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Quantitative Relationship Between MHC Class II-Superantigen Complexes and the Balance of T Cell Activation Versus Death

Pascal M. Lavoie,*† Helen McGrath,‡ Naglaa H. Shoukry,*§ Pierre-André Cazenave,¶ Rafick-Pierre Sékaly,*†.§‖ and Jacques Thibodeau‡‖

The binding of bacterial superantigens (SAgs) is profoundly affected by the nature of the MHC class II-associated antigenic peptide. It was proposed that this limitation in the density of SAgs displayed at the surface of APCs is important for efficient TCR serial triggering as well as for preventing apoptosis of the responding T lymphocytes. Here, we have addressed quantitatively the size of this SAg-receptive pool of HLA-DR molecules that are available to bind and present staphylococcal enterotoxin A (SEA) at the surface of B lymphocytes. Our binding curves, depletion experiments, and quantitative immunoprecipitations show that about half the HLA-DR class II molecules on B cells are refractory to SEA binding. Yet, as compared with typical nominal Ags, an unusually high amount of class II-SAg complexes can be presented to T cells. This characteristic appears to be necessary for SAg-induced T cell apoptosis. When <0.3% of the total cell surface MHC class II molecules are occupied by SEA, T cells undergo a normal sequence of early activation events. However, presentation of a ligand density beyond this threshold results in T cell activation that is readily aborted by apoptosis but only after a few cell divisions. Thus, we confirm the existence of MHC class II subsets that are structurally unable to present SEA and provide a quantitative framework to account for the ability of bacterial SAgs to induce peripheral activation vs tolerance in the host. The Journal of Immunology, 2001, 166: 7229–7237.

Superantigens (SAgs) are bacterial and viral products capable of inducing T cell deletion and anergy through their interaction with both the MHC class II molecule and the TCR. They are produced by a large spectrum of microorganisms, including potential human pathogens (1), and may be insidiously involved in the development of autoimmune diseases by triggering autoreactive T cells (2, 3). The ability of SAgs to stimulate a high proportion of the T cell repertoire can be explained by their distinct interaction with the complementarity-determining regions of the TCR β-chain variable domain (4). T cell stimulation by SAgs leads to suppression of IL-2 production, anergy, and apoptosis of SAg-reactive T cells (5, 6). The determinants responsible for the induction of T cell activation and/or deletion by SAgs remain enigmatic and thus far cannot be explained by their kinetics and/or topology of interaction with the TCR (7). Indeed, it has been demonstrated by surface plasmon resonance that the affinity of SAg-class II complexes for soluble TCRs is well within the range defined for agonist peptide ligands (8, 9). A better understanding of the molecular parameters allowing SAgs to induce a tolerance state in the host should allow a more rational use of these toxins in the development of immunomodulatory therapies.

Bacterial SAgs are presented in their native form and bind conserved regions of MHC class II molecules outside the peptide-binding groove (10). As such, they should bind to the totality of cell surface MHC class II molecules and thus engage the TCR with a higher avidity than nominal Ags. However, we and others have shown that SAg binding and presentation is markedly affected by the structural microheterogeneity of the MHC class II molecules, probably imparted by the groove-occupying peptide (11–16). Whether that phenomenon modulates the activity of SAgs on T cells has not been addressed in previous studies. In vivo, the proportion of MHC molecules bound by a given antigenic peptide appears to be naturally limited by inherent peptide processing or loading mechanisms. This possibly prevents induction of high avidity T cell tolerance against nonself Ags (17). Similarly, the restriction imposed by the groove peptide on the binding of SAgs to class II molecules might limit functional inactivation of the responding T cells at low doses (16, 18). Moreover, given that the TCR machinery is sensitive to very low amounts of antigenic peptide ligands (19, 20) or SAg (21), others have proposed that a limited number of SAg-MHC class II complexes is necessary to insure proper serial triggering of the TCR (16).

In the present study, we investigated quantitatively how the structural microheterogeneity in MHC class II molecules affects the binding of staphylococcal enterotoxin A (SEA) to B lymphocytes. We have determined 1) the proportion of MHC class II-peptide complexes on B cells that allows binding of SEA, 2) the minimal number of complexes required for T cell activation, and 3) the number of SAg-MHC complexes required to induce T cell death. Our results show that the amount of SEA receptors on APCs largely exceeds the proportion of class II-ligand complexes required for T cell activation or deletion.

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2 Address correspondence and reprint requests to Dr. Jacques Thibodeau, Laboratoire d’Immunologie Moléculaire, Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Canada H3C 3J7. E-mail address: jacques.thibodeau@umontreal.ca

3 Abbreviations used in this paper: SAg, superantigen; SEA, staphylococcal enterotoxin A; 7-AAD, 7-amino actinomycin D; FSC, forward light scatter.
Materials and Methods

Cell lines, reagents, and Abs

The human EBV-transformed B cell line LG2 (DR1/DR1) was provided by L. J. Stern (Massachusetts Institute of Technology, Cambridge, MA) and grown in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 5–10% FCS and 2-ME. The pan anti-DR Ab L243 (IgG2a) (HB55; American Type Culture Collection, Manassas, VA) recognizes a conformational epitope situated in the αl domain (22). The anti-DR6 XDS.117 (23) and anti-DRα DA6.147 (24) mAbs have been previously described. In all the biochemical experiments, SEA from three different sources was used and yielded similar results. Purified SEA was obtained from Toxin Technology (Sarasota, FL), and purified recombinant SEA was a kind gift of P. Antonsson (Active Biotech Research, Lund, Sweden) and W. Mourad (Université Laval, Quebec, Canada). The L243 hybridoma was grown in RPMI 1640 medium supplemented with synthetic 2% HY (Life Technologies) and 10 μg/ml LPS (Sigma, St. Louis, MO) and counted.

Intracellular IL-2 and IFN-γ were measured after 8 h of stimulation using procedures previously described (26). In brief, PBMCs (2 × 10⁶/ml) were incubated with no stimulus, PMA (25 ng/ml) and ionomycin (1 μg/ml), or SEA for 8 h. Brefeldin A (10 μg/ml) was added for the final 6 h of stimulation. After stimulation, cells were permeabilized and stained with either anti-IL-2 or anti-IFN-γ, and either anti-CD4 or anti-TCR. Abs, isotype controls, and lysing and permeabilizing solutions were purchased from BD Biosciences. Control Ab for permeabilization was purchased from Mediscorp (Montreal, Quebec, Canada). Flow cytometry analyses were performed on a FACScan using CellQuest software (BD Biosciences). In lymphocyte stimulation experiments, recombinant SEA was used.

Quantitative immunoprecipitations and Western blotting

Cell surface HA-DR1 molecules were immunoprecipitated using SEA-bio or L243-bio. Unless specified otherwise, LG2 cells (3 × 10⁶) were incubated for 4 h in the presence of either SEA-bio (80 μg/ml; 3 μM), or L243-bio (60 μg/ml; 0.6 μM) at 37 or 4°C in culture medium stabilized with 0.01% sodium azide. After incubation, the cells were washed eight times in 1 ml of PBS to eliminate background, as controlled using an excess of SEA-bio and SEA-bio-coupled beads. Cell lysates were then biotinylated in 200 μl of lysis buffer (0.6 M) at 37 or 4°C before washings and immunoprecipitation with streptavidin-coated nonporous beads (Dynal M-280; Dynal Biotech, Oslo, Norway). After 1 h at 4°C, 1 ml of PBS was added, and the beads were centrifuged, washed eight times with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 μM ZnSO₄, 1 mM PefaBloc SC (Roche, Montreal, Canada), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100, and resuspended in 50 μl of 2× Laemmli protein-loading buffer. The samples were boiled for 5 min, loaded on 10% SDS-PAGE, and analyzed by Western blotting using the XDS.117 and/or DA6.147 Abs as previously described (12). A peroxidase-coupled goat anti-mouse secondary Ab reactive to the Fc portion of mouse IgGs was used for chemiluminescence detection (Pierce).

Depletion experiments

LG2 cells (2 × 10⁶) were incubated as indicated above in 50 μl SEA-bio for 4 h at 37°C before washings and immunoprecipitation with streptavidin-coupled beads. The lystate was transferred to an Eppendorf tube containing fresh SEA-bio-streptavidin beads. After a 4-h agitation at 4°C, the beads were centrifuged, washed eight times with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 μM ZnSO₄, 1 mM PefaBloc SC, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100), and resuspended in 50 μl of 2× Laemmli protein-loading buffer. The lystate was then reincubated for two or more rounds of depletion using saturating amounts of streptavidin-coupled Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Quebec, Canada). The Fab portion (L243-Fab) was prepared using a commercially available kit (Pierce, Rockford, IL) and yielded a single band (50 kDa) on SDS-PAGE under nonreducing conditions. The concentration was determined by absorbance at 280 nm on a standard spectrophotometer. The SEA or L243-Fab (referred to as SEA-bio or L243-bio) was biotinylated using the sulfo-NHS-LC-biotin as described by the manufacturer’s protocol. The biotinylated proteins were dialyzed in PBS and stored at −20°C in 50% glycerol. Sepharose-SEA or -L243 was prepared by coupling proteins to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) according to the supplier’s recommendations. In brief, SEA or L243 (600 μg) in 0.1 M NaHCO₃ and 0.5 M NaCl was incubated to cyanogen bromide-activated Sepharose gel (0.75 g) in 0.1 M NaHCO₃ and 0.5 M NaCl. The supernatant was transferred to a fresh Eppendorf tube containing fresh SEA-bio-streptavidin beads. After a 4-h agitation at 4°C, the beads were centrifuged, washed eight times with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 μM ZnSO₄, 1 mM PefaBloc SC (Roche, Montreal, Canada), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100), and resuspended in 50 μl of 2× Laemmli protein-loading buffer. After 1 h at 4°C, 1 ml of PBS was added, and the beads were centrifuged for 10 s. The beads were again washed 10 times (lysis buffer with 0.5% Triton X-100 and resuspended in 50 μl of 2× Laemmli protein-loading buffer. The samples were boiled for 5 min, loaded on 10% SDS-PAGE, and analyzed by Western blotting using the XDS.117 and/or DA6.147 Abs as previously described (12). A peroxidase-coupled goat anti-mouse secondary Ab reactive to the Fc portion of mouse IgGs was used for chemiluminescence detection (Pierce).
a fresh Eppendorf tube containing a pellet of \(3 \times 10^8\) streptavidin beads. The lysate was then transferred to a fresh tube containing 75 μl of L243-coupled Sepharose beads and incubated at 4°C for an additional 4 h before washings. The samples were loaded on SDS-PAGE and analyzed by Western blotting.

**CFSE, annexin V, and 7-aminopterin D (7-AAD) experiments**

The CFSE dye was obtained from Molecular Probes (Eugene, OR). CFSE (0.5–1.25 μM, depending on the preparation) was added to 2 × 10^7 PBMC and incubated with gentle mixing at room temperature for 10 min. The reaction was quenched by addition of an equal volume of FCS, and cells were washed three times with PBS containing 5% FCS. The cells were cultured at 37°C at 5% CO2 at 1.5 × 10^6/ml in RPMI 1640 containing 3% FCS overnight to obtain a stable fluorescence intensity between 10^3 and 10^4 logs. Labeling of cells with 7-AAD and annexin was performed as previously described (27). For the experiment in Fig. 7, cells stained with CFSE according to the above-mentioned method were stimulated with SEA or PHA for 3 days and then labeled with 7-AAD before analysis by flow cytometry.

**Results**

**SEA binds a substantial but restricted fraction of cell surface HLA-DR1 molecules**

**Scatchard analyses.** We have previously shown that B cell lines or primary lymphoid cells expressing the class II-associated invariant chain and HLA-DM can bind and present SAgs most efficiently (12). To determine the fraction of MHC class II molecules that are permissive to SEA binding, we performed Scatchard analyses using radiolabeled SEA. For this purpose, we used the representative DR1-expressing B cell line LG2 because, in those cells, the great majority of MHC class II molecules form SDS-stable complexes containing a mixture of oligopeptides (25). Although B lymphocytes express other class II isotypes, HLA-DR is the major receptor for this SAg (28). The reproducibility of the number of complexes obtained by Scatchard plots is shown by SDs of <2% obtained between each of the independent experiments (see Table I). A representative set of data is shown in Fig. 1A. Of note, in each of these experiments, the binding of either SEA (n = 7) or the L243 anti-DR Fab (n = 4) appeared to follow a first-order binding ligand-receptor interaction as previously described by others (29, 30). Binding of SEA occurs mostly to the high affinity β-chain site, as occupancy of the low affinity of the α-chain site (Kd ≈ 10^-3 M) is insignificant in the range of concentrations used (29–32). Using these data, we calculated that LG2 cells express a total of (3.2 ± 0.5) × 10^8 SEA receptors/cell. An average Kd of (4 ± 2) × 10^-8 M was derived, which is in accordance with affinities reported on a number of cell types (12, 33). Similarly, we found a total number of (5.2 ± 2.6) × 10^6 DR1 receptors/cell as measured using an anti-DR Fab portion (Table I). These experiments show that the proportion of SAg-binding sites averages 60% of the total number of surface MHC class II molecules, suggesting that some class II molecules on the surface of APCs are unable to interact with SEA.

**Immunoprecipitation and depletion analysis.** Given the extent of the experimental variations in the binding of the anti-DR Ab obtained with Scatchard analyses (Table I), we used independent approaches to confirm the number of DR1 molecules expressed at the surface of LG2 cells. As shown in Fig. 1B, the amount of cell surface DR1 molecules immunoprecipitated using saturating concentrations of the L243 Fab portion was compared with DR1 standards of known concentrations. In at least five independent experiments, the total number of HLA-DR1 receptors found (5.3 ± 0.6) × 10^6 molecules/cell) was highly consistent with results obtained on Scatchard plots.

The heterogeneity of antigenic peptides bound to MHC class II molecules at the cell surface may give rise to interactions with SEA of different affinities (13), thus skewing our estimate of the total number of binding sites derived from Scatchard data. In principle, such lower-affinity sites can be discerned from nonbinding sites by using saturating amounts of the ligand. We have confirmed the binding of SEA to a fraction of class II molecules by quantitatively comparing the total surface HLA-DR molecules immunoprecipitated using microgram amounts of L243 vs SEA. The amount of DR1 was determined by Western blotting using the XDS.117 Ab, which recognizes a linear epitope on the DR β-chain (34). Fig. 2A shows that about half (49 ± 8.3%) of MHC class II molecules were immunoprecipitated with SEA as compared with L243. Similar results were obtained in five different experiments independently of the temperature used for incubation, and these are in accordance with Scatchard results (see Table I). Importantly, the nonconformer anti-DRb Ab XDS.117 precipitated an amount of DR1 comparable to that obtained with L243, indicating that the latter does not bind to a limited subset of MHC class II in itself (data not shown).

We reasoned that if a subset of MHC class II molecules is unable to bind SEA, it should not be possible to deplete HLA-DR from a cell lysate using this SAg. Thus, we performed serial rounds of immunodepletion using saturating doses of SEA (a saturating dose was determined from the results of Fig. 1A), followed by a final immunoprecipitation using the anti-DR Ab L243. We specifically chose to use this conformation-dependent mAb, which

<table>
<thead>
<tr>
<th>No. SEA Receptors per Cell* (\times 10^6)</th>
<th>No. DR Molecules per Cell* (\times 10^6)</th>
<th>Percentage of Sites Occupied by SEA</th>
</tr>
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<tr>
<td>3.37 ± 0.05 (R^2 = 1.00)</td>
<td>3.2 ± 0.4 (R^2 = 1.00)</td>
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<td>2.79 ± 0.08 (R^2 = 0.74)</td>
<td>ND (R^2 = 0.99)</td>
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</tr>
<tr>
<td>3.6 ± 0.2 (R^2 = 0.98)</td>
<td>5.8 ± 0.1 (R^2 = 0.99)</td>
<td>41</td>
</tr>
<tr>
<td>2.90 ± 0.09 (R^2 = 1.00)</td>
<td>ND (R^2 = 0.99)</td>
<td>93</td>
</tr>
<tr>
<td>3.5 ± 0.1 (R^2 = 0.99)</td>
<td>8.6 ± 0.4 (R^2 = 0.96)</td>
<td>61</td>
</tr>
<tr>
<td>2.8 ± 0.2 (R^2 = 0.97)</td>
<td>3.0 ± 0.2 (R^2 = 0.98)</td>
<td></td>
</tr>
<tr>
<td>3.2 ± 0.4 (R^2 = 0.86)</td>
<td>ND (R^2 = 0.86)</td>
<td></td>
</tr>
<tr>
<td>3.2 ± 0.50 (n = 7) (R^2 = 1.00)</td>
<td>5.2 ± 2.60 (n = 4)</td>
<td></td>
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*The number of receptors for a given ligand was calculated using a projection on the x-axis of the best fit (as determined by a linear regression) of a plot (bound (B) over the bound/free (B/F)) of Scatchard data. In each case, the correlation \(R^2\) of the regression is indicated in parentheses (see Fig. 1). Each experiment was performed in duplicate, and the variation between the duplicate is represented in the error (\(\pm\)).

*Average number of sites calculated from the different experiments. In this case, the error represents the standard variation in the \(n\) experiments.
recognizes only fully folded post-Golgi αβ heterodimers of importance here (35). Fig. 2B is a representative experiment in which we completely depleted SEA-binding HLA-DR receptors from the LG2 lysate after three to four rounds of immunoprecipitation. However, it was not possible to deplete all of the HLA-DR molecules from the cell extract, as demonstrated by the presence of a strong residual signal for DR molecules immunoprecipitated using L243. Altogether, the results obtained from Scatchard plots, immunoprecipitation, and depletion experiments provide a rigorous quantification of the number of MHC class II-peptide complexes that are structurally competent to bind SEA. These receptors represent 45–60% of the total surface HLA-DR molecules, thus suggesting that some class II-bound oligopeptides might restrict the binding of SAgs on live cells.

The number of cell surface MHC class II-SAg complexes required for T cell stimulation

LG2 cells were used in proliferation assays to characterize the relationship between the number of class II-SAg complexes and the extent of the T cell response. Fig. 3A compares a typical proliferation dose-response curve to the proportion of HLA-DR1 molecules that are occupied by SEA at different concentrations. Independently of the T cell:LG2 ratio, proliferation was detected starting at concentrations as low as 10^{-14} M SEA (Fig. 3A), as previously described (36). Similarly, blastic transformation was evident by flow cytometry at 10^{-15} M SEA (Fig. 3B). These concentrations correspond to an average SEA occupancy of ~0.1 (0.000003125%) to 1 (0.00003125%) DR1 molecules/cell (Fig. 3C). Most likely, at these concentrations, only some APCs are occupied by a few SEA ligands per cell and are therefore able to stimulate a small number of T cells, possibly those of highest affinity for the SAg-class II complex. The potency of SAgs such as SEA is remarkable because an extremely low number of SEA-MHC class II complexes are sufficient to trigger T cell activation in the conditions established in the present assay. Many more potential SAg-MHC class II complexes can be formed than the minimal number required for T cell activation (see Fig. 3A). Maximal T cell proliferation was achieved at SEA concentrations corresponding to ~10,000–20,000 occupied MHC class II molecules (0.2 nM SEA).
We consistently measured decreasing levels of proliferation at SEA concentrations higher than this optimal number of complexes (10,000–20,000 SEA-MHC/cell) (see Fig. 3A), suggesting that a higher density of SAg ligands leads to an impaired T cell response. This decrease in proliferation could not be explained by a lower efficiency of TCR engagement, as evidenced by the strong internalization of the TCR in SEA-reactive V\textsubscript{b}22\textsuperscript{+} T cells (which account for a large part of the SEA-responsive T cells; data not shown) above 0.2 nM SEA. As shown in Fig. 3D, TCR internalization was strictly dependent on the presence of the LG2 cells and on the formation of T cell-APC conjugates (data not shown).

Presentation of supraoptimal number of SEA-MHC class II complexes leads to impaired cytokine production by T cells

The next series of experiments were designed to study the functional response of T cells to supraoptimal doses of SEA as defined above. The decreased T cell proliferation observed after 3 days at high SEA concentrations (Fig. 3A) was not exclusive to presentation by LG2 cells, as the same results were obtained using class II-autologous PBLs as a source of APCs (Fig. 4A). At supraoptimal SEA concentrations, T cells underwent a normal dose-dependent kinetics of induction of early (3–18 h) CD69 and late (24–48 h) CD25 activation markers (see Fig. 4, B and C). However, above...
10⁻¹⁰ M, a selective dose-dependent inhibition of IL-2 production was observed (Fig. 5A). For example, at 10⁻⁷ M SEA (Fig. 5B), we measured a 2-fold reduction in the proportion of cytokine (IL-2 and IFN-γ)-producing cells among activated (CD69⁺) lymphocytes (Fig. 5C) as early as 8 h after stimulation.

**T cell apoptosis at high SEA concentrations occurs as a late event after cell division**

Despite the normal induction of CD69 and CD25, the proliferative response of T cells at high doses of SEA dropped after 3 days (see Figs. 3 and 4). At day 5, the number of cells recovered at 10⁻⁷ M SEA was barely increased over the unstimulated control (Fig. 6A). In contrast, at the optimal dose of 10⁻¹¹ M SEA, considering that SEA triggers only ~10–15% of total population of T cells, a 7-fold expansion was observed. Importantly, the reduction in expansion at high SEA doses was not due to nutrient depletion following a massive expansion, because stimulation with PHA, which roughly triggers proliferation of 10-fold more T cells, yielded a significant expansion (see Fig. 6A).

A likely explanation for the reduced T cell expansion observed at high concentrations is that a high density of SEA ligands results in an abortive activation through programmed cell death, reflecting
the physiological response to SAgs in vivo (5, 6). As shown in Fig. 6B, the proportion of nonviable (7-AAD+) cells after 48 h was significantly increased to >50% as cells were stimulated by supraoptimal (10^{-5} M) doses of SEA. The commitment of activated T cells to apoptosis was further confirmed by the presence of annexin V+7-AAD+ cells (a phenotype associated with early apoptosis (27)) in the cells stimulated by SEA.

In additional experiments, we used the CFSE dye that specifically tracks dividing cells to confirm the relationship between the proliferation of SEA-responding cells and their commitment to apoptosis. As described previously, CFSE is a marker of cell division because the fluorescent dye is halved in each daughter cell (37). A representative experiment is shown in Fig. 7. Activated T cells expanded under all three conditions tested, consistent with our observation that the induction of activation markers is unaffected by supraoptimal levels of SEA in the first 48 h after stimulation (Fig. 4, A and B). Interestingly, we observed a late occurrence of cell death (as measured by staining with 7-AAD) specifically after three to four rounds of division in cells stimulated with SEA. This result is consistent with the absolute requirement for T cell proliferation in SAg-induced apoptosis in vivo (38). Although we detected some degree of cell death in all conditions tested, apoptosis increased in a dose-dependent manner at supraoptimal vs optimal SEA doses (53 vs 13%) (see Fig. 7).

Discussion

The presentation of SAgs such as SEA and toxic shock syndrome toxin-1 is modulated by MHC class II-bound ligands, most likely including Ags present in the MHC class II peptides groove (11–16, 39). For example, using MHC class II molecules loaded with single peptides, Kappler and collaborators have measured by surface-plasmon resonance relatively wide variations in the affinity of these complexes for SEA (13). To better define the extent to which these phenomena are relevant to the presentation of SAgs in vivo, we have used a number of independent biochemical approaches to define unambiguously the proportion of MHC class II molecules acting as SEA receptors on B cells (12). Our Scatchard analysis suggests that, among the 5 × 10^{6} DR1 molecules expressed on LG2 cells, a substantially high proportion (35–60%) is unable to bind and present SEA. We have confirmed that only a fraction of DR molecules bind SEA by performing 1) quantitative immunoprecipitations on cell surface molecules using either the L243 Fab or SEA and 2) depletion experiments on total cell lysates. In these experiments, the maximal concentrations of SEA used were high enough to have picked up any remaining pool of MHC class II complexes with physiologically relevant affinity for the SAg. Therefore, we conclude that there are some class II-bound ligands that are simply nonpermissive for the binding of SEA. Earlier quantification of the proportion of SAg-binding MHC class II molecules, using I-E molecules encapsulated in planar membranes, suggested that most molecules (76%) can bind SEA. These results were interpreted as evidence for the fact that SEA binds MHC class II molecules in an unrestricted fashion (40), although a negative effect of some MHC class II-bound peptides on SAgs binding was not purposely addressed.

The inability of SAgs to bind all the cell surface HLA-DR molecules is a likely a consequence of their indispensable need to interact in close proximity to the polymorphic and structurally diverse peptide groove to reach the TCR (41). Although our results demonstrate that a majority of peptides are permissive to SEA binding, we cannot formally exclude that variations in the affinity of different MHC-peptide complexes may be important for T cell activation. Indeed, among the 35–60% of surface DRIIs that bind SEA, ligand-induced structural variations may give rise to a spectrum of affinities for the SAg (13). If the intrinsic affinity of MHC molecules is high (i.e., >4 × 10^{-8} M), then the existence of progressively lower affinity sites should keep the amount of bound SAg within a range optimal for T cell proliferation. This would be consistent with the idea that a mechanism to limit SAg density is important for inducing a proliferative T cell response as opposed to deletion (16). However, the affinity of SEA as measured herein (4 × 10^{-8} M) is identical with the! The structural difference between SEA-binding vs non-SEA-binding subsets of MHC class II molecules remains to be determined. In addition to MHC class II-bound oligopeptides, other glycoproteins associated in complex with HLA-DR molecules may directly interfere with the binding of SAgs (12, 42). For example, HLA-DR was found to be associated in B cells with molecules such as CD40 (43), HLA-DM (44), and members of the tetraspan protein family (45).

A low amount of SEA, corresponding to 15,000 ligands/APC (0.3% of total cell surface HLA-DR molecules), triggers a strong proliferative response with very little apoptosis. Interestingly, in vivo studies looking at the induction of peripheral deletion by SAgs have used relatively (~1–50 μg/mouse) high doses (6, 46, 47), whereas studies using low doses of SAgs showed that they could stimulate T cell expansion just like antigenic peptides (48, 49). Above a given threshold of SEA-HLA complexes, this balance is tipped toward induction of apoptosis. These observations suggest that the potential for a large pool of MHC-bound SEA is essential to the induction of peripheral tolerance in vivo. In the physiological setting, T cells typically respond to Ags presented by ~10^{2}–10^{3} molecules of the restricting allele occupied by a given peptide (50). Interestingly, these estimates compare well to our calculation of the number of SAg ligands needed to achieve a sustained T cell expansion. This interpretation is consistent with recent experiments showing that the affinity of MHC class II-bound SAgs for the TCR is within the range of purely agonist antigenic ligands (8, 9).

In vivo treatment with high doses of SEA leads to a selective suppression of IL-2 production that occurs before and independently of T cell deletion (6, 46). In addition to IL-2, we observed a selective block in the production of IFN-γ that seemingly occurred before the induction of apoptosis in these cells. Indeed, at the earliest time points of TCR engagement (between 6 and 8 h) and in the absence of any detectable cells undergoing apoptosis, we observed a drop in the IL-2 produced, despite a normal induction of the CD69 activation marker (Figs. 2 and 4). Interestingly, a comparable dose-dependent inhibition of the number of cytokine-producing cells has been reported in specific CD8+ T cells responding to supraoptimal lymphocytic chorioneminitis viral loads (51). It is tempting to speculate that prolonged, sustained engagement of the TCR by SAgs limits the cytokine response of T cells perhaps by virtue of the excess number of TCR down-regulated. In our system, addition of exogenous rIL-2 (50, 100, or up to 500 U/ml) at the beginning or daily in the culture did not rescue these cells from programmed death (data not shown). Finally, apoptosis occurred as a relatively late event after initiation of cell division. Based on the level of CFSE in the apoptotic cells, we estimate that these cells die only after having gone through about
three to four cell divisions. Interestingly, a similar observation has recently been reported using staphylococcal enterotoxin B (52).

Primed or naive T cells may likely differ in their sensitivity to ligand-induced apoptosis (53, 54). Here, a minimal fraction of the PBLs was composed of primed T cells before stimulation with SEA, as evidenced by the expression of either CD69 or CD25 in <1% of these cells (data not shown). Our data mostly reflect the combined response of naive T cells differing with respect to their affinity for SEA. However, it may be interesting to investigate the functional difference existing between memory and naive T cells or cells otherwise differing in their TCR Vβ expression.

In conclusion, our results show that despite the existence of different pools of class II molecules relative to their ability to bind SEA, the potential number of SA-g-HLA class II complexes largely exceeds the prerequisite for inducing peripheral tolerance in the host. This data should help define the optimal dosage for SAgs in anti-tumor therapies (55)

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References

Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan 
molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J. Immu-
hol. 157:2939.

46. Kawabe, Y., and A. Ochi. 1990. Selective anergy of Vβ8⁺, CD4⁺ T cells in 

superantigens acutely trigger distinct levels of peripheral T cell tolerance/immu-

48. Wen, R., S. Surman, M. A. Blackman, and D. L. Woodland. 1997. The conven-
tional CD4⁺ T cell response to staphylococcal enterotoxin B is modified by its 

49. Hofer, M. F., K. Newell, R. C. Duke, P. M. Schlievert, J. H. Freed, 
toxic shock syndrome toxin-1 on B cell apoptosis. Proc. Natl. Acad. Sci. USA 
93:5425.

50. Germain, R. N., and I. Stefanova. 1999. The dynamic of T cell receptor signaling: 
complex orchestration and the key roles of tempo and cooperation. Annu. Rev. 
Immunol. 17:467.

51. Gallimore, A., A. Glithero, A. Godkin, A. C. Tissot, A. Pluckthun, T. Elliott, 
H. Hengartner, and R. Zinkernagel. 1998. Induction and exhaustion of lympho-
cytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using 
soluble tetrameric major histocompatibility complex class I-peptide complexes. 

52. Renno, T., A. Attinger, S. Locatelli, T. Bakker, S. Vacheron, and 
H. R. MacDonald. 1999. Apoptosis of superantigen-activated T cells occurs pref-

53. Lezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic 
stimulation determines the fate of naive and effector T cells. Immunity 8:89.

54. Lee, W. T., and E. S. Vietta. 1992. Memory T cells are anergic to the superan-

55. Hansson, J., L. Ohlsson, R. Persson, G. Andersson, N. G. Ilback, M. J. Litton, 
T. Kalland, and M. Dohlsten. 1997. Genetically engineered superantigens as tol-