High Affinity of Interaction between Superantigen and T Cell Receptor Vβ Molecules Induces a High Level and Prolonged Expansion of Superantigen-reactive CD4+ T Cells

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In mice implanted with an osmotic pump filled with the superantigen (SAG) staphylococcal enterotoxin A (SEA), the Vβ3+CD4+ T cells exhibited a high level of expansion whereas the Vβ11+CD4+ T cells exhibited a mild level of expansion. In contrast, in mice implanted with an osmotic pump filled with SE-like type P (SEiP, 78.1% homologous with SEA), the Vβ11+CD4+ T cells exhibited a high level of expansion while the Vβ3+CD4+ T cells exhibited a low level of expansion, suggesting that the level of the SAG-induced response is determined by the affinities between the TCR Vβ molecules and SAG. Analyses using several hybrids of SEA and SEiP showed that residue 206 of SEA determines the response levels of Vβ3+CD4+ and Vβ11+CD4+ T cells both in vitro and in vivo. Analyses using the above-mentioned hybrids showed that the binding affinities between SEA and the Vβ3/Vβ11 β chains and between SEA-MHC class II-molecule complex and Vβ3+/Vβ11+CD4+ T cells determines the response levels of the SAG-reactive T cells both in vitro and in vivo.

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Supraantigenic toxins (SAGs) bind directly to MHC class II molecules on the surfaces of antigen-presenting cells (APCs) without being processed. Subsequently, the SAG-MHC class II complexes selectively activate virtually all T cells expressing particular Vβ elements in their TCR β chains (1–4). The binding affinity of conventional peptide antigen-MHC complexes to TCRs is thought to play a critical role in triggering specific T cell activation (5–8). This hypothesis is likely applicable to T cell activation by SAGs in light of two lines of experiments using mice. First, in mice implanted with mini-osmotic pumps and continuously exposed to staphylococcal enterotoxin A (SEA, a bacterial SAG), the Vβ3+CD4+ T cells exhibited a high level of protracted expansion, while the Vβ11+CD4+ T cells exhibited a mild level of expansion (9). This experimental system was used to reproduce a mode of T cell response in patients with SAG-induced diseases, such as toxic shock syndrome (TSS) and systemic infection with Yersinia pseudotuberculosis in humans. Recently, we reported that a staphylococcal enterotoxin-like toxin type P (SEiP) has an amino acid sequence that is highly homologous to that of SEA (78.1% identical) and acts as a superantigen to human T cells (10). These results suggested that further studies regarding T cell responses may be of clinical importance. Second, in preliminary experiments using mice, we compared T cell responses to SEiP and we found Vβ3+ and Vβ11+ T cells responded in a manner opposite to that observed after activation with SEA. In mice implanted with mini-osmotic pumps filled with SEiP, the Vβ11+CD4+ T cells exhibited a high level of expansion, whereas the Vβ3+CD4+ T cells exhibited a low level of expansion. These results strongly suggest that the binding affinities of the SEA-MHC class II complexes to TCRs are relatively strong for the TCR Vβ3 β chains and weak for the TCR Vβ11 β chains. In contrast, the binding affinities of the SEiP-MHC class II complexes to TCRs are likely to be relatively strong for the TCR Vβ11 β chains and weak for the TCR Vβ3 β chains. This speculation could be confirmed by measuring the binding affinities of the SEA-MHC class II complexes and of the SEiP-MHC class II complexes to the TCRs of SEA- or SEiP-reactive T cells fractions. The key sites of SEA and SEiP that determine the affinity levels could also be clarified by performing a number of experiments in parallel.

In the present study, a number of the questions posed above were examined in experiments using SEA, SEiP, and hybrid preparations and soluble forms of mouse Vβ3, Vβ11, and I-Aß in in vivo and in vitro systems. The expected results were obtained in experiments examining the binding affinities between the SEA/SEiP and I-Aß complexes and the TCR Vβ3/11 elements. The results also showed that the sites of the two SAGs corresponding to amino acid residue 206 of SEA determined the strength of the binding

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.
2 Both authors contributed equally to this work.
5 The abbreviations used are: SAG, superantigen; SEA, staphylococcal enterotoxin A; TSS, toxic shock syndrome; SEiP, staphylococcal enterotoxin-like type P; APC, antigen-presenting cell; LZ, leucine zipper; PE, phycoerythrin; RMD, resonant mirror detection; YPM, Y. pseudotuberculosis mitogen; TCR, T cell receptor; ANOVA, analysis of variance.

4 K. Imanishi, unpublished data.
affinity. Here, we discuss which factors at the key sites of these two SAGs determine the level of binding affinity.

EXPERIMENTAL PROCEDURES

Preparation of SEA, SElP, and Hybrids—Total DNA of Staphylococcus aureus was purified using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan). Escherichia coli plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. The wild-type SEA gene (sea) fragment was amplified from the genomic DNA of S. aureus FRI722 (11) using PCR with a primer set described by Hu et al. (12), then cloned into plasmid vector pGEM3Zf(+) (Promega, Madison, WI). This clone was named pKOA1. The construction of a mature form of the wild-type SElP gene (selp) encoding plasmid was named pKOP1. All SEA/SElP hybrid expression plasmids were produced using PCR-based site-directed mutagenesis according to the method described by Imai et al. (13). The primers used for site-directed mutagenesis are listed in Table 1. Briefly, pKOA1 and pKOP1 were amplified using Pyrobest polymerase (Takara Syuzo Co., Kyoto, Japan) using mutagenesis PCR primer sets. Each PCR product was self-ligated, then transformed with E. coli DH5α. The nucleotide sequences of all sea/selp hybrids were verified for both strands using 3–4 independent clones and an automatic DNA sequencer (ABI3100avant; Perkin-Elmer Applied Biosystems). The obtained sea/selp hybrid fragments were subcloned into a pGEX-6P-1 glutathione S-transferase (GST)-fusion expression vector (Amersham Biosciences Inc.) to construct hybrid-GST fusion protein expression plasmids. Also, wild-type SEA and SElP were expressed as GST fusion proteins. To construct the SEA expression vector pKAX1, the sea fragment was digested from pKOA1 with BamHI and EcoRI and subcloned into pGEX-6P-1 (12). The construction of the wild-type SElP expression plasmid pKPX1 has been described elsewhere (10). The wild-type SEA, SElP, and hybrids were expressed using an E. coli expression system and purified as described previously (10, 12, 14). Cultures of all E. coli strains intended for the GST-fusion protein expression analyses were performed at 18 °C. Fig. 1a shows the multiple alignments of the amino acids sequences of SEA and SElP. Because the mature form of SElP is equivalent to 3–233 of SEA according to signal peptide prediction (10), we assigned the position numbers based on SEA. b, schematic representations of the SEA/SElP hybrids. The stretches of SEA sequences are indicated by the open boxes, and the stretches of SElP sequences are indicated by the shaded boxes.

FIGURE 1. Multiple alignments of amino acids sequences of sea and selp (a) and schematic representations of hybrid SE molecules (b). The SEA/SElP variable regions close to the TCR binding sites (R1—4) are boxed (8), because the mature form of SElP is equivalent to 3–233 of SEA according to signal peptide prediction (10), we assigned the position numbers based on SEA. b, schematic representations of the SEA/SElP hybrids. The stretches of SEA sequences are indicated by the open boxes, and the stretches of SElP sequences are indicated by the shaded boxes.

TABLE 1

| Primers          | Oligonucleotides sequence (5’-3’) | For construction of |
|------------------|----------------------------------|---------------------|
| SEAtoPregA-S     | TCTTAGACAAACTTATTATCAATG         | SEA/R1-P            |
| SEAtoPregA-A5-S  | TTCTTTAAGCTTCCCTGC              | SEA/R1-P            |
| SEAtoPregH-S     | TATCTGATAGACTTATG                | SEA/R4-P            |
| SEAtoPregH-A5-S  | TTCTTTAAGCTTCCCTGC              | SEA/R4-P            |
| SEIPtoPregA-S    | TCTTTAAGCAATTTATTTATC           | SEIP/R1-A           |
| SEIPtoPregA-A5-S | TTCTTTAAGCTTCCCTGC              | SEIP/R1-A           |
| SEIPtoPregH-S    | TATCTGATAGACTTATG                | SEIP/R4-P           |
| SEIPtoPregH-A5-S | TTCTTTAAGCTTCCCTGC              | SEIP/R4-P           |
| SEAS206P-S       | CAAATACACTTGTGATGTG             | SEA206P             |
| SEAN207D-S       | TCAATACACTTGTGATGTG             | SEA207D             |
| SEAS206P-S       | CAAATACACTTGTGATGTG             | SEA206P             |
| SEAS207D-S       | TCAATACACTTGTGATGTG             | SEA207D             |
| SEAS206P-S       | CAAATACACTTGTGATGTG             | SEA206P             |
| SEAS207D-S       | TCAATACACTTGTGATGTG             | SEA207D             |
| SEAHX-A5-S       | ATACTGTCCTTAGGCCACAAAT          | SEAHX-SEA206P       |
| SEAS206P-A5-S    | TCAATACACTTGTGATGTG             | SEIP206S            |
| SEIP206S-S       | TCAATACACTTGTGATGTG             | SEIP206S            |
| SEIP206S-S       | TCAATACACTTGTGATGTG             | SEIP206S            |
| SEIP206S-S       | TCAATACACTTGTGATGTG             | SEIP206S            |

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extracellular domains of wild-type I-\(\text{A}\)\(^{\alpha}\) and I-\(\text{A}\)\(^{\beta}\) with an HLA-DR51 leader and E\(\alpha\)52–68 between the 3rd and 4th amino acid residues of the mature protein (15) were subcloned upstream of the sequences corresponding to an acidic leucine zipper (LZ) or a basic LZ with a C-terminal BirA recognition site, respectively. The resulting I-\(\text{A}\)\(^{\alpha}\)-LZ and I-\(\text{A}\)\(^{\beta}\)-E\(\alpha\)52–68-LZ-BirA nucleotide constructs were then inserted downstream of P10 or the polyhedrin promoter at Xhol-\(\text{Nsi}\)I or the BSSHII-Xbal site of pFASTBAC Dual (Invitrogen Corp., Carlsbad, CA), respectively. Proteins were produced using a baculoviral expression system (BAC-to-BAC; Invitrogen Corp.) according to the manufacturer’s instructions. The proteins produced in the culture superantigens of infected Sf9 cells were purified using an immunoaffinity technique with anti-I-\(\text{Ab}\) (Y3-P), followed by the manufacturer’s instructions. The proteins produced in the cultures were recovered using Percoll gradient (density, 1.055) centrifugation of mAb-treated cells.

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**Preparation of Cell Fractions**—Detailed procedures for the preparation of splenic lymphoid cells have been described in previous reports (16, 17). Briefly, T cells were obtained by treating C57BL/6 spleen cells with a combination of 28-16-8S, LR-1, and guinea pig serum. T cell-depleted spleen cells were obtained for use as APCs by treating C57BL/6 spleen cells with a combination of 28-16-8S and guinea pig serum. T cell-depleted spleen cells were further purified from T cell preparation using positive selection with CD4 MicroBeads and an autoMACSTM separator (Miltenyi Biotec, GmbH) in two cycles. Purified CD4\(^{+}\) T cells (> 98.5%) were cultured with anti-V\(\beta\)3-3-, anti-V\(\beta\)8-8-3, or anti-V\(\beta\)11-CD4\(^{+}\) mouse T cell blasts were prepared as follows. CD4\(^{+}\) T cells were further purified from T cell preparation using positive selection with CD4 MicroBeads and a combination of V\(\beta\)3-3- and V\(\beta\)8-8-3 and reverse primer CB (5’T-CTC- TAAAGCTTCTTCTGTAGGGCCTGAGG-G-G3’); the resulting cDNAs were cloned into a PCR2.1-TOPO vector (Invitrogen Corp.). The cloned cDNAs were sequenced using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). A DNA fragment encoding the mature form of the TCR \(\beta\) gene was cloned into the corresponding restriction sites of pEG-His1 (MoBiTec, Goettingen, Germany) and transformed into SoloPack Gold Supercompetent Cells (Stratagene, La Jolla, CA). Induction with IPTG resulted in the production of histidine-tagged tCR \(\beta\) chains, which were then purified by chelating with Sepharose 4B (GE Healthcare UK Ltd., Buckinghamshire, UK) preloaded with Ni\(^{2+}\) according to the manufacturer’s instructions. The sequences of the junctional regions of the TCR \(\beta\) chains used in this experiment are shown in Table 2.

**Preparation of T Cells with CFSE and Analysis of T Cell Division**—T cells were labeled with CFSE (Molecular Probes, Leiden, The Netherlands) as described by others (19, 20). Briefly, T cells were suspended in PBS at a concentration of 10\(^{7}\)/ml and CFSE was added to a final concentration of 10 mM. After 10 min of incubation at 37 °C, the cells were incubated in an RPMI 1640 culture medium containing 10% FCS and were expanded by incubating them in the presence of rIL-2 for 2 days. V\(\beta\)3\(^{+}\), V\(\beta\)8\(^{+}\), or V\(\beta\)11\(^{+}\)-CD4\(^{+}\) T cell fractions accounted for >98.0% of each fraction, respectively.

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**Analysis of Intracellular Signaling of SAG-stimulated T Cells**—Spleen cells were stimulated with different concentrations of SAG at 37 °C in a polystyrene round-bottom tube (BD-Falcon). The cultures were terminated by adding Fix Buffer 1 (BD PhosFlow) for 10 min at 37 °C and processed for flow cytomet-

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**Table 2**

| \(\text{V}\)\(^{\beta}\) | \(\text{V}\)\(^{\beta}\) | N-\(\text{D}\)\(_{\beta}\)-N | \(\text{J}\)\(^{\beta}\) |
|---|---|---|---|
| V\(\beta\)3 | CASS | PGPS | GNTLYGEGSRLIVY |
| V\(\beta\)8 | DAQN | NSPLYAAGGTRLTIVT |
| V\(\beta\)11 | CASS | PAP | AEQFFGPTRTLV |

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**References**

Osmotic Pump Implantation into Mice—The osmotic pumps were filled with SEs and implanted into the mice according to the pump manufacturer’s instructions (ALZA Corporation, Palo Alto, CA), as reported previously (18). Briefly, mice were anesthetized with an injection of 0.2 ml of 10% sodium pentobarbital, and a small subcutaneous incision pocket was created between the scapulae. Model 2001 mini-osmotic pumps pre-filled with a 0.2 ml-volume of SE were inserted into the subcutaneous pocket. The skin incision was closed with sutures, and penicillin G (20,000 units) was injected subcutaneously shortly thereafter.

Preparation of Cell Fractions—Detailed procedures for the preparation of splenic lymphoid cells have been described in previous reports (16, 17). Briefly, T cells were obtained by treating C57BL/6 spleen cells with a combination of 28-16-8S, LR-1, and guinea pig serum. T cell-depleted spleen cells were obtained for use as APCs by treating C57BL/6 spleen cells with a combination of HO13 and guinea pig serum. In the final step, viable cells were recovered using Percoll gradient (density, 1.055) centrifugation of mAb-treated cells.

V\(\beta\)3\(^{+}\), V\(\beta\)8\(^{+}\), or V\(\beta\)11\(^{+}\)-CD4\(^{+}\) mouse T cell blasts were prepared as follows. CD4\(^{+}\) T cells were further purified from T cell preparation using positive selection with CD4 MicroBeads and an autoMACSTM separator (Miltenyi Biotec, GmbH) in two cycles. Purified CD4\(^{+}\) T cells (> 98.5%) were cultured with anti-V\(\beta\)3-, anti-V\(\beta\)8-, or anti-V\(\beta\)11-mAb-coated plates in the presence of anti-CD28 in a 24-well culture plate (3047 Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). After 2 days of culture, the recovered cells were applied to a Percoll density gradient (1.055 and 1.075). The large blasts were obtained at the interface of 1.055 and 1.075 and were expanded by incubating them in the presence of rIL-2 for 2 days. V\(\beta\)3\(^{+}\), V\(\beta\)8\(^{+}\), or V\(\beta\)11\(^{+}\)-CD4\(^{+}\) T cell fractions accounted for >98.0% of each fraction, respectively.

Labeling of T Cells with CFSE and Analysis of T Cell Division—T cells were labeled with CFSE (Molecular Probes, Leiden, The Netherlands) as described by others (19, 20). Briefly, T cells were suspended in PBS at a concentration of 10\(^{7}\)/ml and CFSE was added to a final concentration of 10 mM. After 10 min of incubation at 37 °C, the cells were incubated in an RPMI 1640 culture medium containing 10% FCS and were expanded by incubating them in the presence of rIL-2 for 2 days. V\(\beta\)3\(^{+}\), V\(\beta\)8\(^{+}\), or V\(\beta\)11\(^{+}\)-CD4\(^{+}\) T cell fractions accounted for >98.0% of each fraction, respectively.

Analysis of Intracellular Signaling of SAG-stimulated T Cells—Spleen cells were stimulated with different concentrations of SAG at 37 °C in a polystyrene round-bottom tube (BD-Falcon). The cultures were terminated by adding Fix Buffer 1 (BD PhosFlow) for 10 min at 37 °C and processed for flow cytomet-

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**References**

—Male and female C57BL/6 mice were purchased from Japan S.L.C. (Hamamatsu, Japan). These mice were used as osmotic pump recipients or as sources of splen cells. The animal experiments performed in this study were approved by the ethical review committee on animal experiments of The Tokyo Women’s Medical University.
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ric extracellular and intracellular staining. Intracellular phospho-ERK1/2 staining was performed as suggested by the manufacturer of BD PhosFlow stain using Perm/Wash buffer I (BD Biosciences).

Flow Cytometry—Samples were examined using flow cytometry with an EPICS XL flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star, Inc., OR) (10). Three-color cytometric analyses of T-cell division and intracellular signaling events were performed by gating on CD4 and the appropriate TCR Vβ elements.

Binding Analysis by Resonant Mirror Detection—Protein-protein interactions were examined using resonant mirror detection (RMD) with the IAsys® system (Affinity Sensors, Cambridge, UK) (21, 22). In this study, the immobilized protein on the cuvette is referred to as the “ligand,” and the protein in the solution added to the cuvette is referred to as the “analyte.” The immobilization of proteins on the amino silane cuvette has been described in a previous paper (21, 22). The binding assays were conducted in PBS at 25 °C with constant stirring.

A kinetic analysis and calculation of the ratio of analyte binding to ligand were performed using an equation specified in a previous report (21, 22). The dissociation constant in the kinetic analysis (termed $K_{(D)}$) was calculated as $K_{(D)} = k_d/k_a$, where $k_a$ is the association rate constant, and $k_d$ is the dissociation rate constant. $K_{(D)}$ was determined from the mean $k_a$ and $k_d$ using 3–5 measurements. $K_{(D)}$ was confirmed using Scatchard plotting with the binding max ($R_{eq}$) and added molar concentration of the analyte. At least two cuvettes were used to determine the binding constants, and the derived values differed by less than 10% for the two measurements. The cuvettes were reused after being cleaned with 20 mM HCl. The original binding curves were replicated after washing, indicating the absence of a denaturing effect on the bound ligands.

To analyze the interaction between the T cells and SAG-MHC class II-molecules, $2 \times 10^7$ T cells were mixed in the presence or absence of 0.5 μg of SAG in 0.1 ml of PBS for 10 min at room temperature. The mixture was then added to Class II molecules immobilized on an amino silane cuvette. The cell suspension was gently stirred (12.6 Hz) using a Vibro-stirrer® in the cuvette. The binding was monitored as described above. The specific binding was calculated as follows: $R_{eq}$ (without superantigen) − $R_{eq}$ (with superantigen), where $R_{eq}$ was calculated using the software package FAST-fit®. When only 0.5 μg of SAG (without T cells) was added to the MHC class II molecules immobilized on the cuvette, no signal for SAG binding to the MHC class II molecules was observed (data not shown). The cuvettes were reused after cleaning with PBS containing 0.05% Tween-20, 20 mM HCl, and distilled water. The original binding curves were replicated after HCl washes, indicating that the washing procedure itself did not denature the bound ligands.

Computational Modeling of the Three-dimensional Structure of Mutated SEA—The secondary structure of mutated SEA was predicted using the PAPIA software package. The three-dimensional structure of SEA with SEA5206A and SEA5206P was predicted using multiple alignment and Swiss ModelTM software based on the structure of SEA (PDB ID: 1ho5). The electric potential was calculated using eF-surf software.

Statistical Analysis—Statistical comparisons were performed using the GLM procedure of the SAS system (SAS Institute, Cary, NC). The post hoc test with Scheffe option was used for comparison between each group for dose- or time-dependent experiments, and the paired t test was used for comparison within two groups. Differences were considered significant at levels of $p < 0.05$.

RESULTS

SEA and SElP Activate Mouse Vβ3+ T Cells and Vβ11+ T Cells in Quite Different Modes in Vivo—First, SEA and SElP, which exhibits a 78% amino acid homology with SEA and acts as a SAG on human T cells, were tested for mitogenic activity to spleen cells of B6 mice. As shown in Fig. 2, a comparable level of mitogenic activity was observed for the two SAGs over the examined doses.

Second, B6 mice were implanted with mini-osmotic pumps filled with 10 μg of SEA or SElP and examined for the expansion of reactive T cells for 20 days. In mice stimulated with SEA, the Vβ3+ CD4+ T cells exhibited a high level of expansion for more than 2 weeks, while the Vβ11+ CD4+ T cells exhibited a mild level of expansion, as reported previously (9). In contrast, in mice stimulated with SElP, the Vβ11+ CD4+ T cells exhibited a high level of expansion, while the Vβ3+ CD4+ T cells exhibited a low level of expansion (Fig. 3). SEA- and SElP-reactive CD8+ T cells exhibited only a transient expansion (data not shown), as reported previously (9). These results strongly suggest that the binding affinity of the complex of SEA-MHC class II molecules to TCR β chains is stronger for the Vβ3 β chain than for the Vβ11 β chain, and the binding affinity of the complex of SElP-MHC class II molecules to TCR β chains is stronger for the Vβ11 β chain than for the Vβ3 β chain.

In the following experiments, we focused on examining the key sites in the SEA and SElP molecules that determine the reactivity of T cells to these SAG and the binding affinities between complexes of SEA or SElP-MHC class II molecules and Vβ3 or Vβ11 TCR β chains.
Role of Residues at 206 and 207 of SEA and SElP in T Cell Activation—Previous studies have suggested that regions 1 and 4 (R1 and R4) of SEA, especially the latter, have large effects on the responses of Vβ3 + and Vβ11 + T cells (23–27). These two regions are likely involved in the responses of the Vβ repertoire to SElP. In the present study, our experiments confirmed the results of previous studies on SEA and our speculation regarding SElP. Because the mature form of SElP is equivalent to 3–233 of SEA, based on signal peptide prediction, we assigned the position numbers based on SEA (Fig. 1a). Differences in the amino acid sequences of SEA and SElP were only noted at residues 24 and 27 in R1 and at residues 206 and 207 in R4. The preparation of the SEA/SElP hybrids focused on these regions, as shown in Fig. 1b.

First, to confirm which residues in R1 and R4 of the two SAGs determine the responses of Vβ3 + and Vβ11 + T cells, mice were stimulated with SEA, SElP, SEA/R1-P, or SEA/R4-P (Fig. 4a), or SEA, SElP, SElP/R1-A, or SElP/R4-A (Fig. 4b) using the osmotic pump system and the Vβ3 + and Vβ11 + CD4 + T cell responses were examined. A SEA-type T cell response (dominant activation of Vβ3 + T cells over Vβ11 + T cells) was seen in mice stimulated with SEA, SEA/R1-P, or SElP/R4-A, while a SElP-type T cell response (dominant activation of Vβ11 + T cells over Vβ3 + T cells) was seen in mice stimulated with SElP, SElP/R1-A, or SEA/R4-P. These results indicate that residues 206 and 207 have critical roles in determining the SEA-type or SElP-type response.

Second, to examine which of residues 206 and 207 determines the two response patterns, mice were stimulated with SEA, SEA/R4-P, SEAα206P, or SEAN207D (Fig. 4c), or SElP, SElP/R4-A, SElPP206S, or SElPD207N (Fig. 4d). As shown in Fig. 4c, the T cell response induced by SEA markedly changed to a SElP-type T cell response with the replacement of residue 206 (serine) with the SElP-type amino acid (AA) (proline), irrespective of residue 207. A mild response was observed in both Vβ3 + and Vβ11 + T cells after the replacement of residue 207 alone. As shown in Fig. 4d, the SElP-type response changed to a SEA-type response with the replacement of both residues 206 and 207 with the SEA-type AAs. A mild response was induced in both Vβ3 + and Vβ11 + T cells by the replacement of residue 206 with the SEA-type AA. The replacement of residue 207 of SElP with the SEA-type AA had no effect on the SElP responses. These results suggest that residue 206 of both SEA and SElP has a more important role in the responsiveness of Vβ3 + and Vβ11 + T cells than residue 207.

Reproduction of SEA-type Response and SElP-type Response in Vitro—To analyze the differences in the responses to SEA and the SElP-type mutant SEAα206P, we reproduced the above...

FIGURE 3. TCR Vβ-dependent expansion of the SEA/SElP-reactive T cell fractions in mice implanted with SEA/SElP-pumps. C57BL/6 mice were implanted with pumps filled with 10 μg of SEA (a) or SElP (b) (three mice per group) and were analyzed individually to determine the percentage of SEA/SElP-reactive T cells among the splenic T cells. Vβ3 + CD4 + (□) and Vβ11 + CD4 + (●). a and b, effect of Vβ3 + T cells versus Vβ11 + T cells is significant at p < 0.05 using the ANOVA model.

FIGURE 4. TCR Vβ specificities of SEA, SElP, and hybrids. C57BL/6 mice were implanted with pumps filled with 10 μg of SEA, SElP, or a hybrid (three mice per group). a is SEA, SEA/R1-P, SEA/R4-P, or SElP-containing pumps into mice, b is SElP, SElP/R1-A, SElP/R4-A, or SEA-containing pumps into mice, c is SEA, SEAα206P, SEAN207D, or SEA/R4-P-containing pumps into mice, and d is SElP, SElPγ206S, SElPγ207N, or SElP/R4-A-containing pumps into mice. At 6 days after implantation, the percentages of Vβ3 + and Vβ11 + T cells among the CD4 + T cells of the spleen cells were determined using flow cytometry. *, p < 0.05 using the paired t test.

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*in vivo* responses using an *in vitro* experimental system. First, to compare the *in vitro* proliferative activity of Vβ3+ and Vβ11+ CD4+ T cells in response to SEA and SEAS206P, CFSE-stained mouse splenic CD4+ T cells were examined upon stimulation with graded doses of SEA and SEAS206P in the presence of APCs. When a CFSE-stained T cell divides, the intensity of the CFSE fluorescence decreases by one-half, thereby providing an accurate count of the cycles of cell division (19, 20). Data are presented as histograms of the divided cells on day 2 of stimulation (Fig. 5, a and b). Upon stimulation with SEA, the Vβ3+ T cells responded to a lower concentration of the stimulant than the Vβ11+ T cells. In contrast, upon stimulation with SEAS206P, the Vβ11+ T cells responded to a lower concentration of the stimulant than the Vβ3+ T cells.

Mitogen-activated protein kinases ERK1/2 are known to be activated by SAG (28). Thus, to compare the intracellular signaling, the percentages of phospho-ERK1/2-positive cells among the Vβ3+ and Vβ11+ T cells were examined upon stimulation with graded doses of SEA and SEAS206P in the presence of APCs. Upon stimulation with SEA, the percentage of phospho-ERK1/2-positive cells among the Vβ3+ T cells increased at a lower dose than that in the Vβ11+ T cells (Fig. 6a). Upon stimulation with SEAS206P, the results were opposite to those for SEA (Fig. 6b). The percentages of phospho-ERK1/2-positive cells among the Vβ3+ T cells, which are nonreactive to SEA, did not change upon stimulation with graded doses of SEA and SEAS206P (Fig. 6, a and b). These results indicate that we were able to reproduce the SEA-type and SEI-P-type responses observed *in vivo* by monitoring *in vitro* proliferation and intracellular signaling events.

**Analysis of Binding Affinities between TCR Vβ Element and SAGs**—Under physiological conditions, T cell activation by SAGs is observed only in the presence of MHC class II+ APC. However, T cell activation can be induced in the absence of

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**FIGURE 5.** SEA-induced T cell division in populations of CFSE-labeled murine splenic CD4+ T cells. Splenic CD4+ T cells were labeled with CFSE and cultured with graded doses of SEA (a) and SEAS206P (b) in the presence of APCs. On day 2, the cells from individual wells were harvested, stained with PE-conjugated anti-CD4, and PC5-stained appropriate anti-Vβ monoclonal antibodies, and then analyzed using flow cytometry. Histograms show the CFSE profiles of the indicated CD4+ and TCR Vβ-positive subsets of the CFSE-labeled T cells. The numbers appearing at the bottom of the histograms denote the concentration of SEA. The experiment depicted here is representative of more than three separate experiments.
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MHC class II\(^+\) APC using SAG that has been immobilized on a plastic surface (29). SAG reportedly binds directly to the TCR \(\beta\) chain of reactive T cell receptors (23, 29–32).

First, the binding affinities between soluble \(\beta3/\beta11\) \(\beta\) chains to SEA or the SEIP-type mutant SEAS206P were examined at the protein-protein level using IAsys based on RMD. The \(K_D\) value of SEA bound to the \(\beta3\) \(\beta\) chain was 4.6 \(\mu\)M and that of the \(\beta11\) \(\beta\) chain was 21.8 \(\mu\)M. The \(K_D\) values of SEAS206P bound to the \(\beta3\) \(\beta\) chain and the \(\beta11\) \(\beta\) chain were 24.6 \(\mu\)M and 10.2 \(\mu\)M, respectively. No binding signals were observed in the negative controls: both SEA and SEAS206P to the \(\beta3\) \(\beta\) chain and the \(\beta3/\beta11\) \(\beta\) chain were equal (0.17 \(\mu\)M and 0.10 \(\mu\)M, respectively). \(\beta3/\beta11\) and \(\beta8/\beta11\) T cell blasts induced in \(\textit{vivo}\) were added to I-A\(^b\)-immobilized on cuvettes with or without SEA or SEAS206P (Fig. 7). The binding of \(\beta8\) \(\text{CD4}\)^+ T cells to I-A\(^b\) with or without SEA or SEAS206P were used as a control. The binding of the 3 T cell preparations to I-A\(^b\) was similarly low in the absence of SEA or SEAS206P. Much higher responses were observed in the presence of either SEA or SEAS206P. Comparisons of the experimental and control data showed that the specific binding of T cells to SEA-MHC class II was higher in the \(\beta3\) \(\text{CD4}\)^+ T cells than in the \(\beta11\) \(\text{CD4}\)^+ T cells (Fig. 7a), and the specific binding of T cells to SEAS206P-MHC Class II was higher in the \(\beta11\) \(\text{CD4}\)^+ T cells than in the \(\beta3\) \(\text{CD4}\)^+ T cells (Fig. 7b). Taken together, these results indicate that the dominance of the \(\beta3\) \(\beta\) chain over the \(\beta11\) \(\beta\) chain responses induced by SEA is reflected by the higher binding affinity of SEA to the former cell type, compared with the latter.

**DISCUSSION**

In our previous report, we reported a difference in the responses of SEA-reactive \(\beta3\) \(\text{CD4}\)^+ T cells and \(\beta11\) \(\text{CD4}\)^+ T cells upon stimulation with SEA in \(\textit{vivo}\) (9). In the present study, we showed that the mitogenic activity of SEIP was similar to that of SEA (Fig. 2) and that, contrary to SEA stimulation, the continuous exposure of mice to SEIP induced a high level of the protracted expansion of \(\beta11\) \(\beta\) chain T cells and a low level of the protracted expansion of \(\beta3\) \(\beta\) chain T cells (Fig. 3). We speculated that these responses might be caused by the different affinities of the TCR \(\beta\) chains to the SAG-MHC class II complex. The experiments performed in the present studies support our speculation.

First, using an \(\textit{in vivo}\) system (mini-osmotic pumps), we showed that amino acid residues 206 and 207 of SEA and SEIP were important for \(\beta\) specificity (Fig. 4, a and b). This result agrees well with the results of previous reports studying the residues that determine the \(\beta\) specificity of SEA and SEE (23–27). A study of the crystal structure of SEA suggested the presence of four structural components including residues 200–207 in a putative SEA-TCR binding groove (33). SEA induces the dominant activation of \(\beta3\) \(\beta\) T cells over \(\beta11\) \(\beta\) T cells, while SEE, which has a high homology of amino acid sequences with SEA, activates \(\beta11\) \(\beta\) T cells but not \(\beta3\) \(\beta\) T cells (23, 24). In an \(\textit{in vitro}\) culture system, SEA in which residues 200–207 were exchanged for the SEE-type amino acids induced the dominant activation of \(\beta11\) \(\beta\) T cells over \(\beta3\) \(\beta\) T cells, while SEE in which residues 200–207 were exchanged for the SEA-type amino acids induced the dominant activation of \(\beta3\) \(\beta\) T cells over \(\beta11\) \(\beta\) T cells (24). In the present study, we showed that, between the two residues, residue 206 of both SEA and SEIP had a more important role in \(\beta\) specificity. SEA in which residue 206 was exchanged for the SEIP-type amino acids induced a SEIP-type response (dominant activation of \(\beta11\) \(\beta\) T cells over \(\beta3\) \(\beta\) T cells), while SEA in which residue 207 was exchanged for the SEIP-type amino acids induced a SEIP-type response (dominant activation of \(\beta11\) \(\beta\) T cells over \(\beta3\) \(\beta\) T cells), while SEA in which residue 207 was exchanged for the SEIP-type amino acids induced a SEIP-type response (dominant activation of \(\beta11\) \(\beta\) T cells over \(\beta3\) \(\beta\) T cells), while SEA in which residue 207 was exchanged for the SEIP-type amino acids induced a SEIP-type response (dominant activation of \(\beta11\) \(\beta\) T cells over \(\beta3\) \(\beta\) T cells), while SEA in which residue 207 was
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It is important to discuss the meanings of the present study in the pathogenic mechanism of the SAG-induced infectious diseases such as TSS (3) and systemic infection with *Yersinia pseudotuberculosis* (4). T cell activation by SAGs has been implicated in the pathogenesis of them through massive production of various cytokines. In cases with TSS, a massive protracted activation was detected in Vβ2+ T cells, irrespective of CD4+ and CD8+ T cell subsets, by flow cytometric analysis (34, 35). In cases with systemic infection with *Y. pseudotuberculosis*, preferential activation was detected in Vβ3+ T cells from 3 Y. pseudotuberculosis-mitogen (YPM)-reactive T cell subpopulations in the later phase of the disease by a PCR analysis (36). Viewing these responses in patients, it seems reasonable to speculate that, irrespective of the difference of CD4+ and CD8+ subsets, Vβ2+ and Vβ3+ T cells have high affinity interaction to TSS-T and YPM respectively and are mainly involved in generation of pathological changes of the diseases.

The affinities between the TCR β chains and SEA or between the T cells and SEA-MHC class II complexes were changed by replacing the serine at residue 206 with proline. What happens to SEA when the serine at residue 206 is replaced with proline? Serine and proline have notably different effects on protein structure. Serine has an uncharged polar side chain but a polar hydroxyl group, while proline has a nonpolar side chain and its presence creates a fixed kink in the protein chain. To clarify the role of amino acid residue 206 in SEA and its effect on the responses of Vβ3+ and Vβ11+ CD4+ T cells, we created two SEA mutants, SEAS206A and SEAS206T, and examined the response of Vβ3+ and Vβ11+ T cells to the administration of these SEA mutants using an osmotic pump system (supplemental Fig. S1). Replacing residue 206 of SEA with alanine, which does not have a polar hydroxyl group, caused some effect on Vβ reactivity. In contrast, replacing residue 206 of SEA with threonine, which has a hydroxyl group, did not have any effect on Vβ reactivity. These results demonstrated that the hydrogen group in the side chain of serine and threonine is likely a key residue affecting the binding specificity of SEA to Vβ3. Regarding the three-dimensional structure of SEA (PDB ID: 1l05, shown in supplemental Fig. S2), the serine at residue 206 is located in a hole formed by a cluster of tyrosine residues (91YYGY), similar to the structure observed in SEB (37). The mutant SEAS206P, which result in a remodeling of wild-type SEA as predicted using the Swiss Model, also has a pocket structure formed by tyrosine residues. However, replacing residue 206 of SEA with proline forms a hydrophobic surface. Also, the electric potential of the area of residue 206 on the SEA surface was dramatically changed to a negative charge by the replacement with proline (supplemental Fig. S2B). These changes may be important for the binding specificity of SEAS206P to Vβ11. The results of kinetic analyses have shown that differences in

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Replaced serine 206 of SEA with proline, formed a hydrophobic surface. Also, the electric potential of the area of residue 206 on the SEA surface was dramatically changed to a negative charge by the replacement with proline. These changes may be important for the binding specificity of SEAS206P to Vβ11. The results of kinetic analyses have shown that differences in

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The present data are compatible with our recent report (10). Using in vitro experiments, we showed that SEA activates the Vβ3+ CD8+ T cells more strongly than Vβ11+ CD8+ T cells and induces a greater degree of superantigen-dependent cell-mediated cytotoxicity in Vβ3+ CD8+ T cells than in Vβ11+ CD8+ T cells in induction and effector phases. And SEAS206P does the opposite.

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exchange for the SEIP-type amino acids activated both Vβ3+ T cells and Vβ11+ T cells at similar levels (Fig. 4c). For SEIP, residues 206 and 207 of the SEA-type amino acids were required to induce a SEA-type response (dominant activation of Vβ3+ T cells over Vβ11+ T cells). SEIP in which residue 206 was exchanged for the SEA-type amino acids activated both Vβ3+ T cells and Vβ11+ T cells at similar levels, while SEIP in which residue 207 was exchanged did not affect the Vβ specificity of SEIP (Fig. 4d). These in vivo SEA-type and SEIP-type responses were reproduced using in vitro proliferation analyses (Fig. 5) and intracellular signaling events (Fig. 6) using SEA and the SEIP-type mutant SEAS206P.

Second, to demonstrate that the above results were caused by the different affinities of the TCR Vβ chains to the SAG-MHC class II complex, we measured the binding affinities between SAG and the TCR Vβ chains at the protein-protein level and between the CD4+ T cells and the SAG-MHC class II complexes at the protein-cell level. As expected, the Vβ3 β chain bound more strongly to SEA than to SEAS206A and the Vβ11 β chain bound more strongly to SEAS206P than to SEA (Table 3). The results obtained at the protein-cell level were consistent with those obtained at the protein-protein level. The Vβ3+ CD4+ T cells combined more strongly with the SAG-MHC class II complexes than the Vβ11+ CD4+ T cells (Fig. 7a). In contrast, the Vβ11+ CD4+ T cells combined more strongly with the SEAS206A-MHC class II complexes than the Vβ3+ CD4+ T cells (Fig. 7b). A significant correlation between the binding affinity of SEC3 to TCR and the T cell response to SEC3 immobilized on a plastic surface has been reported (29). Together, these results suggest that a high binding affinity between SAG and the TCR β chains induces strong intracellular signaling events in T cells, leading to their proliferation not only in vitro, but also in vivo in the presence of APCs.

The present data are compatible with our recent report (10). Using in vitro experiments, we showed that SEA activates the Vβ3+ CD8+ T cells more strongly than Vβ11+ CD8+ T cells and induces a greater degree of superantigen-dependent cell-mediated cytotoxicity in Vβ3+ CD8+ T cells than in Vβ11+ CD8+ T cells in induction and effector phases. And SEAS206P does the opposite.

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FIGURE 7. In vitro T cell binding to MHC class II molecules with SEA. Mouse Vβ3+, Vβ11+, and Vβ8+ CD4+ T cell blasts were added to I-Aα immobilized cuvettes in the presence or absence of SEA (a), and in the presence or absence of SEAS206P (b). The experiment depicted here is representative of two separate experiments.
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$K_{(D)}$ values depend on the dissociation rate constants, $k_d$, but the association rate constants were not significantly different. Thus, the accessibilities of wild-type SEA and SEA+S206P are almost the same (Table 3); however, the binding of SEA to TCR may cause some structural changes in the TCR receptor. Further analysis of the SEA-TCR complex using x-ray crystallography should provide a structural basis for the Vβ specificity of SEA.

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