T cells accumulating in the inflamed joints of a spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression

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Abstract

Although a number of studies have revealed that T cells expand clonally in the joints of patients suffering from rheumatoid arthritis (RA), the kinetics of T cell clonality in multiple joints of an individual throughout progression of the disease is not known. By employing a TCR β chain gene-specific RT-PCR and subsequent single-strand conformation polymorphism, which enables us to monitor T cell clonality, we analyzed transgenic mice (Tg) carrying the human T cell leukemia virus type I env-pX region. These mice spontaneously develop destructive progressive arthritis similar to RA as they age. In the early stage, the majority of accumulating T cell clones differed in each of four affected feet analyzed. However, in the advanced stage, many of the clones were common to all four feet. The total number of distinct clones gradually decreased as the disease progressed. When splenocytes from arthritic elder Tg were adoptively transferred into either nude mice or young Tg, the clones common to all four feet of the donor were detected again in four feet of the recipients. These findings suggest that, as arthritis progresses, the T cell clones accumulating in the arthritic joints are gradually restricted to certain common clonotypes, some of which are arthrotropic.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent inflammation of synovium, destruction of cartilage and bone, and systemic illness. Based on the association with particular HLA alleles (1–3), massive infiltration of T cells into the synovial tissues (4) and T cell oligoclonal expansion in the joints (5), T cells have been proposed to play an important role in RA pathogenesis: T cells would initiate or be involved in the inflammatory process by recognizing some antigens and producing cytokines (5,6), although this viewpoint has been controversial (7).

We and others have demonstrated that T cells expand clonally in the synovium as well as the synovial fluid of RA patients (5,8–14). Furthermore, some clones were found to be common in multiple joints of the same patient (14). These findings suggest that the oligoclonally accumulated common T cell clones are neither transiently nor randomly recruited into the inflammatory sites, but that they recognize some antigens which are associated with the pathogenesis of RA inflammation. Supporting this viewpoint, Mima et al. reported that T cell clones with an identical Vβ CDR3 sequence frequently detected in the joints of some RA patients induced synovial hyperplasia in SCID mice when these clones were transferred (15).

In autoimmune disease murine models, it has been demonstrated that determinant spreading may be necessary for...
development of the full autoimmune syndrome (16–20). This epitope spreading occurs in experimental autoimmune encephalomyelitis, which is initiated by immunization of autoantigen peptides, as well as in NOD mice, a spontaneous autoimmune diabetes model. Both models are known to be mediated by T cells. This amplification of the autoimmune response by recruitment of T cells with additional specificities would lead to clonality diversification. Recently, it was reported that a shift towards a high-avidity pathogenic T cell population may be the key event in the progression of benign insulitis to overt diabetes in NOD mice (21). Alternatively, the clonality of the lesion might be restricted toward T cells which are able to accumulate preferentially and to surpass other clones, because of their advantage to expand clonally responding to the antigen released from the destroyed inflamed joint. Whether the clonality in the lesion continues to increase the diversity due to determinant spreading or decrease the variety is especially important in relation to T cell clonotype-targeted therapy. If the clonality becomes restricted, then regulation of a smaller number of clones should be sufficient to achieve antigen-specific immunotherapy. Thus, studies of the dynamics of clonality are of great significance.

So far, studies of T cell clonality have been performed using samples from patients with rather advanced disease. Although information on the dynamics of the clonality during the disease course from the early to the late stage could provide more profound insights into the pathogenic clones, kinetics studies of expanded clones in multiple joints at several time points throughout the disease course are not realistic, because of the difficulty of sampling during the long and heterogeneous clinical time course of RA. It is also difficult to appreciate and to compare the significance of expanded clones among patients, since RA patients are diverse and heterogeneous in terms of the disease severity, duration and modification by treatment.

Transgenic mice (Tg) carrying the env-pX region of the human T cell leukemia virus type I (HTLV-I) genome spontaneously develop chronic inflammatory arthritis as they age (22). The histopathologic characteristics of the joints of arthritic Tg closely resemble those found in patients with RA, showing proliferation of synovial lining cells, infiltration of inflammatory cells with lymph follicle-like structures, and formation of pannus-like granulation tissue with destruction of cartilage and subchondral bone (23). Genes for inflammatory cytokines, including IL-1α, IL-1β, IL-6, tumor necrosis factor-α, transforming growth factor-β1, IFN-γ and IL-2, as well as MHC genes are activated in joints. Moreover, these mice develop autoantibodies against IgG, type II collagen and heat-shock proteins (24). A T cell response to type II collagen (25) and loss of T cell tolerance in the periphery are also demonstrated (26). These pathological features are similar to those of RA. Therefore, the Tg are suitable for investigating T cell clonality during the disease course and its contribution to arthritis.

In the present study, using this spontaneous RA model HTLV-I env-pX Tg, we investigated the dynamics of the T cell clonotype in the affected four feet during the disease progression. We found clonally expanded T cells, some of which were common to all four inflamed feet. Surprisingly, as the disease progressed, the number of common clonotypes among the different feet increased, whereas the total number of clonotypes decreased. Adoptive transfer studies revealed that these common clonotypes of the donor feet migrated into the feet of the recipients, whose arthritis was exacerbated by the transfer. These results provide evidence that T cells in the inflamed joints are rather restricted to certain common clonotypes.

**Methods**

**Mice**

HTLV-I env-pX Tg were backcrossed to BALB/c mice for more than eight generations. The mice were maintained under specific pathogen-free conditions in the animal facility of the University of Tokyo, Graduate School of Medicine. All experiments using animals were performed according to the guidelines for animal experiments in our institution.

**Clinical evaluation**

The severity of arthritis was assessed using a clinical scoring system of 0–3: 0 = normal, 1 = redness and swelling, 2 = deformity, and 3 = ankylosis (27). Each foot was scored and the total score was determined; the maximum possible score per mouse was 12. Tg were divided into three groups by the arthritis stage: early stage (age: 5–10 weeks; score: 0–3), mid stage (age: 4–5 months; score: 6–8) and late stage (age: 7–8 months; score: 10–12).

**Analysis of T cell clonality by RT-PCR/single-strand conformation polymorphism (SSCP)**

Joint tissues were obtained from the front and back feet of Tg after removal of skin and muscle. Total RNA was prepared with Isogen (Nippon Gene, Tokyo, Japan) and then converted to cDNA with reverse transcriptase (Superscript II; Gibco/BRL, Gaithersburg, MD) and random hexamer oligonucleotide (Gibco/BRL) at 42°C for 2–3 h. PCR was performed with 100–200 ng of cDNA, 50 pmol of each of the 22 Vb primers and 50 pmol of a common Cb primer, dNTPs, and 1 U of Taq polymerase (Takara, Otsu, Japan) for 35 cycles (94°C for 1 min, 54°C for 2 min and 72°C for 3 min) in a Thermal Cycler 9600 (Perkin-Elmer, Norwalk, CT). The sequences of the Cb primers were obtained from published data (28). The sequence of Cb primer was 5′-GGCTCAAAAAAAGGAG-ACCTTG-3′. SSCP analysis was performed as follows. Amplified DNA was diluted (1:2–1:20) with a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), heat denatured at 90°C for 2 min and electrophoresed on non-denaturing 4% polyacrylamide gels containing 10% glycerol. After electrophoresis, the DNA was transferred onto GeneScreen (NEB, Beverly, MA) and then hybridized with a biotinylated internal common Cb oligonucleotide probe (5′-AGGATCTGAGAAATGTGA-3′). The bound Cb probe was detected using a Phototope-Star detection kit (NEB).

The number of T cell clonotypes was calculated by three researchers independently who did not know the sample sources.
Standardization of TCR mRNA

To adjust the amount of TCR mRNA in spleen to that of joint for SSCP analysis, we conducted real-time quantitative PCR on the iCycler iQ real-time detection system (Bio-Rad, Hercules, CA). PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For the amplification of the Cβ region, 5’-GCTCCCTGTGACCCAT-3’ was used as the 5’ primer and 5’-ACTGATGTTCTGTGACAG-3’ as the 3’ primer. The housekeeping gene β-actin (5’-TCACCCACACTGTGCCCATCT-3’, 5’-ACGATTTCCCTCTCACTGTGG-3’) was chosen for internal normalization.

Sequence analysis

TCR Vβ genes extracted from SSCP polyacrylamide gels were re-amplified using the same TCR Vβ and Cβ primer as primary PCR. The re-amplified TCR Vβ gene was subcloned to a plasmid vector (pGEM-T Easy Vector; Promega, Madison, WI). Nucleotide sequences of the TCR Vβ genes were determined by the dideoxy method using a 310 Genetic Analyzer (Perkin-Elmer/Applied Biosystems, Foster, CA).

Adoptive transfer experiment

Spleens were removed aseptically from arthritic Tg in the late stage and control mice. As a control, we used non-arthritic Tg aged 4–6 weeks which had not developed the arthritis. Single-cell suspensions were prepared by teasing apart the spleens in HBSS and pushing them through a metal sieve with a syringe barrel. After washing with HBSS, 2–5×10⁷ splenic cells were injected into either young Tg (6–7 weeks) with the onset of overt arthritis (the total score was <1–2) or BALB/c nu/nu mice. After 3 weeks, these recipients were sacrificed and then subjected to RT-PCR/SSCP analysis to examine the T cell clonotypes accumulated in the joints.

Statistical analysis

Data was analyzed using statistical software (Statcel, Saitama, Japan) and expressed as the mean ± SEM. The rate of common T cell clonotypes in all four feet and the arthritis score were compared by Student’s t-test, and the total number of T cell clonotypes by Mann-Whitney’s U-test.

Results

Accumulation of T cell clonotypes in arthritic joints of HTLV-I env-pX Tg

Arthritis in the Tg was discerned as swelling and redness of the footpad, including the ankle. The abnormality began to occur at age 4–8 weeks and thereafter the grade of arthritis increased as the animal aged. The clinical score finally reached the maximum at ~6 months (Fig. 1). Thus, since the disease severity was closely related to the age, we divided Tg into three groups according to their age, i.e. early stage (age: 5–10 weeks; score: 1–3), mid stage (age: 4–5 months; score: 6–8) and late stage (age: 7–8 months; score: 10–12).

We analyzed the T cell clonotypes infiltrating the joints of all four feet from HTLV-I env-pX transgenic mice by the RT-PCR/SSCP method (Fig. 2). While the spleen exhibited smear patterns indicating a diverse T cell population consisting of heterogeneous CDR3 regions of TCR, accumulation of several distinct bands was observed in the joints. These results indicate that T cells expand oligoclonally in the joints of all four feet, as has been shown for the joints of RA patients.
The number of common T cell clonotypes increased, whereas the total number of T cell clonotypes decreased as the disease advanced.

To investigate the change in T cell clonality during the progression of arthritis, we compared T cell clonotypes in the early, mid and late stages. The T cell clonotypes which accumulated in each joint of the four feet showed different mobility in the early stage, but they migrated to an identical position in the late stage (Fig. 3). Bands with the same mobility on SSCP gels had been demonstrated to possess the same nucleotide sequences (13,29,30) and we ourselves always confirm this rule by DNA sequencing. Therefore, bands with identical migration in SSCP represent identical clones.

We counted the total number of T cell clonotypes detected in each foot and the number of common clonotypes among the four feet. The results are summarized in Table 1. The proportion of T cell clonotypes common to all four feet was low in each Vb repertoire in the early stage (average 14.1%). However, it increased during progression of the stage. This increase during the transition from the early to late stage was observed in many Vb repertoires (Vb2 and 6, P < 0.0001; Vb7, P < 0.001; Vb8.1 and 8.2, P = 0.001; Vb8.3, 10, 11 and 15, P < 0.05). Finally in the late stage, a high rate of >70% was found for Vb2, 6, 7, 8.1, 8.2, 8.3 and 10 (Fig. 4). Of interest, in contrast to the common clones, the total number of T cell clonotypes accumulated in joints decreased as the disease advanced (P < 0.05) (Table 1). In particular, Vb2, 6, 7 and 8.1 are striking, showing that the total number of T cell clonotypes in the late stage decreased by 40–50% compared with in the early stage. Taken together, the T cell clonotypes became identical while reducing their variety during progression of arthritis.

To examine amino acid sequences of the CDR3 region in common T cell clonotypes among four feet, DNA encoding the Vb genes were collected from SSCP gels and sequenced. As shown in Table 2, conserved amino acid motifs were found, e.g. QGW in the CDR3 region of Vb2 clones, RGTG in Vb9, and SXTGG, LTGG, QGA and YRG in Vb10. In addition, certain conserved amino acid motifs, such as RSG in Vb2 and Vb9, DWG in Vb2 and Vb10, and IQG and QGA in Vb10 and Vb11, were observed in the CDR3 region of different Vb clones. This sequence information implies the possibility that T cell clonotypes in multiple joints might recognize some common epitopes on antigens.

The common clones were able to migrate into the joints of the recipients.

In order to investigate the pathological significance of the common clonotypes in four feet, we conducted an adoptive...
Clonal restriction during arthritis progression

Fig. 4. The rate of common T cell clonotypes in all four feet increased as HTLV-I env-pX transgenic mice aged. The proportions of common T cell clonotypes in all four feet in the early, mid and late stages are demonstrated. Data given as the percentage of the number of bands common to all four feet against the total number of bands in all four feet. Values are expressed as the mean ± SEM (n = 5).

Table 2. The TCR VDJ region of common T cell clonotypes in four feet

<table>
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<th>Vβ</th>
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<th>Jβ</th>
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</tr>
<tr>
<td>Vβ11</td>
<td>CAS</td>
<td>IQG</td>
</tr>
</tbody>
</table>

Underlined letters indicate conserved amino acid motifs.

Fig. 5. The clonotype common to all four feet of a Tg donor migrated to the joints of nude mice. In each experiment, splenocytes from an arthritic Tg were transferred into a nude mouse recipient. Nude mice were sacrificed at 3 weeks after the cell transfer and subjected to T cell clonality analysis. Representative Vβ panels from two independent experiments are demonstrated. S: spleen. Lanes from 1 to 4 represent the right front foot, left front foot, right rear foot and left rear foot respectively. The common clones are indicated by the arrows. TCR mRNAs of spleen and joint were standardized by Cβ products as described in Methods. The amount of TCR mRNA from all joints corresponded to ~1/10 of that from spleen.

Table 3 shows the DNA sequences of SSCP bands from a donor and recipient, shown by arrows in Figs 5 and 7. As expected, the sequences were identical between the donor and recipient. These findings indicate that some of the common clonotypes in four feet of the donor infiltrated again the joints of the recipients.
into the joints of the recipient. There were no clear conserved amino acids among migrated clonotypes in the amino acid sequences of the CDR3 region.

Discussion

We studied the T cell clonotypic change in arthritic joints of HTLV-I env-pX Tg during the development of the disease. In the early stage of the disease, T cell clonotypes diversi®ed among the feet. On the contrary, in the late stage, most of the clonotypes were common to all four feet. The total number of accumulating clones decreased compared with in the early stage. Thus, the T cell clonotypes became identical and decreased in variety as the disease progressed. Some of these common clones remaining in the late stage joints might be arthrotropic, since the transfer studies revealed their ability to migrate into the joints. Due to the restricted availability of clinical samples, it might have been difficult to know the dynamic changes of clonality, which are diverse at the initial stage and finally are restricted to common clonotypes.

Analysis of T cell clonality in the joints of RA patients as well as in the affected organs of autoimmune disease animal models demonstrated the presence of common clonotypes among multiple joints or among different parts of an organ (13,28). Moreover, the amino acid sequences of TCR Vβ junctional regions of clones which are identical in multiple joints of a patient were found to be the same as the sequences of the common clones of different patients (14). Considering that CDR3 interacts with the peptides presented by MHC molecules (32±34), these identical clones might recognize certain common antigens which are involved in the pathogenesis of RA. Our identi®cation of common clonotypes in the four arthritic feet and some conserved CDR3 motifs among them is consistent with these previous findings.

In our present study of HTLV-I env-pX Tg, the accumulation of common clonotypes was distinct after the mid stage, whereas in the early stage it was not so pronounced. How can this change in clonality during disease progression be explained?

Several studies have implicated epitope spreading in the pathogenesis of autoimmune diseases (16±20). Disease progression is associated with a shifting of T cell autoreactivity from primary initiating self-antigenic determinants to de®ned cascades of secondary determinants that sustain the self-recognition involved in disease perpetuation. Intramolecular as well as intermolecular epitope spreading could presumably occur by antigen presentation of autoantigens derived from destroyed tissues (35±37). As recruitment of T cells speci®c for additional determinants continues, T cell clonality should become diversi®ed. In our experiment, the early stage might ®t the epitope-spreading model. The recruitment of T cells with other speci®city proceeded independently in each joint. Since the time of onset of the arthritis is different among the four feet, it is also possible that the degree of autoimmune response propagation differs in each foot, resulting in the accumulation of different clones in each joint. The autoantigens driving the autoimmune response in each joint may differ in this stage. However, epitope spreading may not explain the T cell behavior in our arthritis model throughout the entire course of the disease, since the total number of T cell clones...
decreased and the identity of clonality increased with disease progression. It is shown that the TCR repertoire of memory cells which expand selectively during the recall response are rather restricted (38). Thus, such clones might be increasing while an autoantigen repetitively stimulates and drives the (pathogenic) immune response. Adding to this, some auto-reactive T cell clones might be inactivated or deleted by regulatory mechanisms. Therefore, we may reason that the number of accumulating clones in the lesion of an autoimmune disease might gradually decrease, leaving clones which are able to expand more efficiently or are less susceptible to cell death.

Alternatively, the clonal restriction in the joints might reflect avidity maturation of a T cell population. Amrani et al. demonstrated that a shift towards a high-avidity pathogenic T cell population may be the key event in the progression of benign insulitis to overt diabetes using NOD mice (21). Applying this hypothesis, we tend to prefer that some of the remaining common clonotypes have an advantage in clonal expansion due to their higher avidity for certain autoantigens and are related to the inflammation of the joints. However, it is too early to conclude that the remaining common clonotypes are pathogenic, since other clones might also be involved in the exacerbation of young Tg arthritis after the transfer.

Table 3. The TCR VDJ region of T cell clonotypes common to donor and recipient in the transfer experiments

<table>
<thead>
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<th>Vb</th>
<th>N-Dµ-N</th>
<th>Jµ</th>
</tr>
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<tbody>
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<td>Vb8.2</td>
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<tr>
<td>Vb10</td>
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<tr>
<td>Vb9</td>
<td>TGTCTAACAGT</td>
<td>CASS</td>
</tr>
<tr>
<td>Vb11</td>
<td>TGTGCAACGAG</td>
<td>CASS</td>
</tr>
</tbody>
</table>

aThe similar electrophoretic mobility bands in donor and recipient indicated by arrows in Figs 5 and 7 were cut out and subcloned after PCR amplification. Their sequences were determined. (A) Transfer experiment in nude mice. (B) Transfer experiment in young Tg.

Acknowledgements

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Abbreviations

HIV-1 human T cell leukemia virus type I
RA rheumatoid arthritis
SSCP single-strand conformation polymorphism
Tg transgenic mouse

References


