Inhibitory Effect of (4*R*)-Hexahydro-7,7-Dimethyl-6-Oxo-1,2,5-Dithiazocine-4-Carboxylic Acid (SA3443), a Novel Cyclic Disulfide, on the Production of TNF-Like Factor from *Propionibacterium Acnes*-Primed Rat Liver Macrophages/Kupffer Cells

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ABSTRACT — The effect of (4*R*)-hexahydro-7,7-dimethyl-6-oxo-1,2,5-dithiazocine-4-carboxylic acid (SA3443), a novel cyclic disulfide, on tumor necrosis factor (TNF)-like factor production from *Propionibacterium acnes* (P. acnes)-primed rat liver macrophages/Kupffer cells was investigated. A remarkable increase in TNF-like activity was detected in the culture supernatants of the liver macrophages/Kupffer cells from P. acnes-treated rats. At concentrations of 1×10^{-6} to 1×10^{-4} M, SA3443 significantly inhibited the production/release of TNF-like factor from these P. acnes-primed/activated liver macrophages/Kupffer cells.

Keywords: SA3443, Tumor necrosis factor (TNF)

(4*R*)-Hexahydro-7,7-dimethyl-6-oxo-1,2,5-dithiazocine-4-carboxylic acid (SA3443) is a novel cyclic disulfide compound that provides considerable protection against chemically-induced (1, 2) and immunologically-induced liver injuries (3, 4). SA3443, at doses of 100 to 300 mg/kg, p.o., significantly reduced the lethal acute hepatic failure induced by an injection of lipopolysaccharide (LPS) into BALB/c mice that had been previously treated with heat-killed *Propionibacterium acnes* (P. acnes) (3). SA3443 at concentrations of 1×10^{-6} to 1×10^{-4} M suppressed the production and/or release of interleukin-1 (IL-1)-like lymphocyte-activating factor (LAF) from the liver macrophages/Kupffer cells of P. acnes-pretreated rats (3). Therefore, it is suggested that the efficacy of SA3443 on the mouse liver injury model might be related to an inhibition of cytokine production in the liver macrophages/Kupffer cells.

On the other hand, it is also known that P. acnes-priming followed by LPS treatment causes tumor necrosis factor-alpha (TNF-α) production (5–7). TNF-α is mainly released from activated mononuclear cells or macrophages, and it has a wide range of biological activities, including a role in the synthesis of acute-phase reactants in the liver. It is reported that TNF-α released from activated liver macrophages/Kupffer cells may play a crucial role in the pathogenesis of the P. acnes-LPS-induced murine hepatitis model (7). In fact, when injected into mice, TNF can cause liver cell necrosis (8).

In the present study, therefore, we investigated the effect of SA3443 on the production and/or release of TNF-like factor from the liver macrophages/Kupffer cells of P. acnes-pretreated rats.

Male Wistar rats, weighing 200 to 300 g, were used for the preparation of liver macrophages/Kupffer cells. SA3443 was synthesized at Santen Pharmaceutical Co., Ltd. (Osaka, Japan). *Propionibacterium acnes* (P. acnes) and lipopolysaccharide (LPS) were obtained from Wellcome Biotechnology, Ltd. (Beckenham, U.K.) and Difco (Detroit, Michigan, U.S.A.), respectively. All other chemicals were of the commercially available reagent grade.

The preparation and culture of liver macrophages/Kupffer cells from P. acnes-treated rats were carried out according to the method used by Munthe-Kass et al. (9). In brief, rats that had been injected

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with P. acnes (10 mg/rat) 7 days before were anesthetized with pentobarbital sodium. The abdomen was then opened, and a nylon cannula inserted into the portal vein. The vena cava was immediately transected and the blood drained. The liver was first perfused with 20 ml Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS), then with 20 ml HBSS containing 0.25% collagenase and 5 mM Ca^{2+} (37°C), and finally with 20 ml HBSS containing 0.1% pronase E (37°C). During these treatments, the parenchymal cells were selectively destroyed. The liver cell suspension was filtered through gauze to remove the debris. The suspension was then centrifuged at 500 rpm for 3 min, and the supernatant obtained was further centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended and the suspension was washed 4 times by centrifugation. Finally, the cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS) seeded in plastic petri dishes and incubated for 1 hr at 37°C. Adherent cells were obtained and seeded into 24-well tissue culture plates at 3×10^6 cells/well. They were cultured either with or without SA3443 for 20 hr. The medium was then changed and the cells cultured with LPS for another 20 hr. Culture supernatants were assayed for TNF activity.

TNF-like activity was measured using an inhibition assay for L-M cell proliferation (10). Briefly, L-M cells were suspended at a concentration of 5×10^4 cells/ml in RPMI-1640 medium containing 5% FCS and antibiotics. An aliquot (100 μl) of the L-M cell suspension was then added to each well (5×10^3 cells/well) of the 96-well microtest plate and incubated for 48 hr at 37°C with a 100-μl aliquot of diluted culture sample. Cell proliferation was assayed by measuring the incorporation of tritiated thymidine ([^{3}H]-TdR 0.5 μCi/well; Amersham Radiochemical, Amersham, U.K.) during the last 8 hr of incubation. The L-M cells were harvested onto a glass fiber filter disc, and the radioactivity (cpm) of incorporated [^{3}H]-TdR was measured in a standard liquid scintillation counter. The data are presented as the mean (TNF U/ml) ± S.D. calculated from the standard curve of recombinant human TNF, and they were analyzed by William's t-test for comparing multiple groups.

As shown in Fig. 1, when the liver macrophages/Kupffer cells from P. acnes-treated rats were cultured without LPS, a significant ($P < 0.001$) increase in TNF-like activity was detected in the supernatants, compared with the activities in the culture supernatant of the same cells from normal rats. The TNF-like activities in the liver macrophages/Kupffer cells from normal rats were dose-dependently increased by the addition of LPS (0.04 to 5.0 μg/ml). However, the TNF-like activities in the liver macrophages/Kupffer cells from P. acnes-treated rats did not increase further after additional stimulation with LPS.

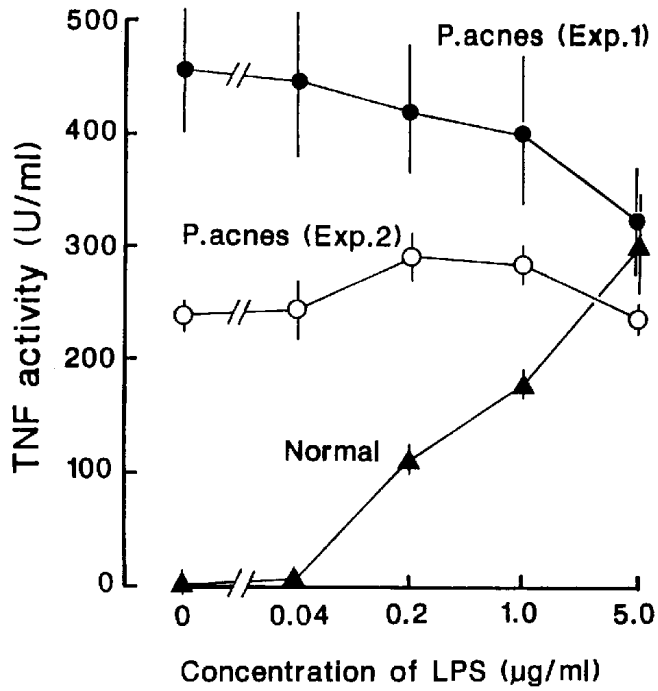

Fig. 1. Production of TNF-like factor from rat liver macropghages/Kupffer cells. Macrophages/Kupffer cells were cultured for 20 hr. The culture supernatants were assayed for TNF-like activity. Each point represents the mean ± S.D. of triplicate cultures.

As shown in Table 1, SA3443 at concentrations of 1×10^{-6} to 1×10^{-4} M significantly suppressed the increase in production and/or release of TNF-like factor in the culture supernatants from the liver macrophages/Kupffer cells of P. acnes-treated rats in the absence of LPS, although the suppression in these experiments did not show constant dose-dependence. The minimal effective concentration and the most effective concentration of SA3443 might be very close. Only slight suppression by SA3443 was observed in the presence of LPS (1.0 μg/ml), compared with that in the absence of LPS.

Recently, TNF-α, IL-1 and IL-6 have been selected for study, because many of their biological effects parallel the clinical and biochemical abnormalities found in patients with both acute and chronic liver diseases (11–14). These studies suggest that the over-secretion of cytokines such as TNF, IL-1 and IL-6 may play a significant role in the pathogenesis and perpetuation of both acute and chronic liver diseases. In particular, it is strongly suggested that TNF is involved in several liver diseases, namely, chronic active hepatitis, acute alcoholic hepatitis and alcoholic cirrhosis (11–14).

In previous studies, SA3443 significantly reduced the

Table 1. Effect of SA3443 on the production of TNF-like factor in the culture supernatant from P. acnes-primed liver macrophages/Kupffer cells

	Concentrations of SA3443		TNF activity (U/ml)	
			without LPS	with LPS (1 μg/ml)
Exp. 1	Control	–	240.2 ± 6.2	283.5 ± 17.2
	SA3443	1×10^{-6} M	283.3 ± 25.9	309.0 ± 45.1
		1×10^{-5} M	7.0 ± 0.2**	123.0 ± 12.5*
		1×10^{-4} M	58.2 ± 2.7**	198.4 ± 31.8
Exp.2	Control	–	461.9 ± 62.4	400.3 ± 71.0
	SA3443	1×10^{-6} M	281.8 ± 44.3**	216.4 ± 21.3**
		1×10^{-5} M	279.8 ± 43.9**	337.0 ± 28.1
		1×10^{-4} M	260.7 ± 62.4**	316.2 ± 20.6

Macrophages/Kupffer cells of the liver treated with P. acnes were cultured with SA3443 for 20 hr. Each value represents the mean ± S.D. of triplicate cultures. *$P < 0.05$, **$P < 0.01$, significantly different from the control.

mortality of mice with acute hepatic failure induced by sequential injections of P. acnes and LPS (3). Furthermore, SA3443 also reduced the production and/or release of IL-1-like LAF from the liver macrophages/Kupffer cells of P. acnes-primed rats (3). Thus, these results suggested that inhibition of cytokine (including IL-1 and TNF) production by SA3443 might be, in part, involved in its inhibitory effect on the lethal hepatic injury induced by P. acnes and LPS.

In this study, we have investigated whether the increased production and/or release of TNF-like factor from liver macrophages/Kupffer cells could be suppressed by the pretreatment of SA3443 in vitro. The results showed that SA3443 significantly suppressed the production and/or release of TNF-like factor from the liver macrophages/Kupffer cells of P. acnes-pretreated rats.

In addition, SA3443 inhibited the antibody forming cell response by inhibiting macrophages/monocytes activity (15). These findings suggested that SA3443 might provide a protective effect against immunological liver injuries through, in part, the inhibition of liver macrophage/Kupffer cell activities.

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