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Hiroaki Takimoto, Yasunobu Yoshikai, Kenji Kishihara, Goro Matsuzaki, Hiroshi Kuga^O, Tsuyoshi Otani^O and

Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka and Research Institute, Daiichi Seiyaku Co. Ltd. O, Tokyo

Stimulation of all T cells bearing $V_{\beta}1, V_{\beta}3, V_{\beta}11$ and V_B12 by staphylococcal enterotoxin A

To determine the molecular mechanisms of T cell stimulation by staphylococcal enterotoxin A (SEA), we examined the expression of Tcell receptor (TcR) V_B on the T cells from four strains of mice stimulated in vitro with SEA, using flow cytometric analysis for the number of Tcells bearing V_B3, V_B6, V_B8, V_B11 and RNA blotting analysis for the amount of transcripts of V₆1, V₆5 and V₆12. The number of T cell blasts bearing $V_{\beta}1, V_{\beta}3, V_{\beta}1$ or $V_{\beta}12$ were increased in the T cell blasts proliferating *in vitro* in response to SEA in C57BL/6 mice. In AKR/J mice, which contain few V_B11- or V_B12-bearing T cells due to a tolerance to the self-MHC class II IE-antigens, Tcells bearing V_B1 or V_B3 responded to SEA. SEA enriched only V₈1-bearing T cells in BALB/c mice carrying Mls-2^a which lack Mls-1ª-reactive V_{β} 3-bearing Tcells as well as V_{β} 11- and V_{β} 12-bearing Tcells. In spite of the presence of V₆1-bearing T cells, C3H/He T cells exhibited a very low responsiveness to SEA. T cell repertoires skewed by clonal deletion of selfreactive Tcells may in part account for the different sensitivity to SEA among the different strains. A tolerance to SEA can be established in C57BL/6 mice which have been primed i.v. with SEA and treated i.p. with 200 mg/kg of cyclophosphamide 2 days later. All mature T cells bearing V₆3 or V₆11 were virtually abolished in the periphery of tolerant mice. These results suggest that most T cells reactive to SEA bear $V_{\beta}1, V_{\beta}3, V_{\beta}11$ or $V_{\beta}12$ and that clonal deletion of mature T cells reactive to SEA may account for the cellular mechanisms for cyclophosphamide-induced tolerance to SEA.

1 Introduction

Bacteria produce a variety of enterotoxins, many of which are involved in either pathogenesis or virulence. Staphylococcal enterotoxins (SE) are a family of molecules implicated in food poisoning and shock in the human, and weight loss and death in the mouse [1, 2]. SE comprise a group of five structurally related but serologically distinct proteins (A, B, C, D and E). Three forms of SEC (SEC1, SEC2, SEC3) have been described [1, 2]. A considerable structural homology is observed between SEB and SEC1, while less similarity exists between SEA and SEB [3, 4]. All SE are powerful Tcell mitogens like other mitogens that cause the activation and proliferation of T cells in the presence of accessory cells [5, 6]. SE are the most potent mitogens, stimulating human or murine T cells at a concentration of < 10⁻⁹ M efficiency and require cells with MHC class II molecules on their surface for presentation to T cells

The murine T cell response to SE, SEB, has recently been shown to involve only those T cells expressing TcR V₈3, 8.1 and 8.3 domains [8]. The specificity of the SE for human TcR with particular V_B elements has also been reported [9]. Self to SE. MHC class II-binding proteins encoded by Mls-1a or by Mls-2a have an avidity for VB6/VB8.1 or VB3 TcR,

Correspondence: Yasunobu Yoshikai, M. D. Department of Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812, Japan

Abbreviations: SC: Spleen cell SE: Staphylococcal enterotoxin(s) Mls: Minor lymphocyte stimulating

respectively [10-13]. A direct linkage between IE molecules and TcR V_B11 and V_B17a is also evident [14-16]. These antigens are called "superantigens" because no variable elements other than particular V_B are required for this peculiarly strong and specific reactivity.

A direct approach to assess the cellular basis of tolerance induction is now available by using the direct linkage between usages of a certain TcR and the reactivity to the "superantigens". MacDonald et al. have shown that neonatally induced tolerance to Mls-1a-encoded antigens was caused by clonal elimination of Mls-1a-reactive T cells in the thymus [17]. Similarly, White et al. [8] have provided evidence that the induction of tolerance to SEB in neonatal mice resulted in virtually a complete deletion of potentially SEB-reactive T cells. Many attempts have been made experimentally to induce tolerance in adult animals, including whole body irradiation [18, 19], total lymphoid irradiation [20], or anti-lymphocyte mAb application coupled with transplantation with donor cells [21, 22]. We have previously reported that a long-lasting skin allograft tolerance was established by a combination of i.v. injection of allogeneic spleen cells and i.p. injection of CY [23, 24]. In our case, clonal deletion of the potentially alloreactive T cells in the antigens in mice such as Mls and IE share properties similar thymus and periphery was also an essential mechanism for tolerant induction [25].

> In this study, in order to determine the molecular mechanisms of T cell activation by SEA, we examined the expression of TeR on the Tcells stimulated in vitro with SEA in mice of 4 different strains, using FCM and RNA blotting analysis. Our results show that the Tcell responses to SEA involve only those T cells expressing TcR V₈1, V₈3, V₈11 or V_B12 domains, a situation which closely mimics the responses to IE- or MIs-encoded antigens and suggests that T cell repertoires skewed by clonal deletion of "super-self

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antigen"-reactive T cells may account for the different sensitivity to SEA among different strains. Furthermore, the fact that induction of tolerance to SEA in mice treated with SEA and CY results in a virtual deletion of V_B3 and V₈11-bearing T cells confirms the existence of linkage between the usage of these TcR and reactivity to SEA.

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2 Materials and methods

2.1 Mice

Female 6- to 8-week-old C57BL/6, BALB/c and C3H/He mice, obtained from Japan SLC, Inc. (Hamamatsu, Japan), were used in this study. Female 6- to 8-week-old AKR/J mice were obtained from Seiwa Experimental Animal Institute (Fukuoka, Japan).

2.2 SEA

SEA was isolated and prepared by the methods of Oda [26]. Briefly, the culture SN of staphylococcus aureus 13N-2909. obtained from the Tokyo Metropolitan Research of Public Health through the courtesy of Dr. H. Igarashi, was centrifuged and applied to an SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatograph. The SEA was eluted with a pH gradient of pH 5 to pH 7.5 and the eluate was concentrated by Amicon UM 10 (Amicon Division, Lexington, MA). The concentrated SEA fraction was further purified by gel filtration on Sephadex G-75 (Pharmacia). The purified sample was shown as a single band by SDS-PAGE. SEA, stored as 2.7 CY-induced tolerant mice lyophilized powder at 4°C, was dissolved in saline.

2.3 Assay for proliferative responses

Proliferative activity was assessed by determining [3 H]dThd uptake into 1×10^{5} spleen cells (SC) which were incubated in quadruplicate either with or without mitogens. Three days later, 1 µCi = 37 kBq of [3H]dThd was added to each well, and the cells were incubated for an 3.1 Different sensitivity to SEA in mice with different additional 4 h before harvest.

2.4 Antibodies

The following antibodies were used for immunofluorescence staining: anti-V_B3 mAb (KJ25), anti- $V_{\beta}8.1 + 8.2 + 8.3$ (F23.1) and anti- $V_{\beta}8.1 + 8.2$ (KJ16; kindly provided by Dr. P. Marrack), anti-V_β6 mAb (44-22-1; kindly provided by Dr. H. Hengartner) [27] anti-V₆11 (KT11; kindly provided by Dr. K. Tomonari) [15], and PE-conjugated anti-CD4 mAb, FITC-conjugated 3.2 T cells bearing V₆1, V₆3, V₆11 and V₆12 respond to anti-CD8 mAb (Becton Dickinson, Mountain View, CA), FITC-conjugated goat anti-hamster IgG, FITC-conjugated IgG (Tago Inc., Burlingame, CA).

2.5 FCM analysis

SEA or Con A blast cells were prepared in the following way: SC were incubated at 1×106 cells/ml with SEA

(0.1 μg/ml) or Con A (3 μg/ml). Three days later, live cells were harvested and expanded for 3 days with 1 U/ml rIL2 (Takeda Chemical Industries, Ltd., Osaka, Japan). Each of the blast or LN cells was stained with FITC-conjugated goat anti-hamster IgG, FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-mouse IgG after treatment with KJ25, 44-22-1, F23.1, KJ16 or KT11, and analyzed with a FACScan (Becton Dickinson).

2.6 Northern blot analysis

Total cellular RNA was extracted from blast cells by the guanidinium thiocyanate and CsCl gradient centrifugation procedure and treated with glyoxal [28]. Twenty micrograms each of total RNA was electrophoresed through 1% agarose in 10 mm sodium phosphate buffer, pH 7.0, and transferred to Gene Screen Plus (NEN, Boston, MA). Hybridization was made to the ³²P-labeled V₈1 probe which was derived from β cDNA (RAB-14-25, 5'0.23-kb Eco RI-Bam HI fragment) [29], V₈5.2 probe from β cDNA (RAB7-48, 5'0.35-kb Eco RI-Alu I fragment) [29], V_B12 probe from βcDNA (RAB-14-90, 5'0.3-kb Eco RI-Hin fI fragment [29] and finally C_β2 probe from β cDNA (NYB2, 3'0.7-kb Eco RI fragment) [30]. After hybridization for 16 h at 65 °C in 1 M NaCl, 1% SDS, 10% dextran sulfate and 100 µg/ml heat-denatured salmon sperm DNA, filters were washed three times in 3 × SSC with 1% SDS at 65 °C and exposed to X-ray film at -70°C in the presence of intensifying

C57BL/6 mice were primed i.v. with 1 µg of SEA and treated i.p. with 200 mg/kg of CY (Endoxan, Shionogi Pharmaceuticals, Osaka, Japan) 2 days later. The day of CY injection is called day 0 here.

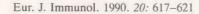
3 Results and discussion

genetic background

We examined the Tcell proliferation of SC from four inbred strains of mice in response to SEA. Unlike Con A, the minimal stimulatory concentration of SEA was as much as 1/100 lower and significant differences in Tcell responses to SEA were observed among the strains. The magnitude of T cell response to SEA occurred in the following order: C57BL/6 > AKR/J > BALB/c > C3H/He (Fig. 1).

SEA in vitro

goat anti-rat IgG and FITC-conjugated goat anti-mouse To determine the expression of TcR on SEA-reactive T cells, we stained the T cell blasts from C57BL/6, AKR/J and BALB/c mice proliferating in vitro in response to SEA with a set of mAb to various V_{β} elements. As shown in Fig. 2, SEA significantly enriched the T cells bearing V_B3 and V_B11 in the SC from C57BL/6 mice as compared to Con A. The percent of CD4+ and CD8+ cells was virtually unchanged before and after stimulation with SEA (data not



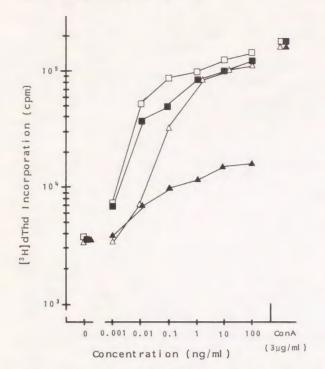


Figure 1. Response to SEA of SC from different mouse strains. SC from C57BL/6 (\square), AKR/J (\blacksquare), BALB/c (\triangle) or C3H/He (\blacktriangle) mice were stimulated with the indicated doses of SEA. Three days later, 1 μCi [3H]dThd was added to each well, and cells were incubated for an additional 4 h before harvest. Results shown are arithmetic mean [3H]dThd uptake (cpm) of quadruplicate cultures.

shown), V₈6- or V₈8-bearing Tcells were nearly undetected in the responding blast population although an appreciable level of such T cells responded to Con A in C57BL/6 mice. In AKR/J mice carrying IE^k, V_B11-bearing Tcells, which are reactive to IE, are eliminated in their pools of mature Tcells [16]. Therefore, only V_B3-bearing T cells were enriched by SEA in this strain. On the other hand, either V₈3 or V₈11-bearing T cells are absent in the mature T cell pool in BALB/c mice because of the super-self antigens including Mls-2a and IEd. However, significant levels of T cell proliferation in response to SEA were observed in the SC of

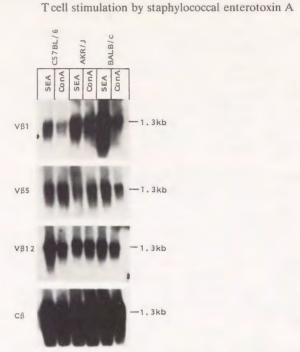


Figure 3. Northern blot analysis of SEA or Con A blast cells. SC of different mouse strains were incubated with either SEA (1 µg/ml) or Con A (3 µg/ml). Three days later, live cells were harvested. expanded for 3 days in 1 U/ml IL 2 and processed as described in

this strain at relatively high doses (Fig. 1). To further determine the expression of TcR V_B on T cells responding to SEA in BALB/c mice and other strains of mice, we examined the expression of TcR V_B genes in the T cell blasts, using Northern blot analysis with probes of specific for $V_{\beta}1, V_{\beta}5, V_{\beta}12$ or C_{β} . As shown in Fig. 3, the amount of V_β1-specific messages was most abundant in the Tcell blasts proliferating in response to SEA in BALB/c mice. The significant increase in the expression of V_B1-specific mRNA was also detected in the T cell blasts from AKR/J and C57BL/6 mice. In addition, V_B12-specific mRNA significantly increased in amount in the T cell blasts stimulated with SEA only from C57BL/6 mice. Similar to V₈5- and

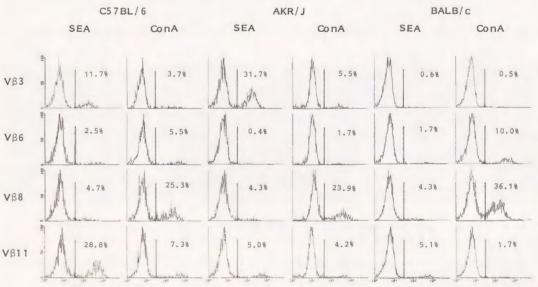


Figure 2. FCM analysis of SEA or Con A blast cells. SC of different mouse strains were incubated with either SEA (1 µg/ml) or Con A (3 µg/ml). Three days later, live cells were harvested and expanded for 3 days in 1 U/ml IL2. The blast cells were stained with a set of mAb to various V_B elements and analyzed.

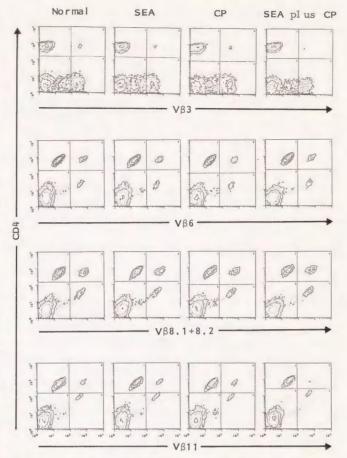
V₆11-bearing T cells, IE-expressing mice such as AKR/J, BALB/c and C3H/He mice contained few, if any, V_B12bearing T cells in their mature T cell pool [31]. Other V_β-specific messages including V_β5, V_β7, V_β9 and V_β10 seemed to remain unchanged or rather decreased inexpression in the T cell blasts stimulated with SEA (Fig. 3, data not shown). These results suggested that SEA stimulate T cells including those bearing V₆1, V₆3, V₆11 and V₆12.

3.3 Tolerance to SEA by CY was due to preferential destruction of SEA-reactive T cells in the periphery

We have previously established a tolerance to alloantigens in mice primed i.v. with allogeneic SC and treated i.p. with CY 2 days later [23, 24]. By using the same protocol, we attempted to induce tolerance to SEA in C57BL/6 mice. Table 1 shows the proliferative response of T cells to SEA in CY-induced tolerant mice. The degree of proliferative responses significantly decreased on day 6 after CY treatment and was restored to a normal level on day 18.

We examined the fate of $V_{\beta}3$ and $V_{\beta}11$ in the thymus and peripheral lymphoid organs in tolerant mice. As shown in Fig. 4, nearly all V_B3 and V_B11-bearing Tcells were virtually eliminated in LN of C57BL/6 mice rendered CY-induced tolerant to SEA on day 6, and the number of these T cells recovered on day 18 in correlation with the restoration of T cell reactivity against SEA. On the other hand, the substantial level of thymocytes bearing a high density of V₆3 and V₆11 elements was detected in the thymus of the tolerant C57BL/6 mice (data not shown). These results suggest that a preferential destruction of SEA-reactive T cells in the periphery occurred in mice rendered CYinduced tolerant to SEA, and also confirmed the linkage between V₈3 and V₈11 usages and SEA reactivity.

It is of interest that the similarity between the T cell response to SEA and to the elusive super-self antigens including IE- and MIs-encoded antigens. The usage of V₈3 or V₈11 domains is directly linked to the reactivity to the products encoded by Mls-2a or IE, respectively [10-13, 17], and tolerance-induced deletion of T cells because of carrying the super-self antigens can lead to the absence of V_{β} 3 and V_{β} 11 [11, 17, 32]. An appreciable level of T cells



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Figure 4. Expression of TcR V₆ elements in LN of C57BL/6 mice rendered tolerant to SEA by CY. C57BL/6 mice were primed i.v. with 1 µg of SEA and treated i.p. with 200 mg/kg of CY 2 days later. LN cells on day 6 after CY treatment were stained with a set of mAb to various V₆ elements, and PE-conjugated anti-CD4 mAb and analyzed.

bearing V_B3 and V_B11 was present in the starting population in C57BL/6 carrying Mls-2b and no IE molecules. C57BL/6 SC exhibit the greatest response to SEA among those in the four strains of mice. In AKR/J. BALB/c and C3H/He mice. T cells bearing V_B11 are absent in the mature T cell pool because of the presence of super-self antigen, IE. In

Table 1. Proliferative responses to SEA or Con A and expression of TcR V_{β} elements in C57BL/6 mice rendered tolerant to SEA

Treated with	Days after CY injection		Proliferative responsesa)		Expression of TcR elements ^{b)} V _B		
		Medium	SEA	Con A	$V_{\beta}3$	$V_{\beta}8$	V _β 11
			(cpm ± SD)			(%)	
Normal		3730 ± 314	121 597 ± 1580	170 906 ± 13 635	3.4	18.2	6.6
SEA + CY ^{c)}	6	3210 ± 124	12 598 ± 1086	176434 ± 4065	2.1	20.8	3.6
	12	3766 ± 193	47 331 ± 3086	179 198 ± 16547	2.1	21.5	2.9
	18	4019 ± 357	84 937 ± 3097	180 383 ± 9 591	4.0	18.9	6.5

a) SC were incubated for 72 h with 0.1 μg/ml of SEA or 3 μg/ml of Con A. The proliferative response is expressed as arithmetic mean [3H]dThd uptake (cpm) ± SD) of quadruplicate cultures. See legend to Fig. 1 for more details.

addition to deletion of V₆11-bearing T cells, BALB/c and C3H/He mice, both carrying Mls-2a, delete V_β3-bearing T cells. Consistent with the deletion of V_B3- or V_B11bearing T cells capable of recognizing SEA, BALB/c and C3H/He mice are less susceptible to the effects of SEA than C57BL/6 mice as assessed by T cell proliferation in vitro. The different sensitivity to SEA among different strains seems to be mainly due to the differences in T cell repertoires skewed by tolerance-induced deletion of Tcells expressing V₈3 or V₈11 in the mature T cell pool because of super-self antigens. The V₈12-bearing T cells, which are only a few, if any, in mature T cell pool of IE-expressing mice, were also stimulated with SEA in C57BL/6 mice. At present, the ligands to V_B12 are not known although it is speculated that the ligand is one of the super-self antigens such as IE [31]. Further studies using recombinant inbred strains are required to elucidate the ligand to V_B12. In spite of the presence of V_B1-bearing T cells, C3H/He SC exhibited very low response to SEA as compared with BALB/c. Recently, Vroegop and Buxser [33] have shown evidence that BALB/c SC were better responders to SEB than C57BL/6 SC, unlike SEA. This might be attributed to the result of a preference of SEB for IE. Although both IE- and IA-like molecules appear to be used in SEA-induced Tcell proliferation, the binding capacity of SEA to MHC class II molecules may also influence the sensitivity to SEA among the various strains.

4 Concluding remarks

We have shown that the stimulation of murine T cells in vitro with SEA enriches Teells carrying V₆1, V₆3, V₆11 and V₈12. If the SEA is injected into adult mice in combination with CY, T cells bearing $V_{\beta}3$ or $V_{\beta}11$ were eliminated from the mature T cell pool in the periphery. Taken together, the target structure of SEA for Tcells is the V region of clonally distributed TcR.

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b) Results shown are gated on CD4+ cells in LN cells.

c) C57BL/6 mice were primed i.v. with 1 µg of SEA and treated i.p. with 200 mg/kg of CY 2 days later. The day of CY injection is called day 0.

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