Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2518–2521, March 1997
Immunology

T cell receptor restriction of diabetogenic autoimmune NOD T cells

(insulin/alpha chain/autoimmunity/type I diabetes)

E. SIMONE, D. DANIEL, N. SCHLOOT, P. GOTTLIEB, S. BABU, E. KAWASAKI, D. WEGMANN, AND G. S. EISENBARTH*

Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, CO 80262

Communicated by David W. Talmage, University of Colorado Health Sciences Center, Denver, CO, December 30, 1996 (received for review October 21, 1996)

ABSTRACT **Restricted use of T cell receptor (TCR) gene segments is characteristic of several induced autoimmune disease models. TCR sequences have previously been unavailable for pathogenic T cells which react with a defined autoantigen in a spontaneous autoimmune disease. The majority of T cell clones, derived from islets of NOD mice which spontaneously develop type I diabetes, react with insulin peptide B-(9–23). We have sequenced the α and β chains of TCRs from these B-(9–23)-reactive T cell clones. No TCR β chain restriction was found. In contrast, the clones (10 of 13) used Vα13 coupled with one of two homologous Jα segments (Jα45 or Jα34 in 8 of 13 clones). Furthermore, 9 of 10 of the Vα13 segments are a novel NOD sequence that we have tentatively termed Vα13.3. This dramatic α chain restriction, similar to the β chain restriction of other autoimmune models, provides a target for diagnostics and immunomodulatory therapy.**

Type I diabetes mellitus, which develops spontaneously in man (1), the NOD mouse (2), and the BB rat, is considered a T cell-mediated autoimmune disorder. In the past 2 years, Wegmann and coworkers (3–5) have isolated $CD4^+$ T cells from islets of prediabetic NOD mice. T cell lines were established after stimulation with whole islets and were later discovered to react with insulin. Most (93%) of these insulin-reactive T cell clones react with an insulin B chain peptide consisting of amino acids 9–23. These T cells are present within islet lesions when the mice were first tested (4 weeks of age). They are pathogenic and rapidly lead to insulitis and diabetes when injected into young NOD mice (6). In addition, the same T cell clones can destroy transplanted human islets in an NOD/*scid* mouse *in vivo* (7).

Several autoimmune disorders have been linked to autoreactive T cells using T cell receptors (TCRs) with restricted variable chains. The best example of this restriction is experimental allergic encephalomyelitis associated with Vβ8.2 and Vα2 in Lewis rats (8), and Vβ8.2 and either Vα2 or Vα4 in B10.PL (9) and PL/J mice (10). The Jα segments share homology as well, with the B10.PL Vα segments rearranged to Jα11, whereas the Pl/J Vα segments were primarily rearranged to Jα40. Other examples of restricted Vβ TCR use include experimental allergic neuritis (11), experimental allergic uveitis (12), and collagen-induced arthritis (13). In contrast to the restricted TCRs of the above autoimmune models, the genetic deletion of Vβ subsets by selective breeding did not prevent diabetes in the NOD mouse (14, 15). Similarly, the transgenic introduction of an anti-ovalbumin Vβ8.2 rearranged chain into the NOD mouse (with allelic exclusion of greater than 98% of endogenous Vβ chains) did not prevent disease (16).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/942518-4$2.00/0
PNAS is available online at **http://www.pnas.org**.

MATERIALS AND METHODS

Antigens. Insulin peptides B-(9–23) (SHLVEALYLVCGERG) and A-(7–21) (CTSICSLYQLENYCN), and tetanus toxin peptide TT-(830–843) (QYIKANSKFIGITE) were synthesized and purified by reverse-phase HLPC (Molecular Resources Center, National Jewish Hospital, Denver).

Proliferation Assay. Each well contained either 2.5×10^4 T cells, T cells plus 1×10^6 irradiated spleen cells (from NOD/bdc, BALB/c ByJ, or C57BL/6J mice), or T cells plus spleen cells and antigen (insulin, B-(9–23), A-(7–21), or tetanus toxin peptide) at a concentration of 100 μg/ml. After 72 h, the cells were pulsed with tritiated thymidine for 6 h and harvested, and incorporated thymidine was determined with a Betaplate 1205 liquid scintillation counter (LKB Wallac). All sample analyses were performed in duplicate or triplicate using a 96-well plate format.

TCR Sequencing. T cell clones were analyzed by flow cytometry with mAbs specific for the different Vβ families (PharMingen, San Diego). Total RNA was isolated from $1–5 \times 10^6$ resting T cell clones using RNAzol B (Tel-Test, Friendswood, TX) and cDNA prepared using random hexamer primers (Pharmacia). The α and β chains were amplified by PCR using degenerate primers for α chains (17) and specific primers for the β chains (sequences provided by X. D. Yang, Stanford University, Stanford, CA). The amplified product was visualized on 1.5% agarose gel and cloned into the TA cloning vector PCRII (Invitrogen). DNA was extracted from positive colonies (Qiagen, Chatsworth, CA) and sequenced on the AB1373A DNA sequencer (Applied Biosystems). Two to four (mean = 2.6) DNA clones from each T cell clone were sequenced in both directions using SP6 and T7 primers. The length of readable variable chain nucleotides ranged from 61 to 182 (mean = 144). The complete N (nDn) regions and the J regions were obtained for all sequences.

RESULTS

NOD T Cell Clones Analyzed. The majority of T cell clones were isolated from spontaneous islet lesions of prediabetic female NOD mice ranging in age from 4 to 12 weeks. In addition, four clones (LN) were derived from cervical lymph nodes after nasal administration of insulin peptide B-(9–23). All of these clones were reactive to peptide B-(9–23) as determined by proliferation assays. As control clones, we sequenced the TCRs of spontaneous, islet-derived NOD clones reactive to an insulin A chain peptide, A-(7–21). We also sequenced the TCRs from a panel of lymph node-derived clones reactive to a tetanus toxin peptide, TT-(830–843), after subcutaneous and nasal immunization with the peptide.

Abbreviation: TCR, T cell receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. U89719–U89742).

*To whom reprint requests should be addressed at: Barbara Davis Center for Childhood Diabetes, 4200 East 9th Avenue, Box B140, Denver, CO 80262.

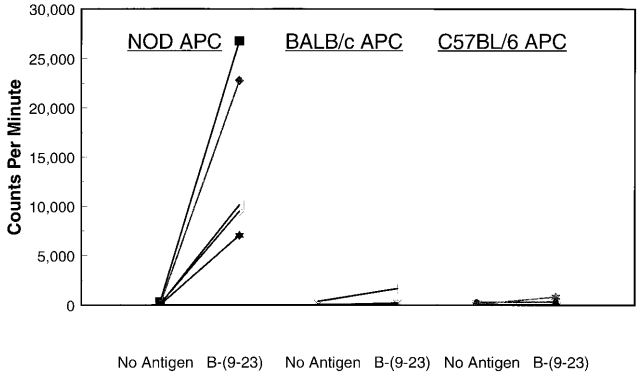

FIG. 1. Proliferation assay results of five different B-(9–23)-reactive T cell clones expressed as mean counts per minute of duplicate or triplicate samples. Each clone was combined with irradiated NOD/bdc, BALB/c ByJ, or C57BL/6J spleen cells as antigen-presenting cells (APC) and then tested with either no antigen or B-(9–23) peptide (100 μg/ml). The response of one of the clones to C57BL/6J spleen cells without antigen is consistent with an alloreactive response for this clone.

Recognition of the Insulin B-(9–23) Peptide by NOD T Cells Is Restricted by the Major Histocompatibility Complex Class II Molecule I-A^{g7}. I-A^{g7} is the only class II molecule of NOD mice because of a natural deletion of I-Eα (18). BALB/c ByJ mice (K^d, I-A^d, I-E^d, D^d) share K^d and C57BL/6J mice (K^b, I-A^b, I-E^o, D^b) share D^b with NOD/bdc mice (K^d, I-A^{g7}, I-E^o, D^b). Antigen-presenting cells from BALB/c or C57BL/6 mice were unable to present B-(9–23) peptide to the clones, a result that is consistent with I-A^{g7} restriction of B-(9–23) peptide recognition (Fig. 1).

TCR Sequences of Insulin B-(9–23)-Reactive T Cell Clones. We observed a wide variety of Vβ, Jβ, and nDn β chain junctions (Table 1). In contrast to the diversity of the β chains, there was a marked restriction of both the Vα and the Jα segments (Table 2). Vα13 occurred in six of nine spontaneous, B-(9–23)-reactive, islet-derived clones, and in four of four B-(9–23)-reactive T cell clones derived from the cervical lymph nodes after immunization. The three other B-(9–23)-reactive clones used Vα1, Vα8, and Vα10. Vα13 was absent in the group of five insulin peptide A-(7–21), islet-derived clones and the three tetanus toxin-reactive clones ($P < 0.001$ B-(9–23) vs. non-B-(9–23)-reactive clones; Fig. 2).

Table 1. Lack of restriction of TCR β chain sequences from insulin B-(9–23)-reactive clones

Clone	Designation		Regions: Vβ	nDn	Jβ
12-4.1	Vβ2	Jβ2.6	LYCTCS	P G L G K	EQY
12-4.4	Vβ12	Jβ1.3	YLCASS	P G Q G T	TLY
4-7.2	Vβ1	Jβ1.3	YFCASS	Q S R T	GNT
6-4.3	Vβ2	Jβ1.1	LYCTCS	A A G G G	TEV
8-1.3 (LN)	Vβ6	Jβ1.6	FLCAS	T S G T G Q G	SPL
12-3.20	Vβ14	Jβ2.5	YLCAWS	R L G G	NQD
8-1.9 (LN)	Vβ4	Jβ1.1	YFCASS	P D N A	NTE
8-1.1 (LN)	Vβ12	Jβ2.6	YLCASS	L G W G D	EQY
12-1.19	Vβ6	Jβ1.1	FLCASS	I L G Q	NTE
12-2.35	Vβ8.3	Jβ1.6	YFCASS	P S G R	NSP
6-6.4	Vβ10	Jβ2.5	YLCASS	W G Q G G	DTQ

The partial amino acid sequences of the TCR β chains include the terminal portion of the Vβ region, the nDn region (underlined), and the proximal portion or the Jβ region. All of the clones react to insulin peptide B-(9–23). The majority of clones were isolated from spontaneous islet lesions of female NOD mice. Clones with the suffix (LN) were isolated from cervical lymph nodes after nasal immunization with the B-(9–23) peptide. This panel represents clones from eight different mice ranging from 4 to 12 weeks of age (first number of the clone indicates the age of the mouse at the time of T cell isolation).

Complete sequencing of these Vα13 chains revealed that 9 of 10 are an identical, novel NOD sequence which we have tentatively termed Vα13.3. This sequence does not appear to be found in BALB/c mice and is either a new Vα13 family member or an allelic variant. The sequence differs from the previously reported Vα13.1 (present in BALB/c, C57BL/6, and NOD mice) at only three nucleotides and two amino acids in the complementary determining region 1 (CDR1) (Table 3). C57BL/6 mice appear to have a similar sequence with the same NOD nucleotide substitution in the CDR1 region but have an additional 2-aa polymorphism near the CDR2 region. We have tentatively termed this sequence Vα13.4.

The B-(9–23)-reactive clones used restricted Jα segments as well. Jα34 and Jα45 are highly homologous, sharing 15 of 20 aa, including the octamer KLTFGKGT. The presence of two positively charged lysine residues at the first and sixth position

Table 2. TCR α chain sequences of insulin B-(9–23)-reactive clones: Predominant Vα13.3 with Jα45 or Jα34

Clone	Designation		Regions: Vα	N	Jα
12-4.1	Vα13.3	Jα45	**MYFCAAS**	E	**SGGSN**YKLTFGKGT
12-4.4	Vα13.3	Jα45	**MYFCAAS**	A	**SGGSN**YKLTFGKGT
6-10.14	Vα13.3	Jα45	**MYFCAAS**	S R	**GGSN**YKLTFGKGT
4-7.2	Vα13.3	Jα45	**MYFCASS**	A N	**GGSN**YKLTFGKGT
6-4.3	Vα13.3	Jα45	**MYFCAAS**	A S G	**SGGSN**YKLTFGKGT
8-1.3 (LN)	Vα13.1	Jα34	**MYFCAAS**	A R G	**SGGSN**AKLTFGKGT
12-3.20	Vα13.3	Jα34	**MYFCAAS**	K I	**GGSN**AKLTFGKGT
8-1.9 (LN)	Vα13.3	Jα34	**MYFCAAS**	R P	**GGSN**AKLTFGKGT
8-1.1 (LN)	Vα13.3	Jα47	**MYFCAAS**	K	**TGGNN**KLTFGQGT
8-1.15 (LN)	Vα13.3	Jα11	**MYFCAAS**	A	**NS**GTYQR**FGTGT**
12-1.19	Vα10	Jα9	**TYLCA**ME	R S	SGYN**KLTFGKGT**
12-2.35	Vα1	Jα1	**LYFCAA**I	Q	NYNQG**KLIFGQGT**
6-6.4	Vα8	Jα48	**LYYCA**PN	Q	**G**GSA**KLIFGEGT**

Partial amino acid sequences of the TCR α chains including the terminal portion of the Vα region, the N region (underlined) and the proximal portion of the Jα region. Areas of homology are in boldface type. All of the clones react to insulin peptide B-(9–23). The majority of clones were isolated from spontaneous islet lesions of female NOD mice. Clones with the suffix (LN) were isolated from cervical lymph nodes after nasal immunization with the B-(9–23) peptide. This panel represents clones from nine different NOD mice ranging from 4 to 12 weeks of age (first number of the clone indicates the age of the mouse). Standard single letter abbreviations for amino acids are used.

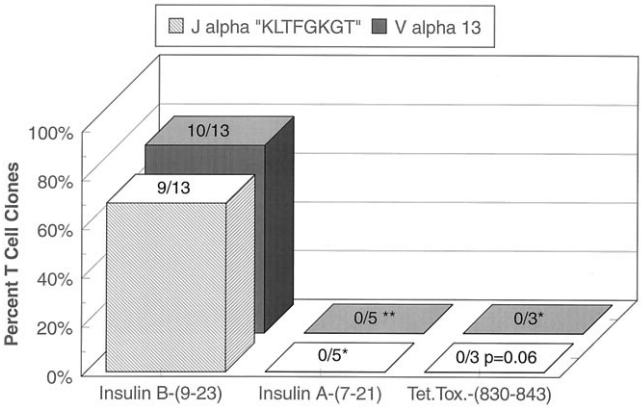

FIG. 2. Analysis of the percent of T cell clones with receptors containing the Jα octamer KLTFGKGT (front) or Vα13 (back) for different panels of NOD T cell clones with reactivity to either insulin peptide B-(9–23) ($n = 13$), insulin peptide A-(7–21) ($n = 5$), or tetanus toxin TT-(830–843) ($n = 3$). The B-(9–23) clones were derived from spontaneous islet lesions ($n = 9$) or cervical lymph nodes ($n = 4$). *P* values were calculated with the Fisher Exact Test (*, $P < 0.05$; **, $P < 0.01$).

of the octamer is unusual (5 of 49 murine germline Jα segments). Jα9 is the only other murine Jα segment that contains this octamer and is also represented in our panel of B-(9–23)-reactive clones. In total, 9 of 13 of the B-(9–23)-reactive clones contain a Jα segment with the KLTFGKGT sequence (Fig. 2). In contrast, none of the insulin peptide A-(7–21) clones or tetanus toxin clones used any of these Jα segments with the KLTFGKGT motif ($P < 0.003$ B-(9–23) vs. non-B-(9–23)-reactive clones).

The combination of Vα13.3 and Jα45 or Jα34 is the dominant motif of B-(9–23)-reactive TCRs and was used by 60% (8 of 13) of the B-(9–23)-reactive clones. The α chain N regions (between the variable and joining segments) were diverse (sequences = E, A, SR, AN, ASG, ARG, KI, and RP). Most (six of eight) of these α chains were the same total length. TCRs with these similar α chains, however, contained diverse β chains (Vβ 1, 2, 4, 6, 12, and 14).

DISCUSSION

Previously reported α chain sequences of NOD TCRs were derived from islet-reactive T cell clones with unknown antigen specificity. Haskins and coworkers (17) described four anti-islet pathogenic T cell clones from spleen and lymph node cells of NOD mice which recognize unknown islet autoantigens (not insulin). One of these clones contained Vα13, but none used the Jα octamer KLTFGKGT (17). Kishimoto and coworkers sequenced the TCRs of five CD4$^+$, anti-islet clones (again of unknown antigen specificity) and reported one clone with a Vα13 segment and a different clone with a Jα octamer (19). Yoon and coworkers (20) sequenced a panel of CD8$^+$, islet-infiltrating clones and similar clones derived from NOD spleen cells. By analyzing the data presented in their tables, 5 of 26 of their islet reactive clones expressed the Jα octamer versus 0 of 31 of spleen-derived control clones ($P < 0.02$; ref. 20). None of their anti-islet clones expressed a Vα13 TCR segment.

Table 3. Amino acid polymorphisms in different Vα13 sequences from the NOD/bdc, BALB/c ByJ, and C57BL/6J mice

Vα	Strain	aa15	aa23	aa26	aa60	aa62
13.3	NOD	**G**	**S**	**D**	**T**	**G**
13.1	NOD, BALB/c, C57BL/6	**G**	T	N	**T**	**G**
13.2a	BALB/c	R	T	N	I	**G**
13.2b	C57BL/6	**G**	T	N	I	E
13.4	C57BL/6	**G**	**S**	**D**	I	E

Amino acid positions 23 and 26 are within the CDR1 region, whereas positions 60 and 62 are near CDR2. Homology to Vα13.3 is indicated by boldface type. The amino acid sequences are identical at the other positions not listed.

Insulin peptide B-(10–20) was recently reported to bind in a nonstandard manner to all class II molecules studied (both human and mouse). It bound outside of the peptide binding groove to the staphylococcal enterotoxin B superantigen binding site (21). This raises the possibility that B-(9–23) might bind to I-A^{g7} at a site outside the usual peptide binding groove. Further study of this interaction is warranted to assess if such nonstandard binding is related to our observed pattern of TCR α chain restriction and the predominance of B-(9–23)-autoreactive T cell clones.

All of the B-(9–23)-reactive T cell clones previously studied produced insulitis and rapidly accelerated diabetes (within 2–3 weeks) when injected into the peritoneal cavity of young (<14 days), nonirradiated NOD mice (6). We have tested two clones from each of the following categories of B-(9–23)-reactive T cells reported in this manuscript: islet-derived Vα13 clones (12-4.1 and 12-4.4), islet-derived non-Vα13 clones (12-1.19-Vα10 and 12-2.35-Vα1), and lymph node-derived Vα13 clones (8-1.1 and 8-1.3). All of these clones rapidly induced diabetes. Thus, the pathogenic activity of these clones is not exclusively associated with either the expression of Vα13 or their site of isolation. Rather, recognition of B-(9–23) peptide is the common factor of these diabetes-inducing T cells.

To date, insulin and the precursor proinsulin (22, 23) are the only islet β cell-specific autoantigens identified. CD4$^+$ T cell clones reacting with insulin B-(9–23) peptide are restricted by I-A^{g7} and use TCRs with restricted α chains. Vα13.3 combined with Jα45 or Jα34 is the dominant pattern in 8 of 13 analyzed clones. We hypothesize that immunodominant insulin B chain peptides, recognized predominantly by restricted TCR α chains, play a major role in the autoimmunity of the NOD mouse, and this relates to the efficacy of preventive insulin B chain peptide therapy (5, 24). The restricted use of TCR α chains in NOD mice should provide a specific target for disease prevention, similar to other experimental autoimmune disorders (25).

We thank Serge Candéias for assistance with the TCR sequencing protocol and for providing TCR primers. This research was supported by National Institutes of Health Grants DK32083, AI39213, and DK47298, and Training Grant DK07446; by Juvenile Diabetes Foundation Grant 195184; and by grants from the American Diabetes Association, the Blum-Kovler Foundation, and the Children's Diabetes Foundation.

1. Bach, J. (1995) *J. Autoimmun.* **8,** 439–463.
2. Bowman, M. A., Leiter, E. H. & Atkinson, M. A. (1994) *Immunol. Today* **15,** 115–120.
3. Wegmann, D. R., Gill, R. G., Norbury-Glaser, M., Schloot, N. & Daniel, D. (1994) *J Autoimmun.* **7,** 833–843.
4. Wegmann, D., Norbury-Glaser, M. & Daniel, D. (1994) *Eur. J. Immunol.* **24,** 1853–1857.
5. Daniel, D. & Wegmann, D. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 956–960.
6. Daniel, D., Gill, R. G., Schloot, N. & Wegmann, D. (1995) *Eur. J. Immunol.* **25,** 1056–1062.
7. Crawford, M., Daniel, D., Wegmann, D., Yang, H. & Gill, R. G. (1997) *Transplant. Proc.*, in press.
8. Chluba, J., Steeg, C., Becker, A., Wekerle, H. & Epplen, J. G. (1989) *Eur. J. Immunol.* **19,** 279–284.
9. Urban, J. L., Kumar, V., Kono, D. H., Gomez, C., Ando, D. G., Sercarz, E. E. & Hood, L. (1988) *Cell* **54,** 577–592.
10. Acha-Orbea, H., Mitchell, D. J., Timmermann, L., Wraith, D. C., Tausch, G. S., Waldor, M. K., Zamvil, S. S., McDevitt, H. O. & Steinman, L. (1988) *Cell* **54,** 263–273.
11. Clark, L., Heber-Katz, E. & Rostami, A. (1992) *Ann. Neurol.* **31,** 587–592.

12. Gregerson, D. S., Fling, S. P., Merryman, C. F., Zhang, X., Li, X. & Heber-Katz, E. (1991) *Clin. Immunol. Immunopathol.* **58,** 154–161.
13. Osman, G. E., Toda, M., Kanagawa, O. & Hood, L. E. (1993) *J. Exp. Med.* **177,** 387–395.
14. Shizuru, J. A., Taylor-Edwards, C., Livingstone, A. & Fathman, C. G. (1991) *J. Exp. Med.* **174,** 633–638.
15. McDuffie, M. (1991) *Diabetes* **40,** 1555–1559.
16. Lipes, M. A., Rosenzweig, A., Tan, K. N., Tanigawa, G., Ladd, D., Seidman, J. G. & Eisenbarth, G. S. (1993) *Science* **259,** 1165–1169.
17. Candéias, S., Katz, J., Benoist, C., Mathis, D. & Haskins, K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6167–6170.
18. Hattori, M., Buse, J. B., Jackson, R. A., Glimcher, L., Makino, S., Moriwaki, K., Kuzuya, H., Imura, H., Seidman, J. G. & Eisenbarth, G. S. (1986) *Science* **231,** 733–735.
19. Nakano, N., Kikutani, H., Nishimoto, H. & Kishimoto, T. (1991) *J. Exp. Med.* **173,** 1091–1097.
20. Santamaria, P., Utsugi, T., Park, B., Averill, N., Kawazu, S. & Yoon, J. (1995) *J. Immunol.* **154,** 2494–2503.
21. Tompkins, S. M., Moore, J. C. & Jensen, P. E. (1996) *J. Exp. Med.* **183,** 857–866.
22. Bohmer, K., Keilacker, H., Kuglin, B., Hubinger, A., Bertrams, J., Gries, F. A. & Kolb, H. (1991) *Diabetologia* **34,** 830–834.
23. Rudy, G., Stone, N., Harrison, L. C., Colman, P. G., McNair, P., Brusic, V., French, M. B., Honeyman, M. C., Tait, B. & Lew, A. M. (1996) *Mol. Med.* **1,** 625–633.
24. Muir, A., Peck, A., Clare-Salzler, M., Song, Y., Cornelius, J., Luchetta, R., Krischer, J. & Maclaren, N. (1995) *J. Clin. Invest.* **95,** 628–634.
25. Offner, H., Hashim, G., Chou, Y. K., Bourdette, D. & Vandenbark, A. A. (1993) in *Molecular Mechanisms of Immunological Self-Recognition* (Academic, New York), pp. 199–230.