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Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A

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Bacterial superantigens (SAgs) are potent activators of T lymphocytes and play a pathophysiological role in Gram-positive septic shock and food poisoning. To characterize potential MHC class II binding sites of the bacterial SAg staphylococcal enterotoxin (SE) A, we performed alanine substitution mutagenesis throughout the C-terminus and at selected sites in the N-terminal domain. Four amino acids in the C-terminus were shown to be involved in MHC class II binding. Three of these amino acids, H225, D227 and H187, had a major influence on MHC class II binding and appeared to be involved in coordination of a Zn^{2+} ion. Alanine substitution of H225 and D227 resulted in a 1000-fold reduction in MHC class II affinity. Mutation at F47, which is equivalent to the F44 previously shown to be central in the MHC class II binding site of the SAg SEB, resulted in a 10-fold reduction in MHC class II affinity. The combination of these mutations in the N- and C-terminal sites resulted in a profound loss of activity. The perturbation of MHC class II binding in the various mutants was accompanied by a corresponding loss of ability to induce MHC class II-dependent T cell proliferation and cytotoxicity. All of the SEA mutants were expressed as Fab–SEA fusion proteins and found to retain an intact T cell receptor (TCR) epitope, as determined in a mAb targeted MHC class II-independent T cell cytotoxicity assay. We propose a model in which the N- and C-terminal sites in SEA cooperate to form a high affinity interaction which involves binding to two separate MHC class II molecules. Considering the previously described SEB–HLA-DR complex, this study indicates that SAgs may bind monovalently or bivalently to MHC class II molecules and could be presented to the TCR as a dimeric or trimeric complex.

Key words: MHC class II/mutants/SEA/superantigens/T cell activation

Introduction

Bacterial superantigens (SAgs) are a group of structurally related proteins which have the ability to activate a large proportion of T lymphocytes. The staphylococcal enterotoxins are the most well characterized among the bacterial SAgs and they have been shown to bind as unprocessed proteins to a region on the MHC class II molecule which is distinct from the peptide binding groove (Scherer *et al.*, 1993; Webb and Gascoigne, 1994). T cells expressing certain T cell receptor (TCR) Vβ families interact with the SAg–MHC class II complex and are activated, resulting in proliferation, release of cytokines and cytotoxicity (Dohlsten *et al.*, 1990; Scherer *et al.*, 1993; Webb and Gascoigne, 1994). Earlier functional studies in our laboratory indicated that SAgs may also interact directly with the TCR in the absence of MHC class II, although with a reduced affinity (Dohlsten *et al.*, 1991, 1994). A direct interaction was recently confirmed by binding studies using gel permeation chromatography and biosensor methodology (Hilyard *et al.*, 1994; Seth *et al.*, 1994). Mutagenesis studies of the SAg staphylococcal enterotoxin (SE) B identified portions of N-terminal domain that mediated binding to MHC class II (Kappler *et al.*, 1992). The structures of SEB and the SEB–MHC class II complex were recently solved by X-ray crystallography (Swaminathan *et al.*, 1992; Jardetzky *et al.*, 1994), which demonstrated that the N-terminal domain of SEB bound to the HLA-DR α-chain. We have previously shown that a recombinant C-terminal fragment of SEA exhibited MHC class II binding activity (Hedlund *et al.*, 1991); in contrast to SEB, the binding of SEA to MHC class II is strictly Zn^{2+} dependent (Fraser *et al.*, 1992). It has been suggested that a Zn^{2+} ion is bridged between H81 on the β-chain of MHC class II and some coordinating residues of the SEA molecule (Herman *et al.*, 1991; Fraser *et al.*, 1992; Karp and Long, 1992). In addition to this, studies on SEA using synthetic peptides (Griggs *et al.*, 1992) and site-directed mutagenesis (Harris *et al.*, 1993) have suggested the existence of an N-terminal MHC class II binding epitope homologous to the SEB binding site (Kappler *et al.*, 1992; Jardetzky *et al.*, 1994). In order to characterize the amino acids involved in the C-terminal binding site and to compare the affinity of the C- and N-terminal binding sites, we have made alanine substitutions of conserved amino acids in the N- and C-termini of SEA. The results indicate that high affinity binding ($K_d \approx 10$ nM) is achieved by the interaction of SEA with MHC class II at two distinct sites. These are a medium affinity, C-terminal site ($K_d \approx 100$ nM) and a low affinity N-terminal site ($K_d \approx 10$ μM). We suggest that a high affinity interaction is obtained by the binding of the N- and C-terminal sites in SEA to α- and β-chains on two separate MHC class II molecules.

Results

Alanine substitution mutagenesis of SEA

We have previously reported that a C-terminal recombinant fragment of SEA which includes amino acids 107–203

retains ~10% of the affinity for MHC class II (Hedlund *et al.*, 1991). In contrast, other studies using SEA–SEE chimeric molecules (Irwin *et al.*, 1992; Hudson *et al.*, 1993) and SEA-derived synthetic peptides (Griggs *et al.*, 1992) have indicated that the N-terminal region of SEA is involved in MHC class II binding. To identify the amino acids involved in MHC class II binding and to determine the relative contribution of the N- and C-terminal sites, we have made alanine substitutions of selected amino acids in the N- (domain 1) and C-terminal (domain 2) regions. Site-directed mutations in the C-terminal were directed to conserved polar or charged amino acids, whereas mutations in the N-terminal part were mainly concentrated in a region homologous to the MHC class II binding region of SEB (Kappler *et al.*, 1992; Jardetzky *et al.*, 1994). These mutations are depicted in a schematic structural model of SEA (Figure 1).

Antibody reactivity with SEA mutants

A panel of monoclonal antibodies (mAb) against SEA were used to determine whether these mutants retained the general native conformation of wild-type SEA (SEAwt). Our results indicate that all of the mutants, with a few exceptions, retained their binding to the panel of mAbs (Table I). For example, mAb 1E did not bind to H187A, D227A and H225A and exhibited a slight reduction in binding to N128A. These four amino acid residues, predicted in our model of SEA (Figure 1) to accumulate in the centre of the C-terminal β-plate, appear to form a binding area for mAb 1E. Previous studies have shown that SEA coordinates a Zn^{2+} ion and that the presence of a Zn^{2+} is required for SEA–MHC class II interaction (Fraser *et al.*, 1992). The binding of mAb 1E was abrogated by addition of EDTA (Figure 2), suggesting that this mAb recognizes a Zn^{2+}-dependent epitope. In contrast, mAb 2A retained its binding in the presence of EDTA (data not shown). The addition of excess Zn^{2+} partially restored the binding of mAb 1E to SEA mutants H187A and N128A, but did not enhance the binding to mutants D227A and H225A (Figure 2).

Characterization of the C-terminal binding site

The binding of SEAwt and SEA mutants to MHC class II^+ Raji cells was examined in two ways. First, a competition assay using FITC-labelled SEAwt was used to determine the inhibition of binding by SEA mutants. Second, the binding of ^{125}I-labelled SEAwt or SEA mutants to Raji cells was determined by Scatchard analysis. The K_d for native SEA was determined to be 13 nM; this agrees with previously published values (Mollick *et al.*, 1991; Irwin *et al.*, 1992; Hudson *et al.*, 1993). Three of the C-terminal SEA mutants showed reduced binding to MHC class II^+ Raji cells (Table II). H225A and D227A exhibited a >1000-fold loss of MHC class II binding, whilst H187A showed only a 10-fold loss. The K_d for binding of D227A and H225A was estimated to be $>10^{-5}$ M. N128A demonstrated a 20-fold reduction in binding as judged by the competition assay, but the Scatchard plot suggested the existence of two distinct binding affinities. The majority of binding sites for N128A (>80%) showed a reduced binding affinity compared with SEAwt, whilst a minority of sites retained a high affinity binding (Table II). The mutant K123A/D132G also exhibited a two site profile.

Table I. Binding of anti-SEA mAb to SEA mutants

SEA mutant	Monoclonal antibody					
	1A	2A	3A	1E	4E	EC-A1
SEA wt +[a]	+	+	+	+	+	
SEA D11A/K14A	+	+	+	+	+	+
SEA D45A	+	+	+	+	+	+
SEA F47A	+	+	+	+	+	+
SEA H50A	(+)	+	+	+	+	+
SEA K55A	+	+	+	+	+	+
SEA H114A	+	+	+	+	+	+
SEA K123A/D132G	+	+	+	+	+	+
SEA N128A	+	+	+	(+)	+	+
SEA K147A/K148A	+	+	+	+	–	+
SEA H187A	+	+	+	–	+	+
SEA E191A/N195A	+	+	+	+	+	+
SEA D197A	+	+	+	+	+	+
SEA H225A	+	+	+	–	+	+
SEA D227A	+	+	+	–	+	+

[a]+ indicates >90%, (+) 10–90% and – <10% binding compared with SEAwt.

Table II. Binding of SEA mutants to MHC class II

SEA mutant	SEA inhibition[a] IC_{50} (nM)	Direct binding[b] K_d (nM)
SEA wt	50	13
SEA D11A/K14A	50	nd
SEA D45A	53	nd
SEA F47A	3150	95
SEA H50A	150	32
SEA K55A	44	nd
SEA H114A	48	nd
SEA K123A/D132G	188	12/237
SEA N128A	1150	3/76
SEA K147A/K148A	58	nd
SEA H187A	1030	97
SEA E191A/N195A	51	nd
SEA D197A	78	nd
SEA H225A	>9000	nd
SEA D227A	>9000	>8000

[a]Concentration of SEA mutant yielding a 50% reduction in binding of 30 nM FITC–SEAwt to MHC class II positive Raji cells. H225A and D227A did not reach half-maximum inhibition at the highest concentrations used. IC_{50} values are the means of at least two separate experiments.
[b]K_d values were calculated from Scatchard plots after binding of ^{125}I-labelled SEAwt and SEA mutants to Raji cells. The D227A mutant did not reach saturation at the highest concentration.
nd, not determined

Apart from these two mutants, all others showed a uniform binding affinity for Raji cells (Table II). The double mutation K147A/K148A, which was predicted to be located close to the α-5 region of SEA (Figure 1), retained activity when analysed for MHC class II binding, T cell proliferation or T cell cytotoxicity (Tables II and III). Furthermore, two mutants in this region, E154A/D156A and R160A, were expressed at levels which prevented further analysis, suggesting the possibility that the α-5 region is important in maintaining the general structure of SEA.

Analysis of the various C-terminal SEA mutants for induction of T cell proliferation and cytotoxicity against MHC class II^+ Raji cells showed a similar loss of activity as demonstrated in the binding assays (Table III). To

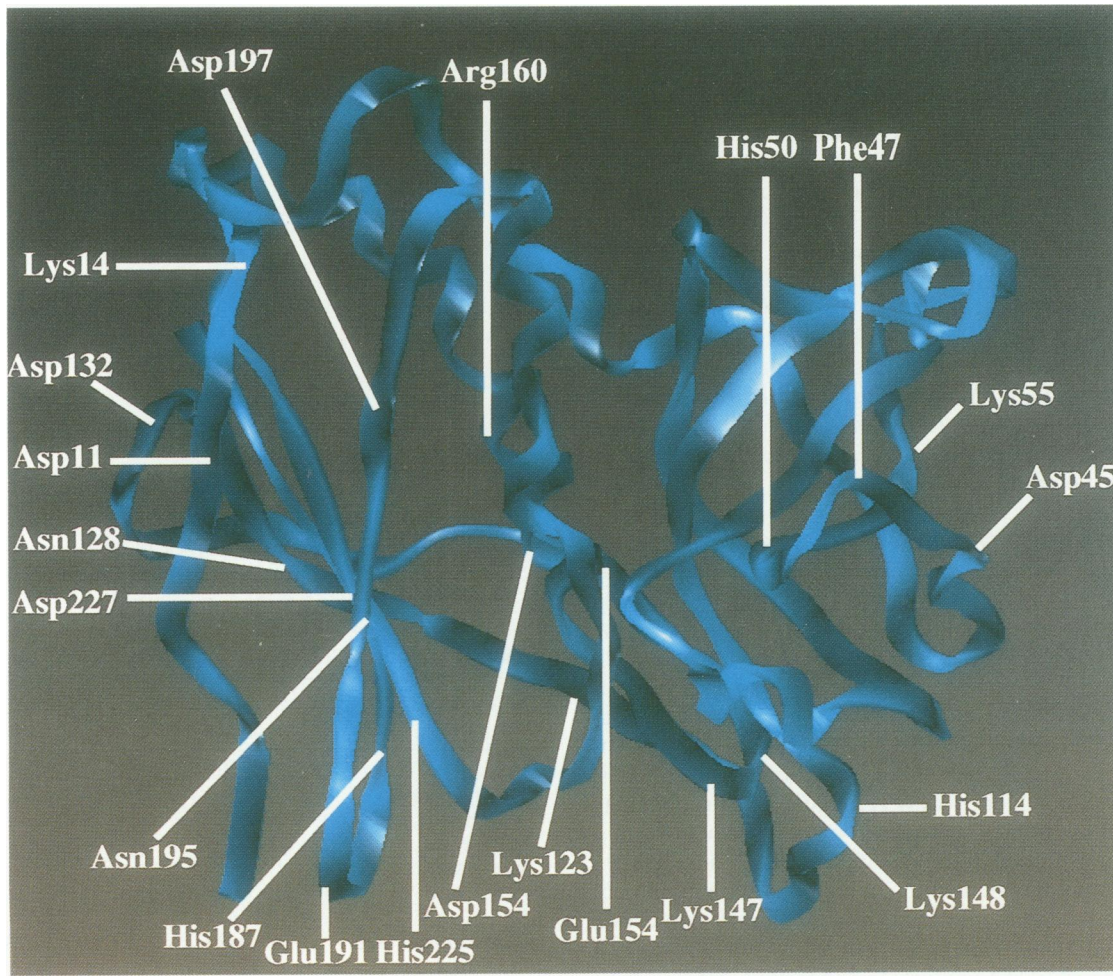

Fig. 1. A model structure of SEA based on the crystal structure of SEB (Jardetzky *et al.*, 1994), given that SEA and SEB have ~30% sequence identity. The model was constructed by means of the COMPOSER program (Blundell *et al.*, 1988), as implemented in the SYBYL 6.1 program (Tripos Associates, St Louis, MO). The structure was modelled by defining homologous stretches in the sequence as structurally conserved with respect to the crystal structure of SEB; ~50% of the structure was defined as structurally conserved. The remaining parts of the protein were modelled from appropriate loops in the Brookhaven Protein Data Bank (Bernstein, *et al.*, 1977) using the COMPOSER program. The resulting protein model was refined using energy minimization.

distinguish between perturbation of MHC class II binding and interference with the TCR site, the SEA mutants were expressed as C215Fab–SEA fusion proteins and examined in a targeted cytotoxicity assay against $C215^+$ MHC class II^- Colo205 cells and C215 transfected CHO-cells (Dohlsten *et al.*, 1994). In this assay the Fab moiety directs the fusion protein to C215-expressing target cells and results in the presentation of fused SEA molecules to cytotoxic T cells (CTL) independent of MHC class II molecules (Dohlsten *et al.*, 1994). The mutants C215Fab–SEAD227A, C215Fab–SEAH225A and C215Fab–SEAH187A retained the ability to target CTL against $C215^+$ MHC class II^- Colo205 cells (Figure 3). The specificity in the cytotoxicity was further confirmed using C215- and HLA-DR-transfected CHO cells. SEAwt and C215Fab–SEAwt induced a strong cytotoxic response against HLA-DR-transfected CHO cells, whilst the SEA mutants or the corresponding Fab–SEA mutants showed a significant reduction in cytotoxicity (Figure 4). No cytotoxicity was seen against parental CHO cells lacking expression of C215 or MHC class II molecules (Figure 4).

Previous studies have shown the binding of Zn^{2+} to SEA and depletion of Zn^{2+} with EDTA abolished MHC

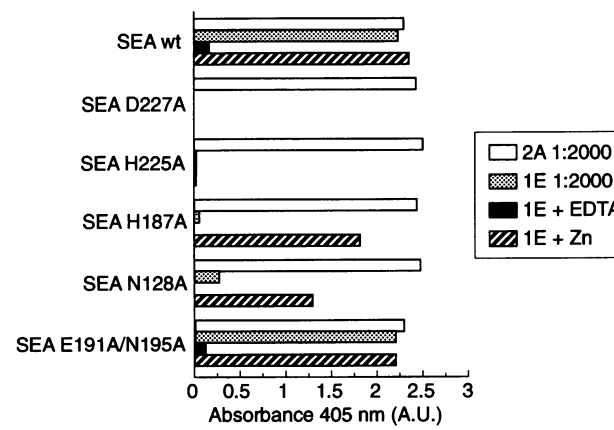

Fig. 2. mAb 1E binds a Zn^{2+}-dependent epitope on SEA and SEA mutants. The binding of mAb 1E to SEA and SEA mutants N128A, H187A, E191A/N195A, H225A and D227A was analysed by ELISA in the presence or absence of EDTA (10 μM) or Zn^{2+} (100 μM) as indicated. mAb 2A, which reacts with all of the mutants (Table I), was used as a control. The binding of mAb 2A to SEA and SEA mutants was not influenced by addition of EDTA or Zn^{2+}.

Table III. Biological effects of SEA mutations

SEA mutant	Proliferation[a] EC_{50} (relative)	Cytotoxicity[b] EC_{50} (relative)
SEA wt	1	1
SEA D11A/K14A	nd	1
SEA D45A	1	1
SEA F47A	52	4
SEA H50A	4	2
SEA K55A	1	1
SEA H114A	nd	1
SEA K123A/D132G	2	2
SEA N128A	2	3
SEA K147A/K148A	nd	1
SEA H187A	11	9
SEA E191A/N195A	1	1
SEA D197A	nd	1
SEA H225A	1272	130
SEA D227A	1057	132

[a]Human PBL were stimulated for 72 h with wild type or mutant SEA. Proliferation was measured as [^{3}H]thymidine incorporation during the last 4 h of stimulation. The SEA mutant concentration resulting in half-maximum proliferation (EC_{50}) was related to the EC_{50} of SEAwt (0.3 ± 0.1 pM; mean ± SD).

[b]Four-hour ^{51}Cr release assay with MHC class II positive Raji cells as target and a SEA activated human T cell line as effector. The concentration of SEA mutant resulting in half-maximum cytotoxicity (EC_{50}) was related to the EC_{50} of SEAwt (0.5 ± 0.4 pM; mean ± SD).

nd, not determined

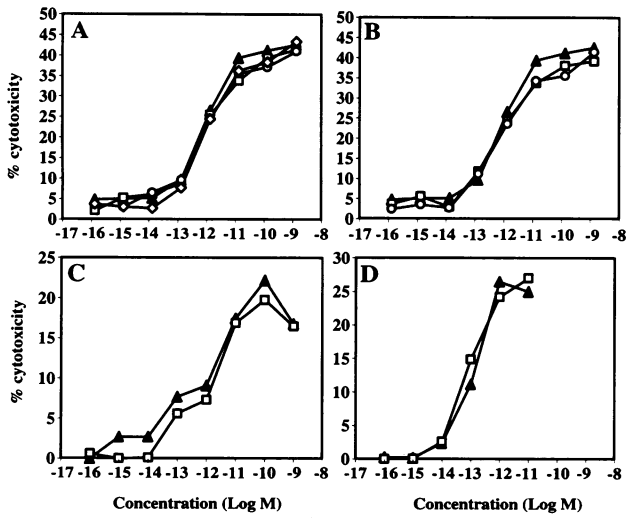

Fig. 3. The SEA mutants have an intact ability to target T cells in a MHC class II-independent cytotoxicity assay. The SEA mutants were expressed as C215Fab–SEA proteins and added at various concentration to the microtitre plate wells. Cytotoxicity was analysed in a 4 h ^{51}Cr release assay using a SEA-reactive human T cell line and the C215$^+$ MHC class II$^-$ Colo 205 cell line as target. The effector:target cell ratio was 30:1. (**A**) C215Fab–SEA(▲), C215Fab–SEAD227A (□), C215Fab–SEAF47A (○) and C215Fab–SEAF47A/D227A (◇). (**B**) C215Fab–SEA (▲), C215Fab–SEAH187A (□) and C215Fab–SEAH225A (○). (**C**) C215Fab–SEA (▲) and C215Fab–SEAF47A/H187A (□). (**D**) C215Fab–SEA (▲) and C215Fab–SEAH50A (□).

II binding (Fraser *et al.*, 1992); this has led to the suggestion that a Zn^{2+} bridge is formed between SEA and H81 on the HLA-DR β-chain (Herman *et al.*, 1991; Fraser *et al.*, 1992; Karp *et al.*, 1992). We have found that a C-terminal fragment of SEA (amino acids 107–233; Hedlund *et al.*, 1991) binds Zn^{2+} and exhibits a Zn^{2+}-dependent interaction with MHC class II; this implies that the Zn^{2+} binding site was located in the C-terminal region of SEA (data not shown). Since studies on other Zn^{2+} binding proteins have demonstrated that aspartic acid and histidine usually participate in Zn^{2+} binding, it seemed reasonable to assume that H187, D227 and H225 were involved in such an interaction. A gel filtration assay using $^{65}Zn^{2+}$ and SEA mutants demonstrated a strong reduction in Zn^{2+} binding compared with SEAwt for mutants D227A, H225A and H187A (Figure 4). In contrast, no effect on Zn^{2+} binding was seen with mutant N128A (Figure 5). The binding of Zn^{2+} to D227A, H225A and H187A was within the range seen for SEB binding (Figure 5). Even though SEB has been reported to bind MHC class II in a Zn^{2+}-independent manner (Fraser *et al.*, 1992), the results are compatible with the view that these mutations almost completely abrogated Zn^{2+} binding by SEA.

The low affinity of D227A and H225A for MHC class II prevented us from determining the affinity constant in a direct binding assay, however, the K_d could be estimated to be ~10^{-5} M by competition assay. In the MHC class II-dependent T cell proliferation and cytotoxicity assays, the activities of the mutants were reduced by 130- and 1300-fold respectively when compared with SEAwt (Table III). Since the affinity of SEAwt for MHC class II$^+$ Raji cells was ~10^{-8} M, it is reasonble to assume that perturbation of the C-terminal site results in a SEA molecule with an affinity in the remaining N-terminal site of ~10^{-5} M. Complete disruption of the C-terminal site by the combined mutation of H225 and H187 (H225A/H187A) or by the insertion of a bulky amino acid such as tryptophan (D227W) confirmed that the remaining affinity was indeed in the range 10^{-5} M (data not shown).

Characterization of the N-terminal binding site

Alanine substitutions at F47 and H50 resulted in K_d values of 95 and 32 nM respectively (Table II). Competition assays revealed 50- and 3-fold respective reductions in MHC class II binding for F47A and H50A when compared with SEAwt (Table II). Furthermore, combined mutation at F47, H50 and L48, which are predicted from the SEB structure to be involved in the N-terminal site (Jardetzky *et al.*, 1994), showed a similar reduction in MHC class II binding as the single F47A mutation (data not shown). This suggests that perturbation of the N-terminal site lowers the affinity from 10^{-8} to ~10^{-7} M. There appeared to be a gross correlation between MHC class II binding, T cell proliferation and T cell cytotoxicity for all N-terminal mutants (Table III). The TCR site was unaffected in F47A and H50A, as judged from the T cell-mediated cytotoxicity of C215Fab–SEAF47A and C215Fab–SEAH50A against C215$^+$ MHC class II$^-$ Colo205 cells (Figure 3). Moreover, the F47A and H50A mutants appeared to retain their conformation, since they were recognized by all tested anti-SEA mAb (Table I).

Combined mutations in the N- and C-terminal sites

Given the fact that the estimated affinity of the N-terminal site was 10^{-5} M and that the mutation F47A substantially reduced high affinity binding of SEA (K_d 10^{-8} M) to MHC class II molecules, we postulated that the N- and C-terminal sites cooperate to form a high affinity interaction.

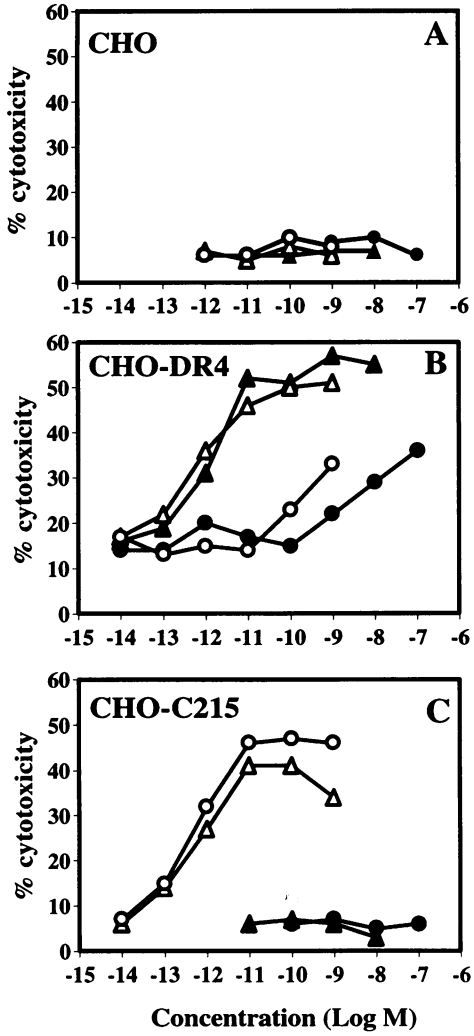

Fig. 4. Comparison of the ability of SEA, C215Fab–SEA, SEAD227A and C215Fab–SEAD227A to direct T cell cytotoxicity to untransfected (**A**), HLA-DR4-transfected (**B**) and C215 antigen-transfected (**C**) CHO cells. SEAwt (▲), C215Fab–SEA (△), SEAD227A (●) or C215Fab–SEAD227A (○) were added at various concentrations to the microtitre plate wells. Cytotoxicity was analysed in a 4 h ^{51}Cr release assay using a SEA-stimulated T cell line as effectors.

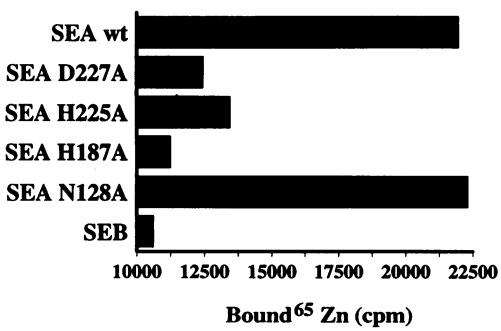

Fig. 5. Reduced binding of Zn^{2+} to D227A, H225A and H187A SEA mutants compared with SEAwt and the N128A mutant. SEAwt and SEA mutants were pre-treated with EDTA, mixed with $^{65}Zn^{2+}$ and incubated for 30 min at 37°C. Free and SEA-bound $^{65}Zn^{2+}$ was separated on a Fast Desalting column using the SMART System (Pharmacia Biotech) and collected fractions were measured in a gamma counter. Recombinant SEB was used as a negative control.

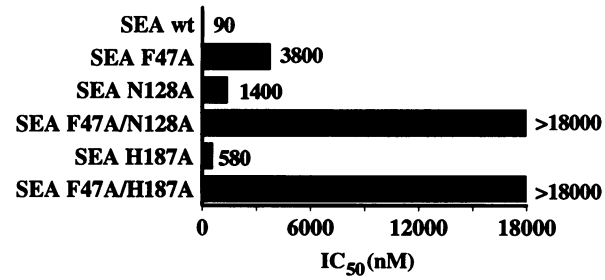

Fig. 6. Combined mutations in the N- and C-termini of SEA resulted in a profound reduction in MHC class II binding. SEA single or double mutants, including the N-terminal mutant F47A and the C-terminal mutants N128A and H187A, were analysed for MHC class II binding in a competition assay using FITC–SEA (30 nM) as tracer and Raji cells as target. MHC class II affinity is expressed as the concentration of SEA mutant resulting in a 50% reduced binding of SEA–FITC to Raji cells. Data from one of two similar experiments are shown.

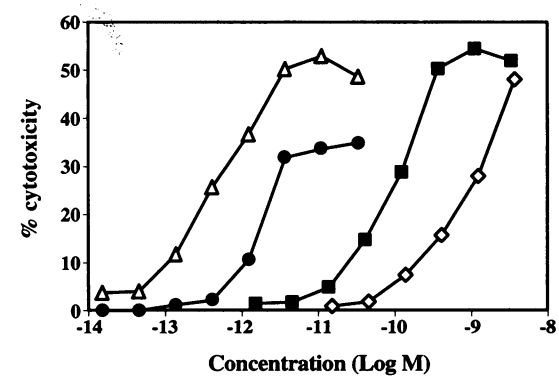

Fig. 7. Effects of combined mutations in the N- and C-termini of SEA on MHC class II-dependent cytotoxicity. The effects on T cell-mediated cytotoxicity of various concentrations of SEAwt (△), SEA mutants F47A (●), D227A (■) and the double mutant F47A/D227A (◇) were analysed in a 4 h ^{51}Cr release cytotoxicity assay. Raji cells were used as target and the SEA-reactive T cell line as effector. Data from one of two similar experiments are shown.

To confirm this hypothesis, we combined mutations in the two sites. The double mutants F47A/H187A and F47A/N128A showed a profound reduction in binding activity, whilst the single mutations F47A, H187A and N128A only resulted in a moderate reduction in binding activity (Figure 6). The double mutant F47A/D227A showed a 3000-fold reduction in the MHC class II-dependent cytotoxicity assay, whereas the D227A and F47A single mutations resulted in only 5- and 300-fold reductions respectively (Figure 7). The double mutants C215Fab–SEAF47A/D227A and C215Fab–SEAF47A/H187A showed intact activity in the MHC class II-independent cytotoxicity assay (Figure 3), confirming that the TCR site was unaffected. These observations support a model in which the individual N- and C-terminal sites form low (N-terminal) and moderate (C-terminal) affinity interactions, but the two sites cooperate to bind MHC class II with high affinity.

Discussion

SEA and SEB are the most extensively studied SAgs and among the staphylococcal enterotoxins they belong to two structurally distinct subfamilies comprising SEA, SED

and SEE and SEB and SEC. (Scherer *et al.*, 1993; Webb and Gascoigne, 1994). Several studies have implied that binding of SEA and SEB to MHC class II show a number of distinct features. SEA binds MHC class II with high affinity and in a Zn^{2+}-dependent manner, whilst the binding of SEB is of moderate affinity and is Zn^{2+}-independent (Mollick *et al.*, 1991; Fraser *et al.*, 1992; Scherer *et al.*, 1993; Webb and Gascoigne, 1994). In addition, mutations in the HLA-DR β-chain have been shown to affect its binding to SEA, but not to SEB (Herman *et al.*, 1991; Karp *et al.*, 1992). Competition experiments have demonstrated that SEA efficiently blocks SEB binding to MHC class II, implying that a part of the binding surface on MHC class II may be shared by these superantigens (Chintagumpala *et al.*, 1991). In the present study we have demonstrated a unique MHC class II binding region in the C-terminus of SEA which involves three Zn^{2+}-coordinating amino acids. In an earlier study we found that a C-terminal fragment of SEA (amino acids 107–233) was bound with high affinity to MHC class II, whilst an N-terminal fragment (amino acids 1–179) lacked this high affinity binding (Hedlund *et al.*, 1994). The affinity of the C-terminal fragment was estimated to be ~10% that of native SEA (Hedlund *et al.*, 1994); this is in complete agreement with the values obtained in the present study. We have now demonstrated, by single amino acid substitutions, that residues H187, H225 and D227 in the C-terminal portion of SEA play a major role in MHC class II binding. Substitution of any of these three amino acids resulted in reduced binding of both MHC class II and Zn^{2+}. Previous studies on MHC class II interactions have shown that SEA and SEE (Fraser *et al.*, 1992) are dependent on Zn^{2+} for MHC class II binding. It is interesting to note that residues H187, H225 and D227 are conserved in SEE, but the Zn^{2+}-independent SAgs SEB and SEC_{1-3} show a weak homology at these positions. Since earlier studies have suggested that H81 in the HLA-DR β-chain may serve as a Zn^{2+} contact residue, it is likely that in SEA the Zn^{2+} ion is coordinated solely by H187, H225 and D227 and that displacement of a water molecule is induced during interaction with H81 in the HLA-DR β-chain. Indeed, the participation of H187, D227 and H225 in ion coordination was recently suported by X-ray crystallography studies (Schad *et al.*, 1995), N128 is located close to this region, but does not directly participate in ion coordination (Schad *et al.*, 1995). This suggests either that the effect of mutation at N128 is due to a local conformational change or that N128 is a Zn^{2+}-independent contact residue. The N128A mutant showed loss of binding to a major population of MHC class II molecules, but retained binding to a minor population. Recently, Thibodeau and co-workers have demonstrated that the SAgs SEB and toxic shock syndrome toxin-1 (TSST-1) bound different subsets of HLA-DR molecules and that SAg binding is disrupted by mutations in the peptide binding groove of HLA-DR (Thibodeau *et al.*, 1994). The crystal structure of TSST-1 complexed with HLA-DR1 confirmed that interaction of SAg with MHC class II may be influenced by bound peptide antigens (Kim *et al.*, 1994). Since the N128A and K123A/D132G mutations demonstrated two distinct binding affinities for HLA-DR molecules, it is tempting to speculate that mutations in this region, corresponding to the β6-strand in SEB (Swaminathan *et al.*, 1992), affect binding of SEA to various peptide–HLA-DR complexes. This hypothesis is presently being examined using empty HLA-DR molecules and loading with distinct sets of peptide antigens.

Previous studies on SEB have shown that there is substantial MHC class II binding activity in an N-terminal recombinant fragment (Buelow *et al.*, 1992) and random mutagenesis of the SEB molecule has revealed an accumulation of mutations with reduced MHC class II affinity in two N-terminal regions (Kappler *et al.*, 1992). Binding studies with SEB have demonstrated a K_d of 10^{-6}–10^{-7} M (Mollick *et al.*, 1991). The recent resolution of the structure of the SEB–MHC class II complex by X-ray crystallography confirmed that interaction between the N-terminal region of SEB and the HLA-DR α-chain occurred outside the peptide binding groove (Jardetzky *et al.*, 1994). The binding interface comprises one ridge of non-polar residues, including the homologue to F47 in SEA, and one mainly polar region (Jardetzky *et al.*, 1994). The polar region of the binding interface between SEB and HLA-DR includes a salt bridge formed between K39 on the HLA-DR α-chain and E67 of SEB. This interaction is surrounded by hydrogen bonds from SEB Y89 and Y115 (Jardetzky *et al.*, 1994). Substitution of K39 by alanine in the HLA-DR1 α-chain reduces binding to SEB, but not to SEA (Thibodeau *et al.*, 1994). The glutamate E67 in SEB is structurally equivalent to an aspartate in SEA. The shorter side chain of the aspartate residue in SEA compared with the glutamate residue in SEB may affect the interaction between K39 in the HLA-DR α-chain and several residues in SEA.Thus the HLA-DR K39 residue may have a more prominent role in binding to SEB compared with SEA. In the present study the K_d of the N-terminal region in SEA was estimated to be 10^{-5} M; it is likely that the N-terminal domain of SEA binds to the MHC class II α-chain in a similar manner to that seen for SEB, although the N-terminal region of SEA seems to bind with less affinity than the corresponding region in SEB. It is the combined binding activity of the two sites in SEA which is the key feature in the high affinity binding of SEA to HLA-DR. Whether the increased potency of SEA compared with SEB in T cell proliferation assays is due to the higher affinity for MHC class II or is a reflection of the potential ability of SEA to interact with two separate MHC class II molecules remains to be clarified. The three-dimensional structure of the SEB–HLA-DR complex clearly indicates that the C-terminal domain of SEB is oriented up and away from the HLA-DR β-chain. Assuming that binding of the N-terminal site of SEA to the HLA-DR α-chain is similar to that of SEB, this strongly suggests that the C-terminal domain interacts with a HLA-DR β-chain on a separate HLA-DR molecule. Crystals of the HLA-DR heterodimer were found to form superdimers (Brown *et al.*, 1993). The suggested contact points between SEA and HLA-DR molecules seem to allow simultaneous formation of HLA-DR superdimers. This implies possible extensive crosslinking between HLA-DR molecules and SEA on the cell surface. The potential ability of the Zn^{2+}-binding SEA and SEE molecules to cross-link HLA-DR complexes implies that the structural differences of these SAgs compared with the SEB/SEC family not only influences the engagement of particular TCR Vβ families (Irwin *et al.*, 1992; Hudson *et al.* 1993; Scherer *et al.*,

1993; Webb and Gascoigne 1994), but also affects the avidity of the HLA-DR–TCR interaction. The mutants described in the present study should serve as useful tools for dissecting the significance of bivalent and monovalent MHC class II binding in the immunobiology of SAgs. Forthcoming studies may involve experimental models for SAg signalling through MHC class II molecules on antigen-presenting cells (APC) (Scholl *et al.*, 1992). Furthermore, whether engagement of the TCR in a dimeric (HLA-DRα–SEA) or a trimeric (HLA-DRα–SEA–HLA-DRβ) complex has relevance for imprinting the TCR Vβ profile of SAgs needs to be addressed.

The question is why did SAg evolve two distinct MHC class II binding sites? A possible explanation is that the N-terminal site, which is characteristic for all staphylococcal enterotoxins, first evolved to allow binding to monomorphic MHC class II α-chains. The appearence of a Zn^{2+} binding site may represent an attempt to use bivalency to increase the potency of the SAg. Alternatively, the Zn^{2+} binding site may represent an ongoing evolution to expand the SAg receptor family from MHC class II molecules to Zn^{2+} coordinating target structures that are distinct from MHC class II. This suggests that SAg presentation may be diverted from MHC class II^+ dedicated APC to non-dedicated APCs. Presentation of antigens on non-dedicated APCs has been implicated as a mechanism for the induction of peripheral T cell tolerance (Schwartz, 1992), which could be used as a potential bacterial strategy to subvert immune recognition.

Materials and methods

Bacterial strains and plasmids

The *Escherichia coli* K12 strains UL635 (*xyl*-7, *ara*-14, T4R, Δ*omp*T) and HB101 were used for expression and cloning respectively. The vector pKP889 was used for expression of fusion proteins, where different SEA variants were fused after the heavy chain portion of the Fab fragment of the murine antibody C215. This expression vector is identical to pKP865 (Dohlsten *et al.*, 1994), except that the spacer between CH1 and SEA is Gly-Gly-Ala-Ala-His-Tyr-Gly. The vectors pKP943 and pKP1055 were used for production of SEA; expression from pKP943 yields SEA with the native N-terminus, while the product from pKP1055 is SEA having a glycine residue added at the N-terminus (Gly-SEA). This extension did not affect MHC class II affinity or T cell activity (data not shown). In both vectors the regulatory elements from staphylococcal protein A (Uhlén *et al.*, 1984) were used for transcription and translation and a synthetic signal peptide was used for secretion (L.Abrahmsén, unpublished results).

In vitro mutagenesis

Mutants were made using the polymerase chain reaction (PCR) on a Perkin Elmer Thermocycler. The reaction mixture (100 μl) contained: 1× PCR buffer (Perkin Elmer Cetus; 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% w/v gelatine), an additional 2 mM $MgCl_2$, 0.4 mM dNTPs (Perkin Elmer Cetus, Norwalk, CT), 2.5 U Ampli Taq DNA polymerase (Perkin Elmer Cetus) and 100 ng DNA template. Primers were added to a final concentration of 0.8 μM. The original template was a plasmid containing a *Staphylococcus aureus* enterotoxin A gene identical to that published (Betley *et al.*, 1988), except that the first triplet (encoding Ser) was changed to TCC to furnish a *Bam*HI site at the 5′-end of the gene. Later a derivative containing more unique restriction enzyme sites introduced by silent mutations was used. For most mutations the PCR was performed in two consecutive steps using two sets of primers; a new restriction enzyme site was introduced together with each mutation to enable facile identification. Oligonucleotides used as primers were synthesized on a Gene Assembler (Pharmacia Biotech, Uppsala, Sweden). To confirm each mutation, the relevant portion of the nucleotide sequence was determined on an Applied Biosystems DNA-Sequenser using the Taq DyeDeoxy Termination Cycle Sequencing Kit®.

Protein expression

Escherichia coli cells harbouring the different gene constructs were grown overnight at room termperature (Fab–SEA vectors) or at 24–34°C (SEA secretion vectors). The broth was 2× YT (16 g/l Bacto trypton, 10 g/l Bacto yeast extract, 5 g/l NaCl) supplemented with kanamycin (50 mg/l). Production of fusion proteins was induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 100 μM, as previously described (Dohlsten *et al.*, 1994); the protein A promoter used in the expression of non-fused SEA is constitutive. The cells were pelleted at 5000 *g* and the periplasmic contents were released by gently thawing the previously frozen cell pellet in 10 mM Tris–HCl (pH 7.5) on ice while aggitating for 1 h. The periplasmic extracts were clarified by centrifugation at 9500 *g* for 15 min.

Protein analysis

The proteins were separated in pre-cast polyacrylamide–SDS Tris–glycine Novcx gels (gradient 4–20% or homogenous 12%; Novex Novel Experimental Technology, San Diego, CA) and either stained with Coomassie Blue or used for Western blotting. Polyclonal rabbit anti-SEA was used to detect SEA in Western blot analyses, followed by porcine anti-rabbit immunoglobulin antibodies and rabbit anti-horseradish peroxidase antibodies and peroxidase. With Fab–SEA fusion proteins, peroxidase-conjugated rat antibodies, recognizing the murine κ chain, were also used (AAC 08P; Serotech Ltd, Oxford, UK). 3,3′-Diaminobenzidine (Sigma, St Louis, MO) was used for visualization of peroxidase.

Purification of SEAwt and SEA mutants

Immunoaffinity purification of SEAwt and SEA mutants from *E.coli* lysates was performed in a single step on anti-SEA immunoadsorbents prepared as described below. Sera from rabbits immunized with SEA were purified on protein G–Sepharose (Pharmacia Biotech, Uppsala) The recovered serum IgG fraction was further purified on a SEA affinity column to select for SEA-reactive antibodies. The affinity-purified anti-SEA antibodies were coupled to 5 ml NHS-activated HiTrap columns (Pharmacia Biotech) according to the manufacturer's recommendations. Wild-type and mutant SEA were eluted as a single peak from the anti-SEA immunoadsorbent with 0.1 M sodium citrate buffer (pH 2.5). Immediately after neutralization with 1 M Tris (pH 9), concentration and transfer to phosphate-buffered saline (PBS) (pH 7.2) was performed in Centriprep-10 concentrators (Amicon, Danvers, MA) and the purified proteins were finally aliquoted and stored at −70°C until used. The affinity-purified SEA mutants were shown to be >95% pure when run on pre-cast 10–15% gradient SDS–poyacrylamide gels (Phast System; Pharmacia Biotech).

Purification of C215Fab–SEA fusion protein

Medium containing C215Fab–SEA (750 ml) was incubated with Q-Sepharose FF (Pharmacia Biotech) in PBS (pH 6.9) and the filtrate was further clarified by centrifugation for 30 min at 20 000 *g*. Following this the supernatant was applied to protein G–Sepharose (90 ml) and eluted with 0.2 M acetate buffer (pH 2.8). The immunoaffinity fraction was injected into a Mono S HR 5/5 column (Pharmacia Biotech) and eluted with a linear gradient of 20 mM ammonium acetate (pH 4.5)–500 mM ammonium acetate (pH 6.0). Fractions containing C215Fab–SEA were pooled and passed through a PD-10 column (Pharmacia Biotech) with PBS (pH 7.4) as elution buffer. The homogeneity of purified fusion proteins was >95% when analysed by SDS–PAGE.

SEA ELISA

Binding of anti-SEA mAb to SEA mutants was measured in a sandwich ELISA. Maxisorb 96-well plates (Nunc, Naperville, IL) were coated with purified polyclonal rabbit anti-SEA antibodies (2.5 μg/ml in 50 mM sodium bicarbonate buffer, pH 9.6) and residual protein binding sites were blocked with 3% fat-free milk powder in PBS. Purified SEAwt and mutants (1 nM) were added to the coated 96-well plates followed by mAbs 1A, 2A, 3A, 1E and 4E (IGEN Inc., Rockville, MD) or EC-A1 (Chemunex Maisons-Afort, France). An SEE-specific antibody, 5E (IGEN Inc.), served as a negative control. Mutants giving $OD_{405\ nm}$ values <10% compared with SEAwt were considered negative for the antibody.

Radioiodination of SEA and SEA mutants

^{125}I-labelling was achieved by the lactoperoxidase method using a radioiodination kit from Amersham (Solna, Sweden). Briefly, the sample to be labelled was concentrated to 10–30 μg/50 μl in PBS and mixed with 1 mCi $Na^{125}I$ in 10 μl PBS, 2 μl lactoperoxidase (2 mg/ml), 10 μl 0.03% H_2O_2. The reaction was allowed to proceed on ice for 75 s under

continuous stirring and stopped by the addition of 5 µl 0.05% sodium azide in 0.43 ml 2% w/v bovine serum albumin (BSA) in Tris–HCl (pH 7.4). Unreacted iodine was separated from the labelled protein by desalting on a PD-10 column previously equilibrated with the BSA–Tris buffer. Conditions were chosen to obtain a sp. act. of 15–35 µCi/µg. The iodination procedure did not affect the binding activity of the mutants as analysed by competition experiments with unlabelled protein (data not shown).

Cell culture reagents

RPMI 1640 medium (Gibco, Middlesex, UK) supplemented with 2 mM L-glutamine (Gibco, Middlesex, UK), 0.01 M HEPES (Biological Industries, Israel), 1 mM $NaHCO_3$ (Biochrom KG, Berlin, Germany), 0.1 mg/ml gentamicin sulfate (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM sodium pyruvate (JRH Biosciences Industries, USA), 0.05 mM 2-mercaptoethanol (Sigma Co., St Louis, MO), 100× concentrated non-essential amino acids (Flow Laboratories, Costa Mesa, CA) and 10% fetal bovine serum (Gibco, Middlesex, UK) was used as complete medium for cell cultures.

Lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBM) were prepared from heparinized blood of normal donors from Lund University Hospital. The cells were isolated by density centrifugation over Ficoll-Paque as previously described (Dohlsten *et al.*, 1991). Following this, 0.2×10^6 PBM/200 µl complete medium were incubated in 96-well microtitre plates with varying amounts of SEA or SEA mutants for 72 h and and tested for DNA synthesis by incorporation of [^{3}H]thymidine as described (Dohlsten *et al.*, 1988).

Cell lines

The human B cell lymphoma Raji, the human colon carcinoma Colo 205 and CHO cells were cultured in complete medium; the EBV-transformed lymphoblastoid B cell line BSM was used as accessory cells for the establishment of T cell lines (Dohlsten *et al.*, 1991). All cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were mycoplasma free. T cell lines were established by stimulation of human peripheral blood lymphocytes with SEA (12 pM). The cultures were maintained for several months by weekly stimulation with mitomycin C-treated BSM cells pre-coated with SEA (12 pM) and the addition of recombinant IL-2 (20 U/ml; Cetus, Emeryville, CA). These T cell lines were all >99% $CD3^+$.

MHC class II binding assay

Determination of the dissociation constant (K_d) of SEA and SEA mutants for binding to MHC class II^+ Raji cells was performed in a cell suspension binding assay using ^{125}I-labelled proteins and Scatchard analysis, as previously described (Dohlsten *et al.*, 1994). The ability of various SEA mutants to displace the binding of FITC-labelled SEAwt to Raji cells was analysed by flow cytometry and the concentration of the mutants giving 50% inhibition of SEA binding (IC_{50}) determined.

Cytotoxicity assay

Cytotoxicity was measured at various effector target cell ratios in standard 4 h ^{51}Cr release assays as previously described (Dohlsten *et al.*, 1991). Colo205, CHO-C215 and CHO-DR cells were used as target cells and SEA-reactive human T cell lines as effector cells. The percentage specific cytotoxicity was calculated as 100×[(c.p.m. experimental release – c.p.m. background release)/(c.p.m. total release – c.p.m. background release)]. ^{51}Cr-labelled target cells were used in the cytotoxicity assay at 2.5 or 5×10^3 cells/0.2 ml complete medium in microtitre wells. SEA or C215Fab–SEA were added at various concentrations directly into the assay.

Transfection of CHO cells with cDNA encoding the C215 or HLA-DR antigens

The expression vector pKGE839 (Dohlsten *et al.*, 1994) contains GA733-2 cDNA, encoding the C215 antigen, and the neomycin resistance gene under transcriptional control of the SV40 early promoter/enhancer. The vector pKGE839 was transfected into CHO cells using Transfectam (Sepracor; IBF SA, Villeneuve la Garenne, France). Following selection in G418, the resistent clones were pooled and harvested, stained with FITC-labelled C215 mAb and subjected to repeated cell sorting. The sorted CHO-C215 cells were kept as a stable cell line. The establishment of CHO cells expressing HLA-DR has been described elsewhere (Dohlsten *et al.*, 1994). Expression of the relevant surface antigen on the transfectants was analysed routinely by flow cytometry.

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