Staphylococcal Enterotoxin D Is a Promiscuous Superantigen Offering Multiple Modes of Interactions With the MHC Class II Receptors

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Staphylococcal Enterotoxin D Is a Promiscuous Superantigen Offering Multiple Modes of Interactions With the MHC Class II Receptors

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Dimerization of MHC class II molecules on the surface of human THP-1 monocytic cell line is a requirement for staphylococcal superantigen (SAG)-induced cytokine gene expression. The capacities of various SAG to induce this response are governed by their modes of interaction with MHC class II molecules. Staphylococcal enterotoxin A (SEA), with its two binding sites, dimerizes MHC class II molecules and subsequently induces cytokine gene expression in THP-1 cells. Here, we demonstrate that staphylococcal enterotoxin D (SED) and staphylococcal enterotoxin E (SEE) induce, similarly, IL-1β and TNF-α gene expression in these cells. Using mutated toxins that lost their binding site with the MHC class II α- or β-chain, we demonstrate that this response is also mediated by the dimerization of MHC class II molecules through two binding sites. Furthermore, SED forms Zn2+-dependent homodimers that allow multiple modes of MHC class II clustering, including ligation of α-chains (α/α), β-chains (β/β), or the α- and β-chains of two different class II molecules. The β/β interaction following Zn2+-dependent SED/SED homodimer formation seems to be mediated by the appearance of a novel binding site on SED that interacts with histidine 81 of the MHC class II β-chain. The different modes of SED interactions also influence SED-induced T cell activation where simultaneous ligation of the α- and β-chains is essential for optimal response. These various modes of SED binding may be used to preserve bivalency regardless of variability in the MHC class II α/β/peptide complexes. The Journal of Immunology, 1998, 160: 225–232.

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4 Abbreviations used in this paper: SAG, superantigen; SEA, staphylococcal enterotoxin A; SED, staphylococcal enterotoxin D; SEE, staphylococcal enterotoxin E; TSST-1, toxic shock syndrome toxin-1; wt, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DSS, disuccinimidyl suberate.
homodimers (SED/SED) in solution (21) that are coordinated by two Zn\(^{2+}\) ions. Each Zn\(^{2+}\) ion is coordinated by H218 from one SED molecule, and D182, H220, and D222 is coordinated from a second SED molecule. Whether this property influences the interactions of SED with class II molecules is not yet clear. In this study we address the above issues and demonstrate clearly that monomeric SED and SEE contain two MHC class II binding sites, have the capacity to dimerize class II molecules, and thus have a similar mode of interaction as SEA. Nevertheless, the SED/SED homodimer seems to be distinct from the other staphylococcal SAGs in its capacity to interact with two class II molecules in three different ways: 1) cross-linking of two \(\alpha\)-chains (\(\alpha/\alpha\)), 2) cross-linking of two \(\beta\)-chains (\(\beta/\beta\)), and 3) cross-linking of an \(\alpha\)-chain and a \(\beta\)-chain (\(\alpha/\beta\)). We also provide evidence that these different modes of interaction play a critical role in T cell activation.

**Materials and Methods**

**Generation of recombinant SEA, SEE, and SED**

SEA, SEE, and SED were cloned using PCR from crude DNA extract isolated from *Staphylococcus aureus* strains producing these enterotoxins. The translated part of the cDNA sequences of SEA and SEE were identical with published sequences (22, 23). The amino terminal of SEE, however, contains three additional amino acid residues (SEK). The SED cDNA sequence was identical with that previously reported (24), except for a single point mutation, C to G, substituting Pro\(^{84}\) to alanine. Direct sequence analysis of the PCR product revealed the same point mutation, indicating that this is a naturally occurring variant of SED. In vitro mutagenesis of SEA mutants were generated: 1) the aspartic acid at position 227 was substituted by alanine (SEA\(^{227A}\)); 2) the phenylalanine at position 47 was substituted by alanine (SEA\(^{47A}\)); and 3) double mutants in which F47 and D227 were substituted by alanines (SEA\(^{47A,227A}\)). With respect to SED, we have generated four different mutants: 1) the phenylalanine at position 42 was substituted by alanine (SED\(^{42A}\)); 2) the aspartic acid at position 182 was substituted by alanine (SED\(^{182A}\)); 3) the histidine 218 was substituted by alanine (SED\(^{218A}\)); and 4) the aspartic acid at position 222 was substituted by alanine (SED\(^{222A}\)). The wild-type (wt) and mutated variants of SED are five amino acid residues shorter than SEA, similar to the published amino terminal sequence of SED (24), and are numbered accordingly. *Escherichia coli* expression and protein purification of wild-type and mutated variants of SED, SEE, and SEA were performed as previously described (10). The purity of these toxins was confirmed by SDS-PAGE followed by Coomassie blue or immunoblot with specific mAbs.

**Cell lines**

The THP-1 monocytic human cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium containing 10% FCS and antibiotics. This cell line expresses low levels of HLA-DR molecules, while it is completely negative for HLA-DQ and -DP (25). DAP-3 cells expressing HLA-DR1, HLA-DR1\(\beta 81\)A, or HLA-DR1\(\alpha 39\)A were previously described and maintained in MEM supplemented with 5% FCS and G418 (26). The murine CD4\(^{+}\) T cell hybridoma K25 that expresses V\(\beta 3\) and responds to SED was maintained in RPMI supplemented with 10% FCS (27).

**Northern blot analysis**

Stimulation conditions for each experiment are detailed in the appropriate figure legends. RNA was purified using Trizol reagent (Life Technologies Products, Burlington, Canada) according to the manufacturer’s procedure, and 10 \(\mu\)g of RNA was loaded onto 1% agarose gels. The RNA was then transferred onto Hybond-N filter paper and was hybridized with random primer-labeled cDNA probes for IL-1\(\beta\) and TNF-\(\alpha\). Equal loadings of RNA were confirmed by hybridization with the GAPDH cDNA probe. The mRNA hybridizing with the cDNA probes was visualized by autoradiography (28).

**Western blot**

Toxins were incubated with different concentrations of Zn\(^{2+}\) or EDTA in 0.1 HEPES buffer, pH 7.5, with 100 mM NaCl for 30 min at room temperature on a rotation platform. The cross-linking agent disuccinimidyl suberate (DSS; Pierce Chemcial Co., Rockford, IL) was added to a final concentration of 0.25 mM, and the mixtures were further incubated for 30 min. The reaction was stopped with SDS sample buffer, then samples were boiled and separated by electrophoresis on precast 12% Tris-glycine gels (Bio-Rad, Hercules, CA). The separated proteins were blotted onto nitrocellulose sheets, then incubated with a polyclonal rabbit anti-SED Abs (1 \(\mu\)g/ml). Blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1/3000; Bio-Rad). ECL detection reagents and Hyperfilm ECL (Amersham, Life Science, Little Chalfont, U.K.) were used for detection.

**Binding assays**

The abilities of different toxins to bind HLA-DR molecules were assessed as previously described (26). Briefly, toxins were labeled with \(^{125}\)I and then \(4 \times 10^6\) DAP-3 transfecteds were incubated with 50 ng of \(^{125}\)I-labeled toxins in 200 \(\mu\)l of binding buffer (RPMI, 10 \(\mu\)M Zn\(^{2+}\), and 0.1% NaN\(_3\)) for 3 h at 37°C. Cells were then pelleted through an oil cushion (84% silicon oil and 16% mineral oil), and their radioactivity in counts per minute was determined using a gamma counter.

**T cell stimulation**

DAP-3 transfecteds were used as APCs to stimulate K25 T cell hybridoma with SED\(_{\alpha}\) or its mutants as previously described (26). Briefly, 2 \(\times 10^4\) APCs were incubated for 20 h in the presence of 8 \(\times 10^3\) T cells and various concentrations of toxins. Cocultures were performed in a final volume of 200 \(\mu\)l in 96-well flat-bottom plates at 37°C in 5% CO\(_2\). Stimulation was evaluated by the amount of IL-2 released by T cells in the coculture supernatants. Levels of IL-2 were determined by the ability of the coculture supernatant to support proliferation of the IL-2-dependent cell line CTLL2.

**Results**

Activation of monocyte cytokine production by Zn\(^{2+}\)-dependent staphylococcal enterotoxins is mediated by MHC class II \(\alpha/\beta\)-chain cross-linking

Certain MHC class II-mediated events can be induced by a monovalent ligand, while others, such as cytokine gene expression, require bivalent ligand that can trigger dimerization or even oligomerization of MHC class II molecules on the cell surface (17, 18, 29). Based on the structural homology among SEE, SED, and SEA, it is highly possible that SEE and SED act as bivalent crosslinkers of MHC class II molecules as described previously for SEA. This possibility was verified by analyzing the capacity of SEE and SED to induce IL-1\(\beta\) and TNF-\(\alpha\) in the THP-1 monocytic cell line. THP-1 cells were resuspended in FCS-free RPMI (Zn\(^{2+}\) free to ensure the absence of homodimeric form of SED) and stimulated with SEE or SED, and the induced responses were compared with those induced by different concentrations of SEA. Both toxins induced dose-dependent IL-1\(\beta\) and TNF-\(\alpha\) gene expression in THP-1 cells comparable to that induced by SEA (Fig. 1A). It is noteworthy that these toxins belong to the Zn\(^{2+}\)-dependent staphylococcal superantigens, and the above experiment was conducted without supplementary ions; however, the Zn\(^{2+}\) that can be released from intracellular storage or the unstable Zn\(^{2+}\) on the MHC class II molecules seems to be sufficient to support their functional binding to their receptors (12). Accordingly, SEE- and SED-induced responses appear to be mediated by dimerization of class II molecules via their \(\alpha/\beta\)-chains.

To confirm this mode of action, inhibition assays were performed using SEA mutants that can interact with a single MHC class II chain, either the \(\alpha\)-chain (SEA\(^{227A}\)) or the \(\beta\)-chain (SEA\(^{47A}\)). We have previously reported that stimulation of THP-1 with these mutants fails to induce IL-1\(\beta\) and TNF-\(\alpha\) expression, and their binding to the \(\alpha\)- and \(\beta\)-chains completely inhibits the SEA\(^{227A}\)-induced response (18). Figure 1B shows that pretreatment of THP-1 cells with a 10-fold excess of SEA\(^{227A}\) or SEA\(^{47A}\) completely inhibited SEE- and SED-induced cytokine gene expression, whereas pretreatment with a double SEA mutant (SEA\(^{47A}/SEA^{227A}\)), which binds neither \(\alpha\)-nor \(\beta\)-chains, did not have any blocking effect. These data clearly indicate that blocking either class II \(\alpha\)-chain or \(\beta\)-chain leads to a complete loss
of SED and SEE activities, supporting that both toxins cross-link an α-chain and a β-chain of two class II molecules.

To further confirm this issue and to identify the residues involved in this response, we generated, by site-directed mutagenesis, SED mutants in which the N-terminal phenylalanine at position 42 (equivalent to SEAF47) was substituted by alanine (SED F42A), or the C-terminal aspartic acid at position 222 (equivalent to SEA D227) was substituted by alanine (SED D222A). Stimulation of THP-1 cells either with SED F42A (expected to interact only with the β-chain) or with SED D222A (expected to interact only with the α-chain) in FCS- and Zn²⁺-free RPMI did not induce any detectable cytokine gene expression (Fig. 2A), confirming the implication of SED₄₂ and SED₂₂₂ in the interaction with class II molecules. Similar results were obtained with SEE mutated at the same residues (data not shown). When the SED mutants were used in inhibition assays, both SED₄₂ and SED₂₂₂ were able to abolish the capacity of SEAₚₚ, SEEₚₚ, and SEDₚₚ to induce cytokine gene expression (Fig. 2B). Hence, the superantigenic activity, at least in inducing cytokine gene expression, of the three Zn²⁺-dependent staphylococcal superantigens is mediated by their interactions with the class II α-chain via F42 (or its equivalent depending on the SAG) and with the class II β-chain via D222 (or its equivalent) that permit an α/β cross-linking of these molecules.

**Interactions of SED/SED homodimer with MHC class II molecules**

The recently elucidated SED crystal structure demonstrated the unique feature of SED (compared with SEA and SEE) in forming...
Zn$^{2+}$-dependent homodimers (21). The SED dimerization, although reported with high Zn$^{2+}$ concentrations, can be detected at concentrations as low as 1 μM. Since the residue D222 is implicated in SED functional binding to class II β-chain and was described as essential for SED/SED dimer formation (21), it was interesting to analyze how these SED homodimers functionally interact with class II molecules. To this end, THP-1 cells were resuspended in FCS- and Zn$^{2+}$-free RPMI, in RPMI supplemented with 1 μM Zn$^{2+}$ (conditions that permit significant SED/SED dimer formation), or in RPMI supplemented with 5% FCS, then stimulated with two concentrations (0.5 or 2 μg/ml) of SEDwt, SED$_{D222A}$, SED$_{H218A}$, or SED$_{D182A}$. It is worth noting that substitution of any of the latter three residues by alanine completely abolishes SED/SED homodimer formation. Figure 3 (A–C) demonstrates that under all the above experimental conditions, SED$_{wt}$, SED$_{H218A}$, and SED$_{D182A}$ induce similar cytokine gene expression, suggesting that the activities of both SED$_{H218A}$ and SED$_{D182A}$ are mediated by α/β class II cross-linking as monomeric SED$_{wt}$. Since D222 of SED is normally engaged in its dimer formation, we were able to detect any dimer formation with SED$_{D222A}$. For D182A, the substitution of histidine 81 by alanine completely abolishes SED binding to class II, indicating that the SED/SED homodimer interaction is necessary for SAG-induced cytokine gene expression. SED/SED dimer interacts with histidine 81 on the β-chain of HLA-DR1 via a novel binding site

Since D222 of SED is normally engaged in its dimer formation, we suggested above that Zn$^{2+}$-induced SED/SED homodimer formation can lead to the appearance of a novel binding site on SED. Indeed, the ability of SED$_{F47A}$, which interacts with the β-chain via H81) to inhibit the SED$_{F42A}$-induced response suggests that this novel SED binding site interacts with histidine 81 of the class II HLA-DR β-chain. Hence, binding experiments were conducted in the presence of 10 μM Zn$^{2+}$ (to permit maximal SED homodimer formation) using radiolabeled SED$_{wt}$, presenting both dimeric and monomeric forms, SED$_{H218A}$, presenting the monomeric form, SEA as a control, and DAP cells transfected with HLA-DR1 or HLA-DR1 β81A. Figure 6 demonstrates that both SED dimers and monomers bind significantly to DAP HLA-DR1-transfected cells. Substitution of histidine 81 by alanine completely abolished SED binding to class II, indicating that the SED/SED dimer that bears the novel binding site and the SED monomer bind to the β-chain of HLA-DR through histidine 81. Similar results
were obtained with SEA, while TSST-1 binding, reported to be mainly via the HLA-DR α-chain, was not affected by the presence or the absence of histidine 81.

Simultaneous ligation of class II α- and β-chains is required for optimal activation of T cell clones by SED

The data provided above indicate that SED interacts with MHC class II molecules in different forms. In the following experiments we investigated the significance of these different modes of interaction on the activation of T lymphocytes. To this end we used a functional assay, IL-2 production, and the murine T cell hybridoma K25 that is known to respond to SED via its Vβ3. Our results (Fig. 7) clearly indicate that SED can trigger the activation of this T cell hybridoma only when presented via α- and β-chains of two different MHC class II molecules. Presenting SED by either α/α or β/β of MHC class II molecules failed to induce any significant...
IL-2 production by this hybridoma. Complementary experiments using DAP-3 cells transfected with HLA-DR1β81A or HLA-DR1α39A showed the failure of the K25 clone to produce detectable IL-2 when stimulated with SEDwt, supporting the idea that interaction of SED with both chains of class II molecules is required for efficient T cell activation.

Discussion

The primary role of MHC class II molecules as receptors able to present the naturally occurring microbial products SAGs to T cells (1–3) has been extended to include the capacity of these molecules to act as signal transducers through which activation of class II-positive cells emerges (28). The panel of cytokines and cell activation accompanying the SAG/class II interaction proposed the implication of these events in the pathology of SAG-producing bacteria (30–34). Although the precise interaction sites between SAGs and class II have not been fully defined, structural and crystallographic studies have provided considerable information, at least for staphylococcal SAGs (8–12, 14, 26, 35–38).

Staphylococcal SAGs can be divided into two major subfamilies based on their interactions with class II: a zinc-coordinated subfamily, including SEA, SEE, and SED, and a zinc-independent subfamily, including SEB and TSST-1. Given the high degree of similarity between the members of the zinc-coordinated subfamily, it was expected that these toxins bind class II with similar orientations. Our previous functional analysis of the SEA/class II interaction confirmed these data and strongly favored the idea that SEA forms a class II/SEA1 trimer configuration that permits dimerization of class II molecules and cell activation (18). In this report we provide functional data supporting the idea that SEE and SED also interact with class II via two distinct binding sites that probably allow dimerization of MHC class II molecules and subsequent cytokine gene expression. Indeed, the capacity of staphylococcal SAGs to induce cytokine gene expression in human monocytes seems to be governed by their capacity to dimerize or oligomerize these receptors.

Results obtained during recent years have given ample evidence that several cell surface receptors, including growth factors and cytokine receptors, are activated by ligand-induced dimerization or oligomerization and that this mechanism is of general applicability for the regulation of signal transduction (39). Cell activation can be obtained either by heterodimerization, as for Ile receptors, or by homodimerization, as for growth hormone receptors (40–42). SAG-induced activation of MHC class II-positive cells seems to be mostly mediated by a Zn2+-dependent receptor homodimerization. Interestingly, binding of human growth hormone to prolactin receptor is also zinc coordinated, and tight binding of zinc occurs only in the presence of receptor and ligand (43). Hence, under normal physiologic conditions it is highly likely that SEA, SEE, and SED interact with class II and activate class II-positive cells by a mode of interaction similar to that reported hereby.

The crystal structure of SED revealed a unique feature of SED to form zinc-dependent SED/SED homodimers, which in the interface coordinates two Zn2+ by participation of amino acid residues D182, H220, and D222 from one molecule and H218 from the other (21). Our results implicate that this particular feature of SED allows its interaction with MHC class II molecules in three different ways: cross-linking of HLA-DRα/SED/HLA-DRα or HLA-DRβ/SED/HLA-DRβ forming tetrameric complexes or cross-linking of HLA-DRα/SED/HLA-DRβ forming trimeric complexes. The SED residues implicated in Zn2+ coordination are at a homologous position as those previously described in SEA (13). In the SEA molecule, zinc coordination by these residues controls the formation of stable SEA/class II complexes. However, in SED these residues control both SED/SED homodimer formation and the interaction with MHC class II molecules. The relative contributions of individual SEA residues to Zn2+ coordination are in the order D227> H225> H187 (11). Our results indicate that the presence of D222 in SED is crucial for monomeric interaction with MHC class II, but is less important for the formation of SED/SED homodimer. In contrast, SED D182, and SED D222, have a minor or no role in regulating the Zn2+-dependent interaction with class II, but they are critical for the formation of SED/SED homodimer. The most surprising observation was the ability of the SED/SED homodimer to interact with both β-chains of different class II molecules. It appears, therefore, that Zn2+-induced SED/SED homodimer formation can also lead to the appearance of a novel site on SED/SED that can interact with histidine 81 on the class II β-chain. Although the SED residue D222 is more important for the interaction with the β-chain than for SED/SED dimer formation, it is still critical for the exposure of the novel dimer binding site, at least under our experimental conditions (1 μM Zn2+). Alternatively, in our recent crystal studies we detected a second bound Zn2+ ion in SED located at the interdomain interface (21). Microcalorimetry demonstrated that binding of Zn2+ to this site required severalfold higher Zn2+ concentrations than that to the classical Zn2+ domain. Thus at high Zn2+ concentrations, it is possible that the second Zn2+ site can be occupied and consequently mediate binding with the class II β-chain. Although the data presented in this paper do not support the interaction of monomeric SED with the β-chain of MHC class II molecules via the novel binding site, one cannot rule out completely that addition of a high concentration of Zn2+ (>1 μM) may lead to the appearance of this novel binding site on monomeric SED. Studies are in progress to clarify these possibilities and to define and characterize the novel SED binding site. Together, these studies support the idea that SED has dynamic ways to interact with MHC class II molecules and can be considered, at present, among the most promiscuous known SAG.

What is the significance of the variety of multimeric binding modes between SAGs and class II receptors? Our present and previously reported results (18, 19) clearly demonstrate that the
modes of interaction of staphylococcal SAGs with the class II molecules control their ability to activate MHC class II-positive cells. The major role of MHC class II molecules in SAG biology has been attributed to their role in the presentation of SAG to T lymphocytes (1). Presentation of SAGs to TCR can also be achieved by immobilization of SAG on solid phases (44), but this response has a much lower amplitude than that observed when SAGs are presented by MHC class II-positive cells. Indeed, presentation of SAG in the absence of class II activates only a fraction of TCR Vβ families, whereas other TCR Vβ families require MHC class II presentation (15). This effect may reflect a role for class II in stabilizing low affinity interactions with certain TCR Vβ-chains. With respect to SEA, recent evidence indicates that binding of this toxin to the α- and β-chains of class II molecules plays a critical role in positioning SEA for appropriate interaction with certain TCR Vβ elements (16). Although SEA can interact with class II molecules using three different modes (αβ, αα, and ββ), only SEA bound to α- and β-chains of two different class II molecules can induce significant activation of T cell hybridoma expressing the Vβ3 element. Regardless of whether the same mode of interaction is required for SEA recognition by other Vβ elements is not yet clear, our data indicate that different modes of SEA interaction can have a major role in T cell activation and point out the importance of toxin positioning in this response. The multiple binding modes between SAG and MHC class II will permit the formation of unique configurations that may allow the SAG to be recognized by certain TCR. Accordingly, it is possible that the T cell repertoire that is expanded by a given SAG is determined by distinct SAG/class II interactions. The induction of stimulatory cytokines, e.g., IL-1, during SAG/class II interactions may further contribute to amplify T cell responses. In the SAG system, monocyte cytokine production is induced following class II ligation, which is controlled, at least in part, by the topology of the SAG/class II interaction. In this context the interaction of SAG with class II will influence directly and indirectly the T cell and inflammatory responses.

Together, these data may contribute to clarify why a microorganism such as S. aureus produces a variety of toxins that share the ability to induce certain effects in the host. The multiple binding modes between SAG and MHC class II may facilitate the survival of the microorganism in a broad range of molecular environments by bypassing variability in the MHC/peptide complex and thus ensure its existence in various hosts and cell types. Increased understanding of how these toxins interact with cells of the immune system will permit an understanding of or further unravel their pathologic effects.

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