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IL-12 RESCUES GALACTOSAMINE-LOADED MICE FROM LETHAL SHOCK TRIGGERED BY STAPHYLOCOCCAL ENTEROTOXIN

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Staphylococcal enterotoxin B (SEB) causes lethal shock in D-galactosamine sensitized mice. The lethal shock triggered by SEB is mediated by T cells. We found that the lethal shock was restricted by MHC class II molecule. In addition, consecutive oral exposures to SEB induced tolerance against the shock in the SEB-sensitive mice. To elucidate the tolerance mechanism, the role of anti-inflammatory cytokines was examined. RT-PCR analysis revealed that $CD4^+$ T cells from the SEB-sensitive mice expressed significant levels of IFN-γ, IL-2, IL-4 and IL-10 mRNA, while those from the tolerant mice exhibited significant levels of IFN-γ but not IL-2 or IL-4 mRNA. These results indicate that polarity of T helper (Th) cells from Th0 to Th1 was involved in the tolerance to the SEB-induced lethal shock . Lymphoid tissues of the tolerant mice generated mRNA of IL-12, a cytokine which favors Th1 response. It was also demonstrated that intraperitoneal administration of IL-12 conferred protection against the lethal shock in the sensitive mice.

Some exotoxins from *Staphylococcus aureus* and *Streptococcus pyogenes* may cause food poisoning and toxic shock syndromes in humans (1-4). These toxins such as staphylococcal enterotoxin B (SEB) have been found to exhibit unique properties of superantigens that bind to MHC class II molecules and activate T cells with specified Vβ segments of the TCR (1-3). Superantigens have been shown to be powerful immunomodulators that hyperstimulate the immune system, which may result in induction of acute and chronic diseases including toxic shock syndrome, rheumatoid arthritis, and Kawasaki disease (5-7). The ability of bacterial exotoxins to induce massive T cell proliferation could result in systemic secretion of various cytokines. These cytokines, in turn, may be critical for initiating systemic toxic shock. The aim of this study is to analyze the mechanisms of tolerance against lethal shock triggered by SEB. Because mice are known to be more resistant than humans to the pathogenic effects of bacterial toxins (8-11), we used D-galactosamine (D-GalN) loaded mice as a model system to evaluate toxic shock triggered by SEB.

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Materials and Methods

Mice: BALB/c, C57BL/6, and C3H/HeN mice were purchased from Charles River Japan (Kanagawa, Japan). B10 congenic mice [B10 (H-2^b), B10.D2 (H-2^d), and B10. BR (H-2^k)] were obtained from Japan SLC (Shizuoka, Japan).

Reagents: SEB was purchased from Sigma (St. Louis, MO). D-Galactosamine (GalN) hydrochloride, cyclosporin A and polymyxin B sulfate were products of Wako Pure Chemicals (Osaka, Japan). IL-12 was a gift from Genetics Institute, Cambridge, MA, and was purified from culture supernatants of Chinese hamster ovary cells transfected with amplified cDNA for the p40 and p35 subunits of murine IL-12. SDS-PAGE analysis revealed that this preparation consisted predominantly of murine IL-12 heterodimeric (98.7%) with a small amount of monomeric p40 subunit (1.3%). The specific activity was 4.6 x 10^6 units/mg. Endotoxin levels as measured by the Limulus amebocyte lysate assay were 17.9 EU/mg. We also used an avirulent strain of *Salmonella typhimurium*, SL1438, for oral immunization of mice, which is a nonreverting aromatic-dependent and histidine-requiring mutant of wild type strain S4454 (12).

Lethal shock triggered by SEB in D-GalN loaded mice: Groups of mice were intraperitoneally (i.p.) administered 20 mg of D-GalN and SEB was titrated for death by its direct action for up to 48 h (8). In this study, we confirmed that the lethal shock triggered by SEB was exclusively mediated by $CD4^+$ T cells, since cyclosporin A and D-GalN-treated mice survived without disease symptoms. In addition, administraition of polymyxin B, an inhibitor of endotoxin shock, did not inhibit the lethal shock triggered by SEB in the D-GlaN-loaded mice (data not shown).

Assay for cytokine production: Cytokine levels in serum and/or culture supernatants were determined by ELISA (13). In brief, flat-bottomed 96-well microtiter plates (Nunc Inc., Naperville, IL) were coated with the anti-IL-4 mAb BVD4-1D11 (Pharmingen, San Diego, CA) and anti-IL-2 mAb JES6-1A12 (Pharmingen), respectively, at a concentration of 1 μg/ml in phosphate-buffered saline (PBS), and incubated overnight at 4°C. The plates were washed three times with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. The serum and/or culture supernatants were two-fold diluted with 1% BSA in PBS containing 0.05% of Tween 20 (PBS-T) and incubated at 4°C overnight. After the plate was washed with PBS-T, biotinylated anti-IL-4 mAb BVD6-24G2 (Pharmingen) or biotinylated anti-IL-2 mAb JES6-5H4 (Pharmingen) at 1 μg/ml in 1% BSA in PBS-T was added to the wells and left for 2 h at 37°C. After subsequent washings with PBS-T, a 1: 2,000 dilution of horseradish peroxidase-conjugated streptavidin in 1% BSA/PBS-T was added and incubated at room temperature for 2 h. The reaction was developed by a substrate solution consisting of 1.1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2 for 15 min. Absorbance was read in a plate reader at 414 nm.

RT-PCR: Reverse transcription (RT)-PCR analysis was carried out to detect antigen-specific cytokine mRNA expression by using RT-PCR Amplimer Sets (CLONETECH, Palo Alto, CA) (14). To isolate RNA from the $CD4^+$ T cells purified by immunomagnetic beads (Miltenyi Biotec, Sunnyvale, CA) charged with anti-L3T4 monoclonal antibody, TRIzol reagent (GIBCO BRL , Gaithersburg, MD) was used. RNA was also isolated from splenic macrophages of BALB/c mice immunized orally with attenuated *Salmonella typhimurium* SL1438. Reverse transcription (RT) was carried out using oligo (dT)16 primer, and the specific cDNA fragment was amplified by using cytokine-specific primers and *Taq* DNA polymerase (Roche Molecular Systems, Branchburg, NJ). PCR products were separated by electrophoresis in 2% agarose gels.

Results

Genetic control of the lethal shock triggered by SEB

In the initial experiments, strain-dependency of the lethal shock triggered by SEB was examined by using various inbred strains and B10 congenic mice. It was found that the 50% lethal dose (LD50) for BALB/c (H-2^d) mice was 40 μg (Table 1). On the other hand, LD50 for C57BL/6 (H-2^b) and C3H/HeN (H-2^k) mice was 500 μg and 218 μg, respectively. We also quantitated the lethal toxicity of SEB in B10 congenic mice. The sensitivity of three strains of B10 congenic mice to the lethal shock of SEB was as follows: B10.D2 (H-2^d) >> B10.BR (H-

Table 1. Lethal effect of SEB on various strains of mice sensitized with D-GalN[a]

Strain	Dose (μg / mouse) 500	250	50	30	LD_{50} (μg)
BALB/c ($H\text{-}2^d$)			5 / 5	1 / 8	40
C57BL/6 ($H\text{-}2^b$)	2 / 4	0 / 8			500
C3H/HeN ($H\text{-}2^k$)		3 / 5	0 / 5		218
B10.D2 ($H\text{-}2^d$)		5 / 5			
B10 ($H\text{-}2^b$)		0 / 5			
B10.BR ($H\text{-}2^k$)		3 / 5			

[a] Mice were intraperitoneally injected with various amounts of SEB and 20 mg of D-galactosamine (D-GalN).

2^k) > B10 ($H\text{-}2^b$). These results indicate that the strain-dependency of the lethal shock triggered by SEB was restricted by H-2 haplotypes.

Cytokine production by T cells from SEB-sensitive and SEB-tolerant mice

When BALB/c mice were fed SEB five times (50 μg each) during the period of five consecutive days, these mice became resistant to the lethal shock of SEB. A total of five i.p. injections of SEB in five consecutive days also resulted in tolerance to the lethal shock. Because release of cytokines is considered to play an important role in the lethal toxicity triggered by SEB, we examined IL-2 and IL-4 levels in serum of SEB-sensitized or tolerant BALB/c mice. Peak levels of IL-2 were observed as early as 2 to 4 h after SEB and D-GalN injection in the sensitive mice (1500-1800 pg/ml). Serum IL-4 was also elevated 8 to 16 h after the injection in BALB/c mice (16-30 pg/ml). On the other hand, SEB-tolerant mice failed to produce detectable quantities of IL-2 and IL-4. We then analyzed the expression of cytokines at the transcriptional level. RT-PCR analysis revealed that $CD4^+$ T cells from the SEB-sensitive (BALB/c) mice generated IFN-γ, IL-2, IL-4 and IL-10 mRNA (Fig. 1A). However, $CD4^+$ T cells from the SEB-tolerant BALB/c mice generated a high level of IFN-γ mRNA and a lower level of IL-6 mRNA, but failed to generate IL-2 and IL-4 mRNA (Fig. 1B). In addition, $CD4^+$ T cells from SEB-resistant B10 and B10. BR mice expressed IFN-γ mRNA alone (Fig. 2). These results indicate that SEB-induced tolerance might be mediated by selective shift of cytokine generation (expression) of $CD4^+$ T cells from Th0 (IFN-γ, IL-2, IL-4, and IL-10) to Th1 (IFN-γ, and weak IL-6), and that production of IL-2 and IL-4 might mediate the lethal shock triggered by SEB.

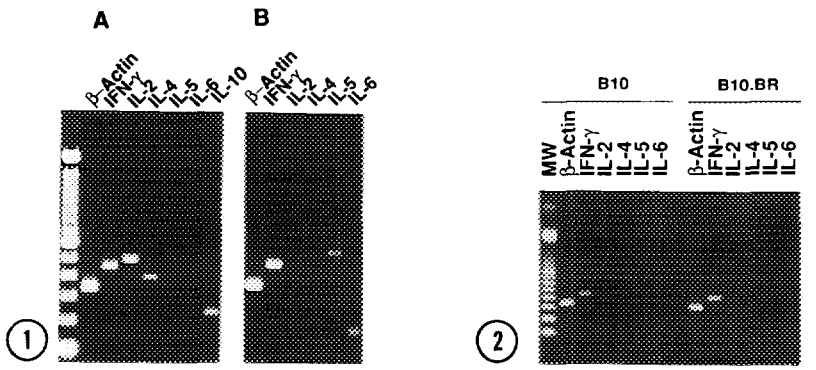

Fig. 1 Cytokine profile of CD4+ T cells from SEB-sensitive (A) and tolerant (B) BALB/c mice. Molecular size of the amplified products of each cytokine mRNA are as follows: β–Actin, 349 bp; IFN-γ, 460 bp; IL-2, 502 bp; IL-4, 399 bp; IL-5, 243 bp; IL-6, 153 bp; IL-10, 237 bp. The left end column shows a 100-bp DNA ladder.

Fig. 2 Cytokine profile of CD4+ T cells from SEB-resistant B10 and B10.BR mice. Molecular size of the amplified products of each cytokine mRNA are as follows: β–Actin, 349 bp; IFN-γ, 460 bp; IL-2, 502 bp; IL-4, 399 bp; IL-5, 243 bp; IL-6, 153 bp; IL-10, 237 bp. The left end column shows a 100-bp DNA ladder.

Cytokine mRNA expression in the lymphoid tissues from SEB-tolerant mice

It is important to know if *in vivo* regulation of selective lymphokine production by CD4+ T cells was controlled by *Salmonella typhimurium* infection. Oral immunization of SEB-sensitive BALB/c mice with 10^{10} CFU of attenuated *Salmonella typhimurium* SL1438 induced tolerance against the lethal shock triggered by SEB (data not shown). CD4+ T cells from the immunized mice expressed significant levels of IFN-γ mRNA and lower levels of IL-6 mRNA; however, neither IL-2 nor IL-4 mRNA was observed in CD4+ T cells from the tolerant mice (data not shown). Furthermore, we analyzed the induction of IL-12 mRNA in the lymphoid tissues from SEB-tolerant and sensitive mice. RT-PCR analysis revealed that macrophages from

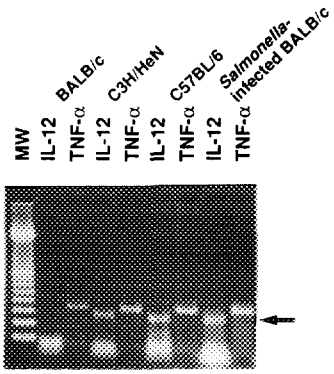

Fig. 3 IL-12 p40 mRNA expression of macrophages from SEB-sensitive and/or resistant strains of mice. Molecular size of the amplified products of IL-12 p40 and TNF-α are 266 bp and 354 bp, respectively. MW, 100-bp DNA ladder.

the spleen of the immunized mice and mice tolerant to SEB generated IL-12 mRNA (Fig. 3). These results indicate that tolerance against the lethal shock by SEB might be mediated by IL-12.

Intraperitoneal administration of IL-12 confers the protection of D-GalN loaded mice against lethal shock triggered by SEB

Finally, we examined the involvement of IL-12 for the preferential induction of SEB-reactive $CD4^+$ T cells and the lethal toxicity triggered by SEB. Consecutive intraperitoneal injections of IL-12 were found to cure the SEB-sensitive BALB/c mice from the lethal shock triggered by SEB (Table 2). RT-PCR analysis revealed that $CD4^+$ T cells from the recovering mice expressed IFN-γ mRNA but not IL-2 and IL-4 mRNA (Fig. 4). These results again indicate that IL-12 is essential for SEB-desensitization via the action of the induction of anergy of SEB-reactive $CD4^+$ T cells producing IL-2 and IL-4.

Discussion

Mice are relatively resistant to bacterial toxins (8, 10). However, it is apparent that gram-positive bacterial toxins including SEB and TSST-1 exhibit a number of pathological effects in susceptible mice such as those loaded with D-GalN, resulting in a rapid weight loss (9), thymus and peripheral lymph node depletion (15, 16), and lethal shock (8, 17). The site of action of staphylococcal enterotoxins has been considered to be the intestinal tract. However, as has been shown in this study as well as toxic shock syndrome in humans, these toxins may directly affect various organs and the cardiovascular system. The results obtained in this study demonstrate that the resistance to the lethal shock triggered by SEB in D-GalN-load mice was mediated, at least partially, by polarization of Th cell differentiation. $CD4^+$ T cells from SEB-sensitive mice expressed IFN-γ, IL-2, IL-4, and IL-10 mRNA, while $CD4^+$ T cells from the SEB-resistant and tolerant mice predominantly synthesized IFN-γ mRNA. These findings indicate that switching from Th0 to Th1 subsets occur, which may account for resistance to superantigen-induced lethal shock in D-GalN-loaded mice. The mechanisms of this Th0 to Th1 switching remain to be

Table 2. IL-12 induction of tolerance to SEB in terms of lethality in D-GalN-loaded BALB/c mice

Pretreatment with IL-12	Dose	Death / Total Mice
-	-	5 / 5
4 i.p. injections	1 μg each	0 / 5[a]
4 i.p. injections	1 μg each	1 / 5[b]

[a] After pretreatment with IL-12, mice were injected intraperitoneally (i.p.) with 50 μg of SEB and 20 mg of D-GalN.
[b] After pretreatment with IL-12, mice were injected i. p. with 250 μg of SEB and 20 mg of D-GalN.

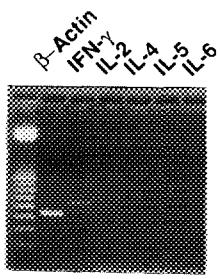

Fig. 4 Cytokine profile of CD4+ T cells from SEB-tolerant BALB/c mice pretreated with IL-12. Molecular size of the amplified products of each cytokine mRNA are as follows: β-Actin, 349 bp; IFN-γ, 460 bp; IL-2, 502 bp; IL-4, 399 bp; IL-5, 243 bp; IL-6, 153 bp. The left end column shows a 100-bp DNA ladder.

elucidated. However, it should be pointed out that SEB-induced anergic Th2 subsets failed to secrete IL-2, but continued to produce other cytokines including IFN-γ and TGF-β (18, 19). It was also suggested that intravenous injection of SEB induced a functional unresponsiveness of peripheral T cells expressing Vβ8 segments as well as partial deletion of the T cells by programmed cell death (15, 16). Furthermore, preferential differentiation of SEB-sensitive Th1 type responses could occur. In this regard, *in vitro* SEB favors Th1 development by stimulation of TGF-β production (20).

Experimental infections by intracellular bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* result in marked polarization to Th1 phenotype, a phenomenon that could be reproduced with IL-12 (21, 22). Attenuated *Salmonella typhimurium* strains have been investigated as a vehicle for carrying foreign antigens into gut-associated lymphoid tissues as well as systemic lymphoid tissues (12, 23). Oral immunization of BALB/c mice with attenuated *Salmonella dublin* carrying the B subunit of *Escherichia coli* heat-labile enterotoxin was demonstrated to induce IL-12 mRNA in Peyer's patches and mesenteric lymph nodes (24). IL-12 is a heterodimeric cytokine composed of 40- and 35- kDa subunits and produced mainly by phagocytic cells. This cytokine has been suggested to play a critical role in the development of cell-mediated immune responses to intracellular pathogens (21). Thus, IL-12 satisfies the requirement for participation in the differentiation of Th1 cells (21).

In this study, we have focused on the involvement of IL-12 on the polarized Th1 development. Oral administration with *Salmonella typhimurium* induced resistance to the lethal shock and CD4+ T cells from the resistant mice expressed significant levels of IFN-γ mRNA but not Th2-type cytokine mRNA. In addition, expression of IL-12 mRNA was observed only in SEB-tolerant and resistant mice carrying the $H\text{-}2^b$ and $H\text{-}2^k$. Furthermore, administration of IL-12 prevented the SEB-sensitive BALB/c mice from the lethal shock by SEB, indicating that IL-12 is committed to Th subset default for SEB-desensitization.

We have found here the genetic control, i.e. MHC restriction, in the the lethal shock triggered by SEB. BALB/c and B10.D2 mice carrying the haplotype $I\text{-}A^d$ were highly sensitive to the lethal shock, while C3H/HeN and B10. BR mice carrying the $I\text{-}A^k$ exhibited a moderate sensitivity to the SEB-lethality. On the other hand, C57BL/6 and B10 mice carrying the $I\text{-}A^b$ exhibited a maximum resistance to the attack of SEB. These findings may reflect the relative

capability of toxin binding to the I-A^d than to I-A^b, I-A^k, as has been suggested by others (9, 17). $CD4^+$ T cells from mice (BALB/c) carrying the MHC class II I-A^d preferentially develop Th1 type response and/or release some factors such as TNF-α and/or TNF-β which could be harmful for the animals. In this regards, it has been reported that C57BL/6 mice are likely to develop a Th1 and less likely to develop a Th2 response than are BALB/c mice (22, 25). Thus, C57BL/6 mice are concluded to be more resistant than BALB/c mice to the cutaneous infection of leishmaniasis and development of insulin-dependent diabetes. These findings are basically in agreement with our findings reported herein.

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