Dissociation of the Stimulatory Activities of Staphylococcal Enterotoxins for T Cells and Monocytes

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Summary

The staphylococcal enterotoxins (SEs) are homologous proteins related in their capacity for stimulating both T cells and monocytes. To assess the importance of conserved structure and sequence to functional activity, the role of the disulfide loop and adjacent sequence in these toxins was evaluated. Contrary to previous reports, we demonstrate here that the disulfide loop was required for the mitogenic activity of SEA and SEB. While T cell–stimulatory activity was compromised, reduced and alkylated SEs retained major histocompatibility complex class II–binding and monocyte-stimulatory activities, suggesting that their inability to induce T cell proliferation was due to failure to interact with T cell receptor (TCR) rather than with class II molecules. Reduction and alkylation did not affect the far-ultraviolet circular dichroic spectrum of SEA, suggesting that the loss of mitogenic activity was not associated with significant changes in secondary structure. The disulfide linkage imparts considerable stability to these toxins as peptide cleavages within the loop of SEB were not associated with detectable loss of function, although cleavage in the conserved sequence outside the loop of SEA resulted in loss of mitogenic activity. This report thus establishes a functional role for a conserved element in SEs, the disulfide loop, and further indicates that their class II– and TCR-binding activities can be dissociated.

The staphylococcal enterotoxins (SEs)[1] are prototypic "superantigens" (for review see reference 1), a family of bacterial exotoxins related in their capacity to stimulate large populations of T cells in a class II MHC-dependent, yet unrestricted manner (2, 3). The SEs bind specifically to class II molecules (4–7) and preferentially activate T cells bearing particular Vβ TCR segments (8, 9). Mitogenic activity thus depends on functional bivalency, as SEs must interact with both class II and TCR molecules to trigger a T cell response. Additionally, the SEs are potent inducers of IL-1 and TNF-α from monocytes (10–13). SE-mediated stimulation of monocytes is a consequence of binding and transducing a positive signal through class II on the monocyte cell surface. This process can be blocked by anti–class II antibodies, and IFN-γ –induced upregulation of class II leads to enhanced responsiveness (14, 15). Our present studies are directed towards understanding how the structure of SEs relates to their dual capacity to stimulate T cells and monocytes.

Previous attempts to elucidate an active region(s) of SEs were largely restricted to examination of T cell–stimulatory activity, and have yielded conflicting results. Ezepchuk and Noskov (16) reported that the mitogenic domain of SEA and SEC_2 resided in the NH_2-terminal region. Spero and Morlock (17) similarly reported that a 6.5-kD NH_2-terminal tryptic fragment of SEC_1 was mitogenic, while the remainder of the molecule (19-kD COOH-terminal fragment) induced emesis in monkeys. Moreover, Pontzer et al. (18) reported the blocking of mitogenic activity of SEA using a synthetic peptide corresponding to the NH_2-terminal 27 amino acids. Conversely, several studies concluded that the NH_2 terminus was not involved in mitogenesis. Bohach et al. (19) reported that the first 59 residues were not required for mitogenic activity of SEC_1. In addition, analyses of cyanogen bromide (CNBr) cleavage fragments of staphylococcal toxic shock syndrome toxin 1 (TSST-1) indicated that a central 14-kD fragment was mitogenic (20, 21).

The similar functional activities of these toxins are paralleled by similarities in both structure and sequence. The SEs are generally small (25–30 kD), basic, heat- and acid-stable, single-chain molecules containing a short centrally located disulfide loop (22). Analysis of computer-aligned protein se-

[1] *Abbreviations used in this paper:* CD, circular dichroism; cm-cys, carboxymethylcysteine; DTT, dithiothreitol; FA, formic acid; GH, guanidine hydrochloride; IAA, iodoacetamide; R/A, reduced and alkylated; SE, staphylococcal enterotoxin (SEA, staphylococcal enterotoxin A, etc.); SPEA, streptococcal pyrogenic exotoxin A; TSST-1, toxic shock syndrome toxin 1.

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quences by Betley and Mekalanos (23) revealed considerable homology in the central and COOH-terminal regions, with the NH_2-terminal region being the least conserved part of these molecules. The SEs appear to segregate into two groups: SEA is most homologous to SEE and closely related to SED, while SEB and SEC_1 are closely related to each other. Two other toxins, streptococcal pyrogenic exotoxin A (SPEA) and TSST-1, display similar functional activities as SEs. SPEA is more similar to SEB and SEC_1 than it is to SEA (23). Finally, TSST-1 is anomalous; it is the least related (24) to the other toxins and the only one without a disulfide loop (22).

Although the disulfide loop is a striking structural feature of the SEs, previous studies (4, 25) examining its importance concluded that it was not required for the mitogenic activity of SEA. Our concern over the experimental conditions used in those studies, however, led us to reexamine this question. We have undertaken a more rigorous evaluation of the loop and its importance for functional activity, and reached the opposing conclusion that it is in fact essential for SE-mediated stimulation of T cells. As previous studies seeking active sites on toxin molecules and peptides primarily examined mitogenicity, they required that the TCR- and class II–binding regions be on the same fragment. Using SEA and SEB we have additionally examined these two activities independently, and describe the contribution of the physical structure of these molecules to their dual capacity for stimulating T cells and monocytes.

Materials and Methods

SEA. SEA was prepared, with modifications, as previously described (26). Casein yeast broth (4 liters) containing casein enzymatic hydrolysate (40 g/liter; Sigma Chemical Co., St. Louis, MO) and yeast extract (10 g/liter; Difco Laboratories, Inc., Detroit, MI), pH 7.2, was inoculated with a high-producer subclone of *Staphylococcus aureus* (No. 722; Food Research Institute, Madison, WI) and incubated at 37°C on a shaker overnight. The toxin-containing broth was clarified by centrifugation, sterile filtered, and passed through a YM-100 membrane (Amicon Corp., Danvers, MA). The filtrate was concentrated to 100 ml on a YM-10 membrane (Amicon Corp.) and dialyzed against 20 mM phosphate, pH 6.8. A 2.5 × 20-cm column of Red Dye A (Amicon Corp.) was stripped with 8 M urea containing 0.5 M NaOH and equilibrated with 20 mM phosphate, pH 6.8, at 23°C. The dialyzed toxin concentrate was filtered, and cycled over the column three times (30 ml/h). After extensive washing with 20 mM phosphate to remove unbound protein, a step-wise gradient (60–500 mM phosphate, pH 6.8) was applied to the column. The eluate was monitored at A_{280}, and protein-containing fractions were collected and analyzed by SDS-PAGE. Fractions containing SEA were pooled, concentrated, and desalted using Centriprep 10 membranes (Amicon Corp.), filtered, quantitated, and stored lyophilized at 4°C. This material migrated as a single band under reducing conditions, and appeared free of any contaminating proteins. Amino acid analysis and NH_2-terminal sequencing of the first 40 residues of one preparation were identical to the published sequence for SEA (27). Typical yield from 4 liters of broth was 40 mg.

Other Toxins and mAb. SEB was obtained in two forms from commercial sources: native toxin from Sigma Chemical Co. and toxin partially "nicked" by endogenous bacterial proteases from Toxin Technology (Madison, WI). NH_2-terminal sequencing of this nicked material indicated two internal cleavages at ser-104 and gln-106, both within the disulfide loop. Using Red Dye A chromatography, we purified nicked SEB from one batch of SEB prepared (from SEB-producing *S. aureus*, No. 1042; Food Research Institute) as described above for SEA. This material was cleaved at one site (thr-107, see Fig. 1), displayed comparable activity to Toxin Technology SEB and was used as the principal source of nicked SEB for these studies. TSST-1 was obtained from Toxin Technology. For mAb, ascites was obtained from hybridomas (American Type Culture Collection, Rockville, MD) and affinity purified on protein G–agarose. The anti–class II mAb L227 recognizes some but not all alleles of HLA-DR, -DP, and -DQ molecules (28), and anti–class I mAb W6/32 reacts with all alleles of HLA-A, -B, and -C molecules.

Characterization of Proteins. Toxins were analyzed by SDS-PAGE on precast 8–25% gradient acrylamide gels (Phast system; Pharmacia Fine Chemicals, Piscataway, NJ) to assess molecular weight and purity. Modification and/or destruction of individual residues was monitored by amino acid analysis (PicoTag system; Waters Associates, Milford, MA), and efficiency of alkylation was assessed using *S*-carboxymethyl-L-cysteine (cm-cys) as a standard (Fluka Chemika-Bio-Chemika, Ronkonkoma, NY). NH_2-terminal sequence analysis (477A protein sequencing system; Applied Biosystems, Inc., Foster City, CA) was carried out to confirm cleavage sites and identity of toxins and fragments. Protein concentrations were determined (except for circular dichroism [CD] analysis) using the bicinchoninic acid (BCA) assay kit (Pierce Chemical Co., Rockford, IL).

Reduction and Alkylation. Toxins were dissolved (5 mg/ml) in reduction buffer of 6 M guanidine hydrochloride (GH), 0.5 M Tris, 10 mM EDTA, pH 8.5, and blanketed with nitrogen. DL-Dithiothreitol (DTT; Sigma Chemical Co.) was added (5 mM final concentration), and tubes were incubated at 37°C for 2 or 3 h for SEA and SEB, respectively. Solid iodoacetamide (IAA; Sigma Chemical Co.) was then added (5–10 and 100 mM final concentration for SEA and SEB, respectively), and alkylation was allowed to proceed at 23°C in the dark for 30 min. The reduced and alkylated toxin was desalted on Sephadex G-25 or dialyzed into PBS, quantitated, diluted into serum-containing media, and stored at 4°C until used (usually within 24 h). A control sample was similarly prepared in 6 M GH buffer in the absence of DTT and IAA and similarly desalted. Aliquots were removed for analysis before the addition of serum.

CNBr Cleavage of SEA. SEA was dissolved (10 mg/ml) in 70% formic acid (FA) containing CNBr (2 mg CNBr:1 mg SEA). A control sample was similarly prepared, but in the absence of CNBr. Reactions were carried out in the dark at 23°C, under nitrogen, for 24 h. Samples were then diluted in water, and lyophilized. SDS-PAGE analysis indicated the presence of undigested SEA (routinely 10%), and it was necessary to remove this material before evaluating the bioactivity of the fragments. CNBr reaction products were resuspended in 6 M GH to ensure complete dissociation of aggregates and individual fragments, and loaded onto a 1.5 × 46-cm column bed (Sephadex G-50 fine) equilibrated with 1 M acetic acid at 23°C. The control sample (exposed to FA in the absence of CNBr) was separately desalted into 1 M acetic acid. CNBr fragments were eluted with a flow rate of 4–5 ml/h and lyophilized. These fragments were insoluble in water at neutral pH and consequently were redissolved in a small volume of 1 M acetic acid (as was the control sample), quantitated, diluted into serum-containing media, and stored at 4°C until used. Aliquots were removed for analysis before the addition of serum.

Mitogenesis Assay. Threefold dilutions of toxins were made in

96-well round-bottomed microtiter plates. PBL were obtained from heparinized venous blood by centrifugation over isolymph gradients, washed twice, and added (5×10^4/well) to the plates. Cultures were maintained in 200 μl RPMI 1640 containing 10% heat-inactivated and defibrinated human AB^+ serum, 2 mM L-glutamine, 10 mM Hepes, and antibiotics (culture medium) in a humidified atmosphere (5% CO_2) at 37°C. Cells were cultured for 3 d, with 1 μCi [^{3}H]TdR (2 Ci/mmol; Dupont Co., Wilmington, DE) added per well for the final 24 h of culture. DNA was harvested onto glass fiber filters, and incorporation of [^{3}H]TdR was assessed by liquid scintillation counting.

Monocyte Production of TNF-α. Human monocytes were obtained from PBL by adherence on polystyrene dishes, and added (5×10^5/well) to 24-well polystyrene flat-bottomed wells in 1 ml culture medium. After incubation at 37°C for 2 h, residual nonadherent cells were removed by vigorous washing. Wells were filled with 1 ml fresh culture medium containing 25 U/ml human rIFN-γ (Genzyme Corp., Boston, MA). Plates were incubated for 24 h at 37°C before the addition of toxins. Supernatants were then collected 18 h later, and sterile filtered; and TNF-α content was measured by ELISA (Quantikine kit; R & D Systems, Minneapolis, MN). Such preparations of doubly adhered cells routinely contained >90% monocytes (Leu M5; Becton Dickinson & Co., Mountain View, CA) and <2% T cells (Leu 3a; Becton Dickinson & Co.), as assessed by flow cytometry. For antibody blocking experiments, monocytes were not pretreated with IFN-γ and mAb were added 1 h before the addition of toxins.

Class II Binding. The ability of toxins to bind MHC class II molecules directly was assessed using HLA-DR–transfected fibroblast lines L165.1 (DR4/Dw14; reference 28; kindly provided by R. Karr) and D.5-3.1 (DR1; reference 29; kindly provided by E. Long). Cells (2×10^5/tube) were preincubated with increasing amounts of competitor toxin in 200 μl of HBSS containing 10% serum at 37°C for 1 h. A pretitered amount of biotinylated SEA was added (~0.5 μg/tube), and cells were incubated for an additional 2 h at 37°C. After washing, cells were incubated in HBSS containing 10% serum and FITC-conjugated avidin (Becton Dickinson & Co.) on ice for 30 min. After washing, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry (Epics Profile; Coulter Electronics Inc., Hialeah, FL).

Circular Dichroism (CD). Toxins were dialyzed (10 mM phosphate, 200 mM KF, pH 6.8) to minimize the presence of Tris and chloride ions, which cause significant interference in the far-UV CD spectrum. Toxins exposed to 6 M GH became cloudy upon extensive dialysis, and these solutions were clarified by centrifugation. For all samples, functional activity was not qualitatively altered by dialysis and centrifugation. Samples so treated displayed a clean UV spectrum, and protein concentrations were adjusted by extinction coefficient ($E^{1\%}_{1\,cm}$277 nm for SEA = 14.3) immediately before analysis. Far-UV CD spectra were obtained at 23°C in a 0.5-mm path length cell (NSG Precision Cells, Inc., Farmingdale, NY) from five repetitive scans (50 nm/min) on a spectropolarimeter (model J-500A; Jasco Inc., Easton, MD). Raw spectra were converted to ellipticity plots, and conformational analysis was performed as previously described by Chang et al. (30). Conformational predictions from amino acid sequence were obtained using conventional Chou–Fasman analysis as previously described (31).

Results

Conserved Sequence and Structural Motif in Bacterial Toxins. We began our studies with the premise that common functional activities among these bacterial exotoxins was a reflection of similarities in protein sequence and structure. Our initial strategy was to consider highly conserved sequence and structural motifs as candidates for regions involved in stimulating T cells and monocytes, namely, regions mediating interactions with TCR and class II molecules. Examination of the protein sequences of several mitogenic toxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes* revealed a striking island of sequence homology adjacent to a short disulfide loop in the central region of these molecules (Fig. 1).

Disulfide Loop Required for Mitogenicity of SEA. Previous studies with SEA in two laboratories concluded that this centrally located disulfide loop was not required for mitogenic activity. Noskova et al. (25) reported that exposure of SEA to 2-ME (in the absence of alkylating agent) did not affect its mitogenic activity on mouse splenocytes. Fraser (4) similarly reported that exposure of SEA to both 2-ME and alkylating agent did not affect its activity on human T cell proliferation, although the efficiency of alkylation was not assessed. The possibilities that the protein was not completely reduced (as these procedures were carried out under nondenaturing

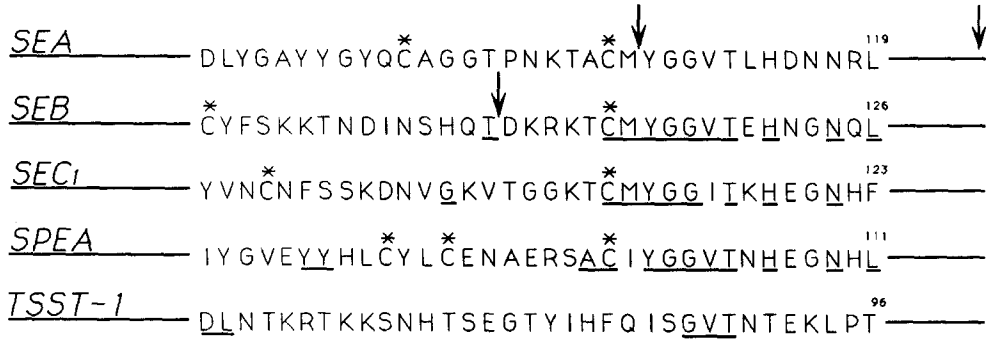

Figure 1. Similar sequence and structural motif in central region of bacterial exotoxins. Amino acid sequences for SEA, SEB, SEC_1, SPEA, and TSST-1 derived from Huang et al. (27), Jones and Khan (37), Schmidt and Spero (38), Weeks and Ferretti (39), and Blomster-Hautamaa et al. (24), respectively. Numbers above last residue correspond to residues in mature protein. Underlined residues indicate identity to residues in SEA. Asterisks indicate cys residues (C) involved in disulfide loops; for SPEA, it is unclear which upstream cys is disulfide linked (22). Solid arrows indicate CNBr cleavage sites in SEA and internal cleavage in nicked SEB.

Table 1. *Reaction Conditions and Efficiency of Reduction/Alkylation of SEA*

Solvent	Reduction	Alkylation	Amino Acid Analysis*			
			his (6)‡	met (2)	tyr (18)	cm-cys (2)
			pmol			
Water	–	–	785	385	2,182	<10
Water	150 mM 2-ME (10 min, 23°C)	–	577	294	1,631	<10
Water	150 mM 2-ME (10 min, 23°C)	180 mM IAA (30 min, 23°C)	694	337	1,866	<10
6 M GH§	–	–	628	354	1,817	<10
6 M GH	5 mM DTT (2 h, 37°C)	–	713	386	2,160	<10
6 M GH	5 mM DTT (2 h, 37°C)	5 mM IAA (30 min, 23°C)	789	423	2,356	257

0.5 mg SEA (in 100 μl) was used for each reaction.
* Approximately 5 μg SEA was hydrolyzed and subjected to amino acid analysis. Recovery of residues (pmol) was based on calibrated standards. Unprotected cys and trp residues are destroyed in hydrolysis, and were not assayed.
‡ Theoretical number of residues per molecule.
§ 6 M guanidine hydrochloride containing 0.5 M Tris, 10 mM EDTA, pH 8.5.

conditions) or that free sulfhydryls reassociated (if not efficiently alkylated) prompted us to reexamine this issue.

We subjected SEA to the previously described reaction conditions and determined both the efficiency of alkylation and effect on mitogenicity. Reduction with 2-ME and alkylation under nondenaturing conditions (4) did not result in alkylation of cysteine residues as reflected by the absence of cm-cys in the amino acid analysis profile (Table 1). As shown in Fig. 2, SEA exposed to such treatment retained full mitogenic activity, in accordance with the previously cited studies (4, 25). A more rigorous treatment (32), however, using denaturing conditions (6 M GH) for both reduction and alkylation, yielded essentially quantitative alkylation of the protein (Table 1). Such treatment abrogated the mitogenicity of SEA (Fig. 2).

We first ruled out the possibility that loss of mitogenicity

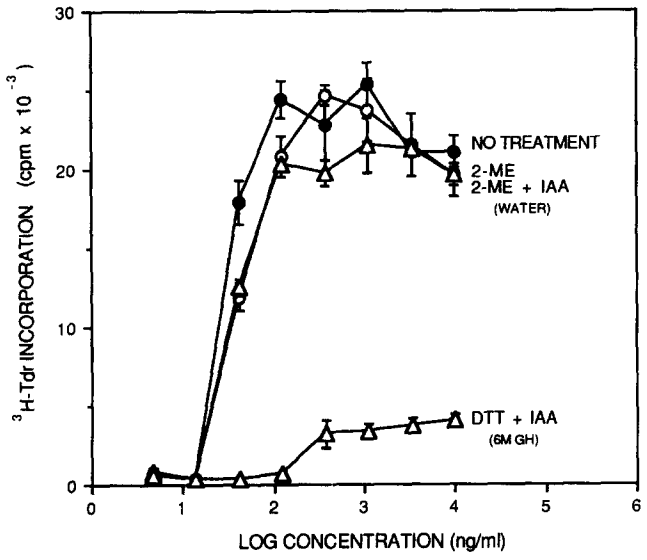

Figure 2. Reduction and alkylation under denaturing conditions abrogates mitogenicity of SEA. SEA was exposed to various reaction conditions, as detailed in Table 1. Error bars indicate SEM of triplicate determinations. Same sample preparations used here and in Table 1.

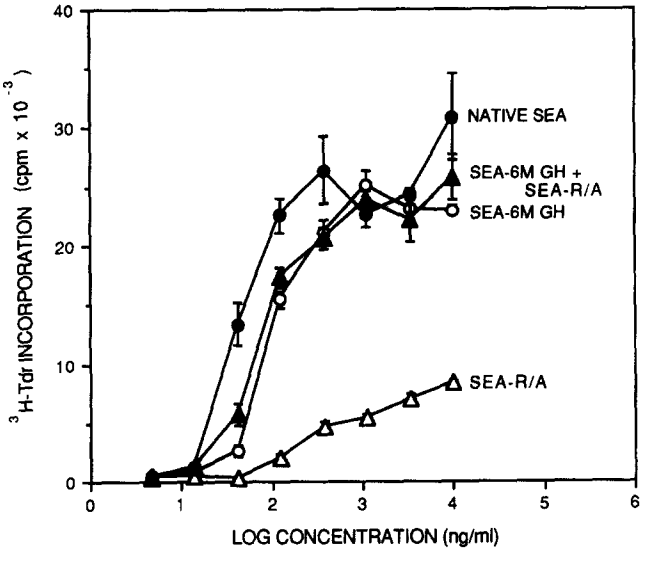

Figure 3. Denaturation in 6 M GH does not affect mitogenicity of SEA. SEA was reduced and alkylated (*SEA-R/A*) as described; control-reacted material (*SEA-6 M GH*) was similarly prepared but in the absence of reducing and alkylating agents. Error bars indicate SEM of triplicate determinations.

was simply a result of denaturation of the protein in 6 M GH. As shown in Fig. 3, control-reacted material (SEA–6 M GH) subjected to similar denaturing conditions in the absence of DTT and IAA was not significantly less mitogenic than native SEA. Furthermore, the inability of the reduced and alkylated (R/A) material (SEA–R/A) to stimulate T cell proliferation was not due to the presence of residual DTT or IAA in the preparation, as it was not inhibitory when mixed with SEA–6 M GH (Fig. 3). The alkylating agent IAA can potentially alkylate met residues (33), as well as liberate free iodine under certain conditions, resulting in modification or damage of his and tyr residues (32). Amino acid analysis profiles of SEA–6 M GH and SEA–R/A, however, were identical except for the presence of cm-cys in SEA–R/A, confirming that no residues had been destroyed or altered by the treatment (data for his, met, tyr, and cm-cys shown in Table 1). SDS-PAGE analysis revealed that SEA–R/A migrated as a single band under reducing conditions (Fig. 4, lane *3*), indicating degradation of the protein had not occurred. Finally, we subjected TSST-1, unique among the staphylococcal exotoxins in lacking a disulfide loop (see Fig. 1), to similar reaction conditions and found that mitogenic activity was not affected (data not shown). Thus, the inability of SEA–R/A to stimulate T cells appears to be a direct consequence of reduction and alkylation of the cysteines forming the disulfide loop.

Reduced and Alkylated SEA Retains Class II Binding and Monocyte-stimulatory Activity. We next examined whether SEA–R/A was also deficient for monocyte stimulation. Previous studies (10–12) demonstrated that TSST-1 is a potent inducer of TNF-α from human monocytes, and recent work from our laboratory (13–15) showed that SEs exhibit similar monocyte-stimulatory activity. We cultured monocytes with native SEA, SEA–6 M GH, and SEA–R/A, and measured TNF-α levels in the supernatants. As shown in Fig. 5 *A*,

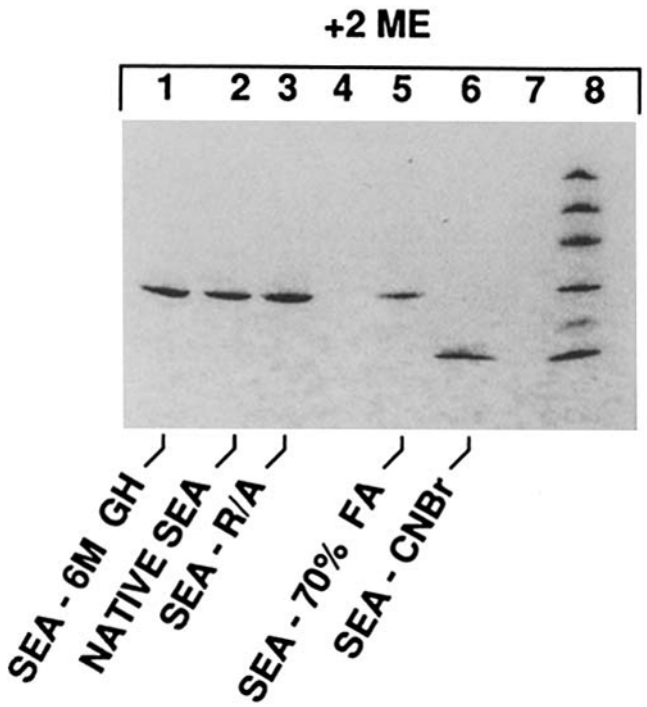

Figure 4. SDS-PAGE analysis demonstrates integrity of SEA and CNBr cleavage fragments. Approximately 0.4 μg of each sample was solubilized in 5% SDS containing 10% 2-ME and subjected to electrophoresis on a 8–25% precast acrylamide Phast gel (10 mA, 70 AVh) at 15°C, followed by staining with Coomassie blue. (Lane *1*) control-reacted SEA; (lane *2*) native SEA (*SEA-6 M GH*); (lane *3*) reduced and alkylated SEA (*SEA-R/A*); (lane *5*) control-reacted SEA (*SEA-70% FA*); (lane *6*) purified CNBr fragments (*SEA-CNBr*); (lane *8*) 94-, 65-, 45-, 30-, 20-, and 14-kD markers. Lanes *4* and *7* contained no samples.

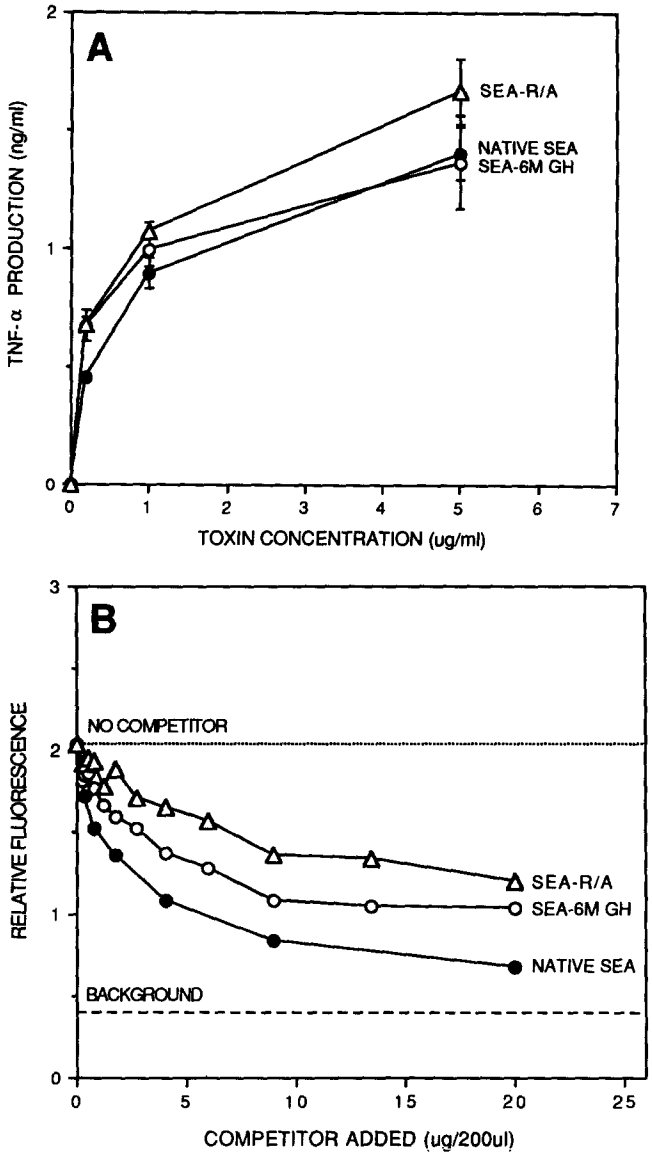

Figure 5. Reduced and alkylated SEA induces TNF-α production from monocytes and binds class II molecules. (*A*) Monocyte production of TNF-α. Error bars indicate SEM. Same sample preparations used here and in Fig. 3. (*B*) Class II binding. Data expressed in linear fluorescence units. Same sample preparations used in *A* and *B*.

reduction and alkylation did not affect the capacity of SEA to induce monocyte TNF-α production. Thus, while reduction and alkylation compromised the T cell–stimulatory activity of SEA, its monocyte-stimulatory activity remained intact.

The ability of SEA-R/A to stimulate monocytes suggests that its lack of mitogenic activity cannot be attributed to an inability to bind class II molecules. To assess class II binding affinity more directly, we examined the ability of SEA-R/A to compete with labeled, native SEA for binding to class II–transfected cells. SEA-R/A was able to block binding of biotinylated SEA to DR1-transfected fibroblasts in a competitive binding assay (Fig. 5 *B*). We consistently observed, however, that the blocking activity of SEA-R/A was slightly less than that of SEA-6 M GH (which in turn was less than that of native SEA). Similar results were obtained using DR4-transfected cells (data not shown).

To investigate whether SEA-R/A was in fact signaling monocytes through its capacity to bind class II molecules, we attempted to block this response with anti–class II antibody. Previous studies in our laboratory (14, 15) showed that monocyte TNF-α production in response to SEA and TSST-1 could be significantly blocked by anti–class II antibodies. As shown in Table 2, antibody to class II but not class I significantly inhibited SEA-R/A-induced TNF-α production from monocytes. This inhibition was comparable to that observed for native SEA and SEA-6 M GH (Table 2). Failure to block the response completely may reflect incomplete blocking of multiple SEA-binding sites on class II, as well as lack of antibody binding to some alleles of HLA-DR, -DP, and -DQ molecules potentially expressed on donor cells.

Consequences of Cleavage in the Central Regions of SEA and SEB. We next examined the role of the highly conserved sequence adjacent to the disulfide loop in SEs and other bacterial toxins. We reacted SEA with CNBr, which cleaves at met-107 just outside the loop (and at the COOH terminus, see Fig. 1), yielding two large fragments of 12.4 and 13.5 kD. We purified these two peptides from residual (unreacted) SEA by gel filtration; SDS-PAGE analysis revealed the absence of starting material (Fig. 4, lane *6*) and amino acid analysis revealed only a loss of met residues (data not shown). We sequenced the first 15 NH_2-terminal residues to confirm the presence of the two expected peptides (data not shown). Despite the extended treatment with FA and acetic acid, control-reacted material (SEA–70% FA) migrated as a single band on SDS-PAGE (Fig. 4, lane *5*) and retained mitogenic activity (Fig. 6). CNBr cleavage, however, completely abrogated the mitogenicity of SEA (Fig. 6), in agreement with results of others (4).

We extended our studies of SEA to SEB because it afforded the opportunity to evaluate the consequences of cleaving the peptide sequence within the disulfide loop. Spero et al. (34) reported that SEB is particularly susceptible to such cleavage, yielding a "nicked" protein. We found one commercial preparation (Toxin Technology) that consisted almost entirely of such molecules, while another (Sigma Chemical Co.) consisted of native molecules and showed no evidence of internal cleavage. SDS-PAGE analysis revealed that nicked SEB migrated as a single band under nonreducing conditions (Fig. 7 *B*, lane 4), but as two smaller bands under reducing conditions (Fig. 7 *A*, lane 4). Nicked SEB was equally as mitogenic as native SEB (Fig. 8), in agreement with a previous report in which

Table 2. *SEA-R/A–Induced Monocyte TNF-α Production Is Blocked by Antibody to Class II but Not Class I Molecules*

Toxin*	Anti-class II‡	Anti-class I§	TNF-α production‖
–	–	–	37 (±2)
–	+	–	69 (±12)
–	–	+	55 (±7)
Native SEA	–	–	1,525 (±49)
Native SEA	+	–	760 (±13)
Native SEA	–	+	1,440 (±34)
SEA-6 M GH	–	–	1,473 (±35)
SEA-6 M GH	+	–	824 (±92)
SEA-6 M GH	–	+	1,568 (±71)
SEA-R/A	–	–	1,555 (±22)
SEA-R/A	+	–	896 (±36)
SEA-R/A	–	+	1,731 (±99)

* 5 μg/ml final concentration.
‡ 10 μg/ml mAb L227.
§ 10 μg/ml mAb W6/32.
‖ pg/ml (± SEM).

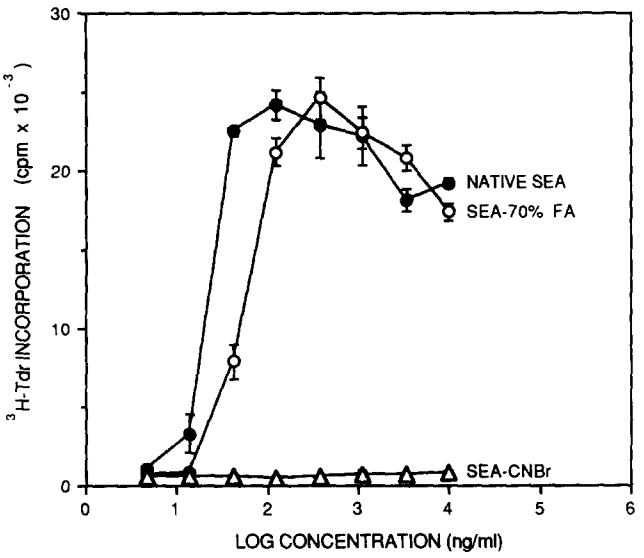

Figure 6. CNBr cleavage outside disulfide loop abrogates mitogenicity of SEA. SEA was cleaved with CNBr (*SEA-CNBr*) as described; control-reacted material (*SEA-70% FA*) was similarly prepared but in the absence of CNBr. Same sample preparations used here and in Fig. 4. Error bars indicate SEM of triplicate determinations.

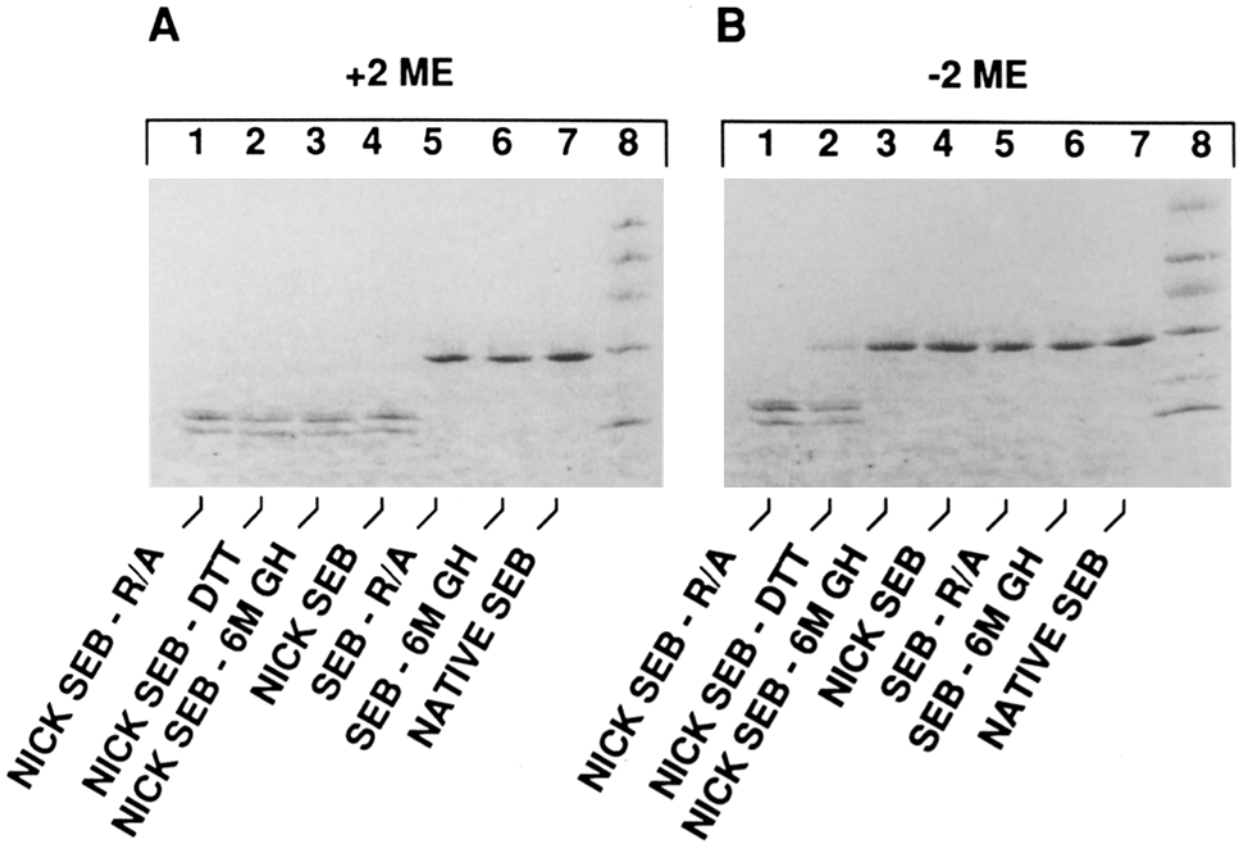

Figure 7. SDS-PAGE analysis of native and nicked SEB under reducing (*A*) and nonreducing (*B*) conditions. Electrophoresis carried out as described in Fig. 4. (Lane *1*) Reduced and alkylated nicked SEB (*NICK SEB-R/A*); (lane *2*) reduced but not alkylated nicked SEB (*NICK SEB-DTT*); (lane *3*) control-reacted nicked SEB (*NICK SEB-6 M GH*); (lane *4*) nicked SEB (no treatment, *NICK SEB*); (lane *5*) reduced and alkylated native SEB (*SEB-R/A*); (lane *6*) control-reacted native SEB (*SEB-6 M GH*); (lane 7) native SEB (no treatment, *NATIVE SEB*); (lane *8*) 94-, 65-, 45-, 30-, 20-, and 14-kD markers.

the nicked molecule was characterized (34). Thus, in the case of SEB, an intact peptide sequence within the loop was not required for functional activity, suggesting the disulfide linkage imparts enough stability to maintain the conformation of the molecule, despite its having been cleaved into two peptide chains.

SEB–R/A Is Deficient for T Cell but Not Monocyte Stimulation. We reduced and alkylated both native and nicked SEB, and similarly found the mitogenic activity of both preparations was significantly reduced (Fig. 8). SDS-PAGE analysis (Fig. 7) revealed that this treatment did not result in cleavage of peptide bonds and, in the case of nicked SEB, allowed visualization of the completeness of the reaction (Fig. 7 *B*, lane *1*). In addition, the treatment did not alter the amino acid analysis profile, with the exception of the appearance of cm-cys in SEB–R/A (data not shown). Interestingly, reduction of nicked SEB in the absence of alkylating agent (SEB–DTT), producing two separate peptide chains (Fig. 7 *B*, lane *2*), did not affect mitogenic activity (Fig. 8 *B*).

We next examined whether nicked SEB could stimulate TNF-α production from monocytes and whether it was affected by reduction and alkylation. As shown in Fig. 9, SEB–R/A was as affective as SEB–6 M GH in inducing TNF-α production. Thus for SEB, like SEA, the disulfide linkage is required for mitogenicity but not for class II–mediated monocyte stimulation. The ability of nicked SEB–R/A to stimulate monocytes suggests that noncovalent interactions stabilize the two peptide chains in their native conformation, or that the putative class II–binding domain resides on either the NH_2^- or COOH-terminal fragment of SEB.

Effect of Reduction and Alkylation on the Structure of SEA. Having established the importance of the disulfide loop for the functional activity of SEs, it was of interest to assess its contribution to conformational structure. We first subjected the primary SEA sequence to conventional Chou–Fasman analysis, which predicted roughly equal amounts (30–40%) of β-sheet and α-helix structure for the native protein (data not shown). In contrast to this prediction, the CD spectrum of native SEA (Fig. 10 *A*) revealed relatively little α-helix (11%) and significant β-sheet (36%) structure, in agreement with previous findings by Singh and Betley (35). We next assessed the effect of denaturation with 6 M GH on the secondary structure of SEA. As shown in Fig. 10 *B*, SEA–6 M GH displayed identical conformation to native SEA, consistent with

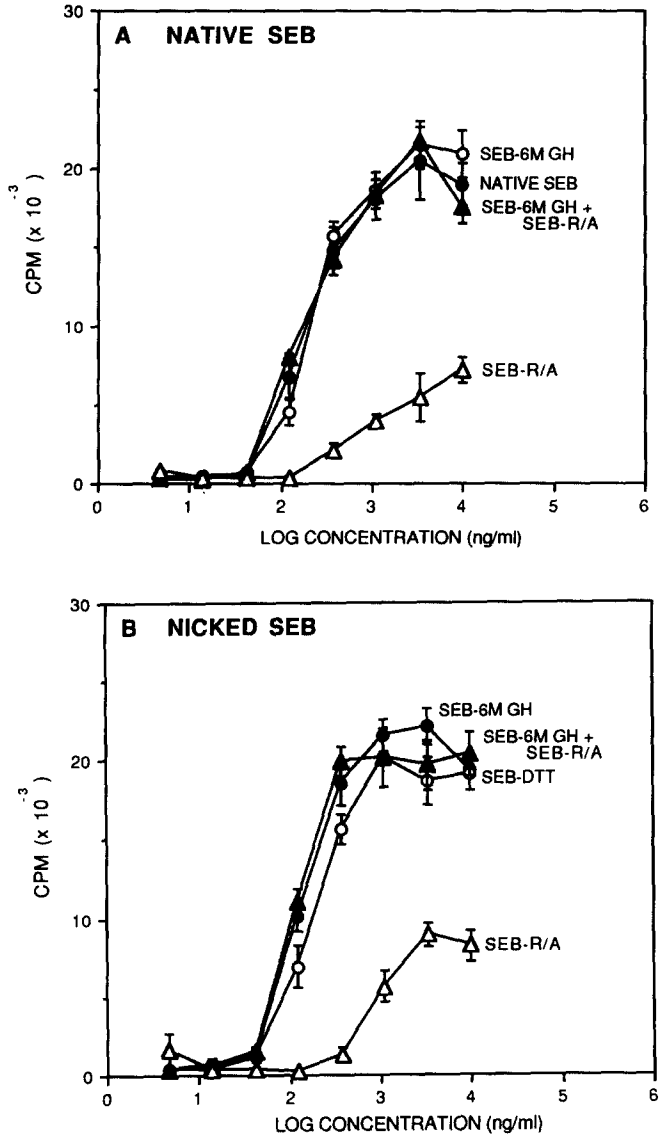

Figure 8. Reduction and alkylation abrogates mitogenicity of both native (*A*) and nicked (*B*) SEB. Error bars indicate SEM of triplicate determinations.

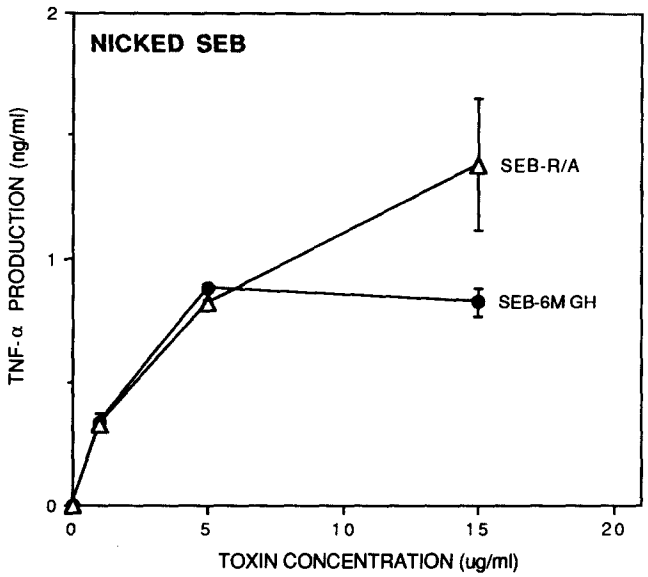

Figure 9. Monocyte-stimulatory activity of nicked SEB is not affected by reduction and alkylation. Error bars indicate SEM. Same sample preparations used here and in Fig. 8 *B*.

the recovery of functional activity upon removal of denaturing reagent. Finally, we obtained the spectrum of SEA–R/A (Fig. 10 *C*) to determine the effect of reduction and alkylation on the structure of SEA. As shown in Fig. 10 *D*, the spectrum of SEA–R/A was virtually superimposable on that of SEA–6 M GH, suggesting that the loss of mitogenic activity resulting from reduction and alkylation was not associated with significant changes in the conformation of the SEA molecule.

Discussion

We investigated the possibility that a conserved sequence and structural motif in the central region of several bacterial exotoxins is essential for interactions with MHC class II or TCR molecules on monocytes and T cells. Contrary to earlier findings (4, 25), we demonstrate that the disulfide loop is critical for the mitogenic activity of SEA and SEB. Reduction and alkylation compromised mitogenicity but spared class II–binding and monocyte-stimulatory activity, suggesting that an intact disulfide linkage is required for interaction of these toxins with TCR but not with class II molecules. These findings indicate a functional role for a conserved element in SEs, and argue that their class II– and TCR-binding activities, both of which are required for T cell stimulation, can be dissociated.

It is likely that the contribution of the disulfide loop to the mitogenicity of SEA was not appreciated by previous investigators because reduction and alkylation was not efficiently carried out in those studies. We demonstrate that quantitative reduction and alkylation of SEA does not take place in nondenaturing solvent. The retention of mitogenic activity in previous studies was probably due to failure to reduce the disulfide loop or to its reformation upon removal of reducing agent. In fact, SDS-PAGE analysis of nicked SEB exposed to reducing agent only (under denaturing conditions but in the absence of alkylating agent) revealed initiation of disulfide bond reformation shortly after the removal of reducing agent (Fig. 7 *B*, lane *2*).

The loss of mitogenic activity resulted directly from reduction and alkylation of the cysteines forming the disulfide loop, and was not an artifact of the reaction conditions. First, the reduction and alkylation treatment did not affect cleavage of peptide bonds, nor destruction or modification of any residues. Second, the mitogenicity of control-reacted toxin was not affected by denaturation in 6 M GH, or when mixed with R/A material. Finally, the experimental conditions used for reduction and alkylation did not affect the mitogenicity of a toxin lacking a disulfide loop (TSST-1).

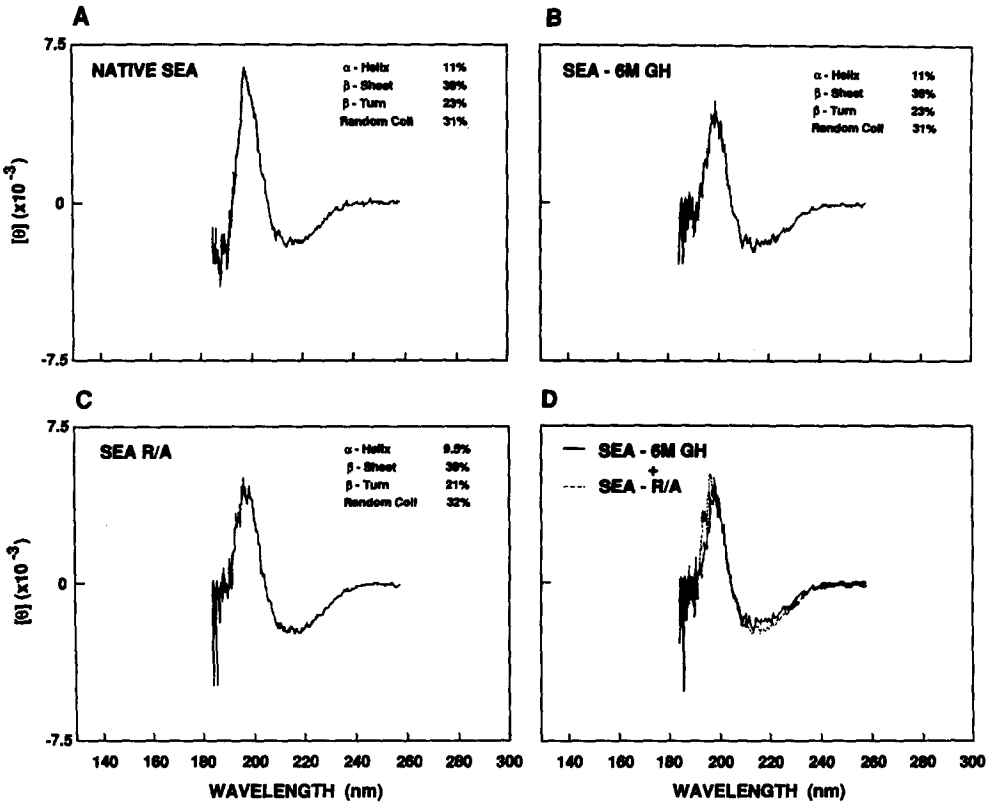

Figure 10. Neither denaturation in 6 M GH nor reduction and alkylation affect secondary structure of SEA. CD spectra of (*A*) native SEA, (*B*), SEA–6 M GH, (*C*) SEA–R/A. Samples are at 0.21 mg/ml in 200 mM KF containing 10 mM phosphate, pH 6.8. (*D*) Overlay of *B* and *C*.

A striking feature of the disulfide loop in these toxins is its short length (see Fig. 1). Consequently, we viewed reduction and alkylation of SEA as a rather subtle modification that would perturb only a localized region. Our analyses of secondary structure from CD spectra support this view. These studies indicate that removal of the disulfide loop did not affect the overall conformation of SEA and suggest that the dramatic effect on functional activity was not accompanied by similarly dramatic alterations in secondary protein structure. Our studies, in fact, offer testimony to the remarkable stability of SEs. First, SEA was largely able to reassume its structure and functional activity after being denatured in 6 M GH. Second, nicked SEB retained full activity despite having been cleaved into two peptides (held together by a disulfide bond). Third, reduction of this disulfide bond in nicked SEB without alkylation (SEB–DTT) did not affect functional activity, suggesting the structural stability of the component fragments was sufficient to direct proper refolding to allow disulfide reformation.

The failure of reduced and alkylated SEA to stimulate T cell proliferation was probably not due to its inability to bind class II molecules, as evidenced both by its capacity to block class II binding of native toxin and stimulate TNF-α production from monocytes in a class II–dependent manner. Our finding that reduction and alkylation had a modest effect on direct binding of SEA to class II molecules without affecting its capacity for class II–mediated monocyte stimulation may at first seem contradictory. Although our recent studies (13–15) with various SEs indicated that induction of TNF-α production by monocytes generally correlated with toxin class II affinity, they also suggested that it may be a relatively insensitive indicator of this interaction. For example, while SEB, TSST-1, and SEC_1 exhibited decreasing affinities for class II (K_d values at 37°C for toxin–DR1 complexes are 2.4×10^{-7}, 4.4×10^{-7}, and 7.4×10^{-7} M, respectively), SEB and TSST-1 were equally potent monocyte stimulators and far better than SEC_1 (13, 36). It is not surprising then that native SEA, SEA–6 M GH, and SEA–R/A did not exhibit identical class II–binding activities, but were equally effective in stimulating monocyte TNF-α production. Although exposure of SEA to 6 M GH caused a slight reduction in class II–binding activity, mitogenic activity was not significantly affected. It can be reasoned then that a similar decrease in class II–binding affinity seen upon reduction and alkylation (SEA–R/A relative to SEA–6 M GH) cannot explain the dramatic difference in mitogenic activity observed with these two preparations. Thus, while the modest reduction in class II–binding activity of SEA–R/A may have contributed to a decreased capacity for T cell stimulation, it is unlikely to have accounted for the striking loss of mitogenic activity observed.

We believe, rather, that disruption of the disulfide loop precludes proper interaction of these toxins with the TCR, thereby rendering them deficient for T cell stimulation. It is unclear whether the domain that interacts with the TCR is the loop itself, or some other part of the molecule the con-

formation of which requires the loop. It is also possible that the introduction of acetamido groups through alkylation interferes with T cell recognition of the cysteines or adjacent residues. The failure to reduce and alkylate SEA in nondenaturing solvent, however, suggests that the disulfide loop is buried within the molecule and not readily accessible. The longer exposure to DTT and higher concentration of IAA used to abrogate the mitogenicity of SEB (see Materials and Methods) may indicate that the disulfide is less accessible (even under denaturing conditions) in SEB than in SEA.

Cleavage of the protein outside the disulfide loop, however, overcomes the inherent stability of these molecules as evidenced with SEA by the loss of mitogenic activity upon CNBr cleavage. This result suggests that either the integrity of the conserved peptide sequence adjacent to the loop or a conformationally dependent domain involving more than one region of the molecule is required for function. There are, however, several considerations that limit interpretation of this result. In addition to cleavage at met-107 in the central region of SEA, CNBr would also be expected to affect cleavage at met-224, nine residues from the COOH terminus (see Fig. 1). It is possible that this second cleavage was responsible for disrupting function, with the COOH-terminal region playing an important role. We also cannot rule out the possibility that the generated peptides were unstable in the solvents used in the cleavage reaction or gel filtration procedure, although native material did retain activity after exposure to these conditions. In a previous report (4) examining CNBr cleavage of SEA, the reduced mitogenic activity associated with the fragments was attributed to the presence of contaminating unreacted material. In our studies, we separated the CNBr peptides from unreacted SEA and clearly established that they were not mitogenic.

Interestingly, one member of this family of bacterial exotoxins, TSST-1, does not contain a disulfide loop. It is also strikingly unrelated by protein sequence to the other SEs, although small regions of homology do exist (reference 24 and Fig. 1). In other studies (13–15, 36), we have found that while this toxin specifically binds class II molecules and effectively induces monocyte TNF-α production, it is considerably less mitogenic than SEA and SEB. Perhaps TSST-1 does not possess a disulfide loop because it already displays a particular structure(s) that the other SEs require the loop to maintain. While the disulfide loop is required for mitogenic activity of those SEs that possess it, it may not be sufficient for full functional activity; it is possible that more complex structural features account for the common ability of these toxins to stimulate T cells, as well as the different patterns of Vβ TCR stimulation that have been observed.

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