STUDY OF TCR Vβ USAGE IN SUPERANTIGEN-REACTIVE HUMAN T CELLS BY THE RT-PCR METHOD

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Human T cells reactive with several bacterial superantigens were examined in terms of the T cell receptor for immunogen (TCR) Vβ repertoires. Human peripheral blood mononuclear cells were stimulated with 10 ng of staphylococcal enterotoxins A, B, C2 and E (SEA, SEB, SEC2 and SEE) or toxic shock syndrome toxin-1 (TSST-1) per ml for 3 days. The large lymphoblasts recovered were expanded for 2 days in the presence of 100 U of recombinant human IL-2 per ml. T cell blasts obtained were examined for TCR Vβ usage by the reverse transcriptase polymerase chain reaction method.

T cells reactive with SEA were Vβ1', Vβ5.2-3', Vβ6.1-3', Vβ7', Vβ9' and Vβ18'. Those reactive with SEB were Vβ3', Vβ12', Vβ13.2', Vβ14', Vβ15', Vβ17' and Vβ20'. Those reactive with SEC2 were Vβ7', Vβ9', Vβ12', Vβ13.2', Vβ14', Vβ15', Vβ17' and Vβ20'. Those reactive with SEE were Vβ5.1', Vβ6.1-3', Vβ8' and Vβ18', and those reactive with TSST-1 were Vβ2' and Vβ4'. While the present study supported principally the validity of the previous studies by other researchers, it revealed the additional TCR Vβ usage in human T cells reactive with several bacterial superantigens. It seems likely that T cells from Japanese in the present study and T cells from Caucasians may respond differently to bacterial superantigens.

Introduction

A number of bacterial exotoxins have been classified as superantigenic. These exotoxins contain toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins A–E (SEA, SEB, SEC, SED and SEE), streptococcal pyrogenic exotoxins A–C (SPE-A, SPE-B and SPE-C), and a recently found Yersinia pseudotuberculosis-derived mitogen (YPM). These exotoxins bind directly to major histocompatibility complex (MHC) class II molecules and activate a vast number of T cell clones in a T cell receptor for immunogen (TCR) Vβ-selective way in association with MHC class II molecules on accessory cells. The potent T cell-stimulating activity of these exotoxins has been implicated as the pathogenic factor in exotoxin-induced illnesses such as toxic shock syndrome, scarlet fever and Y. pseudotuberculosis infection. The last of these has often been reported in Japan. Almost all of the TCR Vβ repertoires in murine and human T cells reactive with these superantigenic exotoxins have been determined during the 6 years since the proposal of the concept of superantigen. In our preliminary experiments using the reverse transcriptase-
polymerase chain reaction (RT-PCR) method, however, we found selective elevation of a certain Vβ element in SEA-reactive human T cells, which was not reported previously. This observation suggests the necessity of reexamination of the previous reports (summarized in reviews19-23). In the present study, we examined the TCR Vβ usage in human T cells reactive with a number of bacterial superantigens, TSST-1, SEA, SEB, SEC and SEE.

Materials and Methods

Superantigens and other reagents
TSST-1 was purified from the culture fluid of Staphylococcus aureus FR1169 as reported previously20). SEA, SEB, SEC, and SEE were purchased from Toxin Technology (Sarasota, FL). The RPMI 1640 culture medium used contained 100 μg of streptomycin per ml, 100 units of penicillin per ml, 10% fetal calf serum and 5 × 10⁻⁸ M 2-ME. Recombinant human interleukin 2 (rIL-2) was kindly provided by Takeda Chemical Industries Ltd. (Osaka, Japan).

Lymphoid cells
Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood of healthy donors by Ficoll-Conray gradient separation as reported previously21,22). PBMC (1–2 × 10⁶/ml) were stimulated with various doses of superantigens or 20 ng of anti-CD3 monoclonal antibody (mAb) OKT3 per ml for 3 days. Recovered cells were subjected to Percoll density gradient centrifugation (Percoll density of 1.068). The large lymphoblasts fractionated were expanded for 2 days in the presence of 100 U of rIL-2 per ml. The cells recovered were fractionated into large lymphoblasts. The large lymphoblasts obtained contained 60–90% CD3⁺ cells as determined by flowcytometric analysis using anti-CD3 mAb.

Assay for IL-2 production
PBMC were stimulated with varying concentrations of the bacterial superantigens in 1-ml quantities in 24-well Falcon plates (Becton Dickinson, San Jose, CA) for 24 hr. Culture supernatants were collected and assayed for IL-2 activity by using IL-2-dependent CTLL-2 as reported previously24). Data are presented as units/ml.

RT-PCR method for determination of TCR Vβ usage
TCR Vβ usage in superantigen-reactive human T cells was determined by the RT-PCR method originally described by Choi et al.16) with slight modifications. Total mRNA was prepared from superantigen- or anti-CD3 mAb-induced T lymphoblasts by using oligo deoxynucleotidylconjugated magnetic beads (Dynabeads Oligo (dt) 25: Dynal, Oslo, Norway). cDNAs were synthesized by incubating the total mRNA obtained from each T lymphoblast sample with 10 units of reverse transcriptase (RAV2: Takara, Kyoto, Japan) in the final volume of 25 μl/tube for 90 min at 42°C. Then aliquots of each cDNA sample (final volume, 20 μl/tube) were amplified with various numbers of incubation cycle by using 22 5’Vβ-specific sense primers and the 3’Cα-specific anti-sense primer in the presence of 1 unit of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT) in Program Temperature Control System PC-700 (ASTEC, Fukuoka, Japan). TCR Ca cDNA as an internal control was co-amplified in each reaction mixture by using the 5’Ca sense primer and the 3’Ca antisense primer. For quantitation of the amplified products, the 3’primers end-labeled with 32P were used. The sequences of the specific primers used were described by Choi et al.16), and are shown in Table 1. All of these primers would be expected to cover at least 80% of the human TCR Vβ gene segments. The amplified products were electrophoresed in 2.5% agarose gels, dried and exposed on imaging plates (Fuji Photo Film Co., Tokyo, Japan). The Vβ and Ca bands identified were examined for radioactivity expressed as counts per minute (cpm) of photostimulated luminescence by a Bioimaging Analyzer, BAS 2000 (Fuji Photo Film Co.). For each sample, the radioactivity in the Vβ : Ca ratio (=1000 × radioactivity in Vβ band/radio-
activity in 5'α band). The two-tailed paired t-test was used for statistical analysis.

**Results and Discussion**

**T cell-stimulatory activity of various bacterial superantigens**

Initially SEA, SEB, SEC₂ and TSST-1 were examined for their ability to induce IL-2 production from T cells of PBMC. Human PBMC were stimulated in vitro with various concentrations of the superantigens for 24 hr and examined for IL-2 production. They induced the production of substantial amounts of IL-2 at 100 pg/ml or more (Fig. 1), and these four toxins and SEE induced blast formation in very high levels at 10 ng/ml on day 3 after stimulation (data not shown). The results show that these five bacterial superantigens are potent T cell activators at similar levels. In the following experiments for determining TCR Vβ repertoires used in the superantigen-reactive T cells, PBMC (2 × 10⁶/ml) were stimulated with 10 ng of superantigen per ml to obtain T lymphoblast-enriched cell preparations.

**Determination of the optimal number of incubation cycles for performing RT-PCR analysis**

Total mRNA was prepared from samples of SEB-induced T lymphoblasts and then single-stranded cDNA was synthesized. We chose one T cell sample with a high RNA content and another with a low RNA content. An aliquot of each synthesized sample of cDNA in the presence of reverse transcriptase was amplified with various numbers of incubation cycles by using the 5'Cα sense primer and 3'Cα antisense primer, using the Program Temperature Controller, and examined for the level of amplified TCR Cα and TCR Vβ 3 genes. The results showed that the Cα gene and the Vβ 3 gene were amplified in a proportional relationship with each other between 23 and 26 incubation cycles in both samples (Fig. 2). The results indicate that the RT-PCR method can be applied to samples containing various amounts of cDNA. In the following experiments, the
amplification was done with 25 cycles without consideration of the RNA content of the samples.

**Identification of TCR Vβ elements used by human T cell populations reactive with SEA, SEB, SEC₂, SEE and TSST-1**

T cell lymphoblasts induced by 10 ng of SEA, SEB, SEC₂, SEE or TSST-1 per ml were examined for their TCR Vβ usage by the RT-PCR method. T cell blasts induced by anti-CD3 mAb were used as a control. The Vβ : Ca ratios calculated from autoradiograms of amplified TCR transcripts of T lymphoblasts induced by these superantigens from a single donor are presented in Table 2. When the ratios in both the control and experimental samples were less than 50, we removed these data from consideration as values too low to evaluate. The ratios of Vβ1 : Ca, Vβ5.2-3 : Ca, Vβ6.1-3 : Ca, Vβ7 : Ca, Vβ9 : Ca and Vβ18 : Ca were higher in the SEA-induced T cell blasts than in the control CD3-induced T cell blasts. This increase in the Vβ18 : Ca ratio was reported previously¹⁹. Vβ : Ca ratios in other Vβs in the experimental samples were far below those of the control. In SEB-induced T cell blasts, an increase in the ratios Vβ7 : Ca, Vβ12 : Ca, Vβ14 : Ca, Vβ17 : Ca and Vβ20 : Ca was observed. The level of the Vβ15 : Ca and Vβ20 : Ca was observed. The level of the Vβ13.2 : Ca ratio in the experimental sample was the same as that in the control. We consider the results as evidence that Vβ13.2⁺ T cells are reactive with SEB in that the ratio in the former was not lower than that in the control. The reactivity of Vβ13.2⁺ T cells with SEB was not reported previously¹⁶. In SEC₂-reactive T lymphoblasts, the ratios Vβ9 : Ca, Vβ12 : Ca, Vβ13.2 : Ca, Vβ15 : Ca, Vβ17 : Ca and Vβ20 : Ca were increased. The Vβ7 : Ca ratio was high in the control sample and increased slightly in the experimental sample. We consider these findings as evidence that Vβ7⁺ T cells react with SEC₂. The increase in the ratios Vβ7 : Ca and Vβ9 : Ca was not reported previously¹⁶. In SEE-induced T lymphoblasts, the ratios Vβ5.1 : Ca, Vβ6.1-3 : Ca, Vβ8 : Ca and Vβ18 : Ca were higher than that in the controls.
Human PBMC from a healthy donor (2 × 10^6/ml) were stimulated with 10ng of SEA, SEB, SEC2, SEE or TSST-1 per ml for 3 days. The recovered cells (2 × 10^5/ml) were expanded for 2 days in the presence of 100U of rIL-2 per ml, The T lymphoblast-enriched preparations obtained were tested for TCR Vβ usage. Data are expressed as Vβ : Ca ratios (cpm Vβ/cpm Ca × 1,000). For the underlined ratios, we consider that reactivity with the corresponding superantigen was present. The parentheses indicate that the superantigen-reactivity was not reported previously.

In TSST-1-induced T lymphoblasts, the ratios Vβ2 : Ca and Vβ4 : Ca were also higher than in the controls. The increase the Vβ4 : Ca ratios was also not reported previously.

The Vβ usage of T cell blasts induced by these superantigens was examined in T cells from different donors and the data are summarized in Fig. 3. The data are expressed as the Vβ : Ca ratio. With regard to the Vβ elements which were reported in the previous studies to be involved in the reactivity with these five superantigens, our data for the several donors are compatible with the results of other studies with one exception. The Vβ7 : Ca ratio in SEA-reactive T cells differed between two donors: an increase in one donor and a slight decrease in another donor; the ratio in the control was high and the decrease is marginal in the experiment samples. We consider that Vβ7+ T cells in the second donor were reactive with SEA. With regard to the Vβ elements which we found newly in the present experiments to be involved in the reactivity with the superantigens, it seems necessary to discuss several points. For the Vβ18+ element in SEA-reactive T cells, the Vβ18 : Ca ratio was increased in both of two donors. For the Vβ13.2+ element in SEB-reactive T cells, the Vβ : Ca ratio was increased in three donors, not changed in two and slightly decreased in one. We consider that the Vβ13.2+ T cells of the last donor would react with SEB on the basis of the previous discussion. With regard to Vβ7+ and Vβ9+ elements in T cells reactive with SEC2, we examined only one donor. Repeated experiments seem to be necessary to conclude that Vβ7+ and Vβ9+ T cells are reactive with SEC2. For the Vβ4+ element in T cells reactive with TSST-1, the Vβ4 : Ca ratio was increased in three donors, not changed in two and slightly decreased in one. We consider that Vβ4+ T cells of the last donor would be reactive with TSST-1 on the basis of the previous discussion.

**Effect of concentration of SEA on the repertoire in the SEA-reactive T cells**

We thought it was important to know whether or not the Vβ repertoire in the superantigen-reactive T cells is influenced by the concentration of the superantigens. Provided that preferential activation is observed in T cells bearing particular TCR Vβ elements at a lower toxin dose, what is meant by the results would be that binding affinity for the complex of superantigen/MHC class II molecules differs among TCR Vβ elements responsible for the recognition of the superantigen.

Human PBMC were stimulated with varying doses of SEA ranging from 0.1 to 100 ng/ml
Fig. 3  Determination of Vβ usage in human T cells reactive with SEA, SEB, SEC, SEE and TSST-1
T cell blasts induced by SEA (A), SEB (B), SEC (C), SEE (D) and TSST-1 (E) from several donors were prepared according to the procedures described in Materials and Methods and analyzed for TCR Vβ usage. Data are expressed as Vβ : Ca ratios. For the underlined Vβ elements, the increase in Vβ/Cα ratio was not reported previously. The symbols represent different healthy donors.
Table 3  TCR Vβ usage in T lymphoblasts induced by various concentration of SEA

<table>
<thead>
<tr>
<th>Vβ element</th>
<th>Ratios of Vβ:Ca</th>
<th>SEA (ng/ml)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>anti-CD3 20ng/ml</td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>5.2-3</td>
<td>152</td>
<td>439</td>
</tr>
<tr>
<td>6.1-3</td>
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<td>18</td>
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<td>296</td>
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</table>

Human PBMC (2×10⁶/ml) from a healthy donor were stimulated with the indicated doses of SEA for 3 days and T lymphoblasts were prepared according to Materials and Methods and tested for TCR Vβ usage. Data are expressed as Vβ:Ca ratios.

and the T lymphoblasts obtained were examined for Vβ usage (Table 3). The results show that the Vβ repertoire observed at a high SEA concentrations. The results suggest that the binding affinity for the SEA/HLA class II complex does not differ among TCR Vβ1, Vβ5.2-3, Vβ6.1-3, Vβ7, Vβ9 and Vβ18.

Several explanations may be possible for the partial discrepancies in the TCR Vβ repertoires used in human T cells reactive with SEA, SEB, SEC2 and TSST-1 between the present study and past studies by other researchers. It seems unlikely that the technical difference between the present study and studies by other researchers caused the discrepancies. The culture method for preparing the toxin-reactive T lymphoblasts and the method of RT-PCR analysis used in the present study were identical to those by other researchers. As a small difference cDNAs were synthesized from total mRNA in the present study and from the total RNA instead of total mRNA in the studies of other researchers. Primers used in the present study were same as those used by other researchers. The most plausible explanation may be as follows. The ratios Vβ13.2:Ca in SEB-induced T lymphoblasts, Vβ7:Ca and Vβ9:Ca in SEC2-induced T lymphoblasts and Vβ4:Ca in TSST-1-induced T lymphoblasts were not elevated in several cases in the present study. These variable results may have led to the different conclusions between the present and previous studies. Second, the Vβ-selective response to superantigens may be influenced in some degree by the difference in human races. T cells from Japanese in the present study and T cells from Caucasians may respond differently to bacterial superantigens.

Acknowledgements

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RT-PCR法を用いた、細菌性スーパー抗原反応性T細胞の
T細胞受容体Vβエレメントの使用頻度の解析

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我々は、種々の細菌性スーパー抗原 staphylococcal enterotoxin A, B, C, E (SEA, SEB, SEC₂, SEE) および toxic shock syndrome toxin-1 (TSST-1) に反応性のヒト T 細胞に発現する、T 細胞受容体 (TCR) Vβ エレメントの使用頻度を調べた。健常人より得られた末梢リッパ球を10ng の SEA, SEB, SEC₂, SEE および TSST-1で3日間培養し、得られた T リンパ芽球を更に2日間100U の IL-2で培養した。この T リンパ芽球から mRNA を抽出し cDNA を合成した後に、RT-PCR 法により20種類の Vβ を決定した。