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Suppression of lipopolysaccharide-induced fulminant hepatitis and tumor necrosis factor production by bisbenzylisoquinoline alkaloids in bacillus Calmette–Guerin-treated mice

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Abstract—The bisbenzylisoquinoline (BBI) alkaloids chondocurine, cycleanine, tetrandrine and berbamine were tested for their capacity to suppress hepatic injury and production of tumor necrosis factor (TNF) induced by lipopolysaccharide (LPS) in mice primed with bacillus Calmette–Guerin (BCG). When administered for three consecutive days before LPS injection, chondocurine, cycleanine and tetrandrine (10 mg/kg/day) strongly suppressed serum alanine aminotransferase (EC 2.6.1.1.) and aspartate aminotransferase (EC 2.6.1.2.); however, berbamine gave only slight protection. Chondocurine, cycleanine and tetrandrine but not berbamine significantly reduced the level of TNF which peaked 2 hr after LPS injection. This study shows that BBI alkaloids prevent BCG/LPS-induced hepatitis at least in part by suppressing TNF production.

Some bisbenzylisoquinoline (BBI*) alkaloids have been used as folk remedies for various diseases including rheumatism in Japan and China. We reported previously that the BBI alkaloids chondocurine and cycleanine prevented lipopolysaccharide (LPS)-induced acute lethal toxicity in bacillus Calmette–Guerin (BCG)-primed mice [1]. In this BCG/LPS model a large amount of tumor necrosis factor (TNF) was induced in a few hr [2] and this was rapidly followed by severe hepatitis [3]. As it is known that TNF causes fulminant hepatitis in combination with galactosamine [4], we examined the effect of BBI alkaloids on BCG/LPS-induced hepatitis and TNF production.

Materials and Methods

Reagents. BBI alkaloids: chondocurine was obtained from E. Merck and berbamine and cycleanine were from the Kaken Drug Co. (Tokyo, Japan). Tetrandrine was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). LPS (*Escherichia coli* 055:B5) was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). BCG was obtained from the Nippon BCG Co. (Tokyo, Japan). BBI alkaloids and BCG were administered in pyrogen-free aqueous solution.

Animals. Male ICR albino mice were purchased from Japan SLC (Shizuoka, Japan) and they were used for experiments at 6 weeks of age. Mice were maintained on food and water *ad lib.*

Assay for the activities of aminotransferases and TNF. Activities of alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) were measured using a commercial transaminase assay kit (Transaminase C-II test, Wako Pure Chemicals, Tokyo, Japan). TNF activity was measured using TNF-sensitive LM cells as described by Flich and Gifford [5]. In brief, serially diluted serum samples and 10^4 LM cells suspended in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 0.5% fetal bovine serum (Gibco) were cultured on a 96-well flat-bottomed plate (37°, 5% CO_2, 48 hr). The plate was washed with PBS and the remaining viable cells were stained with 0.5% methylene blue for 15 min at room temperature. After washing the stained cells were solubilized with detergent. Absorbance of dye was read at 590 nm on a microplate reader (Japan Intermed, Tokyo, Japan). The titer of TNF in sera was defined as a reciprocal dilution of serum causing 50% cell toxicity. All values were expressed as means ± SE and data were analysed according to Student's *t*-test.

Table 1. Dose-dependent suppression by chondocurine of serum GTP and TNF in BCG/LPS-treated mice

Chondocurine (mg/kg/day)	GPT (U/mL)	N	TNF (U/mL)	N
0	1017.4 ± 240.2	9	13,510 ± 1172	10
1	730.0 ± 211.4	5	8640 ± 1433	6
5	515.3 ± 102.3*	8	5683 ± 792*	7
10	367.4 ± 120.2*	8	3126 ± 326*	8

ICR mice were primed i.v. with 1 mg of BCG, and 7 days later, 10 μg of LPS was challenged i.v. Chondocurine was injected i.p. once a day (at 10:00 a.m.) for 3 consecutive days before LPS challenge (the final treatment was carried out 1 hr before LPS injection). Two (for TNF assay) or eight (for GPT assay) hours after LPS injection, animals were killed to obtain blood, to measure GPT and TNF activities.

Data were expressed as means ± SE. Significantly different from control, *$P < 0.01$.

Results and Discussion

As shown in Fig. 1A, an i.v. injection of LPS increased the serum activity of GPT and GOT, markers for hepatic cell damage, within 4 hr in BCG-primed mice. When chondocurine was injected i.p. at 10 mg/kg/day for 3 consecutive days before LPS injection, the serum level of GPT and GOT was greatly reduced throughout the experimental period. The suppression of GPT activity by chondocurine occurred in a dose-dependent manner (Table 1). Of the BBI alkaloids tested, cycleanine and tetrandrine as well as chondocurine strongly prevented the rise in serum level of GPT and GOT (data not shown) at a dose of 10 mg/kg/day; however, berbamine was less effective (Table 2).

After LPS injection of BCG-primed mice the TNF activity in the serum began to increase within 60 min, reached a peak at 120 min and declined toward normal in 180 min (Fig. 1A). Treatment with chondocurine at 10 mg/kg/day significantly reduced TNF activity at 60 min and 120 min after LPS injection, but berbamine did not

* Abbreviations: BCG, bacillus Calmette–Guerin; BBI, bisbenzylisoquinoline; FBS, fetal bovine serum; GOT, aspartate aminotransferase; GPT, alanine aminotransferase; IL-1, interleukin-1; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

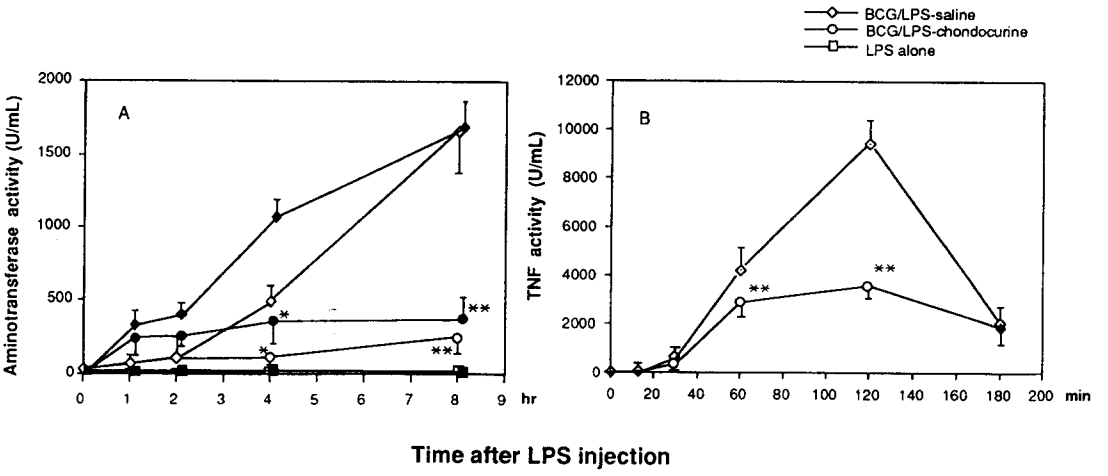

Fig. 1. Time-dependent change in the activities of GPT and GOT (A) and TNF (B) in serum after chondocurine treatment of BCG/LPS-treated mice. ICR mice were primed i.v. with 1 mg of BCG, and 7 days later, 10 μg of LPS was challenged i.v. Chondocurine at 10 mg/kg/day was injected i.p. once a day for 3 consecutive days before LPS challenge. For obtaining blood samples animals were killed at the indicated times after LPS injection. Enzyme activities of GPT (open symbol) and GOT (closed symbol) and TNF content in individual serum samples were measured. Data were expressed as means ± SE. Significantly different from BCG/LPS control group, $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 2. Effect of BBI alkaloids on serum levels of GTP and TNF in BCG/LPS-treated mice

Treatment		GPT	TNF	
BCG/LPS	BBI alkaloids	(U/mL)	(U/mL)	N
—	—	15.2 ± 1.5	ND	4
LPS alone	—	21.1 ± 3.8	ND	4
BCG/LPS	—	1480.6 ± 119.1	6310.0 ± 630.2	10
BCG/LPS	Chondocurine	263.6 ± 71.1†	1786.6 ± 186.1†	8
BCG/LPS	Cycleanine	255.6 ± 14.8†	2446.3 ± 156.8†	8
BCG/LPS	Tetrandrine	420.8 ± 90.0†	3496.5 ± 630.7*	8
BCG/LPS	Berbamine	727.5 ± 198.4*	5780.0 ± 233.5	8

ICR mice were primed i.v. with 1 mg of BCG, and 7 days later, 10 μg of LPS was challenged i.v. BBI alkaloids at 10 mg/kg/day were injected i.p. once a day (at 10:00 a.m.) for 3 consecutive days before LPS challenge. Blood samples were collected and GPT and TNF activities measured as described in Table 1.

Data were expressed as means ± SE. Significantly different from BCG/LPS control group, $^{*}P < 0.05$ and $^{\dagger}P < 0.01$. ND, not detected.

(Fig. 1B). Injection of 1 mg of BCG alone or 10 μg of LPS alone induced no detectable TNF activity in the serum (data not shown). A concentration-dependent suppression of TNF production by chondocurine was observed at 120 min post LPS triggering (Table 1). In addition to chondocurine, cycleanine and tetrandrine also significantly suppressed the increase in serum TNF level in BCG/LPS-treated mice, but berbamine did not (Table 2). Recently, Seow *et al.* [6] reported that tetrandrine inhibited the production of TNF and IL-1 in macrophage cultures *in vitro*. The present results show that BBI alkaloids potently inhibit TNF production *in vivo*.

Animals treated with microorganisms such as BCG and Corynebacterium were sensitive to LPS, which caused lethal toxicity with fulminant hepatitis [7]. TNF is one of the mediators of LPS-induced lethal toxicity as passive immunization against TNF partially protected mice [8], and a submicrogram of human TNFα can substitute for LPS in causing severe hepatitis in galactosamine-treated mice [9]. However, the relationship between TNF production and occurrence of severe hepatitis in BCG/LPS-treated animals has not been made clear. In the present study, it is shown that the three BBI alkaloids chondocurine, cycleanine and tetrandrine which suppressed TNF production also protected against the release of hepatic aminotransaminases into the serum in BCG/LPS-treated mice (Table 2). Since TNF production preceded release of GPT and GOT from the liver (Fig. 1), the protective action of the BBI alkaloids appears to come at least in part from suppression of TNF production.

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