TCRBV20S1 and TCRBV3S1 Gene Segment Polymorphisms in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To compare the frequencies of variants of TCRBV20S1 and TCRBV3S1 gene segments in patients with systemic sclerosis (SSc) and in controls. The null allele (allele 2) of TCRBV20S1 is associated with reduced levels of Vß20+ T-cells in the peripheral blood, while allele 1 of TCRBV3S1 is related to a low frequency of Vß3.1+ T-cells.

Methods. One hundred thirty patients with SSc and 118 healthy volunteer controls were genotyped for TCRBV20S1, and 117 patients and 85 controls were genotyped for TCRBV3S1 variants by PCR-RFLP. Patients underwent clinical evaluation, serology, pulmonary function tests, high resolution computed tomography, and Doppler echocardiography.

Results. The genotypic frequencies of TCRBV20S1 were 0.46 (allele 1/allele 1), 0.43 (allele 1/allele 2), and 0.11 (allele 2/allele 2) in SSc patients; in controls the frequencies were 0.70, 0.26, and 0.04, respectively ($p < 0.001$). The Mantel-Haenszel odds ratio (stratified by race and sex) of the allele 2 carrier state was 3.88 (95% CI 1.94 to 7.75). The allelic and genotypic frequencies of the TCRBV3S1 gene segment did not differ significantly in patients and controls. However, among patients, allele 1 (TCRBV3S1) carriers had a higher prevalence of interstitial lung disease (adjusted $p = 0.032$).

Conclusion. The null allele of the TCRBV20S1 and the allele 1 of TCRBV3S1 gene segments may be considered risk factors for the development of SSc and interstitial lung disease, respectively, suggesting a protective role of Vß20+ and Vß3.1+ cells in the pathogenic immune responses in SSc. (First Release April 15 2008; J Rheumatol 2008;35:1058–63)

Key Indexing Terms:
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Systemic sclerosis (SSc) is a disease characterized by a variable degree of vascular dysfunction, skin and visceral fibrosis, and circulating autoantibodies. The etiology of SSc is unknown, although environmental and genetic factors have been implicated in the susceptibility to this disease[1].

Oligoclonal expansion of T-cells has been reported in skin biopsy specimens[2], bronchoalveolar lavage fluid[3,4], and in peripheral blood[3,4] from patients with SSc. These findings probably reflect the proliferation of particular clones of T-cells in response to certain antigens, suggesting that SSc is an autoimmune disease driven by specific antigens[5]. T-cells recognize antigens through the coupling of the T-cell receptor (TCR) with antigenic peptides bound to MHC molecules in the surface of antigen-presenting cells. The TCR repertoire plays a central role in shaping immune responses. Therefore, variation in the TCR genes may be related to the development of SSc and other autoimmune diseases[6].

There are few studies investigating genetic polymorphisms of TCR gene segments in SSc[7,8]. None addressed polymorphisms in the gene segments encoding the variable beta (Vß) chain of the TCR. We analyzed polymorphisms in 2 different TCR-ß variable (TCRBV) gene segments. The first is located in the TCRBV20S1 gene segment (denoted TRBV30 by the HUGO Gene Nomenclature Committee[9]) and results from a single-nucleotide substitution (C/T) that introduces a stop codon inside the gene segment. Individuals homozygous for this null allele (here denoted allele 2, but previously known as BV20S1A2P[10], BV20S1*4[11], or

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Vß18C[12]) are unable to produce TCR molecules using the TCRBV20S1 gene segment, which results in the absence of Vß20+ T cells in the peripheral pool[12]. An intermediary level of functional Vß20+ T-cells is observed in the peripheral blood of individuals heterozygous for this polymorphism[13].

The second polymorphism is a single-nucleotide polymorphism (C/T) located within the 23-bp spacer region of the TCRBV3S1 gene segment (TRBV3-1 in the HGNC nomenclature[9]) recombination signal sequence (RSS). This variation is associated with the level of Vß3.1+ T-cells in the peripheral pool. Individuals homozygous for allele 1 have a low frequency of Vß3.1+ T-cells (around 1% of the total CD3+ CD4+ T-cells), whereas individuals homozygous for allele 2 have a higher frequency of Vß3.1+ T-cells (8.1%) and heterozygous individuals present an intermediate frequency (4.7%)[14].

In an attempt to identify new factors in susceptibility for SSc we studied the frequencies of these 2 TCRBV polymorphisms in South Brazilian patients with SSc and compared them to the frequencies observed in healthy individuals from the same population.

MATERIALS AND METHODS

Patients and controls. One hundred fifty-four patients with definite or strongly suspected SSc (according to the evaluation of experienced rheumatologists) were prospectively evaluated between April 2000 and December 2004. Patients were referred from the rheumatology units of 4 clinical centers and from private clinics in the city of Porto Alegre. All patients were Brazilian; most had European and/or African ancestry, and the great majority inhabited the metropolitan area of Porto Alegre/RS. The sample was constituted by patients with longstanding or recently diagnosed disease. The evaluation of race was based on observation of phenotypic characteristics (skin color, hair color and type); race was described as white (Caucasians), mixed, or black. There were no individuals of Asiatic or Amerindian origin among the patients. To be included in the study, the patient was required to meet the American College of Rheumatology (ACR) criteria for SSc[15] or the criteria suggested by LeRoy and Medsger for diagnosis of early forms of SSc (objective evidence of Raynaud's phenomenon plus scleroderma pattern on nailfold capillary microscopy or SSc-selective autoantibodies; alternatively, subjective evidence of Raynaud's phenomenon plus scleroderma pattern on capillaroscopy and SSc-selective autoantibodies)[16]. Patients with overlapping syndromes were excluded. However, patients with definite diagnosis of SSc (according to the ACR criteria) who developed inflammatory myopathy or secondary Sjögren's syndrome were not excluded from the analysis. All patients gave signed informed consent before the study.

DNA of healthy volunteer controls was obtained from a bank maintained at the Genetics Department of the Universidade Federal do Rio Grande do Sul (UFRGS). The bank comprised samples of unrelated subjects from the urban population of Porto Alegre/RS (the majority had European and/or African ancestry). These individuals were healthy blood donors, students, professors, or employees from the university. Recruitment of controls, collection of blood samples, and DNA extraction occurred between 1990 and 2000. Individuals with chronic or acute diseases were excluded from the sample, as well as those with family history of genetic diseases (X-linked, autosomal, or chromosomal abnormalities). The assessment of race was performed observing phenotypic characteristics. Amerindians and subjects of Asiatic origin were excluded. All controls gave signed informed consent.

Clinical evaluation. All patients were interviewed and examined according to an extensive questionnaire directed to the evaluation of end-organ damage. The severity and extent of cutaneous disease was quantified using the modified Rodnan skin score[17]. Disease subtype was classified as follows: diffuse cutaneous SSc (truncal and acral skin tautness), limited cutaneous SSc (skin tautness restricted to extremities and/or face), and limited SSc (*sine scleroderma*)[16,18]. Clinical disease characteristics were recorded as described[19]. Pitting scars, reabsorption of the finger fat pad, and nontraumatic finger amputations were considered signs of peripheral ischemia. The presence of "scleroderma pattern" on nailfold capillary microscopy was defined as described by Maricq, *et al*[20]. Blood samples were collected for serology (antinuclear, anticentromere, and antitopoisomerase I antibodies)[19] and DNA extraction.

Pulmonary high-resolution computed tomography (HRCT) was performed in most patients. All HRCT scans were assessed for radiologic evidence of interstitial disease (ground-glass opacities, reticular pattern, and honeycombing) by 2 radiologists. Additionally, they estimated the percentage of parenchyma affected by honeycombing/reticular pattern in both lungs, according to the method proposed by Staples, *et al*[21]. Intraobserver and interobserver agreement (comparing with a third radiologist) for the extent of honeycombing/reticular pattern were moderate (Fleiss-weighted kappa 0.51 and 0.57, respectively). Patients also underwent spirometry and carbon monoxide diffusing capacity (DLCO) test. Forced vital capacity (FVC) and DLCO were considered reduced when < 80% and < 75% of predicted values, respectively. Patients with honeycombing, reticular pattern, ground-glass opacities, or reduced FVC were considered to have interstitial lung disease (ILD). Doppler echocardiography was used to estimate the pulmonary systolic arterial pressure (PSAP). Patients with PSAP ≥ 40 mm Hg were considered to have pulmonary arterial hypertension.

The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre, and consent was obtained according to the Declaration of Helsinki[22].

DNA extraction and genomic analysis. DNA was extracted from peripheral blood as described[23]. Both DNA polymorphisms were analyzed by restriction fragment-length polymorphism preceded by polymerase chain reaction (PCR-RFLP). Samples with defined genotypes (meaning one homozygous individual for the allele containing the restriction site and one homozygous individual for the allele without the restriction site) were included in each analysis to control for restriction enzyme activity. All samples were tested according to established methodology[24,25].

Identification of variants in TCRBV20S1 gene segment. For analysis of TCRBV20S1 polymorphism, the specific primers 5'-ATT CAT CAA TGG CCA GCG AC-3' and 5'-GGA GCT TCT TAG AAC TCA G-3' were used[12,24]. Samples were subjected to 40 cycles of 1 min of denaturation at 94°C, 1 min annealing at 60°C, and 2 min of extension at 72°C, preceded by 1 min at 94°C and followed by 5 min at 72°C in a DNA thermal cycler (MJ Research, Inc., Watertown, MA, USA). The resultant 235-bp fragment was digested with *Kpn* I and the products were visualized by electrophoresis in a 1% agarose gel containing ethidium bromide. A single nucleotide substitution (a C–T base transition) leads to the introduction of a stop codon at the Vß20 gene segment sequence and simultaneously eliminates a restriction site for the enzyme *Kpn* I. Individuals homozygous for the null allele (allele 2) can be identified by the presence of a single band in agarose gel (235-bp) when the PCR amplification product is digested with *Kpn* I. DNA from homozygotes for allele 1 is identified by the presence of 2 bands (100 and 135-bp), whereas heterozygotes show 3 bands (100, 135, and 235-bp)[24].

Identification of variants in the recombination signal sequence (RSS) region of the TCRBV3S1 gene segment. For analysis of the TCRBV3S1 RSS polymorphism, DNA samples were amplified by PCR using the specific primers 5'-CCT TGA TGG CCT GTT TTT CAC-3' and 5'-GTG CCA TCG GAG CCA GCA C-3'[14,24]. PCR was performed under the same conditions described for the TCRBV20S1 polymorphism in 36 cycles of 1 min denaturation at 94°C, 1 min of annealing at 51°C, and 2 min of extension at 72°C, preceded by 1 min at 94°C and followed by 5 min at 72°C. The

resultant 431-bp fragment was digested with *Pvu* II, and the products were visualized by electrophoresis. The 2 allelic variants located at the TCRBV3S1 RSS differ at only a single nucleotide position (C/T); this cytosine to thymine transition creates a *Pvu* II site inside the TCRBV3S1 RSS. Individuals homozygous for allele 1 can be identified by the presence of a single DNA band in agarose gel (431 bp) reflecting the absence of the restriction site. Homozygotes for allele 2 can be identified by the presence of a 352-bp band (the 79-bp fragment cannot be observed in 1% agarose gel), whereas in heterozygotes 2 bands (352 and 431-bp) can be observed[24].

Statistical analysis. Data were analyzed using Epi-Info version 6 and SPSS for Windows version 11.0. The association between categorical variables was tested by Pearson chi-square, Yates corrected chi-square, or Fisher's exact test. Variables with normal distribution were presented as mean (standard deviation). Continuous variables with non-normal distributions were presented as median and 25th/75th percentiles, and the Mann-Whitney test was used for comparisons between groups. Crude and Mantel-Haenszel (for stratified analysis) odds ratios along with 95% confidence intervals were calculated for carriers of allele 2 (the null allele) of TCRBV20S1 and allele 1 of TCRBV3S1. Hardy-Weinberg equilibrium was tested comparing the observed genotypic frequencies with the expected ones (considering the observed allelic frequencies) using the chi-square goodness-of-fit test. A 2-tailed p value $\leq$ 0.05 was considered statistically significant. When testing for associations of the TCRBV20S1 and TCRBV3S1 alleles with clinical and laboratory characteristics (secondary endpoints), Bonferroni correction of the p value was applied considering the number of comparisons performed.

RESULTS

Six of the 154 patients initially evaluated were excluded for not fulfilling the entry criteria; 2 others did not undergo blood collection for DNA extraction. Of the remaining patients, 130 were genotyped for the TCRBV20S1 and 117 for TCRBV3S1 gene segments. One hundred eighteen and 85 controls were genotyped for TCRBV20S1 and TCRBV3S1 gene segments, respectively. The characteristics of patients genotyped for the TCRBV20S1 are described in Table 1. The respective controls were a mean age of 43.8 (SD 6.4) years; 82 (69.5%) were male and 100 (84.7%) were Caucasians. The patients and controls genotyped for TCRBV3S1 had similar features (data not shown), and most of them (96.6% of patients, 74.1% of controls) were also included in the sample genotyped for the TCRBV20S1 gene segment.

The allelic and genotypic frequencies of TCRBV20S1 variants are described in Table 2, and the distributions were significantly different in patients and controls. The crude and Mantel-Haenszel odds ratios (stratified by race and sex) of the presence of the allele 2 (null allele) were 2.66 (95% CI 1.52–4.66; p < 0.001) and 3.88 (95% CI 1.94–7.75; p < 0.001), respectively. Analyzing Caucasians and non-Caucasians separately, the crude OR for the presence of allele 2 were 2.42 (95% CI 1.32–4.47; p = 0.003) and 4.55 (95% CI 0.95-23.57; p = 0.058), respectively. Restricting the

Table 1. Clinical, demographic, and laboratory features of patients genotyped for the TCRBV20S1 gene segment according to disease subtype. Data are presented as number (percentage) of patients, except when indicated otherwise.

Characteristic	Diffuse Forms, n = 36	Limited Forms†, n = 94	All Patients††, n = 130
Female	28 (77.8)	86 (91.5)	114 (87.7)
Caucasian	27 (75.0)	80 (85.1)	107 (82.3)
Age, yrs, mean (SD)	43.3 (14.2)	52.5 (13.1)	49.9 (14.0)
Raynaud's phenomenon	36 (100.0)	94 (100.0)	130 (100.0)
Disease duration, yrs, median (25th, 75th percentiles)	3.2 (1.3, 10.0)	12.0 (4.0, 21.0)	8.0 (3.0, 19.0)
Sclerodactyly	34 (94.4)	75 (79.8)	109 (83.8)
Signs of peripheral ischemia*	28 (77.8)	58 (61.7)	87 (66.9)
Calcinoses	8 (22.2)	23 (24.5)	31 (23.8)
Telangiectases	21 (58.3)	59 (62.8)	80 (61.5)
Puffy hands	3 (8.3)	21 (22.3)	24 (18.5)
Total skin score, median (25th, 75th percentiles)	18.5 (11.0, 31.5)	4.5 (2.0, 9.0)	6.0 (2.0, 14.0)
Scleroderma pattern on NCM**	29/33 (87.9)	89/91 (97.8)	118/124 (95.2)
ANA $\geq$ 1:80	32 (88.9)	78 (83.0)	110 (84.6)
Anticentromere antibodies	4 (11.1)	45 (47.9)	49 (37.7)
Antitopoisomerase I antibodies	13 (36.1)	15 (16.0)	28 (21.5)
Interstitial lung disease on HRCT**	24/35 (68.6)	48/89 (53.9)	72/124 (58.1)
Reduced FVC**	19/32 (59.4)	33/88 (37.5)	52/120 (43.3)
Reduced DLCO**	30/32 (93.8)	73/86 (84.9)	103/118 (87.3)
Pulmonary arterial hypertension**	2/30 (6.7)	15/90 (16.7)	17/120 (14.2)

* Pitting scars, reabsorption of the finger fat pad, or nontraumatic finger amputations. ** Data not available for all patients; values represent number of patients with the indicated abnormalities over number of patients examined (percentages). † Seventy-eight patients with limited cutaneous form and 16 with limited form (*sine scleroderma*). †† Twenty-six patients did not fulfill the ACR criteria (all with limited form of the disease). NCM: nailfold capillary microscopy; ANA: antinuclear antibodies; HRCT: high resolution computed tomography; FVC: forced vital capacity; DLCO: carbon monoxide diffusing capacity.

Table 2. TCRBV20S1 gene segment frequencies in patients with SSc and controls.

TCRBV20S1	SSc	Controls	p*
Alleles, no. alleles (%)			
Allele 1	176 (67.7)	195 (82.6)	< 0.001
Allele 2	84 (32.3)	41 (17.4)	
Genotype, no. subjects (%)			
Allele 1/allele 1	60 (46.1)	82 (69.5)	< 0.001
Allele 1/allele 2	56 (43.1)	31 (26.3)	
Allele 2/allele 2	14 (10.8)	5 (4.2)	

* Pearson chi-square test or Yates-corrected chi-square.

Table 4. TCRBV3S1 gene segment frequencies in patients with SSc and controls.

TCRBV3S1	SSc	Controls	p*
Alleles, no. alleles (%)			
Allele 1	126 (53.8)	96 (56.5)	0.673
Allele 2	108 (46.2)	74 (43.5)	
Genotype, no. subjects (%)			
Allele 1/allele 1	34 (29.0)	30 (35.3)	0.555
Allele 1/allele 2	58 (49.6)	36 (42.3)	
Allele 2/allele 2	25 (21.4)	19 (22.4)	

* Pearson chi-square test or Yates-corrected chi-square.

analysis to patients fulfilling the ACR criteria, the results for the presence of the null allele were similar to those obtained in the whole sample (crude OR 2.37, 95% CI 1.31–4.28, p = 0.003; Mantel-Haenszel OR, stratified by race and sex, 3.82, 95% CI 1.82–8.01, p < 0.001). Patient and control samples were in Hardy-Weinberg equilibrium.

Comparisons of clinical and laboratory features according to the TCRBV20S1 genotype are given in Table 3. No significant differences between carriers and noncarriers of the allele 2 were observed.

The frequencies of TCRBV3S1 variants are described in Table 4. No differences in the distributions were observed between patients and controls. The crude and Mantel-Haenszel OR (stratified by race and sex) of the presence of the allele 1 were 1.06 (95% CI 0.51–2.21) and 0.72 (95% CI 0.29–1.75), respectively. No deviation from Hardy-Weinberg equilibrium was observed in patients and controls.

Table 5 compares clinical features according to the TCRBV3S1 genotype. Allele 1 carriers presented a higher prevalence of ILD than noncarriers, while other features did not differ significantly between the groups. Carriers of allele 1 also had a greater extent of reticular pattern/honeycombing on HRCT than noncarriers: median (25th, 75th percentiles) 0.3 (0.0, 12.5), number of patients = 87 versus median 0.0 (0.0, 0.0), n = 24, respectively (Mann-Whitney, unadjusted p = 0.006).

Considering the description of predominant use of Vß20 in topoisomerase I-specific CD4+ T-cells[26], we also compared the prevalences of antitopoisomerase I antibodies among the carriers of the null allele of TCRBV20S1. Patients homozygous for the null allele had a nonsignificant lower prevalence of antitopoisomerase I (1/14, 7.1%) than those heterozygous (17/56, 30.4%) and homozygous for the allele 1 (10/60, 16.7%). The one antitopoisomerase I-positive patient among those homozygous for the null allele presented low titers of these antibodies (20.9 U/ml, where the minimum value for a positive test is 20.0 U/ml).

DISCUSSION

Several single-nucleotide polymorphisms have been tested in SSc[27], but few studies have addressed polymorphisms involving TCR gene segments. To our knowledge, there are no studies testing TCR-ß variable polymorphisms in SSc to date. Nevertheless, polymorphisms of TCR-ß gene variable regions are of special interest because they may influence the immune response to specific antigens and superantigens. Our results suggest that the null allele of the TCRBV20S1

Table 3. Clinical and laboratory characteristics of patients according to genotype of the TCRBV20S1 gene segment. Values represent number of patients with the indicated abnormalities over number of patients examined (percentages).

Clinical Features	Allele 2 Carriers	Allele 2 Noncarriers	p†
Signs of peripheral ischemia*	46/70 (65.7)	41/60 (68.3)	0.999
Diffuse form	20/70 (28.6)	16/60 (26.7)	1.000
Anticentromere antibodies	25/70 (35.7)	24/60 (40.0)	0.999
Antitopoisomerase I antibodies	18/70 (25.7)	10/60 (16.7)	0.882
Interstitial lung disease**	46/67 (68.7)	41/57 (71.9)	0.999
Pulmonary arterial hypertension	6/63 (9.5)	11/57 (19.3)	0.745

* Pitting scars, reabsorption of the finger fat pad, or nontraumatic finger amputations. ** Honeycombing, reticular pattern, or ground-glass opacities on HRCT, or reduced FVC. † Yates-corrected chi-square; p values were adjusted for multiple comparisons (6 tests).

Table 5. Clinical and laboratory characteristics of patients according to genotype of the TCRBV3S1 gene segment. Values represent number of patients with the indicated abnormalities over number of patients examined (percentages).

Clinical Features	Allele 1 Carriers	Allele 1 Noncarriers	p†
Signs of peripheral ischemia*	63/92 (68.5)	15/25 (60.0)	0.994
Diffuse form	27/92 (29.3)	6/25 (24.0)	0.999
Anticentromere antibodies	37/92 (40.2)	9/25 (36.0)	1.000
Antitopoisomerase I antibodies	20/92 (21.7)	4/25 (16.0)	0.999
Interstitial lung disease**	71/88 (80.7)	12/24 (50.0)	0.032
Pulmonary arterial hypertension	14/86 (16.3)	2/23 (8.7)	0.987

* Pitting scars, reabsorption of the finger fat pad, or nontraumatic finger amputations. ** Honeycombing, reticular pattern, or ground-glass opacities on HRCT, or reduced FVC. † Yates-corrected chi-square or Fisher's exact test; p values were adjusted for multiple comparisons (6 tests).

gene segment is associated with susceptibility to SSc; in a secondary analysis, allele 1 of the TCRBV3S1 was found to be associated with the presence of ILD.

Both polymorphisms tested here are related to important changes in the T-cell receptor repertoire. Allele 2 of TCRBV20S1 is associated with a reduction (or absence, if in homozygosis) of Vß20+ peripheral T-cells[12,13], while allele 1 of TCRBV3S1 is related to reduced levels of Vß3.1+ cells[14]. Therefore, our results suggest that Vß20+ cells may be protective for the development of SSc and that Vß3.1+ cells may exert a protective role against the progression of ILD. A possible explanation is that these subsets could exert regulatory functions, inhibiting the activity of autoreactive cells or altering the production of cytokines. In murine models of experimental autoimmune encephalitis[28] and nonobese diabetes[29], specific regulatory CD4+ cells were found to make preferential usage of certain Vß families — Vß14 and Vß12, respectively.

The cooperation of topoisomerase I-specific CD4+ T-cells is essential for the production of antitopoisomerase I antibody by B-cells[30]. Kuwana, *et al*[26] observed that topoisomerase I-specific CD4+ T-cells of HLA-DR11+ patients and controls showed a predominant Vß20.1a usage (in 14 out of 15 clones analyzed). Therefore, the finding that the null allele of TCRBV20S1 (associated with a lower or absent capacity to produce cells using Vß20) is associated with susceptibility to SSc may be rather surprising. However, it is unlikely that topoisomerase I-specific CD4+ cells are essential for the development of SSc, since antitopoisomerase I antibodies are absent in the majority of patients. The observation of only one patient with antitopoisomerase I antibodies (in low titer) among those homozygous for the null allele, which are incapable of producing Vß20+ cells, is in agreement with the findings reported by Kuwana, *et al*[26].

The appropriate selection of cases and controls is also an important issue surrounding case-control studies[31]. We included some patients who did not fulfill the ACR criteria for SSc, but did fulfill the criteria suggested by LeRoy and Medsger for diagnosis of early forms of the disease. We decided to study these patients since strict application of the ACR criteria would exclude an important and increasingly recognized subset of patients with limited forms of the disease[16,32,33]. However, exclusion of patients not fulfilling the ACR criteria from the analysis did not change the results significantly.

Considering the evidence, we conclude that genetic variations that interfere with the TCR repertoire may be important factors in the susceptibility to systemic sclerosis. We suggest that TCRBV20S1 and TCRBV3S1 polymorphisms are associated with increased risks for systemic sclerosis and pulmonary interstitial disease, respectively.

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