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A Natural Mutation of the Amino Acid Residue at Position 60 Destroys Staphylococcal Enterotoxin A Murine T-Cell Mitogenicity

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A variety of techniques have been used to identify the amino acid residues of bacterial superantigens involved in their interactions with major histocompatibility complex (MHC) class II and T-cell receptor (TCR). In this study, we isolated a naturally mutated staphylococcal enterotoxin A (SEA) from three different *Staphylococcus aureus* strains, in which the amino acid at position 60 has been changed from aspartic acid (D) to asparagine (N). We then studied the influence of this change on the immunological activities of SEA. Our results demonstrated that this mutation does not affect the capacity of SEA to bind MHC class II molecules and consequently activates human monocytes and peripheral blood lymphocytes. In contrast, mutated SEA failed to stimulate the proliferation of murine splenic lymphocytes of two different strains, and when presented by human MHC class II molecules, it also failed to activate murine cell line 3DT, which expresses the SEA-specific TCR Vβ element (Vβ1). These results indicate that this mutation alters the interaction between SEA and murine TCR. The reactivity patterns of the mutated SEA with two specific anti-SEA monoclonal antibodies suggested that the observed effect of the isolated mutation in the murine system might be due to certain conformational changes in the SEA molecule introduced upon changing the D at position 60 to N. Site-directed mutagenesis of the N residue to D or to glycine reconstituted the ability of SEA to stimulate murine splenic lymphocytes. The different effects of this natural mutation at position 60 on the immunological activities of SEA with murine and human cells highlight the relevance of the affinity and avidity in SEA-TCR interactions in the function of different species or may reflect a difference in epitope specificity.

The bacterial superantigens (SAGs) staphylococcal enterotoxin A (SEA), SEB, SEE, and toxic shock syndrome toxin 1 are considered among the most potent challengers of the immune system. Following their interaction with major histocompatibility complex (MHC) class II molecules, they trigger various cellular events in MHC class II-positive cells (32). The complex so formed (MHC class II-SAG) interacts with the T-cell receptor (TCR) and induces polyclonal activation of T cells in a Vβ-restricted fashion (29, 37), although several studies have shown that SAGs can interact with TCR in the absence of MHC class II molecules and that this interaction induces T-cell anergy (20, 33, 39). Presentation of SAGs to T-cell clones by specific immobilized antibodies can induce T-cell activation (15). However, this response is much lower in magnitude than that obtained in the presence of MHC class II molecules (15), indicating that TCR-MHC class II contacts can occur during T-cell stimulation by SAGs to enhance this T-cell response (6, 30).

A variety of techniques have been used to identify the regions of these SAGs involved in their various immunological activities (3, 11–14, 18, 28, 34). Recently, the crystal structures of DR1-SEB and toxic shock syndrome toxin 1 have been solved, and the residues involved in this interaction have been identified (24, 26). Mutational analysis of SEB and resolution of its crystal structure (41) showed that residues 60 and 61 mediate the specificity of the SAG mitogenicity (17, 25). In respect to SEA, it has been demonstrated by using synthetic peptides or mutagenesis that the N-terminal region is required for its ability to induce T-cell proliferation and that the disulfide loop is critical to interactions with TCR (12, 14, 16). Indeed, it has been suggested that the N-terminal region of the SEA molecule is involved in its interactions with MHC class II or with TCR (31). By using similar approaches, it has been demonstrated that binding of SEA to MHC class II molecules involves multiple domains in the N-terminal, central, and C-terminal regions (11, 34). Fraser and coworkers demonstrated that zinc atoms coordinate SEA-MHC class II molecule interactions, suggesting that histidine amino acid residues can play an important role in these interactions (8). Indeed, mutations of histidine residues at positions 187 and 225 and aspartic acid 227 of SEA (7) or of histidine residue at position 81 of the chain β of HLA-DR1 abolish SEA–HLA-DR1 interactions (19). A recent study by Mollick and coworkers proposed that for SEA, the COOH and NH_2 termini together form the contact sites for the TCR and therefore largely determine the Vβ specificity of the toxin (31). Using a series of hybrids of SEA and SEE, Irwin et al. showed that two amino acid residues (206 and 207) near the carboxy terminus are responsible for the discrimination between these molecules by their specific Vβ elements (22).

Different *Staphylococcus aureus* strains can produce SEA, but whether all of the produced toxins have the same T-cell mitogenicity or Vβ specificity remains an unaddressed question. In this study, we produced recombinant SEA (rSEA) from three different *S. aureus* strains. The resulting rSEAs have the same reported SEA molecular weight, but all three differ from the wild type in the amino acid residue at position 60 (aspartic acid or asparagine). Accordingly, we analyzed the

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activity profiles of both toxins with T cells and MHC class II-positive cells. Our results demonstrate that the aspartic acid at position 60 is critical to SEA interactions with murine but not human TCR. Indeed, this mutation does not seem to affect either the capacity of SEA to bind human MHC class II molecules or its capacity to activate MHC class II-positive cells by signaling via these molecules.

MATERIALS AND METHODS

Cloning of SEA. Genomic DNAs of three different *S. aureus* strains, A4 obtained from Toxin Technologie (Sarasota, Fla.), STA 1361 isolated from a patient suffering from toxic shock syndrome, and Cowan 1 used to produce protein A, were all isolated by standard procedures (23). These DNAs, as well as control plasmid pUC19 containing the SEA gene (provided by R. P. Sékaly, Clinical Research Institute of Montreal, Montreal, Québec, Canada), were used as a template in PCR (30 amplification cycles of 1 min of denaturing at 93°C, 1 min of annealing at 55°C, and an extension step of 1 min at 72°C), using oligonucleotide primers corresponding to the NH_2-terminal and COOH-terminal amino acid residues of SEA that flanked the portion of the SEA gene encoding the mature SEA. The 5′ primer (primer 1) was 5′GTAAATGGATCCGAGAAAAGCGAAG3′, which contained a *Bam*HI site placing the SEA gene in frame with the glutathione *S*-transferase (GST) gene of pGEX-2T (Pharmacia Biotech Inc., Québec, Canada). The 3′ primer (primer 2; 5′GGGAATTCAACTTGTATATAAATATATATC3′) contained an *Eco*RI site after the stop codon of the SEA gene. The PCR fragment was digested with *Bam*HI and *Eco*RI and ligated into *Bam*HI-*Eco*RI-digested plasmid pGEX-2T. *Escherichia coli* DH5 was transformed with the plasmid, and transformants were picked and tested for the presence of insert. Positive transformants were sequenced directly by the dideoxynucleotide method (36), using a sequenase dideoxy termination sequencing kit from U.S. Biochemical (Cleveland, Ohio). Several oligonucleotide primers that match the SEA sequence were used.

Targeted mutagenesis. Mutagenesis of SEA was performed by two-step PCR as described previously (10). pGEX-2T containing the SEA-encoding gene from the A4 strain was used as a template in the two-step PCR. In the first PCR, we used primer 1 and a novel 3′ primer, 5′CGAATGATCCTGTAAAAAGCC3′, that allowed changing amino acid residue 60 from N to D (aspartic acid) or G (glycine). In the second PCR, we used the first PCR product of 180 bp as one primer and primer 2. The annealing time of the second PCR was extended to 15 min.

Production of rSEA. Bacteria from an overnight culture were diluted (1:10) in fresh L broth medium and grown to mid-log phase (optical density at 600 nm of 0.8). Then isopropythiogalactopyranoside (IPTG) was added, and the cultures were harvested after 3 to 5 h of incubation. Bacterial cell pellets were collected by centrifugation, resuspended in lysis buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and lysed by sonication at 4°C. Then the supernatants were dialyzed overnight to remove endogenous glutathione. The lysate was passed through a column of glutathione-agarose (Pharmacia Biotech), which specifically binds the inactive 50-kDa fusion protein (GST-SEA). After washing, SEA was cleaved from GST by digestion with bovine thrombin (Sigma Diagnostics, Mississauga, Ontario, Canada) overnight at 4°C according to manufacturer's instructions. Thrombin was removed by a 30-min incubation with *p*-aminobenzeamidine-agarose bead gel (Sigma Diagnostics) and centrifugation. Purified SEA was dialyzed against H_2O buffer and filtered. The purity of produced SEA was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and quantified by a microtitration method using a Micro BCA protein assay kit (Pierce, Rockford, Ill.) and by a sandwich enzyme immunoassay method using specific monoclonal and polyclonal antibodies.

Anti-SEA antibodies. Anti-SEA monoclonal antibodies (MAbs) were prepared in our laboratory by standard methods (27; unpublished data). Two (MAbs 3B9 and 11A8) were used to quantify the produced SEA and to detect the conformational change(s) in mutant SEA. Rabbit polyclonal antibodies were prepared in our laboratory by injection of rabbit with commercial SEA (cSEA; Toxin Technologie).

Enzyme-linked immunosorbent assays (ELISAs). (i) Direct ELISA. SEA-coated microtiter plates (1 μg of SEA per ml in 0.1 M carbonate-bicarbonate buffer [pH 9.6]) were saturated with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 1% gelatin (PBS-Tween-gelatin), washed, and incubated with MAbs at different concentrations for 1 h at 37°C. After washing, the plates were incubated with peroxidase-labeled goat anti-mouse immunoglobulin (Bio-Rad) for another hour at 37°C. The enzyme activity was developed with 2,2-azino-di(-3-ethylbenzthiozoline 6-sulfonate (ABTS) substrate, and the optical density was measured.

(ii) Sandwich ELISA. To quantify and verify the conformational changes of the rSEA sandwich, ELISA was performed. Microtiter plates were coated with a MAb (1 μg/ml) and then incubated with different concentrations of SEA in PBS-Tween-gelatin for 1 h at 37°C. After washing, they were incubated with rabbit anti-SEA antibody, and the reaction was developed as described above, using peroxidase-labeled goat anti-rabbit immunoglobulin.

Class II binding assay. The ability of rSEA to bind MHC class II molecules was assessed as previously described (38), using the Raji human B-cell line (MHC class II positive) from the American Type Culture Collection (Rockville, Md.) and the RM3 cell line (MHC class II negative) derived from Raji (4) and kindly provided by R. P. Sékaly. cSEA or rSEA (20 μg) was iodinated as previously described (42).

For the binding tests, 4×10^5 cells were incubated with 20 to 100 ng of ^{125}I-labeled SEA in 200 ml of binding buffer (RPMI, 2% fetal calf serum, 0.1% NaN_3) for 1 h at 37°C. Cells were then pelleted through an oil cushion (84% silicon oil, 16% mineral oil), and their activity (counts per minute) was determined with a γ counter. In competitive tests, cells were first preincubated for 1 h at 37°C with different concentrations of unlabeled SEA. All tests were performed in triplicate, and the standard error of the mean (SEM) was less than 10% in all assays.

Biological activity of produced SEAs. (i) IL-2 assay. The ability of recombinant mutant SEA to stimulate T cells to produce interleukin 2 (IL-2) was assessed by using murine T-cell line 3DT expressing Vβ1 and Vβ8.1. Briefly, 8×10^4 cells were incubated with different concentrations of cSEA or rSEA in each well of 96-well plates for 24 h at 37°C in the presence of 2×10^4 cells of an HLA-DR1-transfected fibroblast cell line, DAP-3, as antigen-presenting cells or nontransfected DAP cells as a negative control. Supernatants were collected and tested for their levels of IL-2 by using the CTLL cell line. Then 10^4 CTLL cells were cultured in each well of 96-well plates for 24 h in the presence of 100 μl of supernatants or a standard curve of recombinant IL-2. The cells were pulsed with 0.2 μCi of [^{3}H]thymidine for 18 h and harvested, and then the [^{3}H]thymidine incorporated into cellular DNA was counted.

(ii) Proliferation assay. The ability of recombinant mutant SEA to stimulate T-cell proliferation was assessed by culturing murine splenic cells (3.5×10^5 per well in 96-well plates) in the presence of different concentrations of SEA. The cells were cultured for 72 h and then pulsed for 18 h with 1 μCi of [^{3}H]thymidine. Cells were harvested onto glass fiber filters, and incorporation of [^{3}H]thymidine was assessed. Alternatively, human peripheral blood mononuclear cells were isolated by the Ficoll-Hypaque method (5) from human peripheral blood obtained from healthy donors. These cells were incubated (10^5 cells per well) with different concentrations of SEA in 96-well plates for 48 h, pulsed with 1 μCi of [^{3}H]thymidine for 16 to 23 h, and then harvested as described above. Samples were assayed in triplicate, and the data were reported as the mean counts per minute. The SEM was less than 10% in all cases.

IL-1β gene expression by Northern (RNA) blotting. The mRNA levels of IL-1β and IL-1 receptor antagonist (IL-1Ra) following SEA stimulation were determined by Northern blot analysis performed by standard methods (35). Briefly, following stimulation, cells were harvested and cytoplasmic RNA was purified as previously described (35). Then 10 μg of RNA was loaded onto 1% agarose gels, transferred onto Hybond-N filter paper, and hybridized with random-primer-labeled cDNA probes. Equal loading of RNA was monitored by hybridization with a *GADPH* (glyceraldehyde-3-phosphate dehydrogenase) cDNA probe from the American Type Culture Collection (1). All washes were performed under stringent conditions. The mRNA hybridizing with the cDNA probes was visualized by autoradiography. The cDNA probes used in this study were IL-1β from the American Type Culture Collection and IL-1Ra, a generous gift from D. E. Tracey (Upjohn Co., Kalamazoo, Mich.).

RESULTS

Production of rSEA mutant and wild-type toxin. Genomic DNAs of three randomly chosen *S. aureus* strains were isolated and used as a template in PCR to amplify the SEA gene as described in Materials and Methods. The nucleotide sequences of all PCR fragments revealed a single mutation changing the aspartic acid (D) at position 60 to asparagine (N). This mutation seems to occur at a high frequency in *S. aureus* since all of the three strains tested produced the mutated SEA; however, additional strains should be screened to confirm this observation. The rSEA mutant toxin (rSEA-m_{D60N}) was isolated from a clone derived from strain A4 and purified by gel affinity. SDS-PAGE analysis demonstrated that the produced rSEA-m_{D60N} has the same molecular weight as cSEA and also strongly reacts with anti-SEA antibodies as determined by Western blotting (immunoblotting) (Fig. 1). Using the plasmid pUC19 containing the SEA gene as a DNA template for PCR, we produced a wild type rSEA toxin (rSEA-w) in which position 60 was confirmed to be D. rSEA-w possessed the same molecular weight and reactivity pattern to anti-SEA antibodies as cSEA and rSEA-m_{D60N} (Fig. 1). It is worth noting that although cSEA is highly pure, some preparations show two

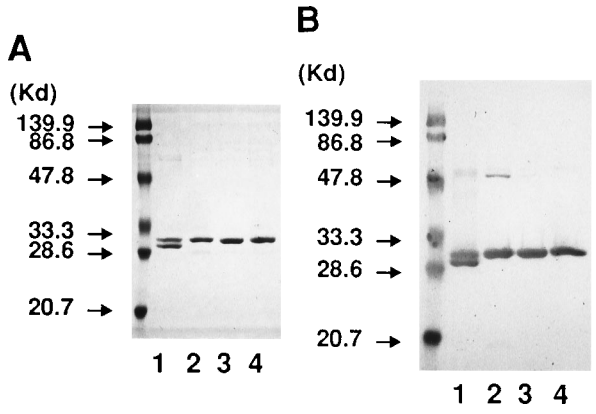

FIG. 1. Coomassie blue-stained SDS–12% polyacrylamide gel under reducing conditions (A) and Western blot (B) as revealed by anti-SEA MAb. Lanes: 1, cSEA; 2, rSEA-m_{D60N} from the A4 strain; 3, rSEA-m_{D60N} from STA 1361; 4, rSEA-w from plasmid pUC19 containing the SEA gene. Each lane was loaded with 1 μg of SEA.

adjacent fragments which may result from proteolysis that do not seem to affect its specificity.

Binding of mutant SEA to MHC class II molecules. SAGs are characterized by the ability to bind MHC class II molecules on antigen-presenting cells, and the complex so formed interacts with the TCR of T lymphocytes in a Vβ-restricted fashion, leading to activation of both cellular populations. The observed mutation may affect one or both of these two functions. To distinguish between these two possibilities, we used Raji and RM3 B-cell lines to evaluate the capacity of rSEA-m_{D60N} to bind HLA class II molecules. Our results demonstrated that rSEA-m_{D60N} binds HLA class II molecules on Raji cells as efficiently as cSEA (Fig. 2A). The absence of binding of both rSEA-m_{D60N} and cSEA on the RM3 cells, which are HLA class II-negative derivatives of Raji cells, confirms the specificity of this binding. Competition assays between rSEA-m_{D60N} and cSEA for binding HLA class II molecules on Raji cells illustrated that rSEA-m_{D60N} inhibits ^{125}I-labeled cSEA binding at the same order of magnitude as cSEA (Fig. 2B). Similarly, cSEA inhibits the binding of ^{125}I-labeled rSEA-m_{D60N} as efficiently as rSEA-m_{D60N}. Binding assays using DR1-transfected L cells similarly demonstrated the capacity of rSEA-m_{D60N} to bind transfected MHC class II molecules in a manner comparable to that of cSEA (data not shown). Together, these results demonstrate clearly that the mutation of the amino acid residue at position 60 from D to N does not affect MHC class II binding.

rSEA-m_{D60N} has the capacity to activate MHC class II-positive cells. Engagement of MHC class II molecules in monocytes by SAGs induces several cellular events leading to gene expression of different cytokines, such as IL-1β and tumor necrosis factor alpha (1, 13, 44). Our previous studies demonstrated the capacity of cSEA to induce selectively IL-1β gene expression over IL-1Ra in the human gamma interferon-treated monocytic cell line THP-1 (2). Hence, we evaluated the capacity of rSEA-m_{D60N} to induce IL-1β gene expression in these cells. Cells were pretreated with gamma interferon for 48 h to a induce high level of expression of MHC class II molecules (THP-1 cells express very low levels of these molecules); then they were washed and stimulated with different concentrations of rSEA-m_{D60N} and cSEA. Both toxins induced, in a dose-dependent manner, significant IL-1β gene expression and, as expected, had no effect on the IL-1Ra mRNA level (Fig. 3). These results confirm that the reported natural mutation does not affect the capacity of SEA to trigger the signaling pathway(s) via MHC class II molecules that leads to cytokine gene expression.

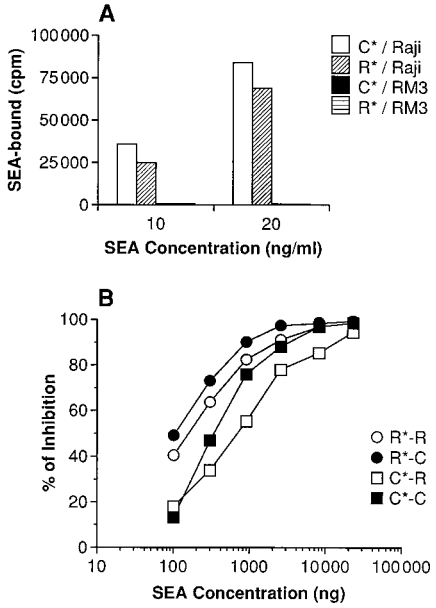

FIG. 2. (A) Binding of cSEA and rSEA-m_{D60N} to Raji (MHC class II-positive) and RM3 (MHC class II-negative) cells. A total of 4×10^5 cells were incubated with 10 or 20 ng of ^{125}I-labeled cSEA or rSEA-m_{D60N} in 200 ml of binding buffer for 1 h at 37°C and then pelleted, and their activities were determined with a γ counter. Data are presented as the means of duplicate measurements. It is worth noting that the mean values for cSEA and rSEA-m_{D60N} binding to RM3 cells are 698 and 745, respectively. (B) Competition assays between rSEA-m_{D60N} and SEA-w. A total of 4×10^5 cells were incubated with different concentrations of unlabeled rSEA-m_{D60N} or SEA-w for 1 h at 37°C and then incubated pretitered ^{125}I-labeled cSEA or rSEA-m_{D60N}. R*, ^{125}I-labeled rSEA-m_{D60N}; R, unlabeled rSEA-m_{D60N}; C*, ^{125}I-labeled cSEA; C, unlabeled cSEA. Data are presented as percent inhibition of means of triplicates. The SEM was less than 10% in all assays.

Effect of the isolated mutation on lymphocyte stimulation. (i) Human PBL. To evaluate the effect of the D60N mutation

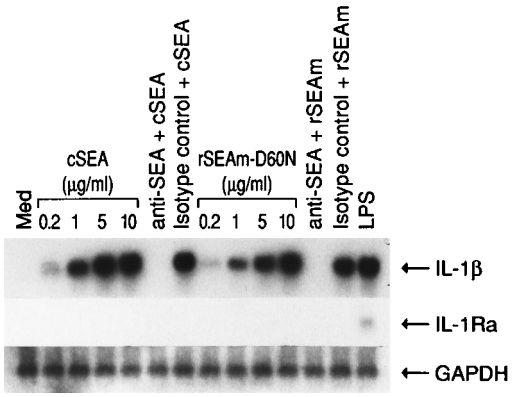

FIG. 3. rSEA-m_{D60N} induces IL-1β gene expression in MHC class II-positive cells. THP-1 cells were treated with gamma interferon for 48 h to reach high levels of MHC class II molecule expression, then washed, and stimulated with different concentrations of cSEA or rSEA-m_{D60N} for 3 h at 37°C. The specificity of the response was verified by stimulating the cells in the presence of anti-SEA MAb or an isotype control antibody. At the end of the incubation period, the total RNA was purified, and the level of mRNA expression of IL-1β and IL-1Ra was evaluated by Northern blotting. Equal RNA loading was controlled by using the housekeeping probe *GAPDH*. Med, medium; LPS, lipopolysaccharide.

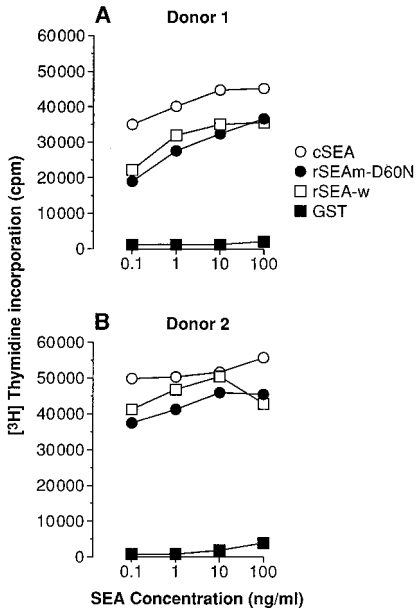

FIG. 4. Human PBL activation following stimulation with the different SEAs. Human PBL cells were purified from donor 1 (A) or donor 2 (B), and then cells (10^5 per well) were stimulated for 72 h with different concentrations of cSEA, rSEA, rSEA-m_{D60N}, or GST as a control. Cellular proliferation was then determined by [^{3}H]thymidine incorporation. Data are presented as the means of triplicate measurements. The SEM was less than 10% in all experiments.

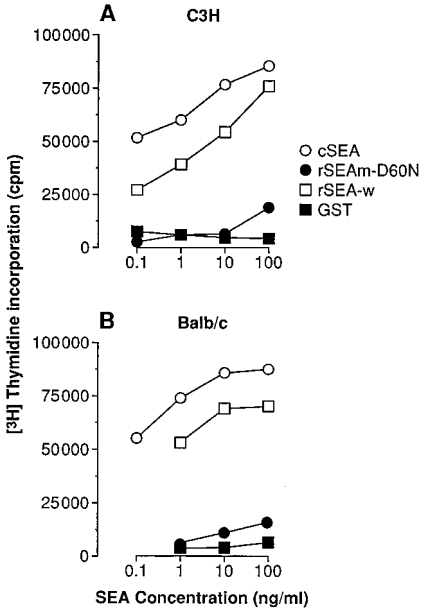

FIG. 5. Stimulation of murine splenic cells by different concentrations of cSEA, rSEA, or rSEA-m_{D60N}. C3H (A) or BALB/c (B) cells (3.5×10^5 per well) were stimulated for 72 h, and then proliferation was measured by [^{3}H]thymidine incorporation. Data are presented as the means of triplicate measurements. The SEM was less than 10% in all experiments.

on human lymphocyte activation, peripheral blood lymphocytes (PBL) were purified from blood samples from several donors. The cells were stimulated with different concentrations of rSEA-m_{D60N}, rSEA-w, or cSEA, and cellular proliferation was evaluated by [^{3}H]thymidine incorporation after 72 h. Our results show that all toxins induce human PBL proliferation (Fig. 4). Stimulation with rSEA-m_{D60N} or rSEA-w appeared to induce a proliferative response that had a magnitude slightly lower than that of cSEA. Accordingly, the change of residue at position 60 from aspartic acid to asparagine did not seem to have pronounced effects on human PBL proliferation.

(ii) Murine splenic cells. The capacity of mutated SEA toxin to stimulate murine lymphocytes was also evaluated by conducting a series of experiments using murine splenic cells from C3H and BALB/c mice. Cells were prepared and stimulated with different concentrations of cSEA, rSEA-w, rSEA-m_{D60N}, or GST in 96-well plates, and [^{3}H]thymidine incorporation was assessed after 72 h. Both cSEA and rSEA-w induced significant cellular proliferation at concentrations as low as 0.1 ng/ml. rSEA-m_{D60N} failed to induce any significant response (Fig. 5). However, when mutated SEA toxin was used at 100 ng/ml, a slight response was detected with both C3H and BALB/c splenic cells. These results suggest that D60N has a pronounced effect on murine T-cell stimulation by SEA, although it may not affect all of the specific Vβ elements recognized by the toxin or may affect the binding affinity of SEA toward Vβ elements; both possibilities might explain the weak stimulation observed at high concentrations of the mutated SEA, and they are currently under investigation.

Reactivity with T-cell clones. SEA interacts with and activates murine T lymphocytes bearing the Vβ1, -3, -10, -11, or -17 element of TCR with variable magnitudes of response. Indeed, this variability seems more pronounced between different species. To assess the effect of the D60N mutation on murine T-cell activation, we used a T-cell clone bearing Vβ1 (3DT) which is known to recognize SEA. DR1-transfected fibroblast cells were used as antigen-presenting cells, since they can bind efficiently cSEA, rSEA-w, and rSEA-m_{D60N}. Although cSEA and rSEA-w stimulated the 3DT cells very well, rSEA-m_{D60N} failed to induce any response (Fig. 6). Because rSEA-m_{D60N} interacts efficiently with DR1-transfected cells, these data indicate that the amino acid residue at position 60 appears to be essential for the interaction of SEA with murine T cells bearing the Vβ1 element. Since rSEA-m_{D60N} purification requires digestion with bovine thrombin, we verified whether this treatment had any inhibitory effect on T-cell activation. cSEA underwent the same thrombin digestion as applied for rSEA-m_{D60N} production. Figure 5 demonstrates clearly that this kind of treatment has no effect on T-cell

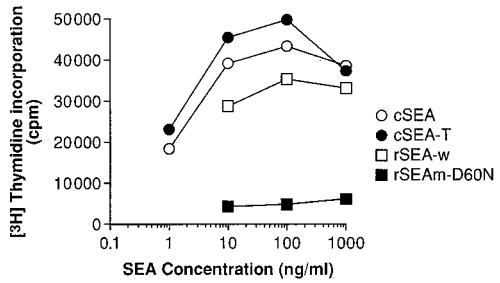

FIG. 6. IL-2 production by 3DT murine T-cells after stimulation with SEA presented by HLA-DR1-transfected cells as determined by the proliferation response of CTLL cells. 3DT cells (0.8×10^5 per well) were cultured for 24 h in the presence of DR1-transfected cells (0.2×10^5 per well) and stimulated by various concentrations of cSEA, cSEA treated with bovin thrombin (cSEA-T), rSEA-w, or rSEA-m_{D60N}. Then 100 μl of supernatant was collected and added to CTLL cells (10^4 per well). Twenty-four hours later, thymidine was added, and CTLL proliferation was determined by [^{3}H]thymidine incorporation after overnight incubation. Data are presented as the means of triplicate measurements. The SEM was less than 10% in all assays.

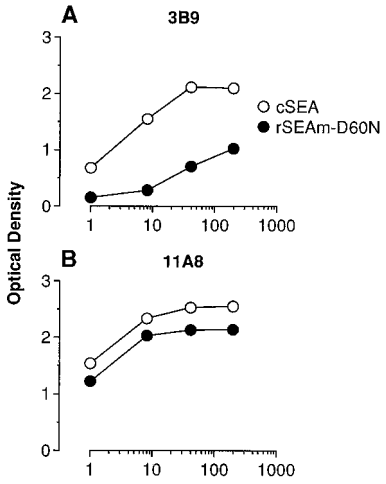

FIG. 7. Reactivities of MAbs 3B9 and 11A8 with cSEA or rSEA-m_{D60N}. Ninety-six-well plates were coated with 1 mg of 3B9 (A) or 11A8 (B) per ml, incubated with different concentrations of cSEA or rSEA-m_{D60N} for 1 h at 37°C, and then incubated with rabbit anti-SEA antibody for another hour. The reaction was completed by an incubation with peroxidase-labeled goat anti-rabbit immunoglobulin and revealed with ABTS substrate. Data are presented as means of optical density counts (at 490 nm) of triplicate measurements. The SEM was less than 10% in all assays.

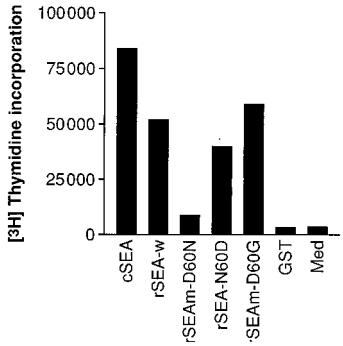

FIG. 8. Proliferation responses of murine splenic cells to rSEA-m_{D60N}, rSEA-m_{N60D}, and rSEA-m_{D60G}. Murine splenic cells (3.5×10^5 per ml) were stimulated with 1 ng of cSEA, rSEA-w, rSEA-m_{D60N}, rSEA-m_{N60D}, or rSEA-m_{D60G}. Proliferation was determined after 72 h by [^{3}H]thymidine incorporation. Data are presented as means of triplicate measurements. The SEM was less than 10% in all assays.

stimulation by cSEA. Accordingly, we can confirm that the failure of rSEA-m_{D60N} to stimulate 3DT was due to the mutation and was not a result of any inhibitory activity from the experimental manipulation.

Reactivities of rSEA-w and rSEA-m_{D60N} with anti-SEA MAbs. To study the influence of the D60N mutation on SEA structure and antigenicity, we compared the reactivities of different anti-SEA MAbs with rSEA-m_{D60N} and cSEA. Our panel of anti-SEA MAbs consisted of 20 MAbs that react strongly but with different affinities with SEA. Certain of these MAbs inhibit the binding of SEA to MHC class II molecules, whereas others affect the SEA reactivity with T cells (unpublished data). By direct ELISA and Western blotting, all of our MAbs reacted with rSEA-m_{D60N} in the same fashion as with cSEA. However, using sandwich ELISA, in which MAbs were immobilized on plates, we distinguished two MAbs according to their reactivities with rSEA-m_{D60N} or cSEA. MAb 3B9, which inhibits T-cell proliferation and IL-2 production following stimulation with SEA (unpublished data), recognizes rSEA-m_{D60N} more weakly than cSEA (Fig. 7A). In contrast, MAb 11A8, which inhibits SEA binding to MHC class II molecules, reacts similarly with rSEA-m and cSEA (Fig. 7B). The significant difference between the reactivity of 3B9 with rSEA-m_{D60N} and its reactivity with cSEA and the absence of this difference for 11A8 all suggest that the D60N mutation introduces conformational changes on the SEA molecule that affect the interactions of SEA with murine TCR without modulating its interactions with MHC class II molecules.

Reactivity of corrected rSEA-m_{N60D} with murine T cells. To confirm that the D60N mutation affects the reactivity of SEA with murine T cells, we corrected this natural mutation by changing N to D by targeted mutagenesis. Murine splenic cells were stimulated with 1 ng of cSEA, rSEA-w, rSEA-m_{D60N}, or rSEA-m_{N60D} for 72 h. Our results show that changing N to D at position 60 reestablished about 75% of the capacity of this rSEA to activate murine splenic cells (Fig. 8). This finding confirms the importance of position 60 of mature SEA toxin to its interactions with T cells.

Effect of substituting N by G at position 60 on T-cell stimulation. Recently, it was reported that substitution of the D residue at position 60 of mature SEA with G had no discernible effect on the serological or biological activity of SEA (15). As N and G belong to the noncharged amino acid group, it was of interest to analyze the effect of substituting the N residue 60 of mature rSEA-m_{D60N} by G. Our results show that G at position 60 restores 100% the wild-type activity (Fig. 8). This observation confirms the previously published report (15) and indicates that the structure of the amino acid residue at position 60 is more important than its charge for the activity of SEA with murine cells.

DISCUSSION

Different approaches, such as use of mutagenesis, synthetic peptides, and chimeric genes, have been used to identify the epitopes involved in the immunological and biological activities of staphylococcal SAGs. It has been shown that SEA interacts with the β chain of MHC class II molecules by its C-terminus via histidines 187 and 225 and aspartic acid 227 (9) and with the α chain by its N-terminus via phenylalanine 47 (43). The amino acid residues at positions 206 and 207 are involved in its interactions with T cells (21). This study describes, for the first time, a naturally occurring mutated SEA molecule and analyzes its immunological activities. The fact that this mutated SEA is produced by three different *S. aureus* strains is of special interest. It may suggest the existence of more than one type of SEA with different activities because the isolated mutation was not limited to one strain. On the other hand, this mutated SEA presents a naturally occurring product with which to study the importance or influence of certain amino acids along with their positions in the immunological activities of this SAG.

Our results demonstrated that mutated SEA binds efficiently to human MHC class II molecules. According to recent findings, different regions of SEB seem important to different haplotypes of human MHC class II molecules (40). Given these data, our observation that the mutated SEA bound DR1-transfected L cells as efficiently as it bound MHC class II molecules (DR3, DR10, DQ2, and DQ1) on Raji cells can be a supplementary indication that this mutation is not involved in MHC class II binding. The capacity of mutated SEA to reproduce the previously reported (2) selective induction of IL-1β gene expression over its receptor antagonist confirms that its

binding to MHC class II molecules is as completely functional as that of cSEA or rSEA-w. Hence, changing the aspartic acid at position 60 of SEA to an asparagine does not affect the superantigenic immunological character of SEA in linking and signaling via MHC class II molecules.

A study by Kappler and collaborators demonstrated the amino acid residues at positions 60 and 61 of SEB to be important for reactivity with some but not all Vβ elements of murine T cells (25). Later, this observation was confirmed by another group, who showed that these two positions are central to conferring the specificity for TCR Vβ gene products for SEB interactions with human and murine TCR (17). Stimulation of human PBL with rSEA-m_{D60N} induced a proliferative response similar in magnitude to that induced with rSEA-w. This result reveals that changing the D to N at position 60 of SEA does not affect the interaction of SEA with human T cells. However, we cannot rule out completely the possibility that the D60N mutation can introduce a switch in the SEA-specific Vβ repertoire, leading to the activation of unusual Vβ gene products that will sustain the observed proliferation. Although it is unlikely that such an event had occurred because the difference between rSEA-m_{D60N}- and rSEA-induced PBL proliferative responses is nonsignificant, this hypothesis is currently under investigation.

In contrast to the human system, this mutation had pronounced effects in the murine system. Stimulation of the murine T-cell clone bearing an SEA-specific Vβ element or total murine splenic cells with rSEA-m_{D60N} did not induce any activation, as evaluated by IL-2 production and thymidine incorporation. These data indicate a disruption in the capacity of SEA to sustain T-cell proliferation in the presence of the D60N mutation. The use of DR1-transfected L cells as antigen-presenting cells in T-cell clone stimulation assay and the fact that rSEA-m_{D60N} functionally binds human MHC class II molecules support the conclusion that the D60N mutation of SEA, by its presence, introduces a defect in the interactions between murine TCR and the toxin.

The mutation may affect the toxin structure in any of several ways; the absence or presence of side chains in the SEA structure may be critical contact points for Vβ or may provoke local perturbations in the secondary or tertiary structure of the toxin, which in turn can displace other amino acids critical in contacting Vβ. The D-to-N mutation changes a negatively charged amino acid to a polar noncharged one. This replacement can create an imbalance of charges, which in turn can affect the structure of the toxin. However, when N at position 60 of the mutated SEA was replaced by G, another member of polar noncharged amino acids, the capacity of this mutant to induce a murine T-cell proliferative response was reestablished, thus excluding the possibility that a change in charge is responsible for the lack of murine T-cell activation. More likely, the N residue, by its structure, at this position affects the capacity of SEA to interact with murine TCR specific for SEA. The reactivity of rSEA-m_{D60N} with a panel of 20 anti-SEA MAbs was not altered, as determined by direct ELISA or Western blotting, which indicates the absence of major conformational changes by this mutation. Nevertheless, the results obtained with sandwich ELISA, which is preferable for studying conformational changes, revealed certain alterations that notably affected the toxin–T-cell interactions. Accordingly, it is proposed that N at position 60 of SEA perturbs the tertiary structure of this toxin in a way that affects the interactions with murine T cells. Indeed, diminution of the toxin affinity toward murine TCR can result from these conformational changes. Consequently, the TCR-MHC class II interactions, described recently as important for SAG T-cell activation (6, 30), can also be affected. This disturbance can lead to the loss of mutated SEA T-cell mitogenicity.

The difference between the reactivity patterns of human and murine TCR with mutated SEA reflects a certain degree of species specificity of SAGs and merits further studies. However, the observed differences can be attributed to the higher affinity of SEA toward the human TCR than toward the murine TCR. In addition, the observed differences can result from the higher number of human TCR Vβ elements that can interact specifically with SEA (at least eight different human Vβ elements [9; unpublished data], compared with six murine Vβ elements [9]).

The different effects of the natural mutation at position 60 on the immunological activities of SEA with murine and human cells suggest that the interactions of staphylococcal enterotoxins are more complex than previously thought and that the relevance or the importance of different epitopes or regions implicated in these interactions can vary in different species.

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