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*J Immunol* 1998; 161:1257-1266;
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Staphylococcal Enterotoxin A Induces Survival of V<sub>H</sub>3-Expressing Human B Cells by Binding to the V<sub>H</sub> Region with Low Affinity

Rana Domiati-Saad and Peter E. Lipsky

Staphylococcal enterotoxins (SE) are bacterial superantigens that bind to MHC class II molecules and to the V<sub>β</sub>-chain of the TCR, and subsequently activate T cells expressing specific V<sub>β</sub> regions. In this study, we have studied the effects of SEA on human B cell activation, and specifically the capacity of SEA to function as a B cell superantigen in vitro. We show herein that SEA failed to induce B cell proliferation and differentiation in the absence of T cells. However, SEA induced survival of B cells uniquely expressing V<sub>H</sub>3-containing IgM, independently of light chain utilization. The sequences of V<sub>κ</sub>3 IgM gene products were determined and found to include a number of members of the V<sub>H</sub>3 family with a variety of different D and J<sub>H</sub> gene segments. Analysis of the sequences of V<sub>H</sub>3 gene products revealed possible sites in framework region 1 and/or framework region 3 that could be involved in SEA-mediated activation of V<sub>H</sub>3-expressing B cells. Binding studies showed that SEA interacts with the V<sub>H</sub>3 domain of Ig with low, but detectable affinity. These results indicate that SEA functions as a B cell superantigen by interacting with V<sub>H</sub>3 gene segments of Ig.

CDR3 or the associated heavy chain (34). These observations suggest the presence of V_{H}^{\text{D}} and V_{K}-specific positive and negative selection of B cells, a process that could be mediated by a superantigen-like mechanism.

We have recently provided evidence that staphylococcal enterotoxin D (SED) functions as a B cell superantigen (35). Moreover, we have proposed a novel mechanism of B cell repertoire selection involving SED-mediated V_{H}^{\text{D}}-specific rescue from B cell apoptosis. The mechanism of rescue of V_{H}^{\text{D}}-expressing B cells appeared to relate to the capacity of SED to counter apoptosis initiated by its engagement of HLA-DR by simultaneously binding the VH 4 complementarity-determining region (CDR3) or the associated heavy chain (34). These observations suggest that SED can rescue from B cell apoptosis initiated by its engagement of HLA-DR by simultaneously binding the VH 4 CDR3 or the associated heavy chain (34). These observations suggest that SED can rescue from B cell apoptosis initiated by its engagement of HLA-DR by simultaneously binding the VH 4 CDR3 or the associated heavy chain.

**Materials and Methods**

**Cell preparation and purification**

Mononuclear cells were prepared by centrifugation of heparinized venous blood of normal healthy donors over sodium diatrizoate/Ficoll gradients (Sigma, St. Louis, MO). PBMC were separated into T cell-enriched and B cell-enriched populations, as previously described (35). The resultant B cell population was >90% CD20+ B cells and <3% CD3+ T cells.

**Cell medium**

All cultures were conducted in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with penicillin G (200 U/ml), gentamicin (10 \mu g/ml), l-glutamine (0.3 mg/ml), and 10% FCS (Life Technologies).

**Culture conditions**

B cells (4 x 10^6/well) were cultured in U-bottom 96-well microtiter plates in the presence or absence of mitomycin C-treated T cells (1 x 10^5/well) and 100 ng/ml of SEDA (a generous gift of Dr. David Karp, The University of Texas Southwestern Medical Center at Dallas). Extensive dose-response curves were conducted with SEDA, and 100 ng/ml was found to be optimal for T cell-dependent B cell proliferation. All cultures were supplemented with 20 U/ml of IL-2 (Hoffmann-La Roche, Nutley, NJ). All cultures were conducted in triplicate. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. After different lengths of culture, B cells were analyzed for activation marker expression, DNA synthesis, Ig production, or mRNA expression.

**Assay of B cell DNA synthesis**

In all assays of DNA synthesis, cultures were incubated for 96 h at 37°C. During the last 18 h of culture, 1 \muCi of [\text{H}]Tdr (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA) was added to the cultures. The cells were harvested onto glass filters, and [\text{H}]Tdr incorporation was measured by liquid scintillation counting.

**Activation marker expression**

For the measurement of activation marker expression, B cells (4 x 10^6/well) were cultured in U-bottom 96-well microtiter plates with 100 ng/ml of SEDA and 20 U/ml of IL-2 for 48 h. Cells were harvested and washed twice in staining buffer containing PBS supplemented with 2% normal human serum. The cells were pelleted by centrifugation and resuspended in 100 \mu l of phycoerythrin (PE)-conjugated anti-CD25 (Becton Dickinson, San Jose, CA), PE-conjugated anti-CD69 (PharMingen, San Diego, CA), or PE-conjugated irrelevant isotype-matched mouse IgM as a control. The cells were incubated for 30 min on ice, washed, and analyzed by flow cytometry with the use of the FACScan (Becton Dickinson).

**Detection of Ig**

After 11 days of cultures, cell-free culture supernatants were removed from each well and analyzed for the presence of IgM, IgG, and IgA by ELISA, as previously described (36).

**RNA extraction**

Cell pellets were harvested after different lengths of culture. Total RNA was extracted by using the Rneasy mini kit (Qiagen, Chatsworth, CA), according to the manufacturer’s instructions.

**cDNA synthesis and PCR amplification**

Total RNA was reverse transcribed into first strand cDNA using 10 pmol of random hexamers in a 40-\mu l reaction (37). mRNA was analyzed by RT-PCR. Each PCR reaction was performed as described previously (35). For V_{H} expression, the 5′ primers corresponded to each of the leader sequences of the six major V_{H} families, and the 3′ primers annealed to the heavy chain C region. For light chain expression, the following Cκ and Cλ primers were used: 5′ Cκ primer, 5′-GGTACCTCGAG GGCGAG-3′; 3′ Cκ primer, 5′-GTTCATCTTCCCGCATT-3′; 5′ Cλ primer, 5′-CCTCTGAGAGGCTTACAAGC-3′; and 3′ Cλ primer, 5′-TGTCATCCCTGCCGTTGCTCC-3′.

PCR products were separated on a 1.2% agarose gel and transferred onto a nylon membrane. Southern blotting was conducted with the appropriate [\alpha-\text{32}P]ATP 5′ end-labeled probe: a consensus Igκ region, 5′-ACCT GAGGAGACCGTGACAGGGT-3′; Cκ probe, 5′-GGTGATACGCG CCTCAATGC-3′; or Cλ probe, GTGGCCTGGAAGCACAGAT-3′. Multiple dilutions of template cDNA were used to demonstrate that the analysis of VH mRNA was semiquantitative, in that the amount of PCR product varied with the amount of template cDNA.

**Subcloning and sequencing of PCR products**

The V_{H}3 Igκ PCR products were purified and subcloned as described previously (35). PCR products were sequenced using the DyeDeoxy Termination Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The VH gene sequences were analyzed using Geneworks software (release 2.3.1; Intelligenetics, Mountain View, CA) and the GenBank and EMBL databases. Assignment of D gene segments was done according to the criteria of Mortari et al. (38). In brief, a D gene segment was identified when it shared at least five consecutive nucleotides with a known D gene segment or six nucleotides of sequence identity interrupted by no more than a one nucleotide substitution.

**Construction of the FabD1.3 myc plasmid with a His6 peptide tag**

The FabD1.3 myc plasmid (generous gift of Dr. E. Sally Ward, The University of Texas Southwestern Medical Center at Dallas) (39) was used to express the cloned V_{H}3 and V_{H}4 gene products as F(\text{ab'})_{2} fragments. This plasmid consists of linked cassettes of a mouse V_{H} and a human C_{H}1 domain with a myc tag coupled to a mouse \kappa and a human \lambda domain. Each cassette is preceded by the pelB sequence from Erwinia carotovora that facilitates secretion of the expressed protein into the periplasm of transformed Escherichia coli, BMH71-18 cells (40). The plasmid also contains the lacZ promoter directly upstream of both cassettes that induces polycistronic transcription of each encoded protein coordinately when the transformed E. coli are induced with isopropyl-\beta-D-thiogalactopyranoside (IPTG). Finally, an ampicillin-resistance gene is included in the plasmid for selection. IPTG induction of transformed E. coli stimulates production of properly assembled F(\text{ab'})_{2} fragments that can be identified by Western blotting with an anti-myc mAb (41). A His6 peptide tag was introduced into the 3′ end of the Cκ portion of the FabD1.3 myc plasmid by PCR. Each PCR reaction was performed with 0.5 \mug of FabD1.3 myc plasmid DNA in a final volume of 100 \mu l containing 1 \mu M of primers, 200 \mu M of each dNTP, 10 mM KCl, 10 mM (NH_4)_2SO_4, pH 8.8, 2 mM MgSO_4, and 2 U Deep Vent DNA polymerase (New England Biolabs, Beverly, MA). The primers used were the following: V_{H}2BACK, 5′-GACATTGAAC TCACCCAGTCTCCA-3′; and His6 tag, 5′-ATCAGAATTCTTTATTAGTGATGGTAGTGGTAGTGCATCTTCCCCCGGTTGAAAGCTTT-3′. The V_{H}2BACK primer anneals to the human \kappa region of the FabD1.3 myc plasmid, and the His6 tag primer anneals to the 3′ end of the plasmid and generates a His6 tag. The PCR product was restricted with XhoI and EcoRI, gel purified, ligated into the XhoI-EcoRI cut and dephosphorylated FabD1.3 myc plasmid to create the FabD1.3 mychis6 plasmid in which the His6 tag was just 3′ of the Cκ domain. E. coli BMH71-18 cells were transformed with the FabD1.3 mychis6 plasmid, and then a single transformant was selected, and the plasmid containing the insert was sequenced to insure its proper orientation.

**Recombinant V_{H}3 F(\text{ab'})_{2}**

The FabD1.3 mychis6 plasmid was used for expression of V_{H}3- and V_{H}4-containing F(\text{ab'})_{2} fragments with a His6 peptide tag that allowed purification of the expressed F(\text{ab'})_{2} fragments with nickel-NTA-agarose. The
VH3 gene products A3-H2 and A3-K1, and the VH4 gene products 83-9E6 and 83-6B3, previously cloned into the pGEM vector, were recloned into the FabD1.3 mycHis6 plasmid to replace the VpD1.3 gene using the following steps. First, the major part of the VH3 or VH4 genes was amplified using Deep Vent DNA polymerase, as described above, and using the following primers: FR1-3-23, 5′-ATACCTGCAGGATCTGGGGGGGAGGG-3′, and 1p-BstEII, 5′-ATCAGGTGACCCAGGGTCCCTGGCC-3′ for the amplification of A3-K1 and A3-H2; FR1-4-34, 5′-ATCTTCGACTG GGGGCCGGAGGA-3′ and 1p-BstEII for the amplification of 83-9E8; and FR1-4-59, 5′-ATCAGGGTACCACGGTTCCCTGGCC-3′ and 1p-BstEII for the amplification of 83-6B3. The PCR products were then digested with PstI and BstEII. The fragments were gel purified and ligated into a PstI-BstEII-digested FabD1.3 mycHis6 plasmid. The VH3 or VH4 genes replaced 95% of VHD1.3 gene, leaving eight to nine nucleic acids at the 3′ and 5′ ends. The appropriate DNA fragments, and the plasmid containing the insert was sequenced.

E. coli. BMH71-18 cells were transformed with the plasmid containing E. coli genes. SEII-digested FabD1.3, leaving eight to nine nucleic acids at the 3′ and 5′ ends of the mouse VH1 gene, which were identical to those of the human VH1 genes.

**Results**

SEA stimulates B cells expressing VH3 gene segments independently of light chain utilization

The first set of experiments was performed to determine whether SEA stimulation induced biased expression of VH3 families. Highly purified B cells were cultured with recombinant SEA and IL-2. After 9 days in culture, IgM mRNA was analyzed by RT-PCR for the distribution of VH3 families. SEA-stimulated B cells expressed the VH3 gene segments only. In unstimulated B cell cultures, IgM mRNA could not be amplified, presumably because of death of B cells after prolonged culture without a stimulus. In contrast, freshly isolated B cells expressed all VH gene products (Fig. 1A). mRNA obtained from SEA-stimulated B cells was also analyzed for the presence of light chain gene products. Both κ and λ light chain mRNA were expressed by SEA-stimulated B cells (Fig. 1B).

**Affinity measurements**

Kinetic measurements were made with the plasmon resonance apparatus (BIAcore 2000; BIAcore, Piscataway, NJ). The limits of accurate determinations of kinetic rate constants of this instrument are $10^{-3} - 10^4 \text{ M}^{-1} \text{s}^{-1}$ for association, and $10^{-3} - 10^3 \text{ s}^{-1}$ for dissociation. Sequential binding was used to measure the on and off rates for the binding of SEA, SED, and SPA (Calbiochem-Behring, La Jolla, CA) to the recombinant VH F(ab′)2. To accomplish this, a mouse anti-human κ light chain mAb (Zymed, San Francisco, CA) was resuspended in 10 mM sodium acetate, pH 4.5, to a concentration of 20 μg/ml and covalently bound to the sensor chips (Sensor Chip CM5; BIAcore) using the BIAcore Amine Coupling Kit, according to the manufacturer’s instructions. The amount of protein bound to the sensor chip was about 2700 resonance units. The recombinant VH F(ab′)2, SEA, SED, and SPA, were diluted in running buffer (HEPES-buffered saline containing 150 mM NaCl, 0.005% Surfactant 20, 10 mM HEPES at pH 7). The recombinant VH F(ab′)2 were injected over the anti-human κ mAb covalently bound to the sensor chip at a flow rate of 5 μl/min or 10 μl/min. SEA or SED was then injected at a flow rate of 10 μl/min. SPA was injected at a flow rate of 5 μl/min. Association and dissociation analyses were conducted in the running buffer. All binding experiments were performed at room temperature. Pulses of 10 mM glycine, pH 2, were used to regenerate the sensor chip surfaces. To determine non-specific binding, the analytes were injected over two flow cells on a single chip, one that had immobilized ligand and the other without an immobilized ligand.

Data sensograms showing relative resonant unit changes caused by injection, buffer changes, and binding of ligand were analyzed to calculate association and dissociation rate constants using the BIAevaluation 2.1 program. The dissociation rate constant (koff) was determined by the AB = A + B regression model to fit the slope of the ln(Re/Ro) vs time plot. The association constant (kass) was calculated using the same model, which assumes a known koff. The AB = A + B regression model is a regression analysis for a first order kinetic reaction. The equilibrium association constant (Keq) was calculated as the dividend of kass over koff. The equilibrium dissociation constant (K′eq) was calculated as the dividend of koff over kass.
**SEA fails to induce B cell proliferation and differentiation**

Human B cells that were cultured with recombinant SEA and IL-2 neither proliferated nor produced measurable amounts of Ig (Fig. 2, A and B). However, SEA-stimulated B cells expressed the activation markers, CD25 and CD86 (Fig. 2C), to a modest degree. Of note, B cells stimulated with SEA in the presence of T cells both proliferated and secreted Igs (Fig. 2, A and B), although the response was not restricted to B cells expressing specific V<sub>H</sub> families (data not shown).

### Table 1. Characteristics of SEA-induced V<sub>H</sub>3 gene segments<sup>a</sup>

<table>
<thead>
<tr>
<th>Clone</th>
<th>Germline Gene</th>
<th>Homology (%)</th>
<th>J&lt;sub&gt;H&lt;/sub&gt;</th>
<th>D</th>
<th>CDR3 Length (aa)</th>
</tr>
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<tbody>
<tr>
<td>A3-K1</td>
<td>3-23/DP-47</td>
<td>99.3</td>
<td>6</td>
<td>D21/9</td>
<td>21</td>
</tr>
<tr>
<td>A3-M2</td>
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<td>98.6</td>
<td>4</td>
<td>D21/10</td>
<td>11</td>
</tr>
<tr>
<td>A3-H5</td>
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<td>97.0</td>
<td>4</td>
<td>DN1</td>
<td>14</td>
</tr>
<tr>
<td>A3-E6</td>
<td>-/-</td>
<td>96.0</td>
<td>6</td>
<td>D5</td>
<td>13</td>
</tr>
<tr>
<td>A3-H2</td>
<td>-/-</td>
<td>96.0</td>
<td>5</td>
<td>DHQ52</td>
<td>9</td>
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<tr>
<td>A3-C1</td>
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<td>4</td>
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<tr>
<td>A3-C9</td>
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<td>4</td>
<td>DN1</td>
<td>12</td>
</tr>
<tr>
<td>A3-J2</td>
<td>-/-</td>
<td>97.6</td>
<td>4</td>
<td>DXP'1</td>
<td>11</td>
</tr>
<tr>
<td>A3-D3</td>
<td>-/-</td>
<td>96.3</td>
<td>4</td>
<td>Unknown</td>
<td>6</td>
</tr>
<tr>
<td>A3-F5</td>
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<td>84.7</td>
<td>6</td>
<td>invDHQ52</td>
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<tr>
<td>A3-E4</td>
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<td>98.6</td>
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<tr>
<td>A3-E5</td>
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<td>4</td>
<td>DXP'1</td>
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<td>6</td>
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<tr>
<td>A3-E2</td>
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<td>93.9</td>
<td>5</td>
<td>DXP'1</td>
<td>18</td>
</tr>
<tr>
<td>A3-J2</td>
<td>-/-</td>
<td>92.8</td>
<td>2</td>
<td>DA1/DA4</td>
<td>17</td>
</tr>
<tr>
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<td>100.0</td>
<td>4</td>
<td>DXP'1</td>
<td>11</td>
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<td>6</td>
<td>DK1</td>
<td>15</td>
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<tr>
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<td>4</td>
<td>D21/10</td>
<td>9</td>
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<td>-/-</td>
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<td>6</td>
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<td>3</td>
<td>D/P/A1</td>
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<td>A3-H6</td>
<td>3-49RB</td>
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<td>4</td>
<td>D23/7</td>
<td>19</td>
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<tr>
<td>A3-E7</td>
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<td>89.1</td>
<td>6</td>
<td>DM2</td>
<td>13</td>
</tr>
<tr>
<td>A3-K3</td>
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<td>4</td>
<td>invDK1</td>
<td>21</td>
</tr>
<tr>
<td>A3-7</td>
<td>-/-</td>
<td>89.3</td>
<td>5</td>
<td>D2</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> SEA-stimulated B cell clones were aligned to their respective germline genes. Homology was calculated by similarity at the nucleic acid level. All D segments are listed that appear in the CDR3; the suffix inv indicates an inverted D segment.

The Ig V<sub>H</sub> gene repertoire expressed by SEA-stimulated B cells consists of a diverse array of V<sub>H</sub>3 gene segments

To determine whether the IgM V<sub>H</sub>3 repertoire expressed by B cells stimulated with SEA had the characteristics of an Ag- or superantigen-driven response, the V<sub>H</sub> regions were cloned and sequenced. Superantigen would be anticipated to stimulate B cells expressing different members of a V<sub>H</sub> family, but with no similarity in D, J<sub>H</sub>, CDR1, CDR2, or CDR3. Analysis of the V<sub>H</sub>3 gene segments expressed by SEA-stimulated B cells revealed the usage of different members of the V<sub>H</sub>3 family (Table I). V<sub>H</sub>3-23/DP-47 was the most frequently utilized, followed by V<sub>H</sub>3-53/DP-42, V<sub>H</sub>3-07/DP-54, V<sub>H</sub>3-15/DP-38, V<sub>H</sub>3-11/DP-35, V<sub>H</sub>3-30/Cos-3, V<sub>H</sub>3-49RB, and DP-58. Some of the V<sub>H</sub>3 sequences were in germ-line configuration, whereas others were mutated (Fig. 3).

Diversity of the CDR3 region of the V<sub>H</sub> genes expressed by SEA-stimulated B cells was noted. Thus, different J<sub>H</sub> and D gene segments (Table I) were used. All six of the known J<sub>H</sub> gene segments were used by the V<sub>H</sub> genes expressed by SEA-stimulated B cells. J<sub>H</sub>4 was most commonly used, followed by J<sub>H</sub>6. The other J<sub>H</sub> gene segments were used less frequently. The distribution of the J<sub>H</sub> length of the CDR3 was highly diverse, and no common motif was discerned. Similarity of amino acids with only nonconservative substitutions was found in FR1 between residues 1–12 and 14–27, in most of FR2 except its 3’ end, in the 3’ end of CDR2 between residues 63–65, and in most of FR3 except for residues 74 and 93–94 (Fig. 4). Because SEA does not stimulate V<sub>H</sub>4-expressing B cells, comparison of the 24 SEA V<sub>H</sub>3-IgM sequences with the known sequences of the most commonly employed V<sub>H</sub>4 family...
members (VH4-34, VH4-39, and VH4-59) was undertaken to identify specific regions of VH3 that might account for SEA stimulation. The comparison shows that the sequences of SEA-induced VH3 gene products differed only modestly with the VH4 family members at residues 1–12 (except 9), 14, 17–26 (except 23), 36–48 (except 40), 63–70 (except 67), and 86–90, making it unlikely that these were related to SEA stimulation. However, the conserved VH3 amino acids in FR1 (9, 15, 16, 23, 27) and in FR3 (71–84, except 72, 80, 82c) were considerably different from the corresponding amino acids of the VH4 genes, indicating these regions might be required for SEA stimulation. These data suggest that the regions critical for SEA responses might lie either in FR1 or in FR3 of VH3. Moreover, the Ag-binding CDR1 and CDR2 regions do not appear to contribute to SEA stimulation, as anticipated from its action as a B cell superantigen.

SEA binds to VH3 F(ab’)_2 with low affinity

Surface plasmon resonance studies were conducted to determine whether SEA bound directly to VH3, and to assess the kinetics and affinity of this interaction. For these studies, recombinant VH3 F(ab’)_2 fragments were used containing VH3 segments cloned from SEA-stimulated B cells. As a control, recombinant VH4-containing F(ab’)_2 fragments were analyzed. As an additional comparison, the interaction of SPA and the VH3 F(ab’)_2 was also analyzed. The VH3 region of A3-K1 F(ab’)_2 was 99.3% homologous to the germline configuration of VH3-23, whereas A3-H2 F(ab’)_2 was 96% homologous to VH3-23. The two F(ab’)_2 fragments displayed very similar binding affinities for SEA (Fig. 5, A and B) or SPA (Fig. 5, C and D). Binding of SEA to VH3 F(ab’)_2 was characterized by a very fast association rate, as well as a very fast dissociation rate, whereas binding of SPA was characterized by a fast association rate with a slower dissociation rate. The affinity of SEA for VH3 F(ab’)_2 fragments was >500-fold lower than that of SPA (Table III). In contrast, VH4 F(ab’)_2 fragments failed to bind either to SEA or SPA, and no interaction was detected between VH4 F(ab’)_2 and SEA or VH4 F(ab’)_2 and SPA (Fig. 5, E and F). Finally, SED did not bind to VH3 F(ab’)_2 fragments, and non-specific binding of VH3 fragments, SEA, or SPA to the flow cell was not detected (Fig. 5H).

Discussion

Staphylococcal enterotoxins are bacterial superantigens that have been shown to bind as unprocessed proteins to regions of MHC class II molecules distinct from the Ag-binding groove (43, 44). Simultaneously, SEA interact with the V region of TCR Vβ-chain (45, 46), resulting in the activation, proliferation, anergy, and deletion of T cells expressing the particular Vβ segments (47, 48). In this study, we have analyzed the capacity of SEA to function as a B cell superantigen. Stimulation of B cells with SEA resulted in the survival of only VH3-expressing B cells, independent of light chain expression. SEA induced only polyclonal responses in the presence of T cells. In the absence of T cells, SEA did not induce the activation and expansion of B cells as might be expected, but rather selective survival of specific VH3-expressing B cells, as was previously shown for SED-induced survival of VH4-expressing B cells (35). Of importance, the bias in detection of VH3-expressing B cells in SEA-stimulated cultures did not appear to reflect a PCR artifact. The primers used are from the leader sequences of each VH family and are completely homologous to all known members of each family (49). In freshly isolated B cells, all families could be amplified, indicating that members of each family could be detected. After 9 days in culture, in unstimulated B cell cultures, no VH gene products could be detected, not even VH3 mRNA that is the largest VH family in the human B cell repertoire (32). Thus, SEA preferentially fosters the survival or slows the rate of death of VH3-expressing B cells. The stimulation of B cells by SEA was found to be independent of light chain utilization, as both κ- and λ-chains

FIGURE 3. Comparison of amino acid sequences of Ig VH3-containing heavy chain segments from SEA-stimulated B cells with VH3 germline genes. Identical amino acids are indicated by dashes. The dot in A3-F5 clone corresponds to the absence of an amino acid at this position. These sequence data are available from EMBL under accession numbers Z96954 to Z96977.
were detected. Thus, the V\textsubscript{H}\textsubscript{3}-specific nature of SEA is consistent with its capacity to function as a B cell superantigen.

In our previous studies, we have shown that SED functions as a B cell superantigen by inducing survival of V\textsubscript{H}\textsubscript{4}-expressing B cells (35). Analysis of the mechanism by which SED induced survival of the V\textsubscript{H}\textsubscript{4}-specific B cells indicated that SED induced apoptosis of purified B cells presumably by binding HLA-DR. Cross-linking of HLA-DR with V\textsubscript{H}\textsubscript{4}-expressing IgM resulted in rescue of V\textsubscript{H}\textsubscript{4}-expressing B cells from apoptosis. In the current studies, SEA was also found to function as a B cell superantigen by inducing the survival of V\textsubscript{H}\textsubscript{3}-expressing B cells by a similar mechanism (50). Thus, SEA is known to bind to HLA-DR. Moreover, non-V\textsubscript{H}\textsubscript{1}-expressing B cells that bind SEA may receive a signal via class II MHC molecules alone that stimulates programmed cell death. This is consistent with findings demonstrating that signaling through MHC class II induces murine B cell activation (51), and that different mAb to HLA-DR induce apoptosis (35, 52, 53). Simultaneous binding to V\textsubscript{H}\textsubscript{3}-containing sIgM appears to be capable of blocking this apoptotic signal, leading to the survival of V\textsubscript{H}\textsubscript{3}-expressing B cells uniquely (50).

When the V\textsubscript{H}\textsubscript{3} gene products were analyzed, eight different V\textsubscript{H}\textsubscript{3} germline genes were found to encode these products. At least 48 V\textsubscript{H}\textsubscript{3} genes have been mapped to the V\textsubscript{H} locus (54–56), but more than half have been found to be pseudogenes (55, 56). It is, therefore, possible that some, but not all members of the V\textsubscript{H}\textsubscript{3} family are bound by SEA-stimulated B cells. It should be noted, however, that the distribution of V\textsubscript{H}\textsubscript{3} genes expressed by SEA-stimulated B cells is similar to the distribution in normal human peripheral blood, in which some V\textsubscript{H}\textsubscript{3} genes are overrepresented, whereas others are less frequently found (32, 33). Thus, four of the V\textsubscript{H} genes used by SEA-stimulated B cells, V\textsubscript{H}3-23, V\textsubscript{H}3-07, V\textsubscript{H}3-30, and DP58, are among the most commonly used genes in the normal adult repertoire (32). Moreover, the sequences of the V\textsubscript{H}3 family members that were not found in SEA-stimulated B cells

### Table II. CDR3 regions of V\textsubscript{H}3 genes expressed by SEA-stimulated B cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3-K1</td>
<td>DVYYYDRGRGRLSPRXSTTGMDV</td>
</tr>
<tr>
<td>A3-M2</td>
<td>VMGVRRVDCGD</td>
</tr>
<tr>
<td>A3-H5</td>
<td>RGYSSWSLRYFDY</td>
</tr>
<tr>
<td>A3-D3</td>
<td>GGHLDY</td>
</tr>
<tr>
<td>A3-E6</td>
<td>SGSTGSTVYD</td>
</tr>
<tr>
<td>A3-H2</td>
<td>YRGDTYDYS</td>
</tr>
<tr>
<td>A3-C1</td>
<td>LEDSATYV</td>
</tr>
<tr>
<td>A3-C9</td>
<td>VGYSSQWSPFD</td>
</tr>
<tr>
<td>A3-J2</td>
<td>DLYSSYYENY</td>
</tr>
<tr>
<td>A3-F5</td>
<td>GVPSSFGNYWTGCPCDV</td>
</tr>
<tr>
<td>A3-E4</td>
<td>GWWRFDY</td>
</tr>
<tr>
<td>A3-E5</td>
<td>GYGGGGYYDSRDFDY</td>
</tr>
<tr>
<td>A3-2</td>
<td>SPSGMD</td>
</tr>
<tr>
<td>A3-E2</td>
<td>TLSRIVGATMVDGDDWFPD</td>
</tr>
<tr>
<td>A3-I2</td>
<td>DQCGPPGMYDPWFDL</td>
</tr>
<tr>
<td>A3-M13</td>
<td>HGSGSYWFDFY</td>
</tr>
<tr>
<td>A3-C8</td>
<td>DRGIVATHTGYMDV</td>
</tr>
<tr>
<td>A3-M9</td>
<td>WVGATSDY</td>
</tr>
<tr>
<td>A3-8</td>
<td>IPRWRPGACDL</td>
</tr>
<tr>
<td>A3-H6</td>
<td>MRGWGEVAFDI</td>
</tr>
<tr>
<td>A3-H6</td>
<td>DSGALYYNLXGGYVLDY</td>
</tr>
<tr>
<td>A3-E7</td>
<td>KWGSNDNYSYMDV</td>
</tr>
<tr>
<td>A3-7</td>
<td>NGNGXQIXLWWWQLLVGSDP</td>
</tr>
<tr>
<td>A3-K3</td>
<td>ERDLVGATYYFDS</td>
</tr>
</tbody>
</table>

![FIGURE 4](image-url)  Nonconservative changes observed among the 24 SEA-induced V\textsubscript{H}3 sequences and between the 24 SEA-induced V\textsubscript{H}3 sequences and V\textsubscript{H}4-34, V\textsubscript{H}4-39, and V\textsubscript{H}4-59. Amino acid positions among the 24 SEA-induced V\textsubscript{H}3 sequences were compared, and the total number of nonconservative amino acid changes at each position was plotted (top). The same analysis was done by comparing the 24 SEA-induced V\textsubscript{H}3 sequences with V\textsubscript{H}4-34, V\textsubscript{H}4-39, and V\textsubscript{H}4-59, respectively. The number of nonconservative changes at each position is plotted (bottom). *Indicates no residue at this position.
were similar in the critical FR1 and FR3 regions to those that were detected (57). This makes it likely that most, if not all, V_{H}3 family members could bind SEA, and that if additional sequences were analyzed, these family members would be detected at a frequency predicted from their distribution in normal human blood.

The different V_{H}3 sequences expressed by SEA-stimulated B cells utilized different CDR3 regions. The CDR3s were encoded by different D and J_{H} gene segments, and the lengths of the CDR3 regions were variable. These features of V_{H}3 genes cloned from SEA-stimulated B cells support the conclusion that the CDR3 region is not crucial in binding SEA. In addition, no similarities in CDR1 or CDR2 were noted in the V_{H}3 sequences cloned from SEA-stimulated B cells. The current results, therefore, are consistent with the conclusion that the interaction between V_{H}3 and SEA is independent of the light chain, D, and J_{H} utilization, as well as CDR1, CDR2, and CDR3. These features of SEA stimulation indicate that SEA is a member of a unique class of B cell superantigens that induce B cell survival by binding to specific aspects of V_{H}3 independent of the CDRs and light chain utilization.

In the current study, we found by plasmon resonance measurements that SEA binds specifically to V_{H}3 F(ab')_{2} fragments. SEA binding to V_{H}3 F(ab')_{2} fragments demonstrated a rapid on rate, a fast off rate, and was of low affinity. Because of these characteristics, the binding of SEA to Ig molecules is difficult to document by other techniques. Levinson et al. (58) failed to detect binding of SEA and other SEs to a panel of monoclonal IgM paraproteins expressing different VH gene segments by ELISA. The current plasmon resonance data indicate that the binding of SEA to V_{H}3 Ig molecules would be difficult to detect by ELISA because of the fast

### Table III. Kinetics measurements of interactions of SEA and SPA with V_{H}3-23 Fabs

<table>
<thead>
<tr>
<th>Fab</th>
<th>V_{H}3-23</th>
<th>SEA</th>
<th>SPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3-K1</td>
<td>V_{H}3-23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(99.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k_{a} (M^{-1}s^{-1})</td>
<td>4.63 × 10^{3}</td>
<td>1.14 × 10^{3}</td>
<td></td>
</tr>
<tr>
<td>k_{d} (s^{-1})</td>
<td>4.20 × 10^{-1}</td>
<td>5.02 × 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>K_{A} (M^{-1})</td>
<td>1.10 × 10^{4}</td>
<td>2.28 × 10^{7}</td>
<td></td>
</tr>
<tr>
<td>K_{D} (M)</td>
<td>9.07 × 10^{-5}</td>
<td>4.41 × 10^{-8}</td>
<td></td>
</tr>
<tr>
<td>A3-H2</td>
<td>V_{H}3-23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(96.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k_{a} (M^{-1}s^{-1})</td>
<td>3.97 × 10^{3}</td>
<td>9.03 × 10^{4}</td>
<td></td>
</tr>
<tr>
<td>k_{d} (s^{-1})</td>
<td>3.21 × 10^{-1}</td>
<td>5.21 × 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>K_{A} (M^{-1})</td>
<td>1.23 × 10^{4}</td>
<td>1.73 × 10^{7}</td>
<td></td>
</tr>
<tr>
<td>K_{D} (M)</td>
<td>8.08 × 10^{-5}</td>
<td>5.57 × 10^{-8}</td>
<td></td>
</tr>
</tbody>
</table>
off rate. Even though the binding of SEA to V_{H3} Ig is of low affinity and transient with a fast off rate, it is sufficient to induce a measurable response by specific B cells. Low affinity functional interactions previously have been reported between surface Ig molecules and membrane self Ags (class I molecules) (59). These low affinity interactions ($K_a = 1 - 6 \times 10^4$ M$^{-1}$) were found to be sufficient to induce complete deletion of immature B cells in vivo and partial deletion of mature B cells. Thus, low affinity interactions between sIg and ligand, in the range noted for the binding of SEA, can lead to biologic effects in vivo. These findings, therefore, support the conclusion that a direct low affinity interaction between SEA and non-Ag-binding regions of V_{H3} molecules could provide a functional signal to specific B cells in vivo. This may account for biased representation of V_{H3}-expressing B cells in the peripheral human repertoire (32, 33).

SPA bound V_{H3} (V_{H3}-23) F(ab')$_2$ fragments with higher affinity than SEA ($1.73 \times 10^7$ to $2.28 \times 10^7$ M$^{-1}$ vs $1.10 \times 10^4$ to $1.23 \times 10^6$ M$^{-1}$, respectively). The affinity of SPA for V_{H3}-23 is similar to that previously reported for SPA binding to F(ab')$_2$ fragments of V_{H3}-23 (60, 61). The marked differences in the affinities for V_{H3} may account for some of the functional disparities between SEA and SPA. Whereas SEA with or without IL-2 has only a limited capacity to activate B cells directly, SPA in the presence of IL-2 directly stimulates B cell activation, proliferation, and differentiation (62). Moreover, the V_{H3} bias of SPA responses is apparent even in the presence of T cells (14), but activated T cells overcome the V_{H3} specificity of SEA stimulation of B cells, resulting in a more polyclonal response. It is likely that these functional differences reflect the marked differences in the affinities of the two B cell superantigens for V_{H3} molecules.

The structural basis of V_{H3} Ig binding to SEA was analyzed, by comparing all of the V_{H3} sequences from SEA-stimulated B cells. Conservation in amino acid sequence was found in FR1, FR2, and FR3. It is unlikely that the 5' ends of FR1 and FR2 could be involved in SEA binding because they are not accessible to solvent. Possible determinants that are critical for SEA binding, therefore, were localized to FR1 and FR3. The comparison between the SEA-induced V_{H3} sequences and those of three of the most commonly utilized V_{H4} germline gene products that do not bind SEA pointed to the possibility that residues 9, 15, 16, 23, and 27 in FR1 and residues 71–79, 81, 82a, 82b, and 83 in FR3 may be critical for SEA binding, as many nonconservative changes were noted at these locations in the V_{H4} gene products. These observations indicate that FR1 and/or FR3 may be important for SEA stimulation.

In an intact folded Ig molecule, FR1 and FR3 regions form two closely adjacent, solvent-exposed $\beta$-pleated sheets (63). Figure 6 shows a model indicating the candidate amino acids of V_{H3} (V_{H3}-23) involved in SEA binding. Some of these may be more important in the overall structure of the molecule rather than direct SEA binding. Thus, amino acid residues 15 and 16, which are glycines and not part of the $\beta$-sheet, are more likely to be related to the flexibility of the loop and maintenance of the conformation of the lateral face of the molecule in general, rather than being directly involved in SEA binding. Of the remainder of the candidate amino acids, those with side chains pointed outward (A23 in FR1, R71, N73, S74, K75, N76, T77, Y79, Q81, N82a, S82b, and R83 in FR3) form a cluster on the lateral face of the molecule in general, rather than being directly involved in SEA binding. Of the remainder of the candidate amino acids, those with side chains pointed outward (A23 in FR1, R71, N73, S74, K75, N76, T77, Y79, Q81, N82a, S82b, and R83 in FR3) form a cluster on the lateral face of the V_{H3} molecule. This suggests that these amino acids, the majority of which are from FR3, may constitute an SEA binding site.

Several groups have shown that the so-called fourth hypervariable region (HVR4) in the $\beta$-chain of the TCR is a crucial site for superantigen binding. Although it has been suggested that TCR sites other than the HVR4 of the TCR V_{\beta}-chain, mainly CDR1 and CDR2, may affect recognition of superantigen, there is a general consensus that the lateral face of the V_{\beta}-chain is important for superantigen binding (64–69). Recently, the x-ray crystal structure of a TCR $\beta$-chain complexed with SEC2 and SEC3 has been solved (70), CDR1 and CDR2 and, to a lesser extent, FR3 and HVR4 were shown to be involved in SEC binding. Moreover, all of the hydrogen bonds between SEC and V_{\beta} were formed between SEC side chains and V_{\beta} backbone carbon atoms. Initial superantigen recognition, therefore, appeared to be dependent on the conformation of the V_{\beta} region, whereas interactions involving V_{\beta} side chains contributed to the binding energetics.
the binding of V_{H}3 to SEA, the side chains pointing toward the solvent might provide additional binding energy, but the possibility that the backbone carbon atoms accounted for the initial superantigen binding must be considered (Fig. 6).

CDR1, CDR2, and HVR4 of the TCR appear to be involved in superantigen binding, whereas FR3 and, to a lesser extent, FR1 of V_{H}3 bind to SEA. The difference may relate to the comparative conformations of the TCR and Ig molecule. HVR4 comprises residues 69 to 75 of the TCR β-chain and forms a loop that folds toward CDR1 and CDR2 (71). This region is analogous to residues 69 to 77 in FR3 of the Ig V_{H} molecule that form a loop directed toward solvent. The continuous face formed by the lateral surfaces of the CDRs and HVR4 in the β-chain of the TCR could explain the observation that certain residues in CDR1 and CDR2 and HVR4 of the β-chain of the TCR may modulate binding to superantigen. In V_{H}3 molecules, by contrast, the analogous loop formed by FR3 is almost always associated with FR1 and the 3’ end of CDR2 forming the external face of the V_{H} domain. These comparative differences suggest that FR3 and FR1 and perhaps CDR2 are more likely to be candidate regions for superantigen binding to Ig molecules. Of note, CDR2 appears to play a role in SPA and SE binding of V_{H}3 and V_{H}4 molecules, respectively (12, 13, 35), whereas no role for CDR2 in SEA binding to V_{H}3 was discerned.

Several studies have investigated the specific portions of V_{H}3 accounting for SPA binding (6–14). The solvent-exposed surface of V_{H}3 created by FR1, CDR2, and FR3 has been implicated in SPA binding. A comparison of amino acid sequences of different IgM proteins with different binding affinities for SPA suggested that V_{H}3-specific residues at and around position 57 in CDR2 were likely to be involved in SPA-binding activity. Moreover, a single amino acid substitution from glutamic acid to lysine at position 57 in CDR2 changed a V_{H}3 family member from an SPA nonbinder to an SPA binder (12). The amino acid sequence comparison of the 24 SEA-induced V_{H}3 clones showed that position 57 was not conserved, especially in clone A3-C1, in which there is an arginine instead of threonine that could affect binding. This suggested that SEA binding to V_{H}3 molecules differed from that of SPA in that CDR2 was not involved. To confirm this, we have examined the binding of SEA to a rheumatoid factor IgM, RF-TS2. This Ab is encoded by V_{H}3-23, has a glutamic acid at position 57, and does not bind SPA (12). RF-TS2, however, bound to SEA (data not shown), confirming that position 57 in CDR2 is not involved in SEA binding. This implies that the binding site for SEA is different from that interacting with SPA, in that CDR2 is not required. Of note, SPA and another V_{H}3-specific binding molecule, protein Fv, have been shown to compete for V_{H}3 binding (61). To confirm that SPA and SEA do not bind to the same site on V_{H}3, we would require competitive binding studies that might not be informative in light of the extremely weak binding affinity of SEA. Finally, and of interest, the binding of SEA to V_{H}3 appears to differ from that of the previously reported SE binding to V_{H}4 in which CDR2 is involved (35). These results imply that SEA binding to V_{H}3 is unique, in that it is defined only by the amino acids of FR1 and FR3 that form the lateral surface of the molecule, clearly distinct from all elements of the classical Ag-binding region.

In summary, SEA binds to V_{H}3-containing Ig molecules with low affinity and can induce survival of V_{H}3-expressing B cells. Thus, SEA or endogenous or exogenous materials with similar binding specificity with enhanced avidity might act as a unique B cell superantigen that may play a role in shaping the expressed B cell repertoire.

Acknowledgments

The assistance of Kathy Potter in the surface plasmon resonance measurements is greatly appreciated. We thank E. Sally Ward for the FabD1.3 mouse plasmid and for the expert assistance in construction of the FabD1.3 mouse plasmid and purification of the V_{H} F(ab')_{2} proteins, David Karp for providing the recombinant staphylococcal enterotoxin A, and Charles Hase mann for his help in generating the immunoglobulin model.

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