Selective Reduction of T Cells Bearing Invariant Vα24JαQ Antigen Receptor in Patients with Systemic Sclerosis

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Summary

A novel subset of T cells characterized by the expression of an invariant T cell antigen receptor (TCR) encoded by Vα24JαQ gene segments was investigated in patients with systemic sclerosis (SSc). Polymerase chain reaction analysis demonstrated that the Vα24 TCR repertoire was selectively used in CD4−CD8− double-negative T cells both in patients and in healthy individuals, while almost all families of TCR Vα were expressed in single-positive T cell fractions. The Vα24+ double-negative T cells were increased by approximately fivefold in patients. However, sequence analysis clearly showed significant differences in the Vα24 TCR repertoire dominating in patients and healthy donors. In healthy individuals, the invariant Vα24JαQ was expanded and comprised 20–50% of the total TCR-α, while their selective reduction was observed in SSc patients who also showed expansion of invariant Vα24 TCR other than Vα24JαQ. Analogous to murine invariant Vα14Jα281 TCR, these results suggest that T cells with invariant Vα24JαQ TCR would function as regulatory T cells, whereas T cells bearing other invariant Vα24 TCR in SSc patients could be autoaggressive T cells in nature.

Materials and Methods

Study Subjects. Four patients diagnosed with SSc (11) were evaluated during the swelling phase of SSc. Three disease-free subjects were also examined as controls. All patients and healthy...
subjects were of Japanese ancestry and were recruited from the Chiba University Hospital.

Flow Cytometry. PBL (1 × 10^7) from 20 ml of peripheral blood were isolated by Ficoll-Paque separation (Pharmacia Biotech Inc., Piscataway, NJ) and incubated with PE-coupled anti-CD4 (Leu-3a) plus anti-CD8 (Leu-2a) mAbs (Becton Dickinson & Co., Mountain View, CA) and FITC-conjugated mAb to α/β TCR. The cells were analyzed by FACScan® with a logarithmic amplifier (Becton Dickinson & Co.).

Purification of DN α/β T Cells from PBL of SSc Patients and Healthy Subjects. DN and SP α/β T cells were sorted by FACStar® (Becton Dickinson & Co.) using PE-anti-CD4 plus anti-CD8 mAbs. The yields of DN and SP T cells were 10^5 and 10^6, respectively. The purity of fractionated DN samples was confirmed by PCR with CD4 or CD8 primers (see Fig. 1 C).

Preparation of RNA and PCR. Total RNA (0.1–10 μg) was prepared using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) from sorted DN α/β T cells. cDNA synthesis and PCR were described elsewhere (12). Briefly, first-strand cDNAs were synthesized with oligo(dT) primer using 0.1–1 μg of total RNA. PCR was performed with 21 different Vα and Cα primers at 95°C for 1.5 min for denaturation, 62°C for 1.0 min for annealing, and 72°C for 1.0 min for extension, for 30 cycles on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The PCR products were hybridized with a 32P-labeled Cα probe of 155 bp in length (13). The sequences of the Vα and Cα primers were published previously (6, 14).

For confirmation of the purity of enriched samples, the cDNA from sorted DN α/β T cells was amplified by PCR with CD4 and CD8α primers (15), and hybridized with the 32P-labeled EcoRI fragment of the human CD4 gene (16) or the PstI/HincII fragment of the human CD8α gene (17). The CD4 or CD8 cDNA (1 ng) was used as a positive control.

Quantitation of Vα24+ DN T Cells from SSc Patients and Healthy Subjects. The relative amounts of Vα24+ DN T cells in PBL were measured by quantitative PCR. RNA was prepared from the sorted DN population derived from 10^7 of PBL T cells from three SSc patients and four healthy subjects. cDNAs (10^-6 diluted) were used for PCR with primers for Vα24 and Cα. For the standardization curve, Vα24+ cDNAs were serially diluted (corresponding to 0.01–10 pg DNA) and subjected to PCR with Vα24 and Cα primers. PCR products were hybridized with a 32P-labeled Cα probe, and the intensities of the bands were quantitated by an automated densitometer (Fujix BAS2000; Fujifilm I & I Co., Ltd., Tokyo).

Cloning and Sequencing of cDNAs Encoding TCR Vα Genes. Vα24+ cDNAs from DN α/β T cells were amplified by primers with an EcoRI restriction site for Vα24 (5'-CGAATTCCTCAGCGATTCGCCCTCCTCCTAC-3') or Cα (5'-CGAATTCGTTGAATAGGCAGACGACTTT-3'). DNA fragments with the expected size after digestion of PCR products with EcoRI were ligated to M13mp19 plasmids and sequenced by the dye primer method.

Plaque Hybridization. The Vα24+ TCR cDNA libraries were generated by PCR using RNA from the DN population with primers for the Vα24 and Cα. Recombinant plaques were transferred from DYT plates to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and were hybridized either with a Vα24 probe (5'-CTCCAGCCATTCCAGCCCTCCTAC-3') or a 53-bp JoQ probe (5'-CAACCTGGGGAGCTATAC-3') or a 3'-JoQ and 5'-AGGCCAGACAGCTAACCTAG-3' for 3'-JoQ).

Statistical Analysis. The statistical significance of the results was determined using the X2-test.

Results and Discussion

Predominant Expansion of TCR Vα24+ T Cells. FACS® analysis of PBL from three patients with swelling-phase SSc clearly showed that their DN α/β T but not DN γ/δ T cell populations were increased in actual cell number (101/ mm^3, 120/mm^3, and 109/mm^3) compared with healthy individuals (average 28/mm^3) (Fig. 1 A and Table 1). The in-
Table 1. Frequencies of Invariant Vα24JαQ TCR Expression in Peripheral DN T

| Source       | Vα24JαQ/ total Vα24 | Cell number mm<sup>3</sup> | Vα24JαQ T | DN α/β T |
|--------------|---------------------|----------------------------|-----------|----------|
| SScDN-1      | 1/284<sup>4</sup>   | 101                        | 0.4       | 101      |
| SScDN-2      | 0/431<sup>4</sup>   | 120                        | 0         | 120      |
| SScDN-3      | 0/165<sup>4</sup>   | 109                        | 0         | 109      |
| Control-1    | 189/379 (49.9%)     | 25                         | 12.5      | 12.5     |
| Control-2    | 31/151 (20.5%)      | 35                         | 7.0       | 7.0      |
| Control-3    | 112/420 (26.7%)     | 26                         | 6.9       | 6.9      |

*Complementary DNA libraries generated by PCR with primers specific for Vα24 and Co were blotted on two separate filters and independently hybridized with the Vα24-specific oligonucleotide probe and the JαQ probe, respectively. The ratio of invariant Vα24JαQ/total Vα24 was calculated by the number of positive plaques.

<sup>1</sup>Actual cell number of DN T cells bearing invariant Vα24JαQ TCR was calculated on the basis of number of DN α/β T cells, since almost all DN α/β T cells were Vα24<sup>+</sup>.

<sup>4</sup>P < 0.001.

crease of DN α/β T cells in SSc patients was calculated to be 3.6- to 4.3-fold compared with those in healthy individuals. Thus, we isolated SP and DN populations from PBL by FACS® (Fig. 1 B). The purity of the fractionated DN samples was confirmed by reverse transcription-PCR with CD4 or CD8 primers (Fig. 1 C), and the TCRVα repertoire was then analyzed. As shown in Fig. 2, almost all families of TCRVα expression were observed in SP T cell fractions. Although individual Vα gene expression varied in each sample, no significant difference was observed between SSc patients and healthy individuals.

On the other hand, very restricted TCRVα expression was noted in the DN T cell population. The only Vα repertoire detected was Vα24, and in some cases Vα24 and Vα23. Other TCRVα expression was below the level of detection by DNA blot analysis even with longer exposures (Fig. 2). The results indicate that the Vα24 TCR repertoire dominates in peripheral DN T cells of both patients and healthy individuals. The dominant expression of Vα24 TCR was further examined by quantitative PCR. Vα24 expression in patients showed a four- to fivefold increase compared with healthy donors (Fig. 3). Together with the FACS® data, the results indicate that the number of DN T cells, particularly Vα24-bearing T cells, is higher in patients, although the TCR-α repertoire in the DN population is restricted to Vα24 in both SSc patients and healthy individuals.

Oligoclonal Expansion of Vα24 TCR in SSc Patients. Porcelli et al. (6) and Dellabona et al. (7, 8) have shown that the Vα24 TCR preferentially used in DN α/β T cells in healthy donors is an invariant TCR encoded by Vα24 and JαQ gene segments. Therefore, we attempted to compare Vα24 TCR sequences in DN T cells between patients and healthy donors. The results are illustrated in Fig. 4. In healthy individuals, invariant Vα24JαQ TCR was dominant at a high frequency (6/11, 6/7, and 5/11). However, among 13 in-frame Vα24<sup>+</sup> cDNA clones in the patient SSc-1, four different Jα genes, IGRJα11, JαG, JαL, and JαAP511, were detected at a frequency of 5/13, 4/13, 3/13, and 1/13, respectively. Although several distinct Vα24 TCR were expressed, we noted clonal expansion of invariant Vα24 TCR other than Vα24JαQ. Interestingly, the most dominant invariant Vα24JαQ detected in healthy donors was not detected in patients. Similarly, in the patient SSc-2, 7 of 12 clones represented the Vα24JαV TCR, three clones used the JαU gene, and two used the JαT gene. Again, we detected dominant expansion of invariant Vα24JαV TCR different from Vα24JαQ. In the patient SSc-3, seven different Jα genes (JαAA17, JαT, IGRJα11, IGRJα10, JαAF211, JαU, and JαAP511) were used at frequencies of 1/15 to 5/15. Among them, invariant Vα24 JαAA17 dominated, while no Vα24JαQ sequences were detected in the patient. Taken collectively, the Vα24 rep-
Figure 3. Quantitative PCR analysis of \( \nu24^+ TCR \). The number of DN T cells isolated was normalized by counting cell numbers. Total RNA was extracted and used for PCR to measure the frequency of \( \nu24^+ \) TCR. PCR products were hybridized with \( \beta\)-P-labeled Ca probe, and the intensities of autoradiographic bands were quantitated by a densitometer. The radioactivity of varying concentrations (corresponding to \( 0.01-10 \) pg DNA) of the standard \( \nu24^\text{cDNA} \) amplified by PCR was compared with that of PCR products from patients and healthy donors: 1, SSc-1; 2, SSc-2; 3, SSc-3; and 4, SSc-4 for SSc patients, and C1, C2, and C3 for healthy donors. The relative radioactivities of samples from four SSc patients are 67.9, 79.5, 89.5, and 93.1, respectively, all of which are significantly higher than the mean value of the control group (19.2 ± 1.2; \( P < 0.0005 \)).

Figure 4. Junctional sequences of \( \nu24^+ TCR \). Obtained from DN \( \alpha/\beta \) T cells in PBL was basically heterogeneous, but with apparent oligoclonal expansion of invariant \( \nu24^+ \) TCR in both patients and healthy individuals. However, in healthy donors, invariant \( \nu24^\alpha\) TCR always dominated, whereas, in SSc patients, invariant \( \nu24^\alpha\) TCR disappeared and oligoclonal expansion of other invariant \( \nu24^+ \) TCR was observed.

$$\text{Selective Loss of T Cells Bearing Invariant \( \nu24^\alpha\) TCR.}$$

As shown in Fig. 4, the invariant \( \nu24^\alpha\) TCR was found in healthy subjects at a high frequency (45–86% of total \( \nu24^+ \) TCR), while it was not detected in SSc patients. To confirm the above findings, cDNA libraries generated by PCR were hybridized with the \( \nu24 \) probe and the \( \alpha \) probe. Frequencies of invariant \( \nu24^\alpha\) TCR among total \( \nu24^+ \) TCR sequences were estimated by the number of positive plaques and expressed as the ratio of invariant \( \nu24^\alpha\) to total \( \nu24^\) TCR. As shown in Table 1, the invariant \( \nu24^\alpha\) TCR was hardly detected in all SSc patients (0.4%, 0%, 0%), while it was expressed at a high frequency of 21–50% in healthy donors. Based on the calculation of actual cell number of DN \( \alpha/\beta \) T cells, the number of T cells bearing invariant \( \nu24^\alpha\) was in the
range of 0–0.4/mm³ in SSc patients and 6.9–12.5/mm³ in the healthy donors (see Table 1). This indicates that the decrease in the expression of invariant Vα24]+Q TCR is due to the selective loss of T cells bearing invariant Vα24]otQ, and not to the relative increase in the number of Vα24]+ T cells in SSc patients.

The predominant expression of invariant Vα14]otQ TCR on the DN T cell population in the periphery has been reported in mice (18, 19). The most characteristic feature of invariant Vα14 TCR with regard to autoimmune diseases is that there is a striking inverse correlation between autoimmune disease development and the expression of invariant Vα14 TCR. In fact, invariant Vα14 expression declines selectively with time after birth and disappears when mice develop autoimmune diseases in NZB × NZW(1), lpr, or gld mice (9). Moreover, the treatment of young lpr mice with anti-Vα14 antibody in vivo induces splenomegaly at least threefold greater than that in untreated lpr mice at the same age, indicating the augmentation and acceleration of lymphoproliferative disorders. These results suggest that invariant Vα14 T cells function as regulatory T cells that control autoimmune disease development. Based on the above analogy to murine invariant Vα14 TCR, it is likely that human invariant Vα24]+Q TCR should play a decisive role in regulating the development of autoimmune disease.

Contrary to the invariant Vα24]+Q TCR, DN α/β T cells in SSc patients showed oligoclonal expansion of invariant Vα24 TCR other than Vα24]otQ, including Vα24 IGR]ot11, Vα24]otG, Vα24]otV, and Vα24]otAA17 (Fig. 4). Our previous studies have shown that the TCR Vβ repertoire in DN T cells is limited to one or two Vβ genes in an individual patient, such as Vβ5, Vβ7, or Vβ11 (15). Because invariant Vα24]otQ TCR has been shown to be associated with skewed Vβ, such as Vβ2, Vβ8, Vβ11, and Vβ13 (6–8, 10), the restricted Vβ usage that predominated in patients was different from that seen in healthy donors. In addition, these invariant Vα24 TCR sequences were not detected in healthy individuals. Thus, they might be unique to SSc patients, suggesting that the expanded oligoclonal invariant Vα24 TCR in SSc patients could be autoimmune T cells in autoimmune status. Sequence differences in the oligoclonal Vα24 TCR that dominate in SSc patients might reflect differences in the polymorphism of restriction elements or in epitope specificities. The establishment of T cells bearing invariant Vα24 TCR other than Vα24]otQ from SSc patients will provide a clue to the mechanisms of autoimmune diseases.

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