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Modulation of macrophage function activity by ethanolic extract of larvae of *Holotrichia diomphalia*

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Abstract

Macrophages play a central role in determining the host response to tumor, in part through the secretion of several potent products. In this study, our work is directed toward studying the in vitro effects of extract from the larvae of *Holotrichia diomphalia* (HD-EX) on the ability to induce cellular and secretory responses in mouse peritoneal macrophages. Macrophages were treated with various doses (0.1, 1, 10 μg/ml) of HD-EX for 20 h. This treatment induced tumoricidal activity and increased the production of tumor necrosis factor (TNF-α) and nitric oxide (NO) by macrophages in a concentration-dependent manner. However, HD-EX had a little effect on phagocytosis and the levels of hydrogen peroxide (H_2O_2), interleukin-1 (IL-1), IL-6 and IL-10 were very low in HD-EX treated macrophages. Thus, the tumoricidal effect of HD-EX appeared to be mainly mediated by NO and TNF-α production from macrophages. Taken together, these results suggest that HD-EX is a differential immunomodulating effect on macrophage secretory and cellular activities. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Holotrichia diomphalia*; Immmunomodulatory activity; Macrophage activation

1. Introduction

A number of natural products are used in the traditional medical systems in many countries. *Holotrichia diomphalia* Bates has been traditionally used to prevent and treat many kinds of diseases such as liver cirrhosis, contusion, edema, furuncle and apoplexy in Korea. This clinical use possibly arises from the induction of immune response, although the precise pharmacological mechanisms of *H. diomphalia* are still obscure.

Macrophages have been shown to play an essential role in host defenses against microbial agents and neoplasia (Hahn and Kaufmann, 1981; Verstovsek et al., 1992). Macrophages can be stimulated by various agents including IFN-γ, lipopolysaccharide, or other microbial products (Dullens et al., 1989; Gautam and Deodhar, 1989; Paulnock and Lambert, 1988) and some of these have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and nitrite and to induce tumoricidal activity in macrophages (Arden et al., 1985; Arenzana-Seisdedos and Virelizier, 1983; Choriki et al., 1989; Stuehr and Marletta, 1987; Keller et al., 1990).

This study was undertaken to investigate the biological effects of an ethanolic extract from larvae of *H. diomphalia* on murine peritoneal macrophages.

2. Materials and methods

2.1. Mice, chemicals and reagents

C57BL/6 mice (6–8 weeks) were obtained from Charles River Breeding Laboratories (Atsugi, Japan). Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). RPMI 1640 medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY). NG-monomethyl-L-arginine (NMMA) was obtained from Calbiochem Co. (LaJolla, CA). All tissue culture

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reagents, the thioglycollate broth and extracts were assayed for endotoxin contamination by the Limulus lysate test (E-Toxate, Sigma) and found to be less than 10 pg/ml.

2.2. Preparation of extract

H. diomphalia (1 kg) was purchased at the herbal drug market in Cheju-Do, Korea in August 1999 and identified by Dr B.G. Lee of the Institute for Traditional Medicine, Sungkyunkwan University, Suwon, Korea. A voucher specimen (SKK-H001) is deposited in the College of Pharmacy at Sungkyunkwan University. One kilogram of *H. diomphalia* was refluxed with 70% ethanol (2 litre) two times for 8 h. The materials were filtered and the clear supernatants were then concentrated under reduced pressure at 40 °C with vacuum rotary evaporator. The concentrated ethanol extract (100 g) was partitioned between water (1 litre) and *n*-hexane (1 litre, $\times 2$). After removing the *n*-hexane fraction, the aqueous layer was partitioned again with methylene chloride (1 litre, $\times 2$), followed by *n*-butanol (1 litre, $\times 2$).

2.3. Isolation of inflammatory peritoneal macrophages

Thioglycollate-elicited peritoneal exudate cells were obtained from 6–8 week-old C57BL/6 male mice following intraperitoneal injection of 1 ml Brewer Thioglycollate broth (4.05 g/100 ml) (Difco Laboratories, Detroit, ML) and lavage of the peritoneal cavity with 5 ml of medium 3–4 days later. The cells were washed twice and resuspended in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (RPMI-FBS). Macrophages were isolated from peritoneal exudate cells as described by Klimetzek and Remold (1980). Peritoneal exudate cells were seeded at densities of $5–6 \times 10^5$ cells/cm^2 on Teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2–3 h in 5% CO_2 humidified atmosphere. Teflon-coated petri dishes were prepared by spraying with aerosolized Teflon (Fisher Scientific, Pittsburgh, PA) and sterilizing with ultraviolet light for 3 h. The nonadherent cells were removed by washing the dishes twice with 10 ml prewarmed medium and dishes were incubated for 10 min at 4 °C. The supernatants were then carefully removed and discarded and the plates were washed once with prewarmed Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO). Cold PBS (15 ml) containing 1.5% FBS (PBS–FBS) was added followed by 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages removed by rinsing 10 times using a 10 ml syringe. The viability of the detached cells was assessed by trypan blue exclusion and proportion of macrophages determined after cytoplasmic staining with acridine orange and examination using a fluorescence microscope. Cell preparations were $>95\%$ viable and contained $>95\%$ macrophages.

2.4. Macrophage-mediated cytotoxicity

The assay for macrophage cytotoxicity was performed by modification of the technique described previously (Flick and Gifford, 1984; Moon et al., 1999). Briefly, macrophages (1.0×10^5 cells/well) were plated in 96 well microplates and co-incubated with B16 melanoma cells (1.0×10^4/wells: an initial effector:target cell ratio of 10:1) in the presence of various doses of HD-EX for 20 h at 37 °C in a 5% CO_2 incubator. In some experiments, antibody to cytokine, the isotype-matched control antibody or inhibitor of metabolic pathway was included. At the extract dosages that we employed, no toxicity was observed. Cell density was then assessed by incubating the cells with 25 μg/ml MTT (3-(4,5-dimethylthiaozle-2-yl)-2,5-diphenyltetrazolium bromide) for another 4 h. Formazan produced was dissolved in dimethyl sulfoxide and the optical density of each well at 540 nm was determined using a molecular device microplate reader (Menlo Park, CA). Cytolytic activity is expressed as the percentage of tumor cytotoxicity where % cytotoxicity = $\{1-[\text{OD of (macrophages + target cells)} - \text{OD of macrophages}]/\text{OD of target cells}\} \times 100$.

2.5. Determination of phagocytosis

The phagocytic activity was measured using the assay system described previously (Okimura et al., 1986). Macrophages were treated with indicated concentration of HD-EX for 20 h and then washed with RPMI1640 to remove HD-EX. Cells were incubated with 5×10^6 particle of zymosan and 600 μg/ml of NBT. After 1 h incubation, plates were centrifuged at 4 °C for stopping ingestion of zymosan and supernatant was removed by flipping. The optical density of the reduction product of NBT, a purple insoluble formazan, was determined at 540 nm using a molecular device microplate reader. It was not required to solubilize the formazan before taking the measurement of absorbance.

2.6. Nitrite determination

The accumulation of NO_2^- in culture supernatants was measured using the assay system described by Ding et al. (1988). Briefly, 100 μl of supernatant was removed from each well into an empty 96-well plate. After the addition of 100 μl Griess reagent to each well, absorbance at 550 nm was measured using a molecular device microplate reader. NO_2^- concentration was cal-

culated from a $NaNO_2$ standard curve. The levels of NO_2^- are indicative of NO production. Griess reagent was prepared by mixing one part of 0.1% naphthylethylene diamine dihydrochloride in distilled water plus one part of 1% sulfanilamide in 5% concentrated H_3PO_4.

2.7. Hydrogen peroxide determination

The secretion of hydrogen peroxide in culture supernatants was fluorimetrically measured by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin to a nonfluorescent product, as described in detail (Nathan and Root, 1977). The 460 nm emission from reduced scopoletin, when excited by light at 350 nm, is extinguished when scopoletin is oxidized by H_2O_2 in the presence of horseradish peroxidase. Under assay conditions, the loss of fluorescence was proportional to the concentration of H_2O_2.

2.8. Cytokine determination by ELISA

The concentration of IL-1, IL-6, IL-10 and TNF-α production in culture supernatants was determined by the use of ELISA kits (Endogen, Woburn, MA) according to the manufacturer's protocol.

3. Statistical analysis

The significance of differences between control and treated test was analyzed using Student's t-test. Differences were considered significant at $P < 0.05$ and significant values were represented by an asterisk. All experiments were repeated at least three times. Data are expressed as means ± SEM.

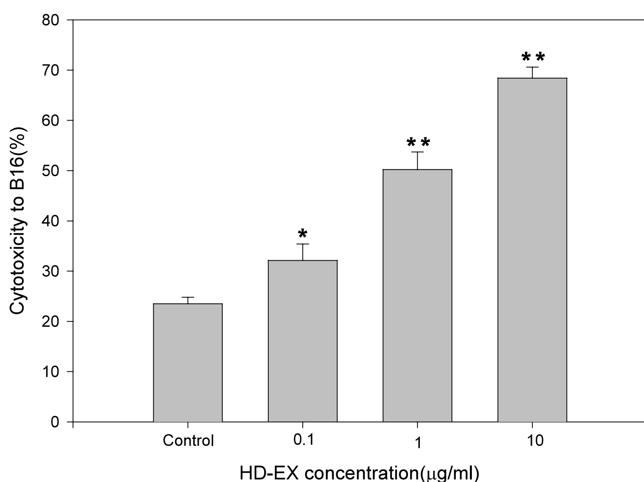

Fig. 1. Tumoricidal activities of HD-EX-treated murine peritoneal macrophages against B16 melanoma cells. Macrophages were treated with various doses of HD-EX for 20 h. Tumoricidal activity was determined as described in Section 2 at an initial effector/target ratio of 10:1. The results are mean ± SEM of quintuplicates from one representative experiment of three. $*P < 0.05$, $**P < 0.01$; significantly different from control (no treatment).

4. Results

4.1. HD-EX-triggered macrophages activation for tumor cytotoxicity

The effect of HD-EX was evaluated for tumoricidal activity in vitro of macrophages against B16 tumor cells. Thioglycollate-elicited macrophages were co-cultured with B16 tumor cells in the presence of various doses of HD-EX for 20 h. B16 tumor cells were used as targets since they are either TNF-α or NO sensitive. HD-EX (0.1–10 μg/ml) increased the cytotoxicity by macrophages in a dose-dependent manner (Fig. 1). The maximum effect was obtained using 10 μg/ml of HD-EX. This amount did not affect the viability of macrophages, but concentrations greater than 10 μg/ml were cytotoxic to cells (data not shown).

4.2. Effect of HD-EX on NO and TNF-α secretion by macrophages

Once activated, tumoricidal macrophages produce a large number of cytotoxic molecules (Sone and Key, 1986; Hibbs et al. 1987). We next examined the ability of HD-EX to induce NO and TNF-α, which are currently believed to be the primary species involved killing tumor cells. HD-EX was observed to activate NO and TNF-α production by peritoneal macrophages in a dose-dependent manner. We found that pretreatment with HD-EX significantly stimulated NO and TNF-α production by inflammatory macrophages compared with the response of untreated macrophages (Fig. 2a and b). In addition, anti-TNF-α antibody and the NO inhibitor were able to abrogate, in part, the production of cytotoxic molecules induced by HD-EX in the culture supernatants (data not shown). These results further demonstrate that the level of NO and TNF-α production in HD-EX-treated macrophages was significantly increased. Moreover, the addition of anti-TNF-α antibody and the NO inhibitor was able to abrogate, in part, tumoricidal activities of HD-EX-exposed macrophages against target (Table 1). At the concentrations employed none of the inhibitors or antibodies affected the growth of the tumor cells and the isotype-matched control antibodies had no effect on cytotoxic activity (data not shown). Taken together these results suggest that TNF-α, and NO are partially involved in the tumoricidal activity of HD-EX-exposed macrophages.

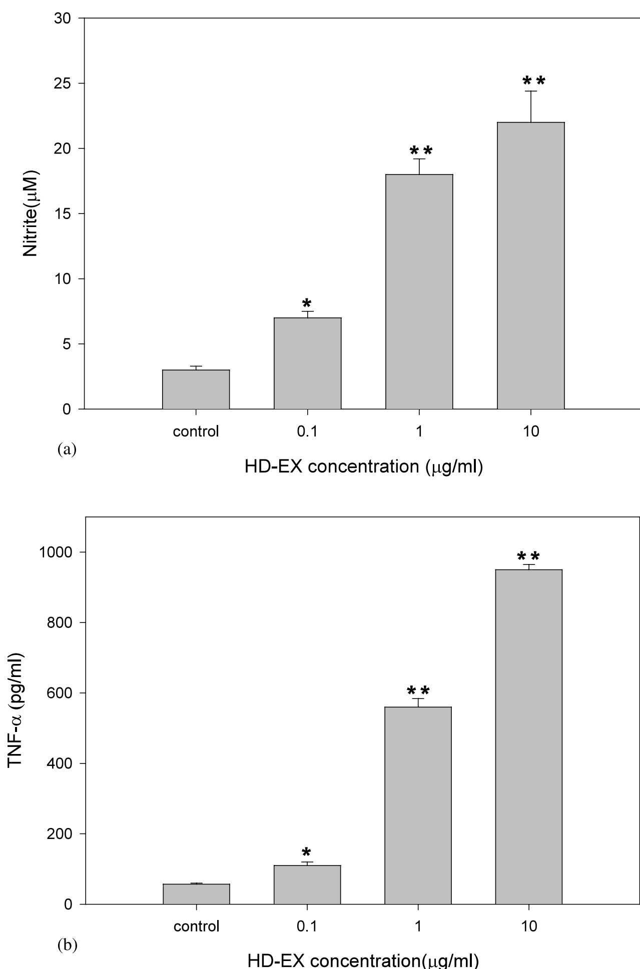

Fig. 2. (a) Nitrite and (b) TNF-α production from peritoneal macrophages stimulated with HD-EX. Macrophages were treated with HD-EX for 20 h. Culture supernatants were collected and the levels of nitrite and TNF-α were measured as described in Section 2. The results are mean ± SEM of quintuplicates from one representative experiment of three. $*P < 0.05$, $**P < 0.01$; significantly different from control (no treatment).

Table 1
Inhibition of tumoricidal activity of HD-EX-exposed macrophages by antibody or inhibitor

Treatment	% Cytotoxicity of target cell (B16)
None	27.2 ± 3.2
HD-EX (10 μg/ml)	70.7 ± 6.3*
HD-EX + NMMA (0.5 mM)	29.2 ± 3.8*
HD-EX + anti-TNF-α (500 units/ml)	37.9 ± 6.9*

HD-EX-exposed macrophages were co-cultured for 20 h with target at an initial effector/target cell ratio of 10:1. Macrophage tumoricidal activity was determined as described in Section 2. Data shown are the results at an initial effector/target ratio of 10:1. The results shown are the mean ± SEM of quintuplicates from a representative experiments. *Significantly different from control (no treatment); $P < 0.05$.

4.3. The effect of HD-EX on phagocytic properties of macrophages

Fig. 3 shows that the incubation of macrophages with 0.1, 1 or 10 μg HD-EX slightly increased the phagocytosis of opsonized zymosan by macrophages, suggesting that there was a little alteration on phagocytosis by HD-EX.

4.4. Production of H_2O_2, IL-1, IL-6 and IL-10 in HD-EX-treated macrophages

To determine the effect of HD-EX on the production of H_2O_2, IL-1, IL-6 and IL-10, macrophages were treated with various doses of HD-EX for 20 h. Culture supernatants were assayed for cytokines by ELISA. Cytokine levels (IL-1, 1L-6 and IL-10) were only slightly elevated at 10 μg/ml of HD-EX compared with background (Fig. 4a–c), suggesting that TNF-α is a more important cytokine in tumoricidal activities of HD-EX-treated macrophages than IL-1, IL-6 and IL-10. The production of H_2O_2 was not greatly increased, either (Fig. 4d). H_2O_2 production in only 10 μg/ml of the HD-EX-treated group was slightly significant in comparison with that of the control group.

5. Discussion

Alternative medicine for treatment of various diseases is getting more popular. *H. diomphalia* larvae have been used as a traditional remedy in Korea and show various important pharmacological roles. Recently, potent antibacterial proteins have been isolated

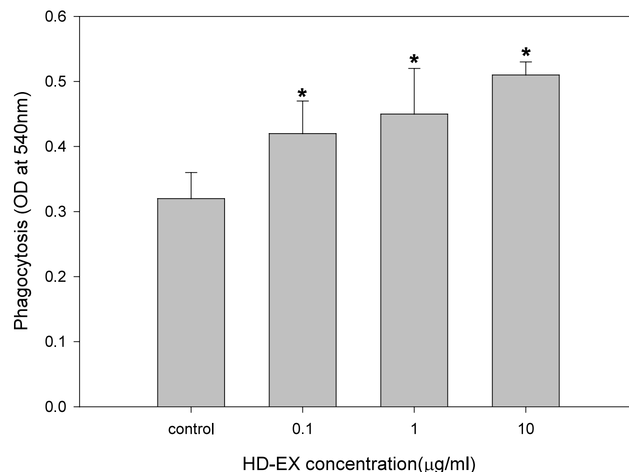

Fig. 3. Effect of HD-EX on the phagocytosis of peritoneal macrophages. Macrophages were treated with HD-EX for 20 h. Macrophages were then incubated with 5×10^6 particle of zymosan and 600 μg/ml of NBT. Phagocytosis was measured as OD 540 nm. The results shown are the mean ± SEM of three independent experiments. $*P < 0.05$; significantly different from control (no treatment).

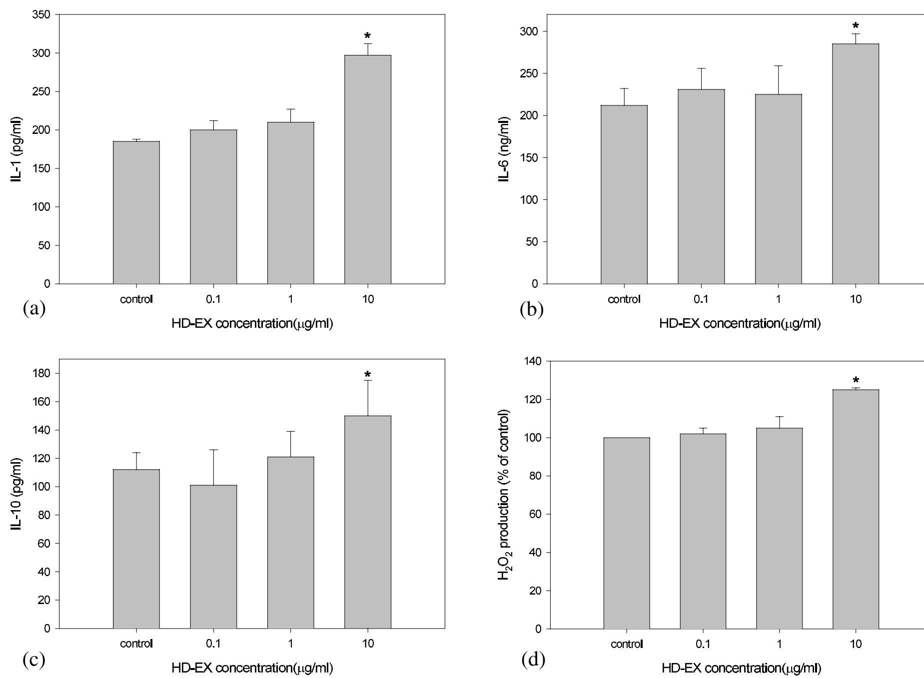

Fig. 4. (a) IL-1, (b) IL-6, (c) IL-10 and (d) H_2O_2 production by peritoneal macrophages stimulated by HD-EX. Macrophages were treated with HD-EX for 20 h. Culture supernatants were collected, and the levels of IL-1, IL-6 and IL-10 were measured by ELISA, respectively. The production of H_2O_2 was measured as described in Section 2. The results shown are the mean ± SEM of three independent experiments. $*P < 0.05$; significantly different from control (no treatment).

from the *H. diomphalia* larvae (Lee et al., 1994) and prophenoloxidase from the hemolymph of *H. diomphalia* larvae has been purified and characterized (Kwon et al., 1997; Lee et al., 1998). Therefore, this in vitro study was undertaken to determine whether ethanolic extracts of *H. diomphalia* larvae (HD-EX) have immunomodulatory properties. The present study provides the first results on the effects of HD-EX on the immune system of mice. Our results show that HD-EX can differentially alter macrophage tumoricidal activity as well as the production of cytokines and cytotoxic molecules.

It has been well known that macrophages play an important role in the defense mechanism against host infection and the killing tumor cells. The modulation of antitumor properties of macrophages by various biological response modifiers is an area of active interest for cancer chemotherapy. It has been shown that antiumor properties of macrophages can be activated by IFN-γ as well as lipopolysaccharide. Treatment of resident macrophages with IFN-γ induces a primed state. Primed macrophages have not only phagocytic activity but readily have become a fully activated cytolytic macrophage when stimulated with LPS. These cytolytic macrophages could be able to secrete various cytokines such as TNF-α, IL-1 and IL-6 (Adams and Hamilton, 1984; Rees and Parry, 1992). Secretion of these mediators was found to require a triggering signal such as LPS, thus providing some evidence to the role of the priming and triggering signals to induce the complete cytolysis function. In this study, HD-EX has also activated murine peritoneal macrophages, resulting in the increased production of various molecules such as NO, H_2O_2, TNF-α, IL-1, IL-6 and IL-10. The level of NO and TNF-α production in HD-EX-treated macrophages was significantly increased, whereas the production of H_2O_2, IL-1, IL-6 and IL-10 and phagocytosis was slightly increased. These results suggest that the beneficial, immunostimulatory activity of HD-EX may be mediated through upregulation of secretory molecules in macrophages and HD-EX plays a role in triggering the activation of macrophages. The present observations are related to the previous report that NO, TNF-α, IL-1, IL-6 and IL-10 were increasingly secreted by activated macrophages at first but H_2O_2 production and phagocytic activity were induced at the primed state not activated state (Lewis et al., 1986). Another previous report showed that H_2O_2 production was not an essential product at the activated state (Cohen et al. 1982). Based on these findings it suggests that the increased production of TNF-α and NO are related to tumoricidal activity of HD-EX-treated macrophages. Additional support for this conclusion came from the observation that anti-TNF-α antibody and the NO inhibitor were able to abrogate, in part, HD-EX-induced cytostasis against target.

Macrophages have been shown to be an important component of host defenses against virus infection by

inhibiting intracellular replication of virus (intrinsic resistance) and by killing virus infected cells (extrinsic cytotoxicity) (Morahan, 1984; Morahan et al., 1985). We have observed that HD-EX did not induce the antiviral activity (data not shown), suggesting that mediators, which play a role in HD-EX-induced tumoricidal activity, are not involved in antiviral activity and also HD-EX has differential effects on efficient activation of macrophages.

In summary, although the data presented in this report yielded an incomplete picture on the effects of the extracts on the immune system, we have demonstrated that HD-EX results in an augmentation of macrophage function and a potent stimulator of both the synthesis and the release of cytotoxic mediators. These results suggest that HD-EX might achieve in vivo enhancement of macrophage function. Further studies with animal models are necessary to clarify how this activation occurs and to what extent it occurs in vivo.

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