBD 1,9 (32). Probe 1(D1) corresponds to the Cα gene and was isolated as a 300-bp Nco I–AVA II fragment from the constant portion of the α chain cDNA clone T 1.2 (33; Fig. 1). The Jα probes are summarized in Fig. 1. Probes 2D1 and 3D1 were isolated from cosmid clone BDFL 2.5, and probes 4F1, 4F2, 6M1, 6F1, and 9F1 from cosmid clone BDFL 7.5 (32). Probes 11M1, 11M2, and 13F1 were isolated from cosmid clone TA 28.1 (34). Probe 10F1 is a 3.0-kb Bam HI fragment isolated from the genomic (EMBL) clone 4B2A1-α-2 (35). Jα region probes 4, 5, 7, 8, 10, and 16(Cδ) were kindly provided by Drs. M. and B. Malissen (Centre d'Immunologie, Marseilles, France). Our Jα4 probe represents a 1.1-kb Sac I–Eco RI fragment that is smaller than the originally described 2.9-kb Sac I fragment (36).

Polymerase Chain Reaction (PCR). The sequences of oligonucleotide primers 5′ to 3′ were the following: Vβ1, AGCGCTGAGAAGCCGCCAG; Vβ4, TTCATGTTTTCCTACAGCTA; Vβ6, CATGGTGATGGTGGCATCATCAT; Vβ8, AACACATGGAGGCTGCAGTC; Vβ14, CCAGGTAGAGTCGGTGGTGC; Vβ15, TGTAAGAGTGGAACTTCCAT; Jβ1.7, CCATGGTCATCCAACACAGG; and Jβ2, TCTCCTACTATCGATTTCCCTCCCG. The Taq polymerase and the Gene Amp Kit used were from Perkin Elmer (Cetus Corp., Norwalk, CT). 1 μg of genomic DNA was subjected to 35 cycles of denaturation at 95°C for 2 min, annealing at 60–70°C for 2 min, and primer extension at 70°C for 6 min. 15 μl of amplified DNA was loaded onto a 2% agarose gel, blotted, and hybridized with the respective ^{32}P-labeled Vβ probes as described above.

RNA Extraction and Dot Blot Analysis. RNA was isolated from frozen cell pellets (37) and applied to nylon filters using a 96-well blotting manifold. RNA from the B cell Hybridoma LK was applied to each filter and was used as a negative control for all probes. The probes were labeled by random priming and hybridized for 18 h at 65°C, as described by Church and Gilbert (38).

Results

Fine Specificities in the Bovine Insulin-specific T Cell Repertoire of H-2^b Mice. To assess the number and relative frequency of BI-specific T cell clonotypes in H-2^b mice, we isolated a large number of clones from C57/BL6 (B6), BALB.B (B.B), and 129 mice carrying the H-2^b haplotype on different genetic backgrounds, and for comparison, a few clones from B10.D2 (H-2^d) mice. This approach has allowed us to detect the possible influences of non-MHC as well as MHC genes on the BI-specific repertoire. To establish clonotypes according to fine specificity, we tested the proliferative response of each clone to BI, EI, OI, and PI, as well as to separated BI chains Ano, Aox, and Box. Three distinct reactivity patterns could be discerned that we designated as clonotypes I, II, and III (Table I). Based on the analysis of 86 clones, clonotype I reactive to BI and Ano, with or without crossreactivity to EI and OI, comprised the bulk (90–94%) of the repertoire. Clonotype II with additional cross-

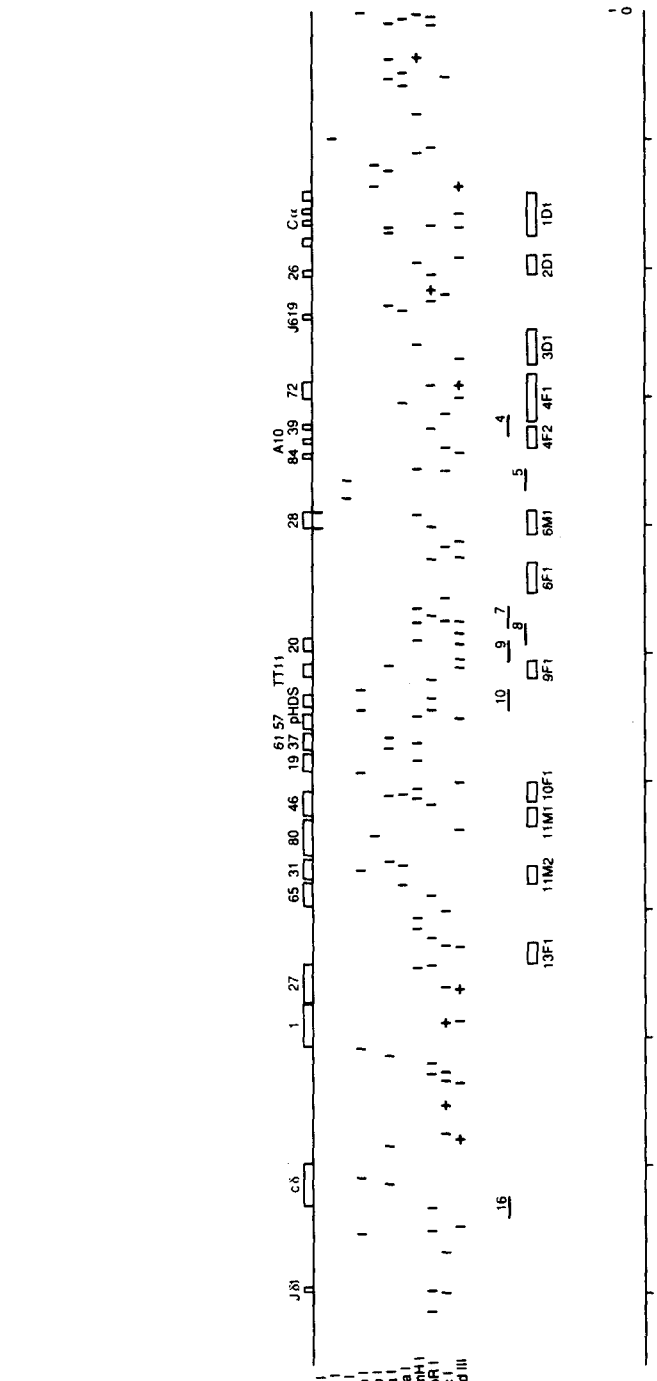

FIGURE 1. Restriction map of the Jα region and location of DNA probes used to detect Jα rearrangements. The positions of Jα gene segments, and the restriction enzyme sites, except of Hind III, are according to Winoto et al. (34) and Malissen et al. (36). Horizontal lines represent probes from Malissen et al. (36), and open boxes are probes isolated in this laboratory (see Materials and Methods). The Hind III sites are as determined in this study. A plus between two restriction sites indicates possible unassigned sites in that region.

TABLE I

Relative Frequency of BI-specific T Cell Clonotypes

Clonotype	Reactivity pattern							Number of clones from strain:				Total
	BI	EI	OI	PI	Ano	Aox	Box	B6	BALB.B	129	B10.D2	
I	+	(+)*	(+)	–	+	–	–	34 (90)‡	17 (94)	26 (93)	2	79 (92)
II	+	+	+	+	+	–	–	2 (5)	0	2 (7)	0	4 (5)
III	+	(+)	(+)	–	–	–	–	2 (5)	1 (6)	0	0	3 (3)
Total								38	18	28	2	86

* Reactivity is detectable only in case of high response to BI.

‡ Numbers in parentheses indicate percent.

reaction to PI, and clonotype III without reactivity to separated A and B chain, represented a minority (6–10%). These or similar clonotypes were also observed by others (25, 39–41). None of the clones tested reacted to rat insulin (data not shown). No qualitative or quantitative differences were detectable in the BI-specific repertoires of the three different H-2^b strains tested (Table I).

A great majority of BI-specific clones (97%, clonotypes I and II) reacted to Ano of BI, demonstrating that the B chain of insulin is not required for the formation of this immunodominant epitope. Irreversible oxidation of Cys residues (to SO_3 in Aox; reference 25) renders the A chain nonimmunogenic for these clones. The lack of immunogenicity is not due to a failure of Aox to bind to MHC molecules, since Aox can induce a T cell response in H-2^b mice (data not shown). It seems, therefore, that in the dominant A chain epitope, the Cys residues either form disulfide bonds, or their sulphur is reduced to sulfhydryl after processing (25). Clonotype III, in contrast, appears to recognize a "conformational" determinant of A and B chain, as also proposed by others (40, 42).

The reactivity pattern of clones subjected to DNA analysis is shown in detail in Table II (one representative experiment per clone). The absence or presence of alloreactivity to bm12 cells resulted in the dissection of each clonotype into two subtypes (i.e., Ia, Ib, etc., see Table II). All clones recognized insulin together with the I-A^b, but not with the I-A^{bm12}, molecule (data not shown).

Rearrangement and Expression of TCR Genes in BI-specific Clones. DNA prepared from 25 clones was digested with Hind III, Eco RI, and Bam HI, and Southern blots were tested for TCR rearrangements, using a series of α and β TCR probes. 5 of the 25 clones (11, 19, 14, 23, and 28) turned out to be repeated isolates, since they were identical in terms of strain of origin, immunization number, and all primary and secondary TCR rearrangements with another clone (Tables II and III). However, the remaining 20 clones could be unequivocally judged as unique by the same criteria.

A large proportion of the clones (8 of 20, 40%) rearranged the Vβ6 gene segment (Fig. 2, Table III). V$\beta6^+$ clones were isolated from B6, B.B., 129, and B10.D2 mice. Three of these clones also rearranged a second Vβ segment (Vβ5, 12, and 14, respectively), however, the Vβ6 protein was expressed on the cell surface of these clones, as demonstrated by immunofluorescent staining with mAb 44-22-1 (anti-Vβ6; reference 28). We tested further 25 BI-specific clones of B6, B.B., and 129 origin with mAb 44-22-1, and found eight of them to express the Vβ6 protein (see Table IV).

TABLE II

Origin and Fine Specificity of Anti-BI T Cell Clones

Clone no.	Strain	Immunization no.	Cloning*	Proliferative response of clones to B6 APC: Medium	BI	EI	OI	PI	Ano	Aox	Box	bm12‡: Medium	Clonotype
				cpm × 10^{-2}									
6	B6	1	L	63	1,997	589	1,595	64	1,411	75	77	−46	Ia
7	B6	1	L	76	2,814	1,497	1,912	111	1,916	50	32	−67	Ia
8	B6	1	L	74	3,163	1,247	1,980	36	1,696	54	25	12	Ia
9	B6	1	L	32	2,261	688	1,450	31	1,457	37	33	−67	Ia
11	B6	1	L	24	400	104	100	6	346	6	6	−9	Ia
13	B6	1	E	5	23	13	7	4	25	6	9	−6	Ia
14	B6	1	E	61	412	365	222	46	424	81	47	−19	Ia
19	B6	1	E	13	1,006	147	166	9	736	11	13	−31	Ia
24	B6	6	E	3	574	10	6	6	7	7	8	130	IIIb
27	B6	6	L	4	14	135	126	73	23	6	7	288	IIb
36	B6	1	L	3	567	9	12	5	41	5	8	−96	Ia
12	B.B	1	E	5	241	12	12	8	186	9	9	3	Ia
17	B.B	6	E	8	340	62	17	14	280	15	15	183	Ib
20	B.B	6	E	5	284	90	95	4	239	4	5	140	Ib
25	B.B	1	L	359	4,327	1,147	2,182	338	3,533	421	395	−20	Ia
28	B.B	1	L	43	541	138	77	24	552	53	27	−14	Ia
1	129	5	E	64	896	271	210	100	777	62	99	−11	Ia
3	129	6	E	3	26	8	7	4	24	7	8	103	Ib
4	129	6	E	3	16	8	5	7	13	6	8	−62	Ia
5	129	6	E	3	45	564	535	545	51	4	3	ND	II
15	129	5	L	4	103	9	6	6	68	4	9	−59	Ia
18	129	6	E	16	254	51	47	16	205	21	24	370	Ib
23	129	5	L	4	63	8	9	6	25	6	9	16	Ia
26	129	5	L	2	10	5	4	4	12	3	6	−3	Ia
22	D2	1	L	17	1,411	212	341	16	1,524	17	16	ND	I

* Cell lines were cloned either 3 d after stimulation with antigen (E = early) or after 6–12 weekly restimulations (L = late).

‡ The difference between proliferative responses to bm12 and B6 spleen cells in the absence of insulin (no additional responses were observed to bm12 cells in the presence of insulin; data not shown).

Thus, altogether, 16 of 45, that is, 36% of the BI-specific clones, tested expressed Vβ6. The Vβ6$^+$ clones rearranged five different Jβ2 and one Jβ1 segment, as shown by PCR (Fig. 3, Table III).

Four clones, all isolated from B6 mice, rearranged Vβ15 to two different Jβ segments (Fig. 2, Table III). This may be an example of strain-specific TCR usage, although the possibility that this clonotype dominated only in one particular immunization could not be excluded. In addition, Vβ1 was rearranged in two clones, and Vβ4, 8.1, 8.2, and 14 in one clone each. In clone 14, two Vβ rearrangements (1 and 5.2) were found, of which only Vβ1 was transcribed as shown by RNA dot blot analysis (Table III). Finally, no Vβ rearrangement was found in two clones (15/23 and 27).

The use of Vα subfamily members was determined in 14 of the 20 clones by the combined use of Southern blot analysis and RNA dot blots (Table III). Five clones used a Vα8 subfamily member, and three clones used Vα1, Vα4, and Vα12 each.

TABLE III

TCR Gene Segment Usage by BI-specific Clones

	Clone		Rearrangement					Secondary rearrangements*			
No.	Strain	Type	Vβ	Jβ	Vα	Jα	RNA dot blot	Vβ	Jβ	Vα	Jα
1	129	Ia	6‡	2.2	4	11M2§	Vα4	5.2 (7.0)	2 (7.0)	-‖	-
4	129	Ia	6	2.7	4	11M2	Vα4	-	2 (4.3)	-	10
17	B.B	Ib	6	1¶	12**	10F1	ND	-	2 (4.4, 3.6)	11**	11M2
18	129	Ib	6‡	2.3	12	10F1	Vα12, 10F1	12 (3.6)	2 (3.6)	4	11M1
20	B.B	Ib	6	2.5	12	10F1	Vα12	-	2 (3.7)	-	-
22	D2	I	6‡	2.3	7**	5	Vα7, Vα2	-	2 (4.2)	2	4F2
26	129	Ia	6‡	2.4	-	11M1	ND	14 (5.6)‡‡	1 (5.6)‡‡ 2 (5.0)	-	-
36	B6	Ia	6‡	2.7	-	10	ND	-	2 (3.4)	-	-
6	B6	Ia	15	2.3	-	13F1	ND	-	2 (4.3) 1 (2 × del)§§	-	-
7	B6	Ia	15	2.3	-	13F1	-	-	1 (7.0)	-	-
8	B6	Ia	15	2.4	8	11M2	Vα8	-	1 (3.7)	-	13F1
9, 11, 19	B6	Ia	15	2.3	1	13F1	Vα1	-	1 (6.0)	-	-
13, 14	B6	Ia	1	2.1	8	9	Vβ1, Vα1, Vα8	5.1 (5.8)	2 (5.8)	-	-
25, 28	B.B	Ia	1	2.1	8**	11M1	Vα8, Vα1	-	2 (5.2)	1**	4F2
5	129	II	8.1‡	2.3	-	13F1	ND	-	2 (3.9)	-	-
12	B.B	Ia	8.2‡	1.2	8	4F2	Vα8	-	2 (3.1)	-	6M1
24	B6	IIIb	4	2¶	8	4F1	Vα8	-	-	-	-
3	129	Ib	14	2.7	4	5	ND	-	2 (3.6)	-	-
15, 23	129	Ia	-	1 (5.0)‡‡	1	10	ND	-	2 (3.7)	-	-
27	B6	IIb	-	2 (5.1)	-	-	ND	-	-	-	-

* Length (kb) of the rearranged Hind III fragment is given in parentheses, unless stated otherwise.

‡ Cell surface expression of the corresponding protein was demonstrated by immunofluorescence, using mAbs 44-22-1 (anti-Vβ6), F23.1 (anti-Vβ8), KJ16 (anti-Vβ8.1, 8.2), and F23.2 (anti-Vβ8.2).

§ See Fig. 4 for mapping of the corresponding Jα gene segment.

‖ Not found.

¶ Not determined by PCR.

** It is uncertain which rearranged gene is expressed.

‡‡ Length (kb) of rearranged Eco RI fragment is given in parentheses.

§§ Gene segment is deleted from both chromosomes.

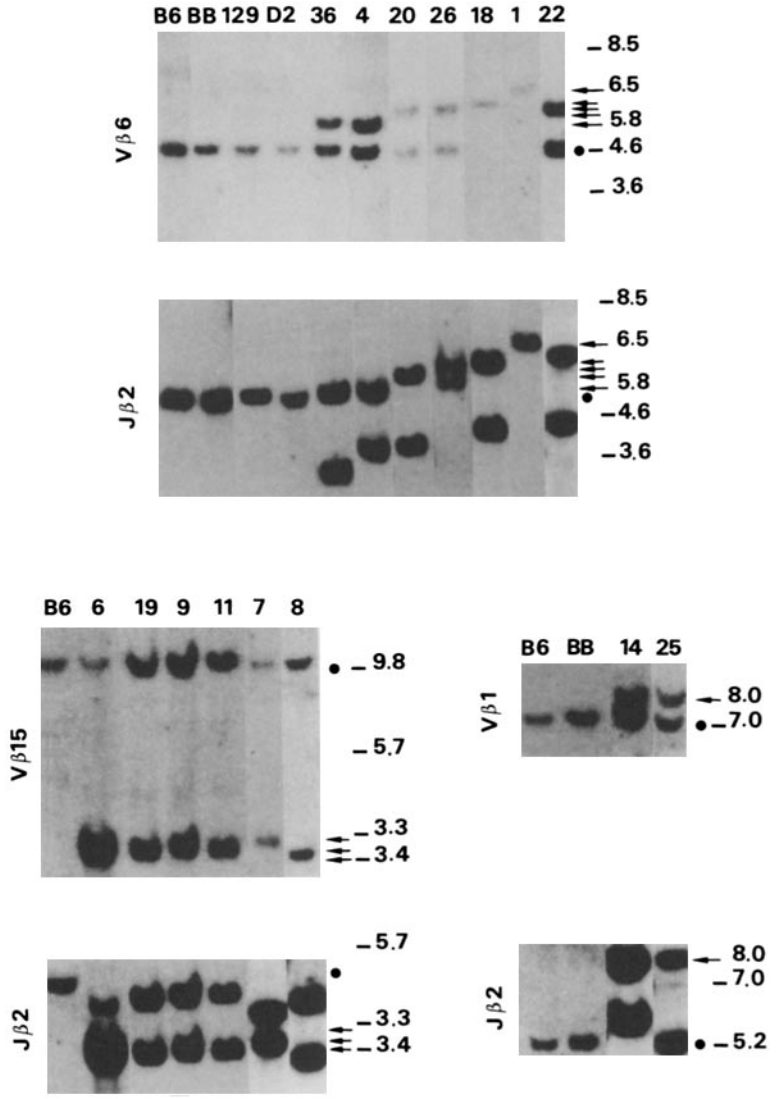

FIGURE 2. Southern blot analysis of TCR β gene rearrangements in BI-specific T cell clones. DNA from B6, BALB.B, 129, and B10D2 liver, and from clones of the indicated numbers (*horizontal*) was digested with Hind III, and hybridized with probes for Vβ1, Vβ6, Vβ15, and Jβ2. Numbers arranged vertically on the left indicate the size of fragments in kilobases. Bands denoted by closed circles represent germ-line fragments, and arrows indicate productive rearrangements.

These Vα genes were rearranged to 10 different Jα segments. A case of correlation between gene usage and fine specificity was also demonstrated, namely, three of the eight Vβ6$^+$ clones that were alloreactive to bm12 cells (clonotype Ib) rearranged the same Vα12 subfamily member to Jα10F1, and at least two of these three clones also expressed Vα12 at the mRNA level (Vα4 in clone 18 was not expressed; see Table III). Three clones (17, 22, and 25/28) rearranged two Vα genes, and at least two of them (22 and 28) appeared also to translate both Vα genes.

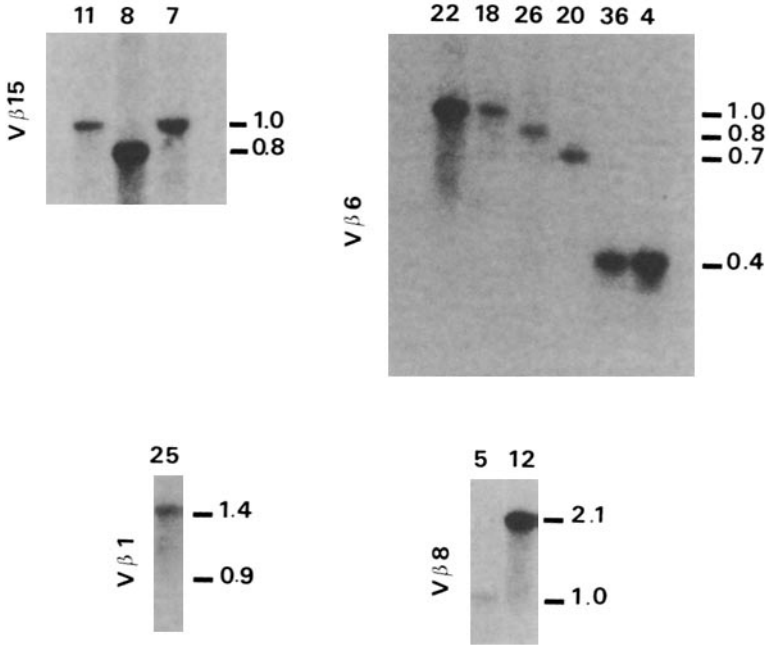

FIGURE 3. Identification of the rearranged member of Jβ gene clusters by PCR. Numbers arranged horizontally indicate the clones, and numbers on the left show the size of bands in kilobases. The Jβ2 primer was a sequence 3′ to Jβ2.7, and the Jβ1 primer (used only for clone no. 12) was a sequence from the Jβ1.7 pseudogene. DNA between the Jβ primer and the appropriate Vβ primer (see Materials and Methods) was amplified, blotted, and hybridized with the indicated Vβ probes. The rearranged member of the respective Jβ cluster was identified by the size of the amplified DNA. For example, clones 7 and 11 rearranged Vβ15 to Jβ2.3 (1.0 kb) and clone 8 to Jβ2.4 (0.8 kb, etc.; see Table V for more details).

In summary, the panel of 20 unique BI-specific clones expressed at least six different Vβ genes with a distinct preference for Vβ6. The use of Jβ segments was virtually random, and no predominant Vα or Jα gene usage could be observed.

Enhanced Vβ6 Expression in BI-specific Polyclonal T Cell Lines. To investigate whether

TABLE IV

Influence of Self Tolerance on the Expression of Vβ6 and Vβ8 by BI-specific T Cell Clones

Strain of origin	T cell clones		Number of T cell clones expressing:				
	Restriction	No. tested	Vβ6	Vβ8	Vβ8.1	Vβ8.2	Vβ8.3
B6, B.B, 129	I-A^b	45*	16 (36)‡	5 (11)	-§	-	-
(B6 × DBA/2)F$_1$	I-A^b	24‖	0	13 (54)	-	-	-
(B6 × DBA/2)F$_1$	I-A^d	35‖	0	21 (60)	-	-	-
(B6 × DBA/2)F$_1$	I-A^b	8‖	-	8	0	7	1
(B6 × DBA/2)F$_1$	I-A^d	16‖	-	16	0	9	7

* Of these clones, 26 were tested by immunofluorescence using the appropriate mAbs (see footnotes to Table III), 19 by Southern analysis (see in Table III), and seven by both methods.

‡ Numbers in parentheses are percent.

§ Not tested.

‖ Tested by immunofluorescence with the relevant mAbs.

the preferential use of Vβ6 in anti-BI response results from antigen selection, we tested Vβ6 expression in uncloned T cell lines after five to six cycles of restimulation with antigen. As demonstrated by immunofluorescent staining using mAb 44-22-1 (Fig. 4), a two- to three-fold enrichment of $V\beta6^+$ cells was detectable in BI-specific lines in comparison with the normal level of Vβ6 expression in unimmunized lymph node cells (23). Five of the eight BI-specific lines tested exhibited this enrichment. In contrast, $V\beta6^+$ cells were depleted from T cell lines specific for Aox of BI, PPD of tuberculin, and HEL, suggesting that these responses use Vβ genes other than Vβ6.

Expression of Vβ6 and Vβ8 Genes in BI-specific T Cell Clones from (B6 × DBA/2)F_1 Mice. The Vβ6 protein is known to confer reactivity to the product of the Mls-1^a gene (23). Thus, T cells expressing Vβ6 are clonally deleted during establishment of self tolerance to Mls-1^a. However, a small proportion (<1%) of $V\beta6^+$ T cells can be demonstrated in the lymph nodes of Mls-1^a mice (23), suggesting that the deletion may not be complete. Since the functional consequences of Vβ6 deletion for immune responses other than the anti-Mls-1^a response were unknown, it was of interest to see whether the residual $V\beta6^+$ clones could be retrieved from Mls-1^a mice by an antigen that induces such clones preferentially. To address this issue, we have isolated T cell clones from Mls-1^{a+} (B6 × DBA/2)F_1 mice immunized with BI. Some of these clones were I-A^b restricted (recognized BI presented by B6 cells), and others I-A^d restricted (recognized BI presented by DBA/2 cells, and their response was inhibited by anti-I-A^d but not anti-I-E^d mAb; data not shown). Vβ expression in these clones was then studied by immunofluorescence using mAbs 44-22-1 (anti-Vβ6) and F23.1 (anti-Vβ8.1, 8.2, 8.3; reference 26). As shown in Table IV, none of the 24 I-A^b-restricted and 35 I-A^d-restricted F_1 clones isolated expressed Vβ6 (in contrast to 36% $V\beta6^+$ clones in non-Mls-1^a strains). However, the majority (54 and 60%, respectively) of F_1 clones expressed Vβ8, as compared with 11% of $V\beta8^+$ clones in the other (non-Mls-1^a) strains. We further tested eight I-A^b-restricted and 16 I-A^d-restricted $V\beta8^+$ F_1 clones with mAbs KJ16 (anti-Vβ8.1, 8.2, reference 27) and F23.2 (anti-Vβ8.2; reference 24), to determine the member of the Vβ8 subfamily they express. We found that, of the 24 $V\beta8^+$ clones tested altogether, 16 clones expressed Vβ8.2, eight clones Vβ8.3, and none Vβ8.1 (Table IV). Thus, the deletion of $V\beta6^+$ and $V\beta8.1^+$ cells by Mls-1^a caused a virtually complete lack of expression of these "forbidden" Vβ genes in the BI-specific T cell repertoire.

In view of the finding that the predominant TCR in the F_1 clones used Vβ8 instead of Vβ6, we investigated whether this difference would also be reflected in differences of fine specificity. The reactivity pattern of several clones to different insulins is shown in Fig. 5. Among the F_1 clones, two reactivity patterns were found that corresponded to clonotypes I and II, demonstrable also in C57/BL, BALB, and 129 mice (see also Tables I and II). The majority of F_1 clones were of clonotype I, although the frequency of clonotype II was elevated in comparison with the other strains tested (data not shown). All clones exhibited individual variability in terms of fine specificity, and this variability could not be correlated with either the strains of origin or the Mls-1 allele expressed by them (Fig. 5). Thus, the shift of Vβ gene usage was not accompanied by a noticeable effect on the fine specificity, as detected with the available panel of antigens.

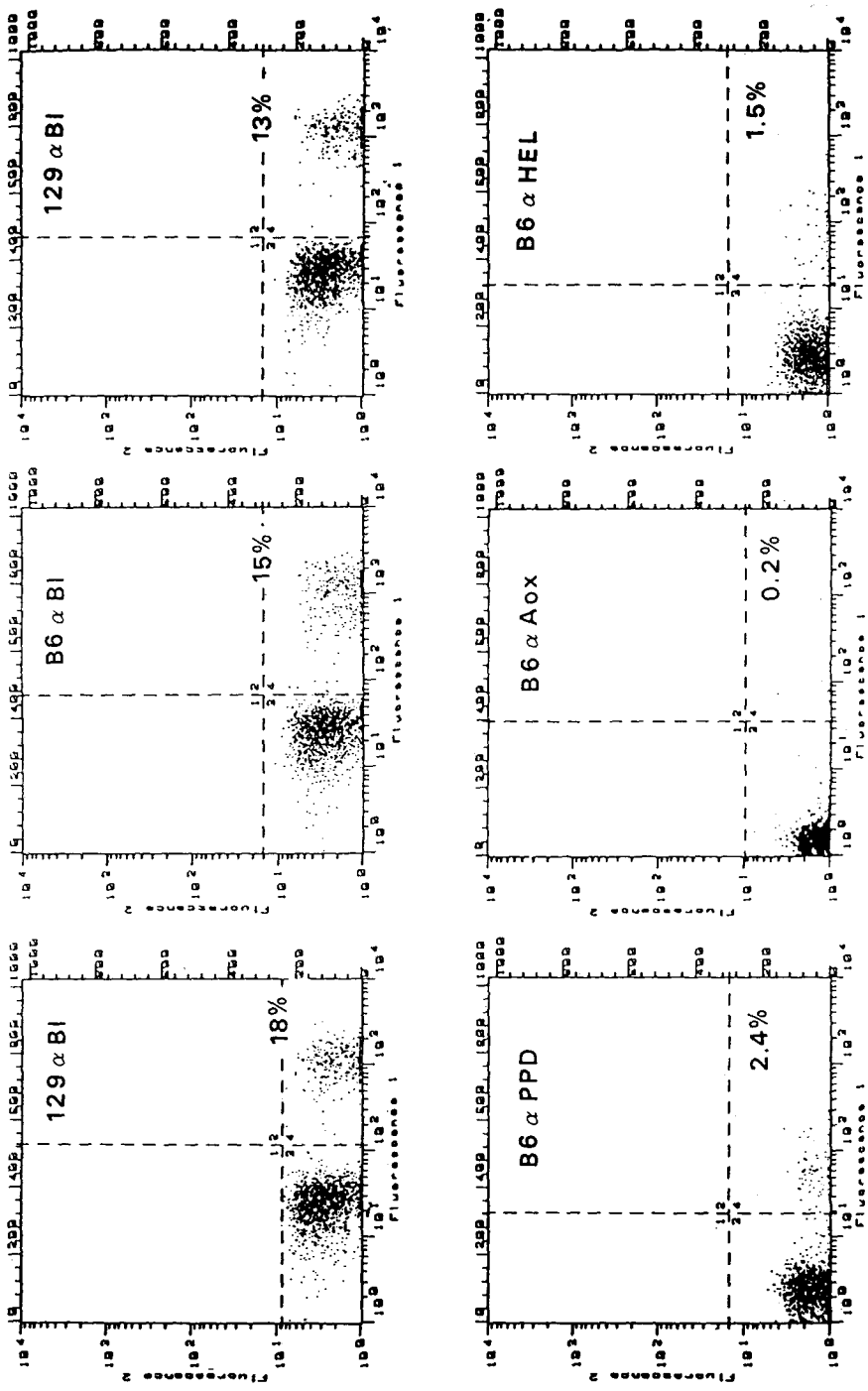

FIGURE 4. Expression of Vβ6 in polyclonal T cell lines. Enriched expression in BI-specific lines (*top*), and decreased expression in lines specific for PPD, Aox, and HEL, respectively (*bottom*). T cell lines were tested by indirect immunofluorescence using mAb 44-22-1. The percent of Vβ6$^+$ cells, after subtraction of background staining with $(Fab')_2$ goat anti-mouse IgG FITC, is indicated in each panel. The Vβ6 expression in lymph nodes of unimmunized mice (determined by double staining with L3T4 and 44-22-1 mAbs) was 2.9–6.2% in B6, and 8.1–8.6% in 129 (data not shown).

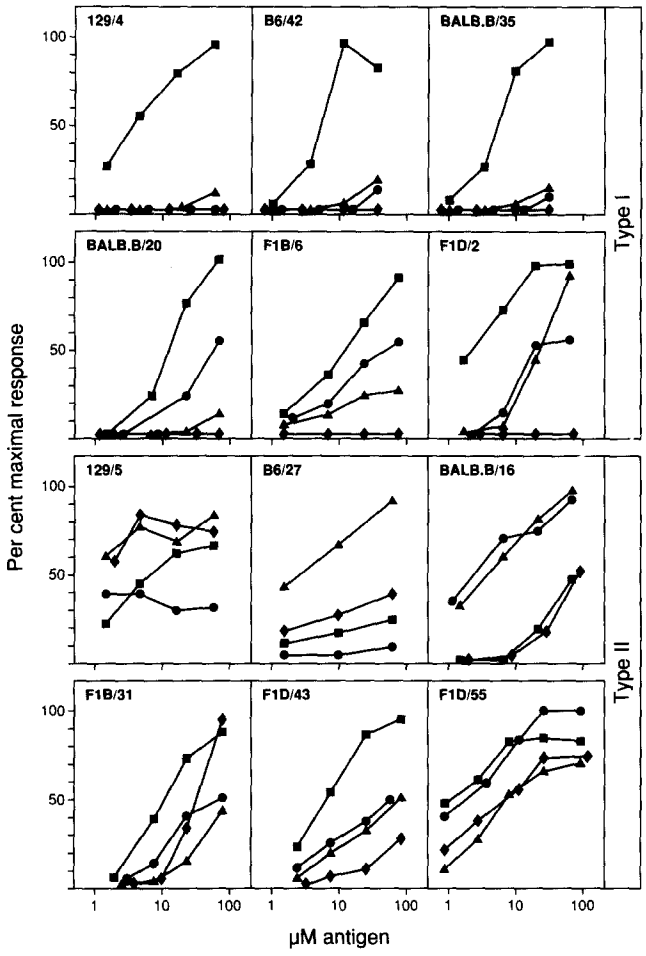

FIGURE 5. Fine specificity of BI-specific T cell clones. Clones were tested for response to different concentrations of BI (■), EI (▲), OI (●) and PI (◆). The strain of origin and serial number of clones is indicated in each panel. F1B refers to I-A^b- and F1D to I-A^d-restricted (B6 × DBA/2)F_1 clones. Clones 129/4, B6/42, BALB.B/20, and BALB.B/16 are Vβ6$^+$; clones F1B/6, F1D/2, 129/5, F1B/31, and F1D/43 are Vβ8$^+$. The results are expressed as percent of maximal response of which percent medium control was subtracted. Type I clones gave maximal response to BI, whereas type II clones gave frequently to another insulin ("heteroclicity"). Maximal responses/medium controls in cpm were: 129/4, 14,800/500; B6/42, 71,200/1,100; BALB.B/35, 79,100/1,100; BALB.B/20, 436,500/430; F1B/6, 23,200/1,300; F1D/2, 416,300/2,000; 129/5, 26,100/4,400; B6/27, 13,900/900; BALB.B/16, 67,600/400; F1B/31, 63,100/2,500; F1D/43, 64,900/4,200; F1D/55, 451,400/1,300.

Discussion

The T cell response to insulin is directed almost exclusively against a small portion of the molecule extending from residue 4 to 11 of the A chain. This immunodominant region includes the intrachain loop formed by the Cys residues at positions 6 and 11. A unique feature of the A chain loop determinant is its apparent conformation dependence. Thus, irreversible oxidation of Cys residues renders the A chain nonimmunogenic for clones generated by immunization with native insulin or nonoxidized A chain (25; F. Falcioni, unpublished results). The implication of this finding is that the A chain determinant may be presented in an unchanged or slightly changed loop form. In fact, the A chain loop has been shown to remain intact even in the acidic late endosomes of rat liver cells (43). This determinant, in contrast to many others presented usually in linear or α helical form (6, 7, 14, 17), attracts a relatively large number of different T cell clones. We assume that the privileged status of this determinant is related to the loop structure, since our preliminary studies suggest that immunization with oxidized (extended) A chain

may induce a T cell response of narrow clonal spectrum (F. Falcioni, unpublished results). There are several possible explanations for the clonal heterogeneity of anti-insulin response. First, the loop determinant may fit in many different ways into the class II MHC groove after processing (41). Second, the loop may protrude from the groove, allowing interaction with many different TCRs. Third, the loop determinant may be presented at high density on the surface of APC, permitting the activation of clones recognizing the epitope at low affinity. Studies are underway to distinguish between these possibilities.

Despite the heterogeneity of antiinsulin response, a predominance of Vβ6-expressing clones has been demonstrated by testing a large sample of clones. This preferential gene usage is not influenced by non-MHC genes in C57BL, BALB, and 129 strains carrying the H-2^b haplotype. The clones expressing Vβ6 appear to be selected by the antigen, since insulin-specific polyclonal T cell lines have high proportions of V$\beta6^+$ cells (two to three times higher than background expression in unimmunized mice). However, less frequent clones using Vβ1, 8, 14, and 15, and exhibiting the same fine specificity as the V$\beta6^+$ ones, have also been identified. Furthermore, the Jβ, Vα, and Jα gene segments appear to be widely interchangeable, without causing noticeable difference in fine specificity. Interestingly, within the V$\beta6^+$ group, the use of Vα12 and Jα10F_1 seems to correlate with alloreactivity against bm12 cells. We therefore assume that more cases of correlation between gene usage and specificity could be identified, were more fine specificity markers available.

We have also investigated whether Mls-1^a, known to cause deletion of T cells expressing Vβ6 and Vβ8.1 (23, 24), would influence the TCR repertoire in the antiinsulin response. In Mls-1^{a+} (DBA/2 × B6)F_1 mice, we could not isolate a single BI-specific clone expressing either of these Vβ genes. Thus, in functional terms, the deletion of these two V genes appears to be complete, even though a small proportion of T cells expressing these "forbidden" V gene products were demonstrated in Mls-1^a mice (23, 24). Instead of the deleted V gene products, the majority of BI-specific F_1 clones expressed Vβ8.2 and Vβ8.3. Despite this shift of gene usage, no difference in fine specificity could be demonstrated in comparison with the other strains tested. Also in quantitative terms, the response to BI was comparable with that of other strains. In a similar study, Fry and Matis (44) have shown that the deletion of Vβ3 by self tolerance fails to change the response to pigeon cytochrome *c* using this Vβ gene preferentially, although a minor shift in fine specificity could be observed. Thus, both studies have shown that the T cell repertoire is sufficiently flexible to compensate for clonal deletions caused by self tolerance. It should be pointed out, however, that in both studies the predominant Vβ gene was expressed in 30–40% of clones. It remains, therefore, to be established whether the deletion of a gene used more extensively for recognition of a particular antigen would lead to unresponsiveness.

Recently, several laboratories have reported on successful treatment of autoimmune diseases in animal models using mAbs directed against TCRs involved in the recognition of autoantigens (14, 15, 45). In these models, a short to medium term cure was achieved, when the majority (~80%) of disease-inducing clones expressed the TCR recognized by the antibody. In another study (46), where only 50% of pathogenic clones expressed the Vβ17a protein recognized by the antibody, the cells not expressing Vβ17a took over the perpetuation of disease after antibody treatment. It appears, therefore, that rare clones comprising <20% of the clonal spectrum cannot

readily take over the response in case of acute peripheral suppression of V gene expression. In contrast, when a deletion was caused by neonatal tolerance, the remaining T cell repertoire may be better adapted to compensate for the deletion, as illustrated in this study by the shift of dominant gene usage from Vβ6 to Vβ8. It remains to be established whether deletions caused artificially in the adult T cell repertoire would be compensated by minority clones in the long term.

Summary

Bovine insulin(BI)-specific I-A^b-restricted T cell clones have been characterized for fine specificity and TCR gene usage. We have demonstrated that mouse strains carrying H-2^b on three different genetic backgrounds (C57BL, BALB, and 129) rearrange and express the Vβ6 gene in a large proportion (36%) of insulin-specific clones. In these strains, the non-MHC background did not seem to influence TCR gene usage in response to BI. The V$\beta6^+$ clones appeared to be selected by the antigen. In contrast, no V$\beta6^+$ clones could be isolated from (B6 × DBA/2)F_1 mice, where V$\beta6^+$ (and V$\beta8.1^+$) T cells are deleted by self tolerance to Mls-1^a. Thus, although a small proportion of residual V$\beta6^+$ cells had been demonstrated in Mls-1^a mice (23), these cells could not be retrieved in a response that uses Vβ6 predominantly. In functional terms, therefore, the deletion of Vβ6 by self tolerance appears to be complete. Instead of Vβ6, the majority (up to 60%) of I-A^b- as well as I-A^d-restricted insulin-specific clones from the (B6 × DBA/2)F_1 mice expressed Vβ8.2 and Vβ8.3. This shift of gene usage was not accompanied by any detectable change in the fine specificity pattern of response. Thus, in the insulin-specific response, the flexibility of T cell repertoire fully compensates for deletions caused by self tolerance.

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