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Staphylococcal Enterotoxin H Induces Vα-Specific Expansion of T Cells

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Staphylococcal enterotoxin H (SEH) is a bacterial superantigen secreted by *Staphylococcus aureus.* Superantigens are presented on the MHC class II and activate large amounts of T cells by cross-linking APC and T cells. In this study, RT-PCR was used to show that SEH stimulates human T cells via the Vα domain of TCR, in particular Vα10 (TRAV27), while no TCR Vβ-specific expansion was seen. This is in sharp contrast to all other studied bacterial superantigens, which are highly specific for TCR Vβ. It was further confirmed by flow cytometry that SEH stimulation does not alter the levels of certain TCR Vβ. In a functional assay addressing cross-reactivity, Vβ binding superantigens were found to form one group, whereas SEH has different properties that fit well with Vα reactivity. As SEH binds on top of MHC class II, an interaction between MHC and TCR upon SEH binding is not likely. This concludes that the specific expansion of TCR Vα is not due to contacts between MHC and TCR, instead we suggest that SEH directly interacts with the TCR Vα domain. ***The Journal of Immunology*****, 2003, 170: 4148–4154.**

The staphylococcal enterotoxins (SEs)[4] (1), secreted by certain strains of *Staphylococcus aureus*, are some of the most potent activators of T cells known. They have for a long time been known to be toxic to humans, which may result in acute toxic shock, food poisoning, or scarlet fever (1). The ability of SEs to specifically activate a high frequency of T cells has led to their designation as superantigens (2). Superantigens simultaneously bind to MHC class II on APCs and to the TCR on T cells. This cross-linking of APCs and T cells results in a strong polyclonal activation of $CD4^+$ and $CD8^+$ T cells (3, 4) followed by massive T cell proliferation (5, 6), T cell cytotoxic activity (7) as well as excessive production of cytokines (8).

During the recent sequencing of the staphylococcal genome, new superantigens have been identified and, to date, the SE family is comprised of SEA, B, C, D, E, G, H, I, J, K, L, and M. Several structural studies have revealed that SEs have a highly conserved three-dimensional fold (9–13). These superantigens bind as unprocessed proteins outside the peptide-binding groove of MHC class II (14) to two completely distinct binding sites. Site-directed mutagenesis studies (15–18) and structural studies of SEA_{D227A}-HLA-DR1 (19) and SEB-HLA-DR1 complexes (20) show that SEA, SEB, SEC, SED, and SEE bind to the α-chain of MHC class II with low affinity using its N-terminal domain. A second high-affinity zinc-dependent interaction via its C-terminal domain with the β-chain of MHC class II has been structurally verified for SEH (21). SEA, SED, and SEE also require coordination of zinc and appears to be presented on MHC class II molecules via a "SEH-like" site in their C-terminal domains (12, 17, 18, 22–24). Hence, these superantigens possess a divalent interaction with MHC class II.

When presented on MHC class II, the SEs are recognized by specific subsets of T cells. All characterized SEs to date interact with the variable region of the TCR β-chain (TCR Vβ) and every SE has its unique TCR Vβ profile (2, 25–27). Both SEB and SEC have been structurally characterized when bound to murine TCR Vβ8.2 chain. Theses studies show that both SEs mainly interact with the complementary determining region (CDR) 2 and framework region 3 in the TCR β-chain (28, 29). In addition, two streptococcal pyrogenic exotoxins (SPE-A and SPE-C), with a three-dimensional fold similar to the SEs, have been structurally investigated when bound to TCR Vβ. The structures show that they bind TCR Vβ in a more extensive manner with additional interactions with the CDR1 and CDR2 on TCR. Moreover, unlike SEB, SPE-C makes specific hydrogen bonds to the side chains of TCR Vβ (30).

SEB and SEC both bind to MHC class II in a way that allows a direct interaction between MHC class II and TCR. This stabilizes the ternary MHC-SE-TCR complex and enhances the T cell activation (31–34). SEH and SPE-C, however, bind on top of the MHC class II molecule and therefore block a similar interaction between MHC class II and TCR (21, 35). SEH interacts with MHC class II with the highest affinity ever measured for a SE (36) and this stable presentation may compensate the absence of a direct MHC class II-TCR interaction.

In this study, we have characterized the interaction between SEH and human T cells. Our results strongly suggest that SEH binds and activates T cells in a way that does not induce TCR Vβ-specific expansion. Instead, we show a Vα-specific activation of human T cells, which has never previously been demonstrated for any superantigen. This contradicts earlier assumptions that superantigens are restricted to TCR Vβ for T cell activation.

Materials and Methods

Protein expression and purification

Recombinant SEA, SEB, SED, SEE, and SEH were expressed as secreted products in *Escherichia coli* K12 strain UL635 (*ara*-14, *xyl*-7, $T4^R$,

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[4] Abbreviations used in this paper: SE, staphylococcal enterotoxin; CDR, complementary-determining region; SPE, streptococcal pyrogenic exotoxin; EC_{50}, half-maximum effect concentration.

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Δ*omp*T). The expression vector contained a gene coding for one of the different superantigens, the constitutive *S. aureus* protein A promoter, a synthetic signal peptide and a kanamycin resistance gene (18). The cultivations were conducted in Bioreactors; the following treatments and the purifications of the SEs were performed as previously described (37). SED was purified using anion exchange chromatography and the running buffer used was 10 mM potassium phosphate (Sigma-Aldrich, St. Louis, MO), pH 6.4, containing 0.02% Tween 20 (ICN Biomedicals, Aurora, OH). The sample containing SED was applied to a SP-Sepharose fast flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) and SED was first eluted with 100 mM sodium chloride added to the running buffer (ICN Biomedicals), then applied again to a SP-Sepharose high-performance column (Amersham Pharmacia Biotech) and eluted with a linear gradient from 0 to 200 mM sodium chloride added to the running buffer. The fractions containing SED were finally applied to a Resource-S column (Amersham Pharmacia Biotech) and once again eluted with a linear gradient from 0 to 200 mM sodium chloride. All of the purified superantigens were >95% pure according to Gelcode (Pierce, Rockford, IL) or Coomassie blue-stained SDS-PAGE and the final yields varied from 30 to 720 mg/L growth medium.

Establishment of human effector T cell lines

Human T cell lines reactive to SEA, SEB, SEC, SED, SEE, and SEH were established as previously described (38). Briefly, PBMCs from the same donor were cultured with the specific superantigen (10 ng/ml) for 3–4 days. The cell cultures were thereafter allowed to grow for 3–6 wk with renewal of the culture medium containing the specific superantigen and IL-2 once a week at a final concentration of 1 ng/ml and 20 U/ml, respectively. Since the activated T cells kill the present MHC class II-expressing cells in the PBMCs, new APCs were added on a weekly basis.

Flow cytometry analysis of the T cell repertoire

Human PBMCs were obtained from EDTA-treated venous blood from two healthy donors by density gradient sedimentation over Ficoll-Paque Plus (Amersham Pharmacia Biotech AB). The isolated PBMCs were resuspended in Dulbecco's PBS without Ca^{2+} and Mg^{2+} (BioWhittaker Europe, Verviers, Belgium) and washed three times. The cells were then resuspended in complete cell culture medium (R10), i.e., RPMI 1640 (BioWhittaker Europe) supplemented with 10% FCS (HyClone, Logan, UT), 1 mM sodium pyruvate (BioWhittaker Europe), and 0.1 mg/ml gentamicin (BioWhittaker Europe) at 2×10^6 cells/ml. Cells were cultured in the presence of either anti-CD3 Ab (0.1 μg/ml; BD PharMingen, San Diego, CA), SEH (1 nM), or SEA (1 nM) for 3 days at 37°C. The dead cells were removed from the living cells by density gradient sedimentation over Ficoll-Paque Plus. The isolated viable cells were then washed in PBS and cultured for 1 additional day in the presence of IL-2 (20 U/ml; Chiron, Amsterdam, The Netherlands). For the flow cytometry analysis, the cells were washed and resuspended in washing buffer (PBS supplemented with 1% BSA; Roche Diagnostics, Bromma, Sweden). To inhibit nonspecific FcR-mediated binding of Abs, anti-CD16 and anti-CD32 Abs (BD PharMingen) were added to a final concentration of 5 μg/ml and incubated at room temperature for 5 min while aliquoting the cells in FACS tubes, 500,000 cells/tube. The cells were stained with the IOTest Beta mark kit (Beckman Coulter, Fullerton, CA) including Vβ1, 2, 3, 4, 5.1, 5.2, 5.3, 7.1, 7.2, 8, 9, 11, 12, 13.1, 13.2, 13.6, 14, 16, 17, 18, 20, 21.3, 22, and 23 conjugated with PE, FITC, or both combined. Anti-CD3 Ab conjugated with PC5 was also added to the samples. The cells were incubated with the Abs for 30 min at 4°C and then washed twice with washing buffer. Finally, the cells were resuspended in washing buffer and analyzed by flow cytometry using a FACSort flow cytometer (BD Biosciences, Mountain View, CA) according to standard settings at 495 nm. Data were analyzed using CellQuest software (BD Biosciences) with the light scatter gate set on the T cell blast population (FSC^{high}). In addition, TCR Vβ analyses by flow cytometry were run on SEA- or SEH-reactive effector cell lines established as described above.

Cytotoxicity assays

T cell-mediated cytotoxicity was measured against superantigen-coated cells in a standard 4-h ^{51}Cr release assay (39). ^{51}Cr-labeled Raji cells were used as MHC class II-positive target cells at a density of 12,500 cells/ml R10 medium in V-shaped microtiter wells. SEA-, SEB-, SED-, SEE-, or SEH-reactive human T cell lines were used as effector cells at an E:T cell ratio of 30:1 along with varying concentrations of different superantigens. To investigate whether SEH and SEA had overlapping T cell binding sites, an inhibitory cytotoxicity assay was performed with a MHC class II-independent system. Dose-dependent T cell-mediated cytotoxicity induced by C215Fab-SEH, C215Fab-SEA, or nonfused superantigens were investigated using ^{51}Cr-labeled Colo205 cells as target cells. The cell line Colo205 is MHC class II^- but expresses the tumor Ag C215, recognized by the C215Fab moiety, and as a result the cytotoxicity is directed against the Colo205 cells (40). SEH- or SEA-reactive human T cell lines were used as effector cells at an E:T ratio of 45:1. In addition, the ability of nonfused SEH or SEA to inhibit either C215Fab-SEH- or C215Fab-SEA-induced cytotoxicity was studied. The T cells were incubated with varying concentrations of the nonfused superantigens for 30 min before addition of 1 pM C215Fab-fused superantigens and the target cells. In both assays, the percentage of specific cytotoxicity was calculated as 100 × (cpm experimental release/cpm total release).

Analysis of TCR Vα and Vβ repertoire using RT-PCR

Analysis of preferential Vα or Vβ expansion was performed by RT-PCR of SEH or anti-CD3-activated cells. The cells were dissolved in RNAwiz (Ambion, Austin, TX) and the RNA was extracted from 1 ml (1×10^7 cells) of cell suspension according to the manufacturer's protocol. First-strand cDNA synthesis was performed using 5 μl of total RNA utilizing oligo$(dT)_{18}$ primers according to the Cells-to-cDNA kit protocol (Ambion). cDNA (5 μl) was aliquoted and amplified in the presence of 1 μl of each primers (10 μM) either with Vα family-specific 5′ primers and 3′ Cα primers to generate a 320- to 410-bp product or Vβ family-specific 5′ primers and 3′ Cβ primers to generate a 170- to 220-bp product (41, 42). As an internal control, two additional reactions were run, one containing 5′ forward and 3′ reverse Cβ primers to generate a 200-bp product (43) and the other containing 5′ forward and 3′ reverse Cα primer to generate a 600-bp product (41). All of the primers were produced by MWG Biotec (Ebersberg, Germany). The reaction mixture also included 5 μl of 10× PCR buffer (PerkinElmer, Wellesley, MA), 1 μl (2.5 mM) of dNTP (Invitrogen, Stockholm, Sweden), 36.5 μl of sterile H_2O, and 2.5 U of AmpliTaq DNA polymerase (PerkinElmer). Amplification was conducted for 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. DNA products where separated on 1.5% agarose gels and the net intensity of the relevant bands were quantified by Image station 440CF, Kodak digital science (NEN Life Science Products, Zanentem, Belgium). PCR values for the Vα and for the Vβ bands were normalized by dividing the net intensity with the net intensity of the internal controls, the amplified Cβ and Cα, respectively.

Analysis of TCR Vα using real-time RT-PCR

The levels of RNA coding for TCR Vα1, 2, 10, 14, and 17 were quantified using real-time RT-PCR with a LightCycler instrument (Roche, Mannheim, Germany). RNA was extracted as described for RT-PCR and the same primers were used. The PCR contained 2 μl of FastStart DNA Master SYBR Green I (Roche), 0.5 μM of each primer, 5 μl of cDNA template, and varying concentrations of $MgCl_2$ in a final volume of 20 μl. The $MgCl_2$ concentration was optimized for each pair of primers: 3 mM was used for TCR Vα1, 5 mM for TCR Vα2, 4 mM for TCR Vα10, 3 mM for TCR Vα14, 4 mM for TCR Vα17, and 2 mM for the internal control. Amplification was conducted for 45 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 5 s, and extension at 72°C for 16 s. All reactions were run with cDNA extracted from either SEH- or anti-CD3-treated cells, where the anti-CD3-treated cells were used as the calibrator. Quantification was performed using the calibrator-normalized relative quantification method and was calculated using the equation: $N = N_O \times E^{Cp}$, where N is the number of molecules at a certain cycle, N_O is the initial number of cycles, E is the amplification efficiency, and Cp is the cycle number at the detection threshold. In this method, the normalized ratio is obtained by dividing the target (TCR Vα1, 2, 10, 14, and 17)/reference (TCR Cβ) ratio of the sample by the target/reference ratio of the calibrator (anti-CD3-treated cells). The calculation was performed without efficiency correction by setting the efficiency of both target and reference PCR to 2, as recommended by the suppliers.

Results

Superantigens are known to activate specific subsets of T cells dependent on various Vβ sequences on TCR and are therefore said to possess a unique TCR Vβ profile (1). In this study, we have explored the ability of SEH to activate human T cells and characterized the properties of these T cells. SEA, SEB, SED, SEE, and SEH were produced at high levels in *E. coli* and specific human effector T cell lines for these superantigens were established.

SEH does not induce a TCR Vβ-specific expansion upon T cell activation

The TCR Vβ stimulation profile of human PBMCs activated by SEH was examined by flow cytometry and compared with cells stimulated with SEA or a T cell-stimulating Ab against CD3 (anti-CD3). Surprisingly, SEH did not induce a TCR Vβ-specific T cell activation (Fig. 1). Cells stimulated with SEH instead showed a similar TCR Vβ distribution as cells activated with the anti-CD3 Ab (Fig. 1), which activates T cells independent of the TCR Vβ. In contrast, a clear TCR Vβ-specific activation was observed for SEA (Fig. 1). The levels of TCR Vβ5.2, 5.3, 9, 16, 21.3, and 22 were increased 2- to 3-fold after stimulation with SEA, which is in accordance with previous findings (44). Due to the expansion of these TCR Vβ subsets, other subsets such as TCR Vβ2, 3, 12, 13.1, 17, and 23 decreased after SEA induced T cell activation. No such reduction was detected in the case of SEH, which further supports a lack of Vβ-specific expansion.

About 80% of the cells in the activated population of the SEH-stimulated cells were stained positive with the TCR Vβ mAbs used in the flow cytometry experiments (Fig. 1). Since this is similar to anti-CD3 stimulation (Fig. 1) or unstimulated cells (data not shown), it is not likely that SEH induces specific T cell activation in one or some of the TCR Vβs not investigated. SEA is known to strongly activate T cells bearing the Vβ6 sequence (45), which was not included in this study and therefore the percentage of stained cells was below 70% in the SEA-stimulated cells (Fig. 1). To exclude differences based on individual variations, the lack of Vβ preference for SEH was confirmed using lymphocytes from two individuals. In addition, SEH effector T cell lines were analyzed to confirm that no TCR Vβ biasing is raised after 4–6 wk of continuous stimulation of SEH (data not shown).

It is clear from earlier published results that SEH activates human T cells (36) and here we demonstrate that SEH does not induce a TCR Vβ-specific expansion of these cells.

SEs with different TCR Vβ specificities mediate cytotoxicity in a SEH effector T cell line

Since no TCR Vβ-specific expansion for SEH was detected in the flow cytometry analysis, it was investigated whether SEH exhibits any specificity at all during induction of a T cell response. Human T cell lines stimulated by SEH, SEA, SEB, SED, or SEE were obtained and their ability to mediate T cell cytotoxicity was examined.

As expected, each superantigen induced high cell-mediated cytotoxicity in cell lines reactive to that specific superantigen (Fig. 2). However, all investigated SEs induced high T cell cytotoxicity in the SEH-reactive T cell line (Fig. 2*A*). All of the zinc-coordinating superantigens showed an EC_{50} value in the order of 1 pM, which is in the same range as SEH while SEB showed a half-maximum effect concentration (EC_{50}) value of ~30 pM. Since the different SEs stimulate distinct TCR Vβ subsets of T cells, this strongly indicates that the SEH-activated T cells carry several, if not all, TCR Vβ structures.

SEH induced cytotoxic responses in all T cell lines reactive to the other SEs, although it was sometimes a low response (Fig. 2, *B–E*). Except for the SEH-reactive cell line, SEH mediated the most pronounced responses in the SED- and the SEE-reactive cell lines. In the SEE-reactive T cell line, SEH induced cytotoxicity to the same extent as SEE at the highest concentration tested (Fig. 2*E*). Plateau and EC_{50} values for SEH in the SEA, SEB, SED, or SEE T cell lines (Fig. 2, *B–E*) are difficult to determine because slight increases were observed with rising concentrations of SEH.

In contrast to the SEH-reactive T cell line, a much more superantigen-specific response was shown in the other T cell lines. Only SEB induced efficient cytotoxicity in the SEB-reactive cell line, whereas the other SEs induced no or low responses (Fig. 2*C*). With the exception for the SEH-reactive cell line, SEB was not able to mediate any significant cytotoxicity in any of the other cell lines. This is in accordance with published TCR Vβ specificities (45) showing that SEB utilizes a TCR Vβ repertoire that is markedly different from the other SEs. SEA and SEE mediated proper cytotoxicity when using the SEA-reactive cell line (Fig. 2*B*), whereas the SED- and SEE-reactive cell lines seemed to be activated by a broader range of SEs (Fig. 2, *D* and *E*).

SEH and SEA do not compete for T cell interaction

The experiments above demonstrate that SEH-activated T cells possess a broader range of TCR Vβ subsets than T cells activated by other SEs. One reason for this could be that SEH interacts in a different way with T cells compared with other superantigens. To investigate this hypothesis, a competition assay was performed using SEA or SEH presented on tumor cells as tumor-reactive fusion proteins. These SE fusion proteins (C215Fab-SEH and C215Fab-SEA) bind simultaneously to T cells via the TCR and tumor cells via the Ab fragment. Since the tumor cells do not express MHC class II, MHC-independent T cell-mediated cytotoxicity can be

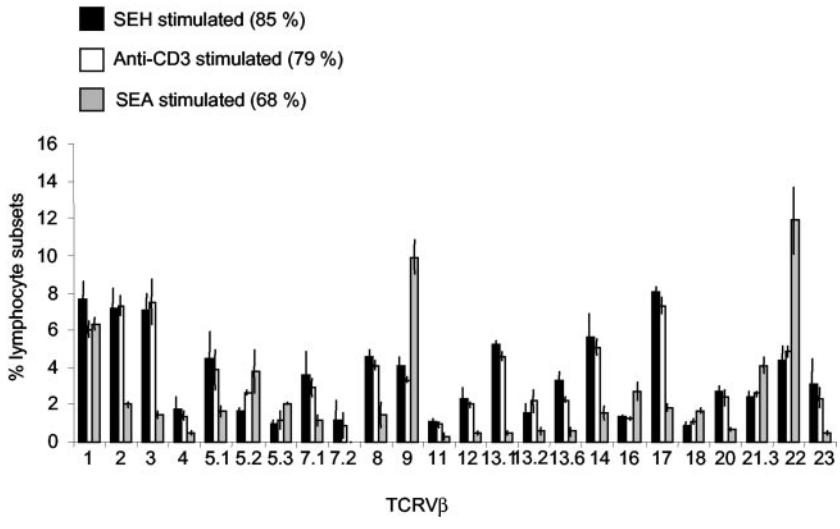

FIGURE 1. TCR Vβ profile of anti-CD3-, SEH-, or SEA-stimulated human PBMCs. The cells were stained with Abs against different TCR Vβs and analyzed by flow cytometry. SEH-, SEA-, and anti-CD3-stimulated cells gated for the blast cell population. The percentage of stained cells in the blast cell population is indicated in parentheses. SEM was calculated from four different experiments using lymphocytes from two individuals analyzed in duplicates.

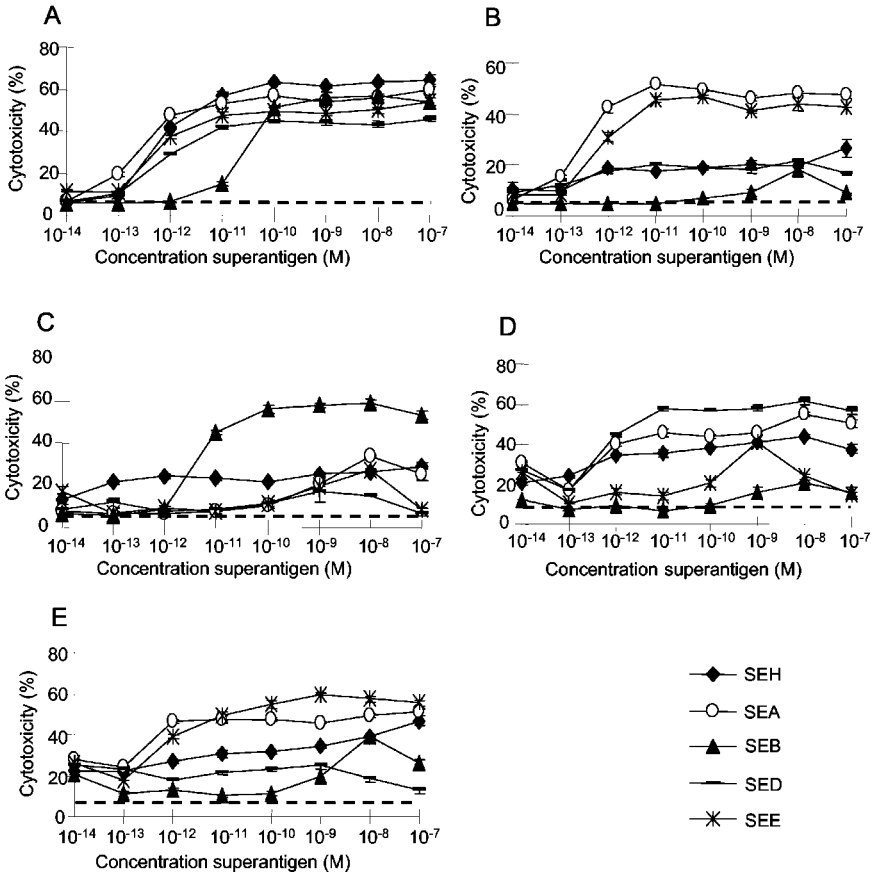

FIGURE 2. Superantigen-dependent T cell-mediated cytotoxicity against MHC class II-expressing Raji cells was analyzed in a standard 4-h ^{51}Cr release assay. SEH-reactive (*A*), SEA-reactive (*B*), SEB-reactive (C), SED-reactive (*D*), SEE-reactive (*E*) human T cell line as effector cells. Background level of spontaneous lysis of target cells is represented as a dotted line. Data from one representative experiment of three is shown with SEM from triplicates.

observed (40) Hence, the system was designed to only investigate competition on the T cells by adding increasing concentrations of unfused SEH or SEA to inhibit cytotoxicity induced by the fusion proteins.

Both C215Fab-SEH and C215Fab-SEA were able to induce T cell-mediated responses in a dose-dependent manner in both the SEH-reactive and SEA-reactive T cell line (Fig. 3, *A* and *C*) although the ability of C215Fab-SEA to induce a response in the SEH-reactive cell line was less potent in this MHC class II-independent system (Fig. 3*A*). Differences in plateau values between this assay and the MHC class II-dependent system described above have previously been observed (39). The unfused SEH or SEA did not induce any cytotoxicity since they are not presented on the target cells (Fig. 3, *A* and *C*).

In the competition experiments, an excess of unfused SEH inhibited C215Fab-SEH-mediated responses of SEH-reactive T cells due to competition for the binding to the T cells (Fig. 3*B*). Corresponding competition was observed for unfused SEA on

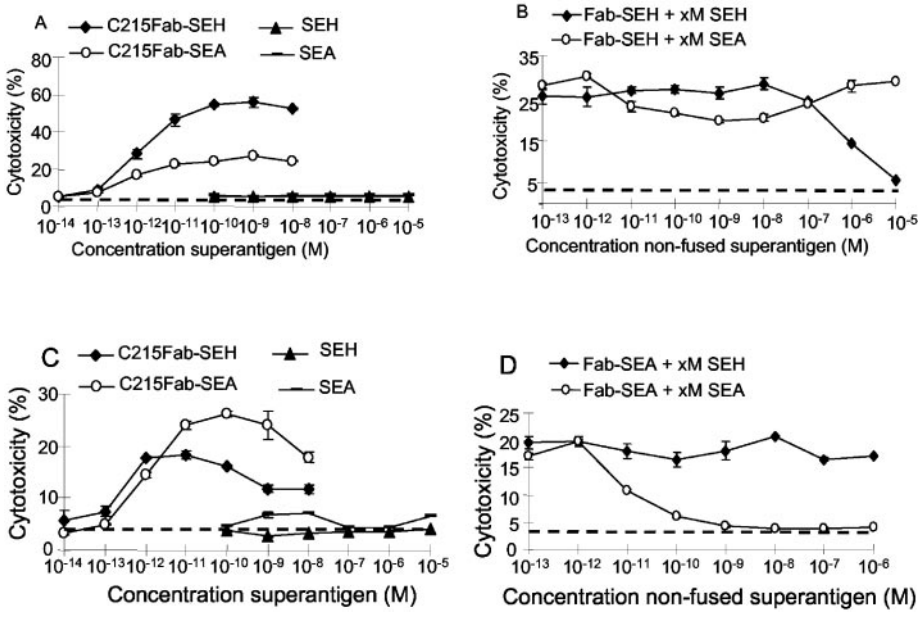

FIGURE 3. MHC class II-independent superantigen-induced cellular cytotoxicity against Colo205 cells was analyzed in a standard 4-h ^{51}Cr release assay. SEH-reactive (*A* and *B*) or SEA-reactive (*C* and *D*) human T cell line as effector cells. *A* and *C*, Dose-dependent T cell-mediated cytotoxicity induced by C215Fab-fused superantigens and nonfused superantigens. Competition using increasing concentrations of nonfused superantigens with 1 pM C215Fab-SEH (*B*) or 1 pM C215Fab-SEA (*D*) for T cell-mediated cytotoxicity. Background level of spontaneous lysis of target cells is represented as a dotted line. Data from one representative experiment of three is shown and SEM from triplicates.

C215Fab-SEA-mediated cytotoxicity of SEA-reactive T cells (Fig. 3*D*). The concentration of SEA giving rise to 50% inhibition was markedly lower than the corresponding value of SEH. Strikingly, SEA was not able to inhibit C215Fab-SEH-mediated T cell cytotoxicity (Fig. 3*B*) and the same was observed for SEH on the cytotoxic response induced by C215Fab-SEA (Fig. 3*D*). Consequently, no competition was demonstrated between SEH and SEA for T cell binding although the T cells were reactive to both superantigens.

SEH induces a TCR Vα-specific expansion of activated T cells

From the experiments above, no TCR Vβ-specific expansion was detected. The T cell competition experiments suggested that SEA and SEH bind to different sites on the T cell; therefore, a possible TCR Vα-specific expansion by SEH was investigated. A semi-quantitative PCR technique was used to investigate the relative levels of mRNA coding for various TCR Vαs and TCR Vβs. The number of cycles was optimized by investigating when all the PCR products reached log phase and 35 cycles were found to be optimal. To compensate for individual variations, lymphocytes from five individuals were investigated. The PCR analysis clearly showed that SEH induces a Vα-specific expansion of human T cells (Fig. 4*A*). TCR Vα10 (TRAV27) was specifically expanded in all individuals tested compared with anti-CD3-stimulated cells. The levels were increased 2- to 4-fold (Fig. 4*A*). Vα14 and Vα17 (TRAV24 and TRAV23) also appear to be moderately expanded after SEH activation (1- to 3-fold) in different individuals. Furthermore, a down-regulation of a number of different Vαs, such as TCR Vα2, 3, 7, 8, and 16 was observed (Fig. 4*A*). The TCR Vβ distribution was similar to that of the anti-CD3-activated cells and no specific expansion was detected, which is in clear contrast to the results for the TCR Vαs (Fig. 4*B*). The only alteration compared with anti-CD3 was a slight down-regulation of Vβ16; however, this was not seen in the flow cytometry analysis. These observations led to the conclusion that there is no specific Vβ expansion upon SEH stimulation, instead SEH induces a TCR Vα-specific expansion of T cells.

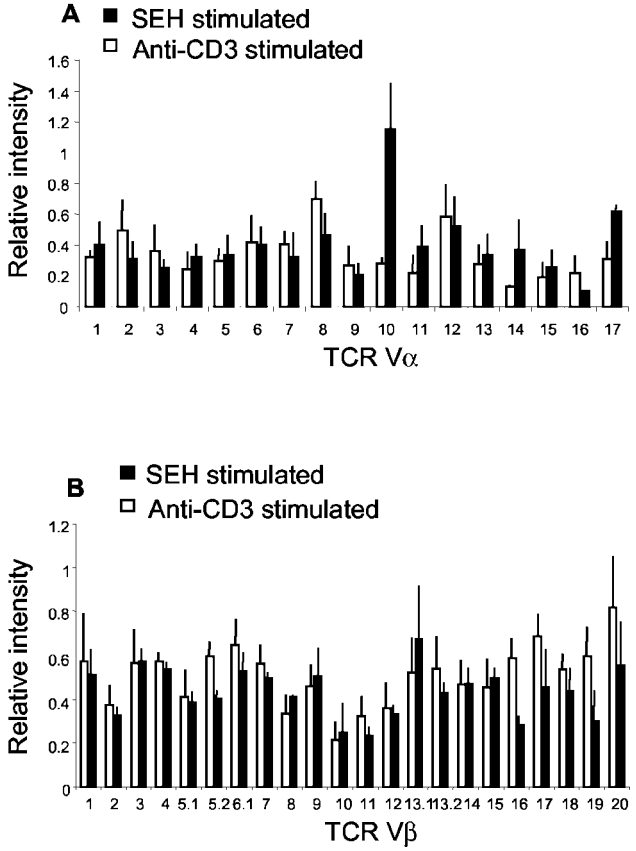

FIGURE 4. TCR Vα and Vβ profile of SEH- or anti-CD3-stimulated human PBMCs. Vα and Vβ analyses were performed with RT-PCR and normalized with the total amount of mRNA of TCR. *A*, Levels of mRNA coding for different Vαs after SEH or anti-CD3 stimulation. The average value and SEM of the five individuals is shown. *B*, Levels of mRNA coding for different Vβs after SEH or anti-CD3 stimulation. The average value and SEM of the three individuals is shown.

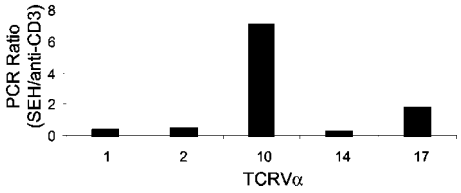

FIGURE 5. Normalized ratio after real-time RT-PCR analyses for TCRVα1, 2, 10, 14, and 17. The ratio between anti-CD3-stimulated cells and SEH-stimulated cells was obtained by dividing the target:reference ratio of each sample by the target:reference ratio for the calibrator (as described in *Materials and Methods*). One representative experiment of three is shown.

Real-time RT-PCR shows specific expansion of TCR Vα10 after SEH activation

To verify the results from the RT-PCR, real-time RT-PCR was performed where the log phase values of the amplifications are obtained. This is more quantitative than the end point analysis performed by RT-PCR. The real-time RT-PCR analysis was focused on TCR Vα10, 14, and 17, which appeared to be expanded after SEH stimulation in the RT-PCR analyses. TCR Vα1 and 2 were included as controls. The real-time RT-PCR clearly showed that TCR Vα10 is specifically expanded after SEH stimulation (Fig. 5). A normalized ratio between anti-CD3-stimulated cells and SEH-stimulated cells was obtained by dividing the target:reference ratio of the SEH-stimulated sample by the target:reference ratio for the anti-CD3-stimulated sample for each of the targets analyzed. The ratio for TCR Vα10 varied between 5 and 10 in different experiments. In contrast to TCR Vα10, TCR Vα1, 2, and 14 did not seem to expand after SEH stimulation and can most likely be ruled out as targets for SEH activation. A slight increase of the ratio was detected for TCR Vα17.

Discussion

A characteristic hallmark for all superantigens has so far been a TCR Vβ-restricted activation of T cells (46). Many studies have contributed to define different TCR Vβ repertoires of bacterial superantigens, as reviewed by Fraser et al. (45). In this study, we show that SEH differs from other superantigens by not having a specific TCR Vβ activation profile. Instead, SEH appears to use the Vα domain of TCR to activate T cells since a specific expansion of human cells bearing TCR Vα10 (TRAV27) was detected. Several studies have speculated about Vα-reactive superantigens (31, 34, 47, 48), but this has previously not been observed for any of the known bacterial superantigens.

A common method to investigate the Vα or Vβ expression of a T cell population is to extract the mRNA of activated T cells and quantify different transcripts by using RT-PCR or real-time RT-PCR (41–43, 49). In addition, flow cytometry can be used to detect different T cell populations at the protein level. In this study, all of

these techniques were combined and they showed that SEH activates T cells without any preference for certain Vβ structures (Figs. 1 and 4). Instead, the Vβ activation profile resembled that of anti-CD3 activation. In addition, cytotoxicity assays showed that SEA, SEB, SED, and SEE were able to induce an efficient response in the SEH-reactive T cell line (Fig. 2*A*). Hence, SEH-reactive T cells are not limited to certain TCR Vβs. SEH has, however, a varying ability to stimulate the different SE-reactive T cell lines and must therefore possess some kind of specificity during T cell stimulation. This specificity is obtained by certain Vα expansions. One interpretation of the cytotoxicity assays as well as competition experiments is that SEH directly interacts with TCR to cause this expansion. The Vα specificity of SEH contradicts all previously published data on T cell stimulation by superantigens, but SEH still has to be regarded as a bacterial superantigen since it exhibits all other characteristics of the protein family; e.g., like a similar three-dimensional fold (13), the ability to bind MHC class II (21, 36), potent T cell mitogen properties (36), and the ability to induce acute toxic shock in mammals (50). A striking difference between SEH and other SEs is, however, the incapability of SEH to induce T cell activation in mice (37). Several SEs appear to have low affinity for murine MHC class II, but SEH is different since it does not interact with murine T cells (37). A possible explanation is that murine TCR Vα adopts a different three-dimensional conformation than human TCR Vα10. The toxicity of SEH is comparable to other SEs in monkeys (51, 52), whereas SEH seems to be less potent in rabbits (1, 50, 53). This suggests that SEH has evolved a more species-specific response with a comparable toxicity in primates but a much lower response in rodents relative to other SEs.

The interaction between T cells and previously characterized superantigens is governed by the TCR Vβ repertoire but skewed TCR Vα repertoires after stimulation with SEA, SEB, SEC, or SEE have been detected (31, 34, 47, 48). This is most likely an indirect result since Vα specificity can be induced by the direct interaction between TCR and MHC class II. Mechanistically it is believed that superantigens like SEB or SEC, which exclusively interact with the α-chain of MHC class II, form a ternary complex with MHC and TCR with an additional contact between the α-chain of TCR and the β-chain of MHC class II (31, 34). This contact is not likely to be formed for superantigens like SEH or SPE-C, which directly binds to the β-chain of MHC class II, since these superantigens take the position of TCR (21, 35) when they bind on top of MHC class II. Therefore, we suggest that SEH binds to the Vα part of TCR. This hypothesis leads to the question whether other superantigens can bind to TCR Vα in an analogous way. SEA is known to cross-link MHC class II molecules by binding both to the α-chain and β-chain of MHC class II and in this way activate T cells in a Vβ-restricted manner (54, 55). It is known that SEA induces TCR Vβ specificity although it is presented only by the high-affinity site (56). Even so, it is unknown whether this interaction can simultaneously introduce TCR Vα reactivity. In T cell stimulation studies with SEA and a K39A variant of MHC class II, which presents SEA bound to its β-chain only (19, 48), it was observed that the IL-2 production by the activated T cells was dependent on TCR Vα. Thus, SEA may, like SEH, exhibit a Vα-restricted activation of T cells, in addition to the Vβ-restricted activation, when presented in a "SEH-like" mode by MHC class II. It is important to note that the affinity between SEA and the β-chain of MHC class II is 100 times higher than that between SEA and the α-chain (18). Hence, SEA molecules will be presented by the high-affinity site and therefore have the possibility to activate T cells dependent on TCR Vα. It is not possible to predict which part of SEH that interacts with TCR Vα, but one interesting observation by Grossman et al (57) may be a guide. They observed that the disulfide loop of SEA is important for the induction of T cell mitogenicity but it is not involved in the TCR Vβ specificity (57). One may speculate that the disulfide loop instead can be involved in an interaction with the Vα part of TCR. This disulfide loop is also present in SEH and according to the structure of the complex between SEH and MHC class II, no structural restraints would make an interaction between the disulfide loop and TCR impossible (13, 21). In addition, structural alignments between SEH and other SEs show that SEH differs in the loop regions that are involved in the binding to TCR Vβ for these SEs (13).

As long as superantigens have been investigated the fundamental questions have been why bacteria produce molecules that cause activation or deletion of T cells expressing certain Vβ structures and how these agents cause human diseases. Potential approaches to treat or prevent such infections are drugs that interfere with the interactions between superantigens and molecules in the immune system. The findings presented here show that blocking the TCR Vβ-superantigen interaction may not be universally functional. It also shows the divergence that is needed to induce such a potent T cell stimulus and reflects the complexity of T cell activation by superantigens.

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