Published in final edited form as:

J Neuroimmunol. 2008 May ; 196(1-2): 49–59.

Relationship of varying patterns of cytokine production to the anorexic and neuroendocrine effects of repeated Staphylococcal enterotoxin A exposure

Daniella Urbach-Ross¹, Beth Crowell², and Alexander W. Kusnecov^{1,2,*}

¹ Joint Graduate Program in Toxicology, Rutgers University, Piscataway, NJ, and University of Medicine and Dentistry of New Jersey, Piscataway, NJ

² Department of Psychology, Rutgers University, Piscataway, NJ, and University of Medicine and Dentistry of New Jersey, Piscataway, NJ

Abstract

Staphylococcal enterotoxin A (SEA) is a superantigen that stimulates T cells and induces the production of multiple cytokines. Previous studies have shown that SEA augments gustatory neophobia and activates the hypothalamic-pituitary-adrenal (HPA) axis. This study aimed to determine if the cytokine response, behavioral effects, and HPA axis activation persisted after repeated SEA treatment. Male C57BL/6J mice were given 1–4 intraperitoneal injections of 5 µg SEA, after which food intake, corticosterone, or peripheral cytokines were measured. In a series of experiments, it was found that secondary exposure to SEA two or three days after priming increased corticosterone, but attenuated splenic TNFα, while augmenting IL-1β, IL-2, and IFNγ. The anorexic response was intact after secondary exposure, but absent after a third injection, which was still able to elevate corticosterone. It is unlikely that IL-1 mediated the persistent effects on corticosterone, since this was increased in groups lacking corticosterone elevations. Similarly, TNFα was only modestly elevated under repeated SEA conditions that elevated plasma corticosterone. This attenuation appeared to be inversely related to the levels of IL-10, the production of which incrementally rose with each successive injection. In conclusion, repeated exposure to SEA activates the HPA axis and alters behavior. However, there may be dissociation between the behavioral and endocrine effects of SEA with increased SEA exposure. Furthermore, it is possible that while TNFα was previously shown to be important in response to acute SEA-induced HPA axis activation, further exposure to SEA elicits other cytokines that may exert neuromodulatory effects through sensitization and/or synergistic mechanisms.

1. Introduction

Staphylococcal enterotoxin A (SEA) is a well recognized immunogenic protein that belongs to the class of bacterial toxins known as superantigens (SAGs). A major property of SEA is the ability to stimulate T cells bearing the Vβ3 and Vβ11 T cell receptor phenotypes, resulting in pronounced cytokine production and T cell proliferation. Similar effects on cytokine production are observed with other staphylococcal enterotoxins, such as staphylococcal

* Corresponding Author: Alexander W. Kusnecov, 152 Frelinghuyen Road, Piscataway, NJ 08854, kusnecov@rci.rutgers.edu, Phone #: 732-445-3473, Fax#: 732-445-2263.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

enterotoxin B (SEB) (Bette et al., 1993). Indeed, rodents challenged with SEA or SEB produce high levels of interleukin-2 (IL-2) and tumor necrosis factor α (TNF α), and these changes are associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis (Bette et al., 1993; Goehler et al., 2001; Gonzalo et al., 1993; Kusnecov and Goldfarb, 2005; Sundstedt et al., 1994).

A single injection of SEA to C57BL/6J mice activates limbic brain regions and produces behavioral alterations, such as anorexia, neophobia and altered nociception (Kawashima and Kusnecov, 2002; Rossi-George et al., 2005). Moreover, the ability of SEA to stimulate the HPA-axis relies on the presence of reactive T lymphocytes, as well as endogenous TNF α (Kawashima and Kusnecov, 2002; Rossi-George et al., 2005). Similarly, in response to a single exposure of SEA, animals lacking TNF α showed an attenuated c-Fos response in brain regions involved in mediating fear and anxiety, a blunted CORT response, and abrogated sickness-like behavior (Rossi-George et al., 2005). Interestingly, IL-1 does not appear to mediate these effects, as suggested by undetectable plasma levels of IL-1 β following SEA treatment, and persistent SEA-induced anorexic responses in IL-1 receptor knock-out mice (Kawashima and Kusnecov, 2002; Rossi-George et al., 2005).

It has been shown that the pronounced stimulatory effects of acute SAg exposure on T cell activation are distinct from those seen after repeated exposure, whereby the initially expanded population of T cells either undergoes apoptosis or becomes unresponsive to subsequent SAg exposure (Feunou et al., 2003; Kawabe and Ochi, 1991; MacDonald et al., 1991; Miller et al., 1999). This tolerance may be induced by T cell anergy and a shift towards a Th2-type response (Miller et al., 1999; Watson et al., 2003). The mechanism for this is not fully understood, although regulatory cytokines may play a role. For example, Sunstedt et al. (1997) demonstrated an inverse relationship between the concentration of serum IL-10 and IL-2 after SEA restimulation. Therefore, given the well known anti-inflammatory effects of IL-10 (Strle et al., 2001), it is possible that repeated exposure to SEA leads to a progressive inhibition of proinflammatory cytokines, and coincident loss of neuromodulatory changes associated with SEA exposure.

While acute exposure to SAg has repeatedly been shown to induce tolerance to subsequent exposure, there are far fewer studies addressing repeated SAg exposure. Moreover, there is no evidence for the effects of repeated SEA exposure on HPA-axis activation and alterations in sickness behavior. If repeated SEA exposure continues to exert neuromodulatory effects, it is important to determine the relative pattern of cytokine responses, since the nature of the cytokine response to initial and subsequent exposures to SEA may vary. Where studies have looked at repeated SEA exposure, most have focused on endpoint cytotoxic and proliferative *in vitro* measures subsequent to *in vivo* injections (Kuroda et al., 1996; Miller et al., 1999; Sundstedt et al., 1994). However, little is known about *in vivo* variations after repeated SEA in the composition and magnitude of different cytokines initially induced by an acute SEA injection.

In the present study, the endocrine, behavioral and cytokine-inducing effects of acute and repeated injections of SEA were determined under conditions that varied the temporal parameters between succeeding SEA injections. This was based on previous reports that the nature of the T cell and cytokine responses to SEA varied as a function of different time intervals (measured in days) between initial and subsequent challenges with SEA (Sundstedt et al., 1994). These previous observations were conducted in T cell transgenic mice, but have yet to be repeated in normal C57BL/6 mice. Moreover, given that dynamic T cell alterations (eg., proliferation) persist after a single injection of SEA, it is conceivable that further introduction of SEA will impact on these and possibly other non-activated lymphocyte populations, resulting in altered patterns of cytokine production. Furthermore, the persistence

of the neuroendocrine and behavioral responses to SEA, in the context of changing patterns of cytokine production has not previously been determined. Therefore, given that repeated exposure to SAGs results in T cell tolerance (Feunou et al., 2003; Kawabe and Ochi, 1991; MacDonald et al., 1991; Miller et al., 1999), it was important to determine whether tolerance occurs also to the neurobehavioral effects of SEA after repeated treatment. To this end, the present study sought to determine the persistence of plasma corticosterone and anorexic effects in response to repeated challenges with SEA. Moreover, splenic and plasma cytokine measures were conducted to determine whether loss of endocrine and behavioral effects of SEA was associated with a similar cessation of cytokine reactivity.

2. Materials and Methods

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5–6 weeks of age and housed four per cage under 12:12 hr light:dark illumination (lights on 0600h). This mouse strain was used because the T cell receptor V β 3 phenotype, which reacts strongly to SEA, is highly expressed on T cells from C57BL/6J mice. For this reason, C57BL/6 mice have been the strain of choice for studies with SEA (Kusnecov and Goldfarb, 2005). Animals were fed milled mouse chow *ad libitum* for five weeks. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Rutgers University.

Reagents

Staphylococcal enterotoxin A was purchased from Sigma-Aldrich (St. Louis, MO). All injections of SEA were given intraperitoneally (IP) at a dose of 5 μ g/mouse in pyrogen-free physiological saline in a volume of 0.2 ml.

Schedules for SEA Injection

Experiment 1: Two Injections of SEA two and three Days Apart: In this experiment, the effects of two SEA exposures separated by two and three days was tested. Sundstedt et al. (1994) demonstrated that a single injection of SEA resulted in proliferation of spleen cells, however secondary *in vitro* exposure within a short time interval (7days) resulted in T cell anergy (Sundstedt et al., 1994). Further, it has also been reported T cell expansion peaked at two days after a single injection of SEA and continued to decline thereafter (Kuroda et al., 1996; McCormack et al., 1993). Consequently, this particular study chose to look at the effect of a second injection of SEA that coincided with a time when T cells were actively dividing, as based on the literature.

The design of the experiment and the temporal distribution of injections over a four day period are shown in Table 1. Although no group was given more than two injections of SEA, some groups received saline injections to control for handling and injection of SEA in other groups subjected to different temporal parameters. Subjects were given an acute injection of SEA (Total of N = 11), two injections of SEA spaced two days apart [SEAx2 (2d)] (N = 6), and two injections of SEA spaced three days apart [SEAx2 (3d)] (N = 5). As already noted, there were two different acute SEA and saline groups to control for the different injection schedules.

Experiment 2: Injections of SEA three and five days apart: In contrast to Experiment 1, this experiment assessed the effects of different regimens of SEA exposure on the anorexic response. As noted by previous literature, repeated exposure to SEA significantly reduces T lymphocyte reactivity (Miller et al., 1999; Sundstedt et al., 1994), although the nature and magnitude of the cytokine responses after repeated *in vivo* SEA is less well known. However, it could be hypothesized that the refractory nature of the T cell response after repeated SEA treatment may underlie a loss in behavioral reactivity to SEA. Therefore, in this current

experiment, the schedule of injections involved spacing two SEA injections three or five days apart, in order to capitalize on the effects of the three day separation in Experiment 1, as well as determine whether SEA still has a behavioral effect if administered after the proliferative peak reported to occur in response to an initial SEA injection (Kuroda et al., 1996; McCormack et al., 1993).

Table 2 summarizes the injection schedule. All animals received a total of three injections. Control animals received injections of saline on days 0, 2, and 5. Animals given a single SEA injection (Acute SEA) were injected with saline on days 0 and 2 and given an injection of SEA on day 5 (N = 8). The group of animals that were given two injections of SEA spaced three days apart [SEAx2 (3d)] was given saline on day 0 and SEA on days 2 and 5 (N = 8). The other group that received two injections of SEA spaced five days apart [SEAx2 (5d)] received SEA on days 0 and 5 with saline on day 2 (N = 8). Finally, a group of animals were given three injections of SEA on days 0, 2, and 5 (SEAx3) (N = 8). On day 5 of the experiment, and two hours after injection, animals were tested for anorexia, by assessing the consumption of a commercially available and highly palatable baby liquid formula (Prosobee, Mead Johnson, Evansville, IN).

Experiment 3: Repeated SEA injection protocol: In order to extend the findings of Experiment 2, the animals from that experiment were not sacrificed on the day of consumption testing, but were given further injections of SEA. This allowed for the assessment of the cytokine profile and the CORT response in animals given two injections of SEA spaced five days apart (which was not measured in Experiment 1). In addition it was possible to examine the influence of three and four injections of SEA on these parameters. Table 3 summarizes this extended regimen of treatment, and for the most part, the treatment schedule is self-explanatory (See Table 3). Briefly, for the animals that were sacrificed after the final SEA injection, the SEAx2 5d group represented two injections of SEA given 5 days apart, with the corresponding control receiving a single injection of SEA on day 5 and saline on day 10 (Control 5d). Groups designated as SEAx3a and SEAx4 received three and four injections respectively with the controls receiving a saline injection on the day of sacrifice instead of SEA (Controlx3a and Controlx4 respectively). The SEAx3b group also received three injections of SEA, but the spacing of the three injections for this group was every 5 days (see Table 3). The control for this group received SEA injections as shown in Table 3, but was given saline on the day of sacrifice (see Table 3, Controlx3b). For all of the post-hoc tests each group was compared to their respective control as indicated above. Finally, there were N = 4 animals in each group. This reduction in N was inevitable, since this experiment involved splitting each of the groups in Experiment 2. As we have repeatedly demonstrated, SEA is a powerful stimulus for corticosterone and cytokine production ((Kaneta and Kusnecov, 2005; Kawashima and Kusnecov, 2002; Rossi-George et al., 2004; Rossi-George et al., 2005), and therefore does not require exceedingly large numbers of animals to demonstrate a clear difference between saline and SEA-treated groups. Considerable care was taken to minimize the presence of non-specific stress to ensure that endocrine parameters were not unduly confounded. Moreover, for at least cytokine determinations, four animals per group provided sufficient statistical power, which was borne out by the statistical results. Moreover, this experiment revealed the expected increases in corticosterone that is a routine feature of SEA challenge (Rossi-George et al, 2005).

Behavioral Testing for Liquid Consumption

Prosobee is a substance that is readily consumed by mice and can therefore be used as a measurement of sickness induced anorexia (Kaneta and Kusnecov, 2005; Rossi-George et al., 2005). The Prosobee solution was made following the manufacturer's instructions. For testing, animals were placed in a white opaque box along with a preweighed bottle of Prosobee. After

one hour the animals were returned to their home cage and the bottles were reweighed in order to measure the total consumed by each animal.

Blood and Tissue Collection

Animals were sacrificed by decapitation two hours after the last injection and blood and tissue collected. This time point was previously shown to be optimal for increased pituitary-adrenal activation and cytokine production (Kawashima and Kusnecov, 2002). Trunk blood was collected by rapid decapitation into heparin-treated vacutainer tubes (Becton Dickinson, Rutherford, NJ). The blood was centrifuged immediately at 2000 rpm for 15 min, and the plasma was collected and stored at -70°C . Tissue was flash frozen in 2-methylbutane and stored at -70°C until protein extraction.

Protein extraction and quantification

The spleens were dissected and placed in 1 ml of 1mM Phenylmethanesulfonyl fluoride (PMSF) in 0.1M phosphate buffer to inhibit protease activity. The tissue was homogenized and centrifuged at 4000 RPM for 30 minutes after which the supernatant was collected. Total protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL). Absorbance was read at 562 nm and concentrations were calculated off a standard curve generated using bovine serum albumin (BSA). Total protein was expressed as $\mu\text{g/ml}$.

Cytokine enzyme linked immunosorbent assay (ELISA)

Spleen homogenates were centrifuged and the supernatant assayed for immunoreactive IL-2, $\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-1 β , IL-10, and IL-4 using OptEIA ELISA kits according to the manufacturer's instructions (BD Biosciences, San Diego, CA). Portions of plasma not dedicated to CORT assays, were also assayed for select cytokines. Plasma was diluted at 1:4 dilutions for all cytokines except IL-2 which was run at a 1:40 dilution. All standards were run in duplicate. The absorbance was read at 450 nm using EL800 universal BioTek microplate reader. Concentrations were calculated off a standard curve using KC Junior software (Biotek). The cytokine data were expressed as a ratio of total protein (pg of cytokine/ μg of protein).

Corticosterone Radioimmunoassay (RIA)

Plasma corticosterone was measured by ImmunoChemTM Double Antibody Corticosterone ^{125}I kit following the manufacturer's instructions (MP Biomedicals, Irvine, CA). All standards and samples were run in duplicate and counted using a Cobra II Auto Gamma counter. The corticosterone data were expressed as ng/ml.

Statistical Analysis

Most experiments conformed to a factorial design and were therefore analyzed by ANOVA using Statview, a statistical software package. In the event of significant main effects, appropriate post hoc comparisons were conducted with the inbuilt protection for Type I error. Post hoc comparisons were conducted using the Fisher's least significant difference test when the omnibus F achieved a 0.05 level of significance. In some cases, in which *a priori* predictions based on previous published data were warranted (Rossi-George et al, 2005), an unpaired t test was conducted.

3. Results

Experiment 1

Table 1 presents the design for this experiment. It should be noted that both groups of animals that received saline injections were included in the ANOVAs as separate groups. However, for purposes of clarity, they were collapsed in the figures, since in all cases animals sacrificed after

a saline injection (whether pretreated with saline or SEA) did not show any differences in cytokine or corticosterone production. This was a universal observation, being the case also for experiments 2 and 3.

Splenic Cytokines

Figure 1 shows the cytokine concentrations for TNF α , IL-1 β , IL-2 and IFN γ as measured in the non-dissociated spleen. It is evident that for all cytokines, except IFN γ , there was a marked increase in response to an acute injection of SEA. Variations in response, however, were noted in response to the second injection of SEA given two or three days after the initial treatment. These analyses are summarized below.

Tumor Necrosis Factor α —Treatment with SEA significantly increased the level of TNF α ($F_{(3,29)} = 66.314, p < .0001$). However, both secondary injections of SEA showed an attenuated TNF response compared to the first injection (SEA 2d, $p < .0001$; SEA 3d, $p < .0001$).

Interleukin-1 β —There was a significant increase in IL-1 after injection with SEA in all of the groups ($F_{(3,28)} = 26.433, p < .0001$). Interestingly, there was no attenuation of the IL-1 β response to another SEA injection two days after initial SEA exposure (SEA 2d, $p = .1694$), although a significant reduction relative to the acute response was observed if SEA was given again three days later (SEA 3d, $p = .0203$).

Interleukin-2—Acute or secondary administration of SEA increased the level of IL-2 ($F_{(3,29)} = 48.615, p < .0001$). Neither of the secondary injections of SEA attenuated the IL-2 response, but rather appeared to increase the level of IL-2 above that of an acute injection. (SEA 2d, $p < .0001$; SEA 3d, $p = .0428$).

Interferon- γ —There was no significant production of IFN γ after a single injection of SEA, although a second injection of SEA given two days after the initial injection caused a significant increase in IFN γ (SEA 2d, $p < .0001$). This was no longer evident, however, if the second injection was given three days after the initial SEA injection.

Corticosterone—Figure 2 shows the plasma corticosterone concentrations associated with the above treatments and cytokine responses. It can be seen that there was a significant increase in the corticosterone response after treatment with SEA ($F_{(3,26)} = 5.998, p = .0030$). This was also the case after secondary exposure to SEA two or three days after the initial administration.

Experiment 2

Table 2 shows the design for this experiment. As described in the materials and methods, the rationale for this experiment was to extend the cytokine and corticosterone data from Experiment 1 to a confirmation of whether the well-documented anorexic response to SEA is retained after two or more SEA treatments. Testing for anorexia involved exposure to a palatable liquid diet (Prosobee), which is consumed without the need for water or food deprivation. Previous studies have shown that this reveals significant anorexic or hypophagic effects subsequent to a single SEA injection. In the present experiment, the retention of this anorexic response was determined after multiple injections of SEA. Figure 2 shows that treatment with SEA decreased consumption of Prosobee ($F_{(4,34)} = 5.060, p = .0026$), this being evident after an acute injection of SEA ($p = .0010$). Interestingly, two injections of SEA either three or five days apart continued to decrease consumption of Prosobee ($p = .0151$; $p = .0130$). However, three injections of SEA abrogated the anorexia, showing no difference to controls ($p = .7303$). Therefore, while two injections spaced at different intervals continue to exert a behavioral effect, the effect of a third injection is lost, in spite of the three injections in this

particular group occurring within the same five-day period. Whether this is related to changes in cytokine production and loss of a corticosterone response after the third injection was assessed in the next experiment.

Experiment 3

Table 3 shows the design for this experiment, which extended the treatments of surviving animals in Experiment 2. It is acknowledged that the effects of additional SEA treatments may have interacted with prior behavioral testing. However, it was anticipated that this would be without major effect, given that animals were already habituated to multiple handling and intraperitoneal injections. In any case, all groups from Experiment 2 were subdivided into those that received a Saline or SEA injection, and sacrificed two hours later and corticosterone, and splenic and plasma cytokines were then determined. For all ANOVAs, the respective control groups for each regimen of SEA treatment were included in the analysis, and are also indicated in the figures.

Corticosterone—Figure 4 shows the plasma corticosterone concentrations for the various SEA treatment groups. A significant SEA treatment effect was obtained ($F_{(9,30)} = 4.667, p = .0006$), with the acute SEA treatment showing the strongest response ($p = .0009$). Although significantly attenuated from acute SEA, two and three injections of SEA continued to increase the level of CORT (SEA 5d, $p = .0452$; SEAx3a, $p = .0149$). Interestingly, three injections spaced at five day intervals (SEAx3b) and four injections (SEAx4) of SEA failed to increase CORT.

Splenic Cytokines

Interleukin-2—There was a significant effect of SEA treatment on the concentration of splenic IL-2 ($F_{(9,27)} = 56.754, p < .0001$). All of the animals that received SEA on the day of sacrifice showed an increase in IL-2 when compared to their respective controls. Acute exposure to SEA and two injections of SEA showed the greatest increases of splenic IL-2 compared to their respective controls ($p < .0001$), while three injections (SEAx3a and SEAx3b) and four injections of SEA showed increased, but attenuated, IL-2 levels ($p < .0001$; $p = .0040$; $p = .0003$). Therefore, as the number of exposures to SEA exceeds two, the IL-2 response to additional SEA challenges is substantially diminished.

Interferon- γ —Irrespective of the type of SEA regimen, there was a main effect of SEA treatment on splenic IFN γ concentrations ($F_{(9,27)} = 9.585, p < .0001$). Post hoc analysis revealed that acute SEA, along with two and three injections of SEA all increased the level of splenic IFN γ compared to their respective controls ($p = .0099, p < .0001; p = .0017; p = .0412$). Four injections of SEA did not increase IFN γ ($p = .0844$). Interestingly, two injections of SEA seemed to exacerbate the level of splenic IFN γ compared to acute exposure to SEA ($p = .0015$) and seems to represent the time point in which there is a maximum increase due to the fact that two injections of SEA show a significantly higher level of IFN γ compared to three and four injections of SEA (SEAx3a, $p = .0075$; SEAx3b, $p = .0002$; SEAx4, $p < .0001$). It should also be noted that in Experiment 1, a similar augmenting effect of two injections, spaced two days apart, was also observed for IFN γ .

Interleukin-1 β —There was a significant effect of the differential regimen of SEA on IL-1 production ($F_{(9,27)} = 9.585, p < .0001$). Post hoc analysis revealed that acute SEA did not cause an elevation in IL-1 ($p = .5294$). After the second injection of SEA, however, IL-1 was drastically increased in all treatment groups when compared to their respective controls (all groups: $p < .0001$). While not as extreme as IFN γ , the trend suggests that three and four injections of SEA did not elevate IL-1 as much as two injections (SEAx3a, $p = .1085$, SEAx3b, $p = .0003$; SEAx4, $p = .0575$).

Tumor Necrosis Factor α —There was a significant effect on the level of TNF after treatment with SEA ($F_{(9,30)} = 35.190, p < .001$). Acute SEA significantly increased the level of TNF compared to the respective control ($p < .0001$). While two or three injections of SEA (SEAx3a) continued to show an elevated level of TNF compared to their controls (SEA 5d: $p = < .0001$; SEAx3a: $p = .0139$), the levels were significantly attenuated compared to an acute injection of SEA (SEA5d, $p < .0001$; SEAx3a, $p < .0001$). Interestingly, three injections of SEA every 5 days (SEAx3b) and four injections of SEA (SEAx4) did not increase splenic TNF.

Interleukin-10—There was a significant SEA treatment effect on the levels of IL-10 ($F_{(9,29)} = 30.983, p < .0001$), which was associated with a progressive increase in IL-10 output as the number of SEA injections increased (SEA5d, $p = .0010$; SEAx3a, $p < .0001$; SEAx3b, $p = .0270$; SEAx4, $p < .0001$). Interestingly, there was a marked difference between the SEAx3a and SEAx3b groups, suggesting that the timing of injections was critical in determining whether a third injection of SEA would induce a strong IL-10 response.

Interleukin-4—There was no significant SEA treatment effect on the IL-4 response ($F_{(9,30)} = .957, p = .4931$).

Plasma cytokines

Figure 6 summarizes the plasma IL-1 β and IFN γ concentrations measured in response to different numbers of SEA injection. These cytokines showed augmented splenic responses in response to secondary injections of SEA. Therefore, it was important to determine whether this also reflected considerable extracellular release of the cytokines. In particular, IL-1 β was of interest since it plays an important role in promoting anorexic responses and HPA axis activation.

Interleukin-1 β —The plasma IL-1 β results were surprising in that they failed to parallel those obtained for the spleen, there being no significant effect of SEA treatment ($F_{(9,24)} = 2.085, p = .0730$). Perhaps the most notable suggestion of an increase was after four injections of SEA, which was actually significant using a post hoc comparison (SEAx4, $p = .0026$). However, the relevance of this to neuromodulation by SEA cannot be determined, since the plasma corticosterone response was not elevated in this group.

Interferon- γ —In contrast to IL-1 β , there was a significant effect of SEA treatment on plasma IFN γ after differential regimens of SEA ($F_{(9,28)} = 17.786, p < .0001$). Post hoc analysis revealed that acute SEA, along with two, three, and four injections of SEA, increased the level of splenic IFN γ compared to their respective controls (Acute SEA, $p = .0063$; SEA5d, $p < .0001$; SEAx3a, $p = .0019$; SEAx3b, $p = .0225$; SEAx4, $p = .0024$). This mirrored findings for the spleen, especially the augmenting effect of a second injection of SEA ($p < .0001$).

4. Discussion

Previous studies have confirmed that systemic SEA challenge produces significant neural, behavioral and endocrine effects. These include induction of c-Fos immunoreactivity in limbic brain regions, activation of the HPA axis, and augmentation of neophobic behavior (Gonzalo et al., 1993; Kawashima et al., 2002; Kawashima and Kusnecov, 2002; Rossi-George et al., 2004). These effects have been observed only in relation to a single challenge of SEA, there being no evidence for dynamic changes in the relationship between cytokine production and neurobehavioral alterations after multiple exposures to SEA. This question is important in light of evidence that superantigens can induce immunological tolerance, which may result in the elimination of any neurobehavioral effects subsequent to SEA challenge. Consequently, the

present study sought to determine the persistence of plasma corticosterone and anorexic effects in response to repeated challenges with SEA.

Since acute injection of SEA increases plasma corticosterone in a CRH-dependent manner (Rossi-George et al., 2005), the current study focused on this glucocorticoid as a measure of HPA axis activation. It is understood, however, that in relation to multiple exposures to SEA, the central mechanisms responsible for the corticosterone response may shift, as has been demonstrated for chronic psychogenic stressors. As shown in the results, repeated SEA treatment continued to elevate plasma corticosterone, suggesting that this endocrine effect is not confined to acute SEA exposure. However, the interval between SEA challenges may be an important determining factor of the magnitude of this effect. In Experiment 1, challenge with SEA two or three days after an initial priming injection, produced a corticosterone response that was equal in magnitude to a single exposure. In contrast, Experiment 3 demonstrated that when the priming injection was given five days earlier (SEA 5d group, Table 3), the CORT response to the second SEA challenge was reduced by approximately 50%. Interestingly, this 5 day interval between injections did not affect the anorexic response to the second SEA challenge, as tested in Experiment 2 [see Fig 3, SEAx2 (5d)]. This suggests a possible dissociation between the behavioral effects of SEA and its capacity to affect the neuroendocrine system.

This dissociation is also implied by the results obtained for three injections of SEA. Two three-injection regimens were instituted in Experiment 3. For one group (SEAx3a), a reinterpretation of Table 3 indicates that SEA injections essentially occurred on time point days 0, 3 and 8, while for the second group (SEAx3b), SEA exposures occurred on days 0, 5 and 10 (as actually indicated in Table 3). Under these two separate regimens, the latter group failed to show a significant increase in the corticosterone response to the final SEA injection. This was not the case for the SEAx3a group which showed a significant increase in plasma corticosterone (see Figure 4). Therefore, if three injections are spaced at 5 day intervals, the neuroendocrine effect is lost. However, this does not appear to be a function of there being a 5 day interval between the final two injections, but rather a function of the interval between the initial two injections, which varied between these two groups. That is, a second injection given 5 days, rather than 3 days, after the first priming dose, enables refractoriness to the neuroendocrine effects of a third SEA injection. Whether this third injection needs to be given 5 days later or can be delivered earlier does not appear to be critical. This is suggested by the behavioral effects of Experiment 2, where it was demonstrated that a third injection of SEA given 3 days after the second injection failed to elicit an anorexic response. It is not known whether this was also associated with a deficient corticosterone response. These data suggest that an accumulation of SEA exposures within 2–3 days of a priming injection may facilitate continued CNS reactivity to subsequent challenges with SEA. However, as the interval between the first two injections increases, the facilitatory influence of these initial injections dissipates.

The immunological mechanisms underlying these effects remain elusive, but are likely to be dependent on the kinetics of the T cell proliferative response after an acute injection to SEA. The primary cells responding to SEA bear the T cell receptor V β 3 and V β 11 phenotypes, and display peak proliferative capacity two days after injection (Kuroda et al., 1996; McCormack et al., 1993), after which expansion declines and enters a phases of clonal deletion and T cell anergy (Kawabe and Ochi, 1991; MacDonald et al., 1991). In the present study, in vivo measures of cytokine production were taken to determine whether the endocrine and behavioral effects of SEA injections before, during and after these stages of T cell activity could be related to shifting patterns of cytokine output. In particular, it was of interest to monitor the TNF α response, since the CNS effects of an acute SEA challenge were shown to be dependent on the presence of TNF α (Rossi-George et al., 2005).

As expected, an acute injection of SEA induced a strong TNF α response, which was significantly attenuated after secondary exposure to SEA. In contrast, IL-1 β , IFN γ , and IL-2 all remained elevated after secondary exposure, which for IFN γ was consistently augmented if given two days after priming (see results for Experiment 1 and 3). The augmentation of the IL-2 response observed in Experiment 1 was not observed in Experiment 3, although it did not change in magnitude from that to a single injection of SEA. However, three or four injections of SEA led to substantially attenuated IL-2 and IFN γ responses, which is consistent with the literature (Florquin et al., 1995; Gaus et al., 1994; Noel et al., 2001; Sundstedt et al., 1994). There is evidence to suggest that the source of IFN γ may be CD8 $^{+}$ T cells, which, unlike CD4 $^{+}$ T cells, fail to enter an anergic state subsequent to superantigenic exposure (Hoiden and Moller, 1996; Kawabe and Ochi, 1991; Sundstedt et al., 1994). However, in the context of the current study, there is little evidence that IFN γ is a primary stimulus for the HPA axis and modulation of behavior. Nonetheless, this has yet to be tested in the superantigen model, and in the present case may represent a viable factor that affects the CNS. Indeed, it has been demonstrated that shock induced by a secondary injection to SEB is IFN γ dependent (Plaza et al., 2007). Whether this implies a role for IFN γ in CNS signaling, is not known.

The anergic response that is observed after SEA exposure is partially mediated by the anti-inflammatory cytokine IL-10. For example, IL-10 has been shown to inhibit production of IL-2, TNF, and IFN γ and directly inhibit T cell proliferation (de Waal Malefyt et al., 1991; Sundstedt et al., 1997; Taga and Tosato, 1992). The current findings showed that primary exposure to SEA led to a progressively enhanced IL-10 response after each successive exposure to SEA. This suggests that repeated exposure to SEA produces a shift from a Th1 to a Th2 cell response, although it might be expected that other Th2 cytokines, such as IL-4, might also show greater prominence with repeated SEA exposure. This was not the case, since the splenic concentration of IL-4 after acute or repeated SEA remained unchanged. This lack of an IL-4 response to an acute SEA challenge is not inconsistent with others superantigen models (Assenmacher et al., 1998; Krakauer, 1995; Rink et al., 1996), but contrasts with other findings of an increase in IL-4 after a single SEA or SEB exposure (Lagoo et al., 1994). Still others have found enhanced IL-4 responses after repeated exposure to SEA or SEB, although this is not a consistent finding (Florquin et al., 1995; Miller et al., 1999; Sundstedt et al., 1997). Consequently, the role of IL-4 is still an unresolved issue, but at least in the present study does not appear to be of relevance to the corticosterone and behavioral effects that were observed in response to acute or repeated SEA. At present it is not clear whether the progressively increased IL-10 response to succeeding injections of SEA is derived from Th2-like cells or whether it is emerging from accessory cells (eg., macrophages or dendritic cells). This needs to be determined in future studies.

The pattern of cytokine responses obtained in the current study is simultaneously compelling and potentially complex. Tumor necrosis factor, which plays an important role in mediating the behavioral and endocrine effects of acute SEA exposure (Rossi-George et al., 2005) is substantially reduced (by 50%) in response to secondary and tertiary injections of SEA, which still activate corticosterone, but not necessarily anorexia (recall that in Experiment 2, three injections of SEA failed to reduce food intake). Therefore, TNF α may be more relevant to gustatory reactions to SEA, while the increased corticosterone may be still promoted by additional cytokines.

Among neuromodulatory inflammatory cytokines, IL-1 β has been the best characterized. However, in relation to superantigenic effects on CNS function it has not been considered a prominent factor, given the difficulty to detect plasma IL-1 β after acute SEA or SEB injections (Kawashima and Kusnecov, 2002; Rossi-George et al., 2005). Moreover, the anorexic effects of SEA were not seen in IL-1 receptor knockout mice (Rossi-George et al., 2005). The current study also suggests a tenuous relationship between IL-1 β and the neurobehavioral effects of

SEA. Plasma levels of IL-1 β (as seen in Figure 6) did not hold a positive relationship with SEA exposure conditions that were found to produce anorexia or increased corticosterone production. Moreover, while the SEA-induced plasma IL-1 β was variable, the cytokine was more consistently elevated in the spleen, relative to saline-injected controls. This suggests that either splenic IL-1 β does not achieve endocrine levels that would be apparent in the circulation, or it is quickly sequestered in tissues and/or bound by receptors.

It should be noted that Experiment 1 showed that an acute injection of SEA increased splenic IL-1 β , although this was not replicated in Experiment 3. A possible explanation for this may be due to the greater amount of handling, behavioral testing and ip injections imposed on animals prior to the acute challenge in Experiment 3 (see Table 3). Indeed, repeated handling has been shown to modify immune responses and under the current circumstances may have inhibited the IL-1 β response to SEA (Moynihan et al., 1990; Moynihan et al., 1989; Moynihan et al., 1992). While this explanation would require further empirical testing, it was evident that splenic IL-1 β concentrations increased in response to repeated injections of SEA, and this also occurred in plasma. The capacity for IL-1 β to be synthesized in response to additional SEA administration suggests that the lack of an increase to acute SEA was an inherent feature of the differences in experimental design between Experiments 1 and 3 (eg., amount of handling and testing). These differences notwithstanding, it was evident that up to two, three and four exposures to SEA were capable of driving up IL-1 β responses to a significantly greater extent than a single SEA treatment. To our knowledge, we are the first to report these findings.

It is also interesting to note that while TNF α levels were inversely related to IL-10 production, splenic IL-1 β was not inversely related to IL-10. It has been shown that Interleukin-10 can inhibit both IL-1 β and TNF, and that IL-10 deficient mice treated with SEB show enhanced TNF, IL-1 β , and IL-2 responses (Hasko et al., 1998; Moore et al., 2001). The current results, however, suggest that IL-1 β may have escaped IL-10 regulation after repeated treatment with SEA. Additionally, while IL-1 β spleen and plasma levels remained elevated, this was observed when the corticosterone response to SEA was attenuated or absent. Although IL-1 β is a strong activator of the HPA-axis, this suggests that IL-1 β may not be mediating the HPA axis activation after repeated SEA challenge.

In conclusion, secondary exposure to SEA continued to produce CNS reactivity, in that animals continued to display anorexia and an increase in corticosterone. Repeated exposure to SEA attenuated TNF while other cytokines such as IL-1 β , IFN γ , and IL-10 remained elevated. While TNF α has previously been shown to be important in mediating the effects of SEA, it remains to be determined which cytokines are mediating the behavioral and endocrine effects during repeated conditions. While TNF remains a likely candidate, its impact may occur in an additive and/or synergistic fashion. Moreover, it is possible that the somewhat attenuated, but still significant levels of TNF α may still have neuromodulatory effects, given that there is evidence for TNF α mediated sensitization of behavioral and endocrine effects (Hayley et al., 2002). Similarly, IL-2 alters behavior and activates the HPA axis, may therefore be a significant contributor to the continued corticosterone and anorexia to secondary and tertiary SEA challenge (Karanth et al., 1994; Pauli et al., 1998; Zalcman et al., 1998; Zalcman, 2001).

Acknowledgements

Support Contributed By: Grants MH60706, NIEHS P30 ES05022 and NIEHS Graduate Training grant 5T32 E507148

References

- Assenmacher M, Lohning M, Scheffold A, Manz RA, Schmitz J, Radbruch A. Sequential production of IL-2, IFN-gamma and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes. *Eur J Immunol* 1998;28:1534–1543. [PubMed: 9603458]

- Bette M, Schafer MK, van Rooijen N, Weihe E, Fleischer B. Distribution and kinetics of superantigen-induced cytokine gene expression in mouse spleen. *J Exp Med* 1993;178:1531–1539. [PubMed: 8228806]
- de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991;174:915–924. [PubMed: 1655948]
- Feunou P, Poulin L, Habran C, Le Moine A, Goldman M, Braun MY. CD4+CD25+ and CD4+CD25– T cells act respectively as inducer and effector T suppressor cells in superantigen-induced tolerance. *J Immunol* 2003;171:3475–3484. [PubMed: 14500643]
- Florquin S, Amraoui Z, Goldman M. T cells made deficient in interleukin-2 production by exposure to staphylococcal enterotoxin B in vivo are primed for interferon-gamma and interleukin-10 secretion. *Eur J Immunol* 1995;25:1148–1153. [PubMed: 7774618]
- Gaus H, Miethke T, Wagner H, Heeg K. Superantigen-induced anergy of V beta 8+ CD4+ T cells induces functional but non-proliferative T cells in vivo. *Immunology* 1994;83:333–340. [PubMed: 7835956]
- Goehler LE, Gaykema RP, Hansen MK, Kleiner JL, Maier SF, Watkins LR. Staphylococcal enterotoxin B induces fever, brain c-Fos expression, and serum corticosterone in rats. *Am J Physiol Regul Integr Comp Physiol* 2001;280:R1434–1439. [PubMed: 11294765]
- Gonzalo JA, Gonzalez-Garcia A, Martinez C, Kroemer G. Glucocorticoid-mediated control of the activation and clonal deletion of peripheral T cells in vivo. *J Exp Med* 1993;177:1239–1246. [PubMed: 8478606]
- Hasko G, Virag L, Egnaczyk G, Salzman AL, Szabo C. The crucial role of IL-10 in the suppression of the immunological response in mice exposed to staphylococcal enterotoxin B. *Eur J Immunol* 1998;28:1417–1425. [PubMed: 9565382]
- Hayley S, Kelly O, Anisman H. Murine tumor necrosis factor-alpha sensitizes plasma corticosterone activity and the manifestation of shock: modulation by histamine. *J Neuroimmunol* 2002;131:60–69. [PubMed: 12458037]
- Hoiden I, Moller G. CD8+ cells are the main producers of IL10 and IFN gamma after superantigen stimulation. *Scand J Immunol* 1996;44:501–505. [PubMed: 8947602]
- Kaneta T, Kusnecov AW. The role of central corticotropin-releasing hormone in the anorexic and endocrine effects of the bacterial T cell superantigen, Staphylococcal enterotoxin A. *Brain Behav Immun* 2005;19:138–146. [PubMed: 15664786]
- Karanth S, Lyson K, McCann SM. Cyclosporin A inhibits interleukin-2-induced release of corticotropin-releasing hormone. *Neuroimmunomodulation* 1994;1:82–85. [PubMed: 8528889]
- Kawabe Y, Ochi A. Programmed cell death and extrathymic reduction of Vbeta8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *Nature* 1991;349:245–248. [PubMed: 1670963]
- Kawashima N, Fugate J, Kusnecov AW. Immunological challenge modulates brain orphanin FQ/nociceptin and nociceptive behavior. *Brain Res* 2002;949:71–78. [PubMed: 12213301]
- Kawashima N, Kusnecov AW. Effects of staphylococcal enterotoxin A on pituitary-adrenal activation and neophobic behavior in the C57BL/6 mouse. *J Neuroimmunol* 2002;123:41–49. [PubMed: 11880148]
- Krakauer T. Inhibition of toxic shock syndrome toxin-1-induced cytokine production and T cell activation by interleukin-10, interleukin-4, and dexamethasone. *J Infect Dis* 1995;172:988–992. [PubMed: 7561220]
- Kuroda K, Yagi J, Imanishi K, Yan XJ, Li XY, Fujimaki W, Kato H, Miyoshi-Akiyama T, Kumazawa Y, Abe H, Uchiyama T. Implantation of IL-2-containing osmotic pump prolongs the survival of superantigen-reactive T cells expanded in mice injected with bacterial superantigen. *J Immunol* 1996;157:1422–1431. [PubMed: 8759722]
- Kusnecov AW, Goldfarb Y. Neural and behavioral responses to systemic immunologic stimuli: a consideration of bacterial T cell superantigens. *Curr Pharm Des* 2005;11:1039–1046. [PubMed: 15777253]
- Lagoo AS, Lagoo-Deenadayalan S, Lorenz HM, Byrne J, Barber WH, Hardy KJ. IL-2, IL-4, and IFN-gamma gene expression versus secretion in superantigen-activated T cells. Distinct requirement for

- costimulatory signals through adhesion molecules. *J Immunol* 1994;152:1641–1652. [PubMed: 8120374]
- MacDonald HR, Baschieri S, Lees RK. Clonal expansion precedes anergy and death of V beta 8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. *Eur J Immunol* 1991;21:1963–1966. [PubMed: 1907921]
- McCormack JE, Callahan JE, Kappler J, Marrack PC. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. *J Immunol* 1993;150:3785–3792. [PubMed: 8473733]
- Miller C, Ragheb JA, Schwartz RH. Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms in vivo. *J Exp Med* 1999;190:53–64. [PubMed: 10429670]
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765. [PubMed: 11244051]
- Moynihan J, Brenner G, Koota D, Breneman S, Conen N, Ader R. The effects of handling on antibody production, mitogen responses, spleen cell number, and lymphocyte subpopulations. *Life Sci* 1990;46:1937–1944. [PubMed: 2362549]
- Moynihan J, Koota D, Brenner G, Cohen N, Ader R. Repeated intraperitoneal injections of saline attenuate the antibody response to a subsequent intraperitoneal injection of antigen. *Brain Behav Immun* 1989;3:90–96. [PubMed: 2765687]
- Moynihan JA, Brenner GJ, Ader R, Cohen N. The effects of handling adult mice on immunologically relevant processes. *Ann N Y Acad Sci* 1992;650:262–267. [PubMed: 1605482]
- Noel C, Florquin S, Goldman M, Braun MY. Chronic exposure to superantigen induces regulatory CD4 (+) T cells with IL-10-mediated suppressive activity. *Int Immunol* 2001;13:431–439. [PubMed: 11282982]
- Pauli S, Linthorst AC, Reul JM. Tumour necrosis factor-alpha and interleukin-2 differentially affect hippocampal serotonergic neurotransmission, behavioural activity, body temperature and hypothalamic-pituitary-adrenocortical axis activity in the rat. *Eur J Neurosci* 1998;10:868–878. [PubMed: 9753154]
- Plaza R, Rodriguez-Sanchez JL, Juarez C. Staphylococcal enterotoxin B in vivo modulates both gamma interferon receptor expression and ligand-induced activation of signal transducer and activator of transcription 1 in T cells. *Infect Immun* 2007;75:306–313. [PubMed: 17074848]
- Rink L, Luhm J, Koester M, Kirchner H. Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J Interferon Cytokine Res* 1996;16:41–47. [PubMed: 8640450]
- Rossi-George A, LeBlanc F, Kaneta T, Urbach D, Kusnecov AW. Effects of bacterial superantigens on behavior of mice in the elevated plus maze and light-dark box. *Brain Behav Immun* 2004;18:46–54. [PubMed: 14651946]
- Rossi-George A, Urbach D, Colas D, Goldfarb Y, Kusnecov AW. Neuronal, endocrine, and anorexic responses to the T-cell superantigen staphylococcal enterotoxin A: dependence on tumor necrosis factor-alpha. *J Neurosci* 2005;25:5314–5322. [PubMed: 15930379]
- Strle K, Zhou JH, Shen WH, Broussard SR, Johnson RW, Freund GG, Dantzer R, Kelley KW. Interleukin-10 in the brain. *Crit Rev Immunol* 2001;21:427–449. [PubMed: 11942558]
- Sundstedt A, Dohlsten M, Hedlund G, Hoiden I, Bjorklund M, Kalland T. Superantigens anergize cytokine production but not cytotoxicity in vivo. *Immunology* 1994;82:117–125. [PubMed: 7913912]
- Sundstedt A, Hoiden I, Rosendahl A, Kalland T, van Rooijen N, Dohlsten M. Immunoregulatory role of IL-10 during superantigen-induced hyporesponsiveness in vivo. *J Immunol* 1997;158:180–186. [PubMed: 8977189]
- Taga K, Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. *J Immunol* 1992;148:1143–1148. [PubMed: 1737931]
- Watson AR, Mittler JN, Lee WT. Staphylococcal enterotoxin B induces anergy to conventional peptide in memory T cells. *Cell Immunol* 2003;222:144–155. [PubMed: 12826084]
- Zalcman S, Murray L, Dyck DG, Greenberg AH, Nance DM. Interleukin-2 and -6 induce behavioral-activating effects in mice. *Brain Res* 1998;811:111–121. [PubMed: 9804916]
- Zalcman SS. Interleukin-2 potentiates novelty- and GBR 12909-induced exploratory activity. *Brain Res* 2001;899:1–9. [PubMed: 11311862]

Figure 1.

Mean splenic TNF α , IL-1 β , IL-2 and IFN γ concentrations ($\pm$ SE) after acute SEA, two injections of SEA spaced two days apart (SEAx2 (2d)) or three days apart (SEAx2 (3d)) and the control. N = 5–11/group; $p < .05$. Error bars represent standard error of the mean.

Figure 2.

Mean plasma corticosterone levels after acute SEA, two injections of SEA spaced two days apart (SEAx2 (2d)), three days apart (SEAx2 (3d)), and the control. $N = 5-11/\text{group}$; $p < .05$. Error bars represent standard error of the mean.

Figure 3.

Consumption of a novel liquid diet (Prosobee) after acute SEA, two injections of SEA spaced three (SEAx2 (3d)), five days apart (SEAx2 (5d)), three injections of SEA (SEAx3), and the control. N = 8/group; $p < .05$. Error bars represent standard error of the mean.

Figure 4.

Mean plasma corticosterone levels after acute SEA, two injections of SEA spaced five days apart (SEA 5d), three injections of SEA given three and then five days apart (SEAx3a), three injections of SEA given every five days (SEAx3b), and four injections of SEA (SEAx4). See Table 3 for control group designation. $N = 4/\text{group}$; $p < .05$. Error bars represent standard error of the mean.

Figure 5.

Mean splenic TNF α , IL-1 β , IL-2, IFN γ , IL-10 and IL-4 concentrations after acute SEA, two injections of SEA spaced five days apart (SEA 5d), three injections of SEA given three and then five days apart (SEAx3a), three injections of SEA given every five days (SEAx3b), and four injections of SEA (SEAx4). See Table 3 for control group designation. N = 4/group; $p < 0.05$. Error bars represent standard error of the mean.

Figure 6.

Mean plasma IFN γ and IL-1 β after acute and repeated SEA. See legend to Figure 5 for group designations. N = 4/group; $p < .05$. Error bars represent standard error of the mean.

Table 1

Summary of experimental design for Experiment 1

	D0	D1	D3
Acute SEA	Sal		SEA
Acute SEA		Sal	SEA
SEAx2 (2d) (2 days apart)		SEA	SEA
SEAx2 (3d) (3 days apart)	SEA		SEA
Saline		Sal	Sal
Saline	Sal		Sal

Table 2

Summary of experimental design for Experiment 2

	D0	D2	D5
control	Sal	Sal	Sal
Acute SEA	Sal	Sal	SEA
SEAx2 (3d)	Sal	SEA	SEA
SEAx2 (5d)	SEA	Sal	SEA
SEAx3	SEA	SEA	SEA

Table 3

Summary of experimental design for Experiment 3

	D0	D2	D5	D10
Acute SEA	Sal	Sal	Sal	SEA
SEAx2 5d	Sal	Sal	SEA	SEA
SEAx3a	Sal	SEA	SEA	SEA
SEAx3b	SEA	Sal	SEA	SEA
SEAx4	SEA	SEA	SEA	SEA
Acute control	Sal	Sal	Sal	Sal
Control 5d	Sal	Sal	SEA	Sal
Controlx3a	Sal	SEA	SEA	Sal
Controlx3b	SEA	Sal	SEA	Sal
Controlx4	SEA	SEA	SEA	Sal