Staphylococcal food poisoning is a result of ingestion of Staphylococcal enterotoxins (SEs) produced by the bacterium Staphylococcus aureus. SEs cause gastrointestinal illness and also cause activation of T cells and massive cytokine release. A current method for the detection of active SEs relies on its emetic effect on monkeys or kittens. However, this costly procedure has low sensitivity and raises ethical concerns. This present study overcomes the limitations of such bioassays by providing an alternative method based on the alteration of TCR Vβ8 protein levels expressed on Jurkat T cell-line. We demonstrated that increasing concentrations of SEE, the causative agent in foodborne outbreaks in France, UK, and USA, reduced TCR Vβ8 protein levels in a dose dependent manner and similarly alters the luciferase gene expression under the regulation of nuclear factor of T-cell activation (NFAT). Unlike previous studies that show accessory cells are not required for T cell activation by SEA or SEB, this present study demonstrated that accessory cells are required for T cell activation by SEE and SEE has greater affinity for the accessory cells than the Jurkat T cell. It is advantageous to use fixed dead cells where possible to reduce cell culture work. In this study we show that fixed accessory cells lacking any metabolic function without processing can present intact SEE and consequently alter TCR Vβ8 levels and the reporter gene expression.

**Keywords:** Staphylococcus aureus; Staphylococcal enterotoxin type E (SEE); TCR Vβ8 protein; Jurkat T cell-line

**Introduction**

*Staphylococcus aureus* is a prevalent cause of clinical infection and food-borne intoxication. This bacterium produces a wide variety of staphylococcal enterotoxins (SEs) that target the gastrointestinal tract, produce an emetic response and can also activate the immune system. These actions seem to be related. The loss of the immune response has been shown to correlate with the loss of emetic response [1-3]. It was shown that SE levels have been correlated with the severity of atopic eczema [4-6], and rheumatoid arthritis [7,8]. Staphylococcal enterotoxin E (SEE) was detected in the blood of patients with rheumatoid arthritis [9]. It has also been associated with foodborne outbreaks in the UK [10], France [11], and the USA [12]. SEE belongs to group III, which has one binding site to T-cell Receptor (TCR), to the T Cell Receptor Beta Variable (TRBV) domain [13], and two distinct binding sites to major histocompatibility complex (MHC) class II [14-16].

Current methods for the detection of active SEs focus on its emetic effect on monkeys or kittens [12,17-20]. However, these expensive methods raise ethical concerns. Immunological techniques have also been used for SEE detection [18,21] yet are unable to discern biologically active SE from inactivated toxin, which poses no concern. We seek to develop fast sensitive and quantitative bioassays as an alternative to the monkey and kitten bioassay for detection of biologically active SEE. To that end we have focused on utilizing the immune response through cell based assays as an alternative means to detect and quantify toxin activity. The present study examines the use of Raji B cells, both viable and fixed, lacking any metabolic function, as accessory cells in combination with a Jurkat T cell-line and measuring TCR Vβ8 protein levels for the rapid, sensitive and quantitative detection of biologically active SEE.

**Materials and Methods**

**Materials**

SEE toxin and biotinylated SEE toxin were purchased from Toxin Technology, Sarasota, FL, RPMI 1640, fetal calf serum (FCS), MEM non-essential amino acids, sodium pyruvate, hygromycin B, and penicillin/streptomycin, were purchased from Gibco/Invitrogen, Carlsbad, CA. Bio-Glo reagent was obtained from Promega Sunnyvale, CA. Biotinylated SEE was labeled with Streptavidin Allophycocyanin (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s suggestions and incubated in the dark for 30 minutes at room temperature. As a control, biotin coated microspheres (Bangs Laboratories, Fishers, IN) were also labeled with Streptavidin Allophycocyanin.

**Paraformaldehyde fixation**

Raji B cell were fixed by incubation in PBS containing 0.5, 1 and 1.5% paraformaldehyde at room temperature for 15 min. Fixed cells were washed three times in PBS and washed three more times in media before being used in the activation assays.

**SEE assay**

To quantify active SEE, the toxin was incubated with Raji cells and Jurkat reporter cells in black 96 well microplates with clear bottoms. Each well contained $1 \times 10^4$ Jurkat cells in a 50 μL volume and 5 $\times 10^4$ Raji cells in a 25 μL volume plus a 25 μL sample containing 4x SEE of

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final concentration. The incubation stage was 5 hours at 37°C followed by 10 to 15 minute incubation at room temperature. Detection of luciferase expressed by the Jurkat reporter cells in response to SEE was by the addition of 100 μL Bio-Glo luciferase substrate per well and a 5 to 10 minute incubation at room temperature. The luciferase enzyme activity was detected according to the Bio-Glo manufacturer’s directions. The emitted light was measured by a microplate reader.

Flow cytometry

Flow cytometric analysis was performed using a FACSAria Fusion cytometer (BD Biosciences, San Jose, CA) equipped with 488 nm (blue) and 633 nm (red) lasers. Samples of Jurkat and Raji cells were stained with PE labeled anti-CD 19 mAb (BD Pharmingen) to discern the two cell types. Dead cells were identified and excluded from analysis by staining with the dead cell stain eFluor 780 (Thermo Fisher, Waltham, MA). TCR Vβ levels were measured by staining with FITC labeled anti-V mAb (BD Pharmingen). SEE binding to cells was assayed using APC labeled SEE (BD Pharmingen). With blue laser excitation, FITC fluorescence was quantified in the 530/30 nm bandpass channel and PE fluorescence was quantified in the 585/42 nm bandpass channel. With red laser excitation, APC fluorescence was quantified in the 670/30 nm bandpass channel and eFluor 780 fluorescence was quantified in the 780/60 nm bandpass channel. Compensation and data collection was performed using FACSDivA software (BD Biosciences). Data analysis was performed using FlowJo software (FlowJo, Ashland, OR). Debris and other contaminants were excluded from analysis by gating the cell population based on forward and side light scattering. Levels of TCR Vβ expression were reported as histogram distribution of FITC fluorescence or mean fluorescence with calculated 99% confidence interval of mean for cells which were CD19 negative and eFluor 780 negative (where dead cell stain was used).

Results

Expression level of TCR Vβ-chain, responsible for recognizing SEs in splenocyte cell

In previous work we developed a murine splenocyte assay based on differential expression of the CD4+ T cell surface receptor CD154 that was more sensitive than in vivo monkey or kitten bioassays for the detection of the related toxin SEA [22]. The purpose of the present study is to examine if detection of the TCR variable region β-chain (Vβ), responsible for recognizing SEs, can be used for detection of biologically active SEE. In the process we explored which of the repertoire of T cell surface receptor (TCR) Vβ families might be useful for SEE detection by an examination of the expression of Vβ immunophenotypes on murine splenocytes. Splenocyte cells were probed with fluorescently labeled monoclonal antibodies to the TCR Vβ receptor repertoire and examined by flow cytometry. Results show that the V88 receptor was the most highly expressed in splenocyte cells with Relative Fluorescence Units (RFU) of 13.9 followed by Vβ4, Vβ6, Vβ10, Vβ3 and Vβ5 with RFU of 5.6, 3.5, 3.3, 2.4 and 1.5, respectively.

Accessory cells are required for T cell activation by SEE but not by lectin PHA

Utilizing the human Jurkat T cell line that contains genes encoding the TCR Vβ8 is a convenient alternative to the isolation and culture of splenocyte cells expressing Vβ8. Here we utilized a human Jurkat T cell line engineered to express the luciferase gene under regulation of a nuclear factor of activated T cell response elements (NFAT-RE) that mediates CD154 expression, to explore SEE detection. Fleischer and Schrezenmeier [23] reported that a T-cell clone could respond to SEA in the absence of accessory cells that process and present SEA molecules to T cells. Our results in Figure 1 show that the Jurkat T-cell line does not react to SEE in the same way as to phytohaemagglutinin (PHA), which induced T cell activation in the absence of accessory cells. On the other hand, the Jurkat T-cell line did not respond to SEE without Raji B cells which are shown to be efficient accessory cells for SEE. This interaction between PHA, a lectin found in plants, and Jurkat T-cells does not result in maximal T-cell activation. Activation with PHA alone, the relative light unit (RLU) was lower by 48% than activation with SEE mixed with Raji B cell. The importance of the inclusion of the Raji B cell line as accessory cells for the activation of Jurkat T cells by SEE is confirmed by the results in Figure 1b showing that the relative response is a function of the concentration of Raji B cells. At a ratio of 2 Jurkat to 1 Raji (100,000 Jurkat cells and 50,000 Raji cells in 100 μl) the RLU was 5,361. At a ratio of 20 Jurkat to 1 Raji the RLU decreased to 1,388. In the absence of Raji B cell the luciferase level decreased to 273 RLU essentially the background levels.

Increasing concentrations of SEE produce a dose dependent reduction in detection of Vβ8

In this experiment we examined the effect of biologically active SEE on levels of TCR Vβ8 protein measured by flow cytometry. The Jurkat T cell line contains genes for the mRNA transcripts TRAV8-4-TRAJ3, TRBV12-3-TRBJ1-2 that encode the TCR Vβ8. Use of this clonally pure cell line precludes interference from other TCR Vβ families expressed on the cell surface of nonhomogeneous splenocytes. Our flow cytometry results, illustrated in Figure 2, show that following a two hour incubation of SEE with Jurkat T cells and Raji B cells there is a dose-dependent reduction over a 10-log range in TCR Vβ8 protein detection in the T cell population. The detection limit is much lower than 1 fg/mL which is a million times more sensitive than a typical ELISA assay at a detection limit of 1 ng/mL, and a billion times more sensitive than the rabbit and mouse bioassays in which vomiting occurs after administration of 10 mg. The result shows that the Raji B cell line is essential and highly effective as accessory cells for SEE. In their absence, SEE did not affect TCR Vβ8 protein detection levels in the T cell population.

SEE has higher binding affinity to accessory cells than to T cells

One possible explanation for the apparent reduction of TCR Vβ8 protein in the T cell population could be the binding of SEE molecules to the TCR Vβ8 protein, consequently interfering with the ability of the fluorescently conjugated antibody to bind TCR Vβ8. To explore this possibility, SEE was replaced with fluorescently labeled SEE and incubated for two hours with Jurkat T cells combined with Raji B cells. The data presented in Figure 3 show that the amount of fluorescent label associated with cells in the B cell population increased preferentially to that associated with the T cell population as the concentration of SEE was increased from 1 ng/mL to 1 μg/mL. The preferential association of the labeled SEE with the B cell population is demonstrated by the shift in mean of Allophycocyanin (APC) fluorescent signal of the B cell population from 54 RFU to 200 RFU compared with 34 RFU to 59 RFU for the T cell population. Background APC signal levels were 52.5 and 38.2 RFU, respectively, for the two cell types. Jurkat T cells have a relatively lower affinity to SEE compared with Raji B cells. Although we observed binding of SEE to T cells, it is only at the highest concentrations that we observe a significant association of labeled SEE with T cells compared with background signal levels whereas decreased
Jurkat T cells and SEE. We used fluorescently labeled anti-Vβ8 antibody to determine TCR Vβ8 levels. The data suggest that this assay does not require live accessory cells. The flow cytometry results in Figure 4a show that fixed Raji B cells lacking proteolytic processing or any metabolic function act as antigen-presenting cells that present intact SEE molecules to Jurkat T cells and consequently reduce the TCR Vβ8 protein expression. As demonstrated in Figure 4b, using those dead fixed cells as accessory cells to present intact SEE, the toxin mediated signals necessary to trigger early gene transcription and induce expression of the luciferase gene in a genetically engineered Jurkat T cell-line expressing the luciferase reporter gene under the regulation of nuclear factor of activated T-cells response element (NFAT-RE). However, Raji B cell fixed with 0.5%, 1% and 1.5% paraformaldehyde and used to present 1 µg/mL SEE produced lower activation of T-cells.

TCR Vβ8 expression was observed at much lower concentrations of SEE. At 1 ng/mL SEE, measurable cell associated SEE is essentially at background levels. This finding is in contrast to what has been reported by others that did not observe any binding of SEB to T cells [24].

**Fixed accessory cells lacking metabolic function present intact SEE altering TCR Vβ8 levels and reporter gene expression**

It is advantageous to use fixed dead cells where possible to reduce cell culture work. To that end we examined whether fixed Raji B cells, retaining their cellular morphology, can be used to replace live Raji B cells as accessory cells. Raji B cells fixed with paraformaldehyde retain their surface features but cannot actively internalize and proteolytically degrade SEE and display the short fragments of the degraded SEE on the Raji B cell surface. Such cells were incubated with the engineered Jurkat T cells and SEE. We used fluorescently labeled anti-Vβ8 antibody to determine TCR Vβ8 levels. The data suggest that this assay does not require live accessory cells. The flow cytometry results in Figure 4a show that fixed Raji B cells lacking proteolytic processing or any metabolic function act as antigen-presenting cells that present intact SEE molecules to Jurkat T cells and consequently reduce the TCR Vβ8 protein expression. As demonstrated in Figure 4b, using those dead fixed cells as accessory cells to present intact SEE, the toxin mediated signals necessary to trigger early gene transcription and induce expression of the luciferase gene in a genetically engineered Jurkat T cell-line expressing the luciferase reporter gene under the regulation of nuclear factor of activated T-cells response element (NFAT-RE). However, Raji B cell fixed with 0.5%, 1% and 1.5% paraformaldehyde and used to present 1 µg/mL SEE produced lower activation of T-cells.
than unfixed, as measured by luciferase assay with light production falling from $23273 \pm 1656$ RLU with no fixation to $22150 \pm 470$, $18409 \pm 1223$ and $13841 \pm 3970$ RLU, respectively.

SEE but not SEB stimulate Jurkat T-cell activation

To demonstrate the specificity and cross reactivity of SEs to Jurkat T-cells we compared T-cell activation by SEE with Staphylococcal Enterotoxin subtype B (SEB) that belongs to a different serotypical group and has low amino acid sequence homology with SEE. The result show that unlike the mitogen lectin PHA that in a non-specific way activates Jurkat T-cells and SEE. The intact SEE mediated early gene transcription and reduced the TCR Vβ8 protein level. Error bars represent standard errors.

Figure 3: SEE more strongly associated with B cells than T cells. Fluorescently labeled SEE at concentrations of 1 ng/mL or 1 µg/mL incubated for two hours with Jurkat T cells combined with Raji B cells. Flow cytometric analysis was used to measure the binding affinity to the cells.

Figure 4: Paraformaldehyde fixed Raji B cells lacking any metabolic function can replace live Raji B cells as accessory cells. Raji B cells fixed with paraformaldehyde were incubated with the Jurkat T cells and SEE. The intact SEE mediated early gene transcription and reduced the TCR Vβ8 protein level. Error bars represent standard errors.

Discussion

Our results show that Jurkat T-cell line activation induced by the plant lectin PHA is clearly distinguishable from activation induced by SEE. Jurkat T-cell line responds to PHA in the absence of accessory cells, but this interaction does not result in maximal T-cell activation. The specific sites of interaction between PHA and T-cell are still poorly understood, although some literature suggests that PHA may initiate activation via the CD2 receptor [25]. In contrast, SEE activation is more specific, while SEE lacks the capacity to directly bind to T cells in the absence of accessory cells. The binding of SEE to Raji B cells may assist the binding of SEE to TCR Vβ8 and increase the stability of the SEE Vβ8 interaction.

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With added Raji B cells, SEs are able to significantly stimulate T-cell activation at five thousand times lower concentrations than PHA. This fact further suggests that activation of T cell by PHA is less specific than activation by SEE. Higher T-cell activation and IL-2 secretion play an important role in the clinical manifestations of SEs mediated toxic shock syndrome. The finding that accessory cells play a significant role to induce T cell activation is in accordance with our previous work [26]. Depletion of accessory cells from splenic tissue using positive selection to enrich concentration of CD4+ T cell from 22% to 90% resulted in similar proliferative response as the splenocyte cell. However, adding negative selection to further increase the purity of CD4+ T cells to >95% resulted in a reduction in T-cell proliferation by 42% compared to splenocytes cells. Proliferation reduction was measured by BrdU incorporation falling from 1.1170 ± 0.0878 to 0.4700 ± 0.0718 (OD 450 nm). Those results also showed that antibodies against MHC class II subregion glyco-proteins -I-Ab, I-Ad, I-Aq, I-Ed, I-Ek block the interaction between MHC class II and Vβ regions of the TCR and further blocked T cells proliferation. This fact suggests that T cells purified from splenocytes or from peripheral blood mononuclear cell (PBMC) which are frequently used to study the response to SEs, are not pure T-cells. Contamination of T-cell, even by small numbers of residual accessory cells of less than 5%, trigger T-cell activation of 42% compared to splenocytes cells. The use of impure T cells which are contaminated with residual accessory cells can lead to the wrong conclusion that T-cell can respond directly to SEs in the absence of accessory cells. In the present study we used pure cell lines and demonstrated that T cell activation could not be achieved by using SEE unless accessory cells were added and the level of stimulation depends upon the ratio between T cells and accessory cells. At a ratio of two T-cells to one accessory cell the activation was 3.9 times higher than at ratio of twenty T-cells to one accessory cell. In the absence of accessory cells the activation was no different than background. This finding is not in accordance with the findings of Fleischer and Schrezenmeier [23] and Spertini et al. [27], who reported that a T-cell clone could respond directly to SEA and SEB (which are in different class than SEE) in the absence of accessory cells. This finding also contrasts with Niedergang’s et al. [24], observation of a biological effect of SEB on Vβ3-transfected Jurkat cells in the complete absence of accessory cells. It is worth noting that the fluorescently labeled SEE was able to reduce TCR Vβ8 protein expression and induce expression of the luciferase gene with the same efficiency as unlabeled SEE. This indicates that the fluorescently labeled SEE was biologically active. We do not observe a reduction in TCR Vβ8 protein expression in the absence of Raji B cells. This indicates that SEE cannot interact directly with TCR Vβ8. Our result in Figure 3 showed that Jurkat T cells have a relatively lower affinity to SEE compared with Raji B cells. This lower affinity of SEE to the T-cell presumably allows a higher rate of release of SEE. Such behavior would permit B cells to present SEE to a multiplicity of T-cell receptors effectively amplifying the biological response and extending the range of response to very low concentrations of toxin.

In this study we terminated any ongoing biochemical reactions in Raji B cells by using parafomaldehyde fixation that is known to function by reacting with proteins; reduce methylation and cross-link lysine residues by reaction with the free 6-amino groups [28]. The morphology of the cells is retained by fixation and the antigenic sites remain. Further, fixed Raji B cells lack any metabolic function and are incapable to uptake and process SEE, yet they have unimpaired capacity to bind unprocessed SEE and are able to present intact SEE to T cells, altering TCR Vβ8 levels and reporter gene expression. Fixation of B-cells with parafomaldehyde prevents those cells from processing antigens and abolishes their degradation ability. It seems that activation of T cells by SEE is not similar to conventional antigens and did not require internalization. Viable accessory cells and cellular processing of SEE into antigenic peptides are not needed. Presentation of the intact SEE to the Vβ8 regions of the T cell receptor did not affect the co-stimulatory signal that modulates early gene expression and toxin-driven T cell activation. Two hours after SEE interaction the TCR Vβ8 protein level was reduced in a dose-dependent fashion. Five hours after exposure of these mixed cultures to SEE modulation of early gene expression in T cells was demonstrated by the expression of the reporter gene luciferase. The light production was proportional to SEE concentration. Unlike PHA that activates jurkat T-cell line in a non-specific way, the upregulation of the early gene expression and the down regulation of TCR Vβ8 expression were specific to SEE not to SEB.

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