Clonal prevalence of T cells infiltrating into the pancreas of prediabetic non-obese diabetic mice

Yoshinori Komagata¹,²,⁵, Kayo Masuko³, Fumi Tashiro¹,⁵, Tomohiro Kato³, Koichi Ikuta¹, Kusuki Nishioka³, Koji Ito², Jun-Ichi Miyazaki¹,⁵ and Kazuhiko Yamamoto⁴

¹Department of Disease-related Gene Regulation Research (Sandoz), and ²Department of Medicine and Physical Therapy, Faculty of Medicine, The University of Tokyo, Tokyo, Japan
³Division of Rheumatology and Molecular Immunology, Institute of Medical Science, St Marianna University, Kawasaki, Japan
⁴Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan
⁵Present address: Institute of Development, Aging and Cancer, Tohoku University, Seiryo-machi 4-1, Aoba-ku, Sendai 980-77, Japan

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Abstract

The non-obese diabetic (NOD) mouse spontaneously develops T-cell-mediated autoimmune insulitis. We analyzed the clonotypes of T cell infiltrates of the NOD mouse islets using a new method we have developed recently, which consists of RT-PCR amplification of the CDR3 region of the TCR β chain mRNA and subsequent single-strand conformation polymorphism (SSCP) analysis. NOD mice of 10–32 weeks of age were shown to accumulate oligoclonal T cells in the pancreas. To examine whether each T cell clone stays in a small area of the pancreas or spreads over the whole pancreas, a pancreas was divided into two pieces, which were then subsequently analyzed in a pair by the above PCR-SSCP method. When a pair produces common bands with the same mobility in SSCP gel, they are likely to represent the presence of the same T cell clones between these two parts of the pancreas. Aged mice (24–32 weeks old) with severe insulitis obviously produced more common bands for most of the Vβ subfamilies than younger mice (10 weeks old) with only perilsulinitis. DNA sequencing verified that these common bands have the same TCR junctional sequences, suggesting that they were derived from the same T cell clones. These results suggest that clonal prevalence of T cells infiltrating into the pancreas occurs in the late stage of insulitis development and that a limited number of T cell clones finally predominate over the whole pancreas.

Introduction

Human insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease targeted at insulin-secreting β cells in the pancreatic islets of Langerhans (1). Non-obese diabetic (NOD) mice are known as an excellent model for human IDDM (2), and histopathological studies on NOD mice have revealed sequential steps in the destruction of islets (3,4). Lymphocyte infiltration into islets, i.e. insulitis, starts from 4 weeks of age and gradually extends, leading to overt diabetes after 15 weeks of age. The incidence of insulitis reaches almost 100% in female NOD mice at 30 weeks of age and ~80% of female mice are diagnosed as IDDM at that point. Adoptive transfer experiments, using lymphocytes from diabetic NOD mice, have suggested a vital role of T lymphocytes in the disease development (5–7). The class II MHC molecule of the NOD mouse consists of a unique I-Aβ7 molecule, while the I-E molecule is not expressed because of a deletion in the I-Eα gene. Transgenic expression of I-Eα was shown to completely prevent insulitis and diabetes (8).
and transgenic expression of both I-A^*^ and I-A_9^*^ effectively suppressed the incidence of insulitis (9). These studies are consistent with the notion that T cells bearing a limited repertoire of TCR are involved in the development of autoimmune diabetes in NOD mice. Both CD4^+^ and CD8^+^ T cell subsets have been implicated in the development of overt diabetes (5,6).

A number of groups have analyzed the TCR V_a and V_p repertoire of T lymphocytes involved in the development of autoimmune diabetes by using various methods, i.e. establishment of autoreactive T cell clones (10-13), administration of mAb against specific V_p (14) and PCR analysis of TCR mRNA (15,16). However, preferential usage of specific V_a or V_p TCR has not been conclusive in NOD mice. Another related and important issue is the clonality of islet-infiltrating T cells. It has not yet been determined whether they are composed of a small or large number of clones, or whether each clone stays in a small area of the pancreas or spreads over the whole pancreas. Studies dealing with these problems have been hampered by methodological limitations.

We have recently reported a novel method that can distinguish each T cell clone bearing a different TCR in a T cell population (17-21), using RT-PCR amplification (22) of specific V_p-C_p regions of TCR mRNA followed by single-strand conformation polymorphism (SSCP) analysis (23,24). This method has allowed us to detect certain T cell clonotypes accumulated in a large heterogeneous population, based on the difference in nucleotide sequences in the CDR3 region. We have applied this method to the temporal and spatial analysis of the T cell clonotypes infiltrating into pancreatic islets of the NOD mouse. Our results suggest that oligoclonal T cells spread over the whole pancreas during the progression from mild to severe insulitis.

**Methods**

**Mice**

NOD female mice (NOD/Shi) purchased from Clea Japan (Tokyo, Japan) were used throughout this study. Mice were maintained under specific pathogen-free conditions in the animal facility at the University of Tokyo, Faculty of Medicine.

**Islet isolation**

The procedure used was adapted from the protocol of Lacy et al. (25). Briefly, collagenase solution (type II; Sigma, St Louis, MO; 5 mg/ml in HBSS) was injected into a common bile duct of an NOD mouse anesthetized with sodium pentobarbital, then the pancreas was carefully dissected free of adjacent pancreatic lymph nodes. The pancreas was divided into two pieces in some experiments. The pancreas was vigorously shaken in a 50 ml beaker for 20 min at 37°C and washed in HBSS. Islets were enriched by Ficoll gradient centrifugation and picked up by a Pasteur pipette, leaving connective and exocrine tissues. Between 30 and 40 islets were isolated from each pancreas of 20-week-old NOD mice.

**RNA isolation, cDNA synthesis and PCR-SSCP analysis**

Total RNA was extracted from a spleen, a whole pancreas or a part of a pancreas by the AGPC method described previ-
clusively (26) and poly(A)^+ RNA was isolated from total RNA with oligo-dT-coupled magnetic beads (Serotape, Serotec, Germany). Poly(A)^+ RNA of islets was directly extracted by binding to oligo-dT-cellulose (Microprep kit; Pharmacia, Uppsala, Sweden). Poly(A)^+ RNA (~1 μg) was converted to cDNA with reverse transcriptase (Superscript; Gibco/BRL, Gaithersburg, MD) and random hexamer oligonucleotides (7.5 pmol/μl; Amersham, Amersham, UK) at 42°C for 2 h in 20 μl. The procedure of PCR-SSCP analysis applied to TCR study was described previously (17-21,27). In brief, cDNA was amplified by PCR with a common C_p primer, one of the V_p subfamily-specific primers, dNTP and Taq DNA polymerase (AmpliTaq; Perkin-Elmer, Norwalk, CT) for 36 cycles (94°C for 1 min, 58°C for 2 min and 72°C for 2 min) in a Thermal Cycler (Perkin-Eimer). The sequences of the V_p and C_p primers were as follows: V_p1, 5’-TTCGAAAATGAGACGTGGCCCC-3’; V_p2, 5’-AGAGGTCAAATCTCCTCCCGC-3’; V_p3, 5’-CTTCAACAAATGACAGTAC-3’; V_p4, 5’-TGGAACATGACGTGGCCTCA-3’; V_p5, 5’-GAGATAAAGGAAACTGCCC-3’; V_p6, 5’-G-GAAGACAAAGGATCTATTCAACC-3’; V_p7, 5’-CGACAGACCTACGGAAAGAG-3’; V_p8, 5’-CATATGGTGCTGGCAGACAT-3’; V_p9, 5’-CATATGGTGCTGGCAGACAT-3’; V_p10, 5’-AAAGGAAATGACGTGGCCCC-3’; V_p11, 5’-GGATCCTGGATTCTTTC-3’; V_p12, 5’-AAGATGATGGGAGTCTCAAG-3’; V_p13, 5’-CTTAACTGGTGGCTCGG-3’; V_p14, 5’-CTTCAACAAATGACAGTAC-3’; V_p15, 5’-CGGTCCTAAAAGGCATTTGAA-3’; V_p16, 5’-ACAGCAACATGACGGTCTTCTC-3’; V_p17, 5’-ACAGAATTGTCAGTGCAAGAAG-3’; V_p18, 5’-GGTCCAGGAACAGAGCTTGA-3’; V_p19, 5’-GAAACCGGAGAAGAATCACTCA-3’; C_p 5’-GGCTCAACAAAGGACATTTGAA-3’). The nomenclature of V_p subfamily, while the ICR mouse pancreas gave no distinct bands for any of the V_p subfamilies examined, although both samples were similarly treated in this analysis. Each band was considered to be derived from a T cell clone bearing a unique TCR (20). This result suggested that lymphocytes infiltrating into NOD pancreas consist of a limited number of T cell clones.

Clonal prevalence of NOD T cells in the pancreas

To examine whether each T cell clone stays in a small area of the pancreas or spreads over the whole pancreas, we compared the TCR clonotypes between two different parts of a pancreas from young (10 weeks old) and aged (24-32 weeks old) NOD female mice. A brief scheme of the experimental design is shown in Fig. 1. A pancreas was divided into three pieces and the central small part was subjected to histological examination. RNAs were extracted separately from two other parts and analyzed by the PCR-SSCP method. PCR products from these two parts were electrophoresed in one of the lanes of the SSCP gel.

A typical pattern of PCR-SSCP analysis of a young and aged NOD mouse is shown in Fig. 1. The pancreas from the young NOD mouse (10 weeks of age) showed only perinsulitis (insulitis score: 0.97), while that from the aged mouse (24 weeks of age) showed severe insulitis (insulitis score: 2.67). The criteria for insulitis score are described in the legend to Fig. 3. If two separate parts of a pancreas give common bands with the same mobility in SSCP gel, they are likely to represent the prevalence of the same T cell clones over the whole pancreas. Each pair of lanes was examined and determined for such coincidence as described in the legend to Fig. 3. In this experiment, the coincidence was observed in 36.8 and 72.2% of the V_p subfamilies examined in the young and aged NOD mice respectively (Fig. 3). Thus, the aged mouse with severe insulitis obviously showed more common bands between two separate parts of its pancreas for most of the V_p subfamilies than the young mice. We further examined the coincidence in other NOD mice using a V_p8.1-C_p primer set. Figure 4(A) shows a part of these PCR-SSCP analyses. The coincidence was observed only in half of the young NOD mice (10 weeks old; n = 8), but in all of the aged NOD mice (24-32 weeks old; n = 9). It should be noted that all of the major bands have a corresponding band in the other lane in the aged NOD mice. These results strongly suggest that the frequency of common TCR transcripts between two separate parts of a pancreas is...
Clonal prevalence of NOD T cells in the pancreas

A 10-week old

Insulitis score 0.97

Score + + - + - - + + N + - - N + + - - - - -

8/19 (36.8%)

B 24-week old

Insulitis score 2.67

Score - - - + + + N + + + + - + + N + + + -

13/18 (72.2%)
higher in aged NOD mice than in young NOD mice. Therefore, it is suggested that a limited set of T cell clones infiltrating into the pancreas spreads all over the pancreas during the progression from mild to severe insulitis.

To verify that these common bands represent the same TCR \( \beta \) chain sequences, \( V_{\beta} 8.1-C_{\beta} \) PCR products corresponding to bands 1 and 2 in the left and right lanes were extracted from the membrane (Fig. 4A), and were cloned into a plasmid. DNA sequences of 10 or 11 independent clones were determined for each band. The VDJ junctional sequence which most frequently appeared in each band (45–80%) was identical in each pair and is shown in Fig. 4(B). The rest of the DNA sequences differed from each other. This result suggests that most of the bands with the same mobility which appeared in a paired analysis (Figs 3 and 4A) were derived from the same T cell clones.

Fig. 4. T cell clonality analysis of two separate parts of a pancreas. (A) PCR-SSCP analysis of two parts of a pancreas with \( V_{\beta} 8.1 \) and \( C_{\beta} \) primers. The PCR-SSCP analysis depicted in Fig. 2 was applied with \( V_{\beta} 8.1 \) and \( C_{\beta} \) primers to the pancreas from young NOD mice (10 weeks old) with mild insulitis and from aged NOD mice (24–32 weeks old) with severe insulitis. Common bands between two separate parts are indicated by arrowheads. (B) VDJ junctional sequences of common bands. \( V_{\beta} 8.1-C_{\beta} \) PCR products corresponding to bands 1 and 2 in the left and right lanes were extracted from the membrane, and were cloned into a plasmid. VDJ junctional sequences of 10 or 11 independent plasmid clones were determined for each band. Only the sequence which most frequently appeared was identical in each band pair and is shown. The rest of the DNA sequences differed from each other. The frequency of the indicated DNA sequence in each band is shown on the right. N/P, N or P nucleotides.
To confirm that the PCR-SSCP analysis using the whole pancreas represents the T cells infiltrating into islets, we isolated islets from two separate parts of the pancreas of a 20-week-old NOD female mouse. RNA extracted from the islets was subjected to PCR-SSCP analysis with several Vp primer sets (Fig. 5). For these Vp subfamilies, most of the bands were commonly observed between two pancreatic parts in accordance with the results using the whole tissue. Therefore, the data obtained by the whole pancreas are considered to represent the clonality of T cells infiltrating into islets.

Discussion

The PCR-SSCP analysis used in this study has been shown to be so sensitive that a corresponding band can be detected when a T cell clone exists at a frequency of <1 in 1000 (17). Therefore, the presence of a small number of T cells of heterogeneous population, e.g. T cells from circulating blood, does not disturb the detection of significant bands under our experimental conditions. This method has been effectively used to compare accumulating T cell clones among different parts of a single animal (21). Our results show that oligoclonal T cells accumulate in the islets of the NOD mouse (Figs 1 and 3). The usage of the TCR Vp repertoire did not appear to be restricted. This situation differs from other autoimmune diseases such as experimental allergic encephalomyelitis (38,39). To analyze the prevalence of T cell clones in the pancreas, a pancreas from an aged NOD mouse was divided into two pieces and analyzed separately by PCR-SSCP (Fig. 2). The result suggests that a limited number of T cell clones predominates over the whole pancreas in the late stage of insulitis development (Figs 3-5). In this analysis, preferential use of specific Vp segments was not observed in any NOD mice. It should also be emphasized that we did not detect preferential use of any particular CDR3 sequences among NOD mice so far analyzed. Although a number of studies have been reported on the Vp usage of islet-infiltrating T cells of NOD mice, few of them have dealt with T cells at the clone level in vivo. Recently, Sarukhan et al. (40) reported the analysis of the CDR3 region of T cells infiltrating into islets by cloning and subsequent sequencing. Their study suggested that T cells in the pancreas of young NOD mice were oligoclonal and that there were few common T cell clones among different islets. Our results are consistent with their data and have further provided evidence for the clonal prevalence of T cells in the pancreas in the later stage of insulitis development.

Our present study suggests that insulitis development is divided into two stages from a viewpoint of clonal change of T cell infiltrates. In the early stage, probably starting from ~4 weeks of age, islets are spontaneously seeded with a small number of T cells presumably reactive with some islets autoantigen(s) and these T cells propagate only within an islet or in a small area of the pancreas. In this stage, the diversity of the infiltrating T cells may further be expanded by the appearance of T cell clones reactive with a broader spectrum of β cell autoantigens, which are exposed by the initial invasion of T cells. In the late stage, a limited set of T cell clones progressively spreads all over the pancreas and many of them become commonly seen over the whole pancreas. Interestingly, the overall diversity of the T cell clones appears to rather decrease in this stage (Fig. 4A). Although it is not known whether they are pathogenic for overt diabetes, the fact that these common clones appear prior to overt diabetes may suggest that they are directly involved in the destruction of β cells.

Finally, the PCR-SSCP method used in this study provides a powerful tool with which to study temporal and spatial changes of T cell clonality during various immune responses. The NOD mouse is known to suffer from lymphocyte infiltration not only into islets but also into other organs, such as salivary gland, thyroid and adrenal cortex. The PCR-SSCP method can be applied to determine whether the same T cell clones infiltrate into the pancreas and also into these other organs.

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Abbreviations

IDDM insulin-dependent diabetes mellitus
NOD non-obese diabetic
SSCP single-strand conformation polymorphism

References

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