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Inhibitory Activity of Bumblebee Worker (*Bombus terrestris* L.) Venoms on Nitric Oxide, TNF-α and IL-6 Production in Lipopolysaccharide-Activated Macrophages

Sang-Mi Han*, Kwang-Gill Lee, Joo-Hong Yeo, HaeYong Kweon, Soon-Ok Woo, Hyung-Joo Yoon, Ha-Ju Baek[1] and Kwan-Kyu Park[2]

Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea.
[1]Gyeongsang Buk-Do Government Public Institute of Health and Environmental, Daegu 701-702, Korea
[2]Department of Pathology, School of Medicine, Catholic University of Daegu, Daegu 712-702, Korea

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To elucidate the composition of bumblebee (*Bombus terrestris*) venom (BBV) and the anti-inflammatory effect of BBV. The major components of BBV by LC chromatography and SDS-PAGE were identified. The production of nitric oxide (NO) and proinflammatory cytokines was examined by lipopolysaccharide (LPS) in a macrophage cell line, RAW 264.7 cells, with BBV. BBV inhibits LPS-induced NO in a dose dependent manner. We also found that BBV inhibits proinflammatory cytokine, tumor necrosis factor (TNF)-α and interleukin (IL)-6 production. These findings mean that BBV can be used in controlling macrophages mediated inflammation related disease. Additional studies on the pharmacological aspects of the individual components of BBV are recommended for future trials.

Key words: Bumblebee venom, *Bombus terrestris*, Nitric Oxide, TNF-α, IL-6

Introduction

Hymenopteran venoms are quite complex mixtures of peptides and small molecules, frequently containing histamine that causes the inflammatory response associated with many stings (Habermann, 1972; Dotimas and Hidr, 1987). Many of these are described in a work by Piek and colleagues, including their chemistry and pharmacological actions (Piek, 1986). Peptides isolated from hymenoperan venoms include melittin and apamin from honeybees (*Apis melifera*) interacting with lipid membranes (Dempsey, 1990) and potassium channels (Hugues *et al.*, 1982), respectively, the mast cell degranulating mastoparans (Hirai *et al.*, 1979) and bombolitins (Argiolas and Pisano, 1985) from social wasps (*Vespula* sp.) and bumble bees (*Bombus* sp.) that cause presynaptic inhibition of cholinergic neurotransmission (Piek, 1991). Especially, honeybee venom has been utilized to relieve pain and to treat inflammatory diseases since ancient ages (Billingham *et al.*, 1973). Honeybee venom has been tested to date for its components and their possible anti-inflammatory function (Dotimas and Hider, 1987; Choi *et al.*, 2003; Kim *et al.*, 2005). But, the present knowledge of bumblebee venom other than the honeybee venom is rather poor. This is because of the relatively small quantities from the limited amount of venom available and the difficulty of completely separating the components. Bumblebees are increasingly used in Korea to pollinate vegetable crops grown in greenhouses. This has led to an increased mass rearing of bumblebees.

We now report the components and pharmacological effects of bumblebee (*Bombus terrestris* L.) venom. Both nitric oxide (NO) and proinflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-6 plays an important role during the various phases of inflammation (Schimmer and Parker, 2001). Because of their pivotal role in inflammatory disease, a significant effort has focused on developing therapeutic agents that interfere with NO, TNF-α and IL-6 production (Manogue *et al.*,

*To whom correspondence should be addressed.
Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea. Tel: 082-031-290-8510; Fax: 082-031-290-8516;
E-mail: sangmih@rda.go.kr

1992; Faris-Eisner *et al.*, 1994). As a potential therapeutic agent, bumblebee venom needs to be evaluated its anti-inflammatory effect in inflammatory environments. Inflammatory diseases, such as rheumatoid arthritis, result in significant morbidity and mortality (Lang *et al.*, 1995). Hence, there is currently considerable interest in inhibiting agents of NO, TNF-α and IL-6 production such as a therapeutic intervention in inflammatory diseases (Kondo *et al.*, 1993; Park *et al.*, 1993).

This study was carried out to determine the composition of bumblebee venom and to evaluate the inhibitory activity of NO, TNF-α and IL-6 production in LPS-stimulated RAW 264.7, macrophage cell line.

Materials and Methods

Bumblebee venom (BBV) preparation

Bumblebees, *B. terrestris*, workers were collected from artificially mass rearing in National Institute of Agricultural Science and Technology (Suwon, Korea). Worker bees were killed by freezing and then their venom was obtained by squeezing venom sacs. The extracted venom was diluted twice in cold water and then centrifugated at 10,000 g for 5 min at 4°C to discard cellular residues from the supernatant. The venom was lyophilized by freeze dryer and stored at refrigerator for later use.

BBV analysis

10 mg freezing dried whole BBV was dissolved in 0.1 M ammonium formate (pH 4.5). Particles were removed by centrifugation and filtering prior to sample application to a Sephadex TM200 column (AKTA explorer, Pharmacia, USA) equilibrated in 0.1 M ammonium formate. Honeybee (*A. mellifera*) and bumblebee (*Megabombus fervidus*) venom standard proteins were purchased from sigma (Sigma, USA). Protein concentration was determined by the Bradford method (BioRad, CA, USA). The purity of proteins and peptides was assessed by SDS-PAGE on 4-20% gradient tricine gels (Novex Tricine Gels, Invitrogen. USA). Proteins and peptides were stained with Coomassie blue R-250.

Cell culture and stimulation

The murine macrophage cell line, RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% penicillin (10,000 U/ml)/streptomycin (10,000 U/ml). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. For experiments, the cells were added to 24-well plates at a density of 5×10^5 cells/well and adhered overnight for assay. Various amounts of bumble bee venom and LPS (100 ng/ml) were added and incubated. The supernatants were then collected for nitric oxide, TNF-α and IL-6 assays.

MTT assay of cell viability

The effect of BBV on cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. RAW 264.7 cells were seeded in 96-well plates and treated with various reagents. They were cultured in a CO_2 incubator for the indicated time periods. The medium was removed and MTT was added followed by incubation at 37°C for 24 hrs in an incubator. Supernatants were carefully removed and DMSO was added. They incubated at 25°C for 30 min and measured by a microplate reader (UV max, Molecular Devices, USA) at 540 nm. Percent viability was calculated as follows (Orsolic *et al.*, 2003):

$$\text{Viability}(\%) = \frac{A_{540} \text{ of experimental}}{A_{540} \text{ of control}} \times 100$$

NO generation analysis

Nitrite was carried out the method reported by (Stuehr *et al.*, 1991). 100 μl of supernatant from a 24-well plate culture at 22 hrs was transferred into new 96-well plate and mixed with an equal volume of Griess reagent (Sigma, USA). The new plate was then incubated for 10 min at room temperature and measured by a microplate reader. The standard calibration curve was prepared using sodium nitrite as a standard.

TNF-α and IL-6 measurement assay

TNF-α and IL-6 secretion was measured using an ELISA kit (Endogen, USA). 50 μl of either various samples was added in 96-well ELISA plate. Biotinylated antibody reagent to each well was incubated in the plate at room temperature for 2 hrs. After washing the plate with PBS-tween 20, diluted streptavidin-HRP was added, and the plate was incubated at room temperature for 30 min. After washing the plate, premixed TMB substrate solution was added, and then the plate was developed in the dark room for 30 min. The plate was read at 490 nm using a microplate reader. The concentration of TNF-α and IL-6 was calculated using murine rTNF-α and rIL-6 as a standard.

Statistical analysis

Nitrate, TNF-α and IL-6 production is expressed as a mean ± S.D. of 3 to 5 independent experiments. The statistical significance was determined using Student's t-tests (SAS Institute, 2004).

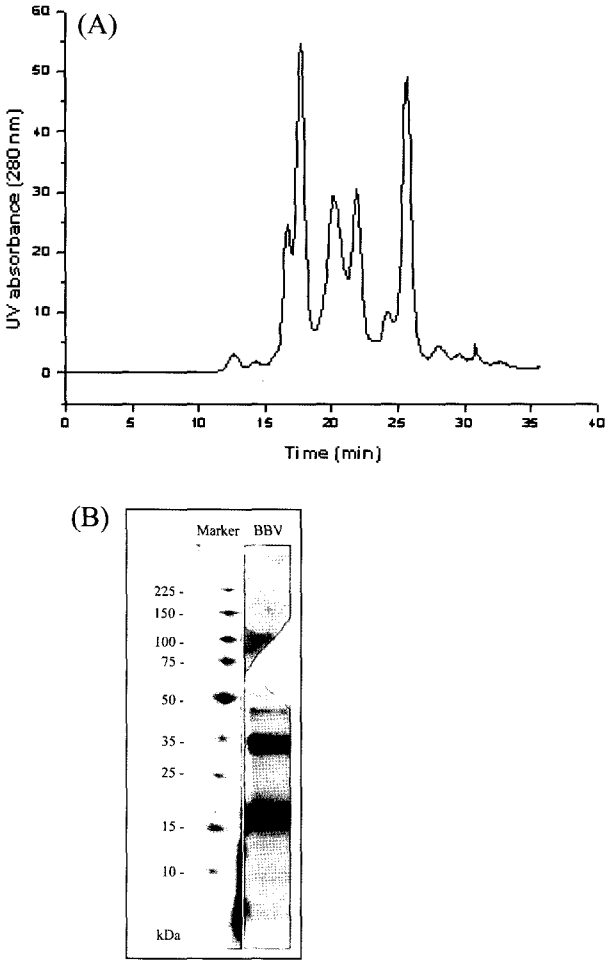

Fig. 1. Gel filtration of 10 mg freeze-dried whole BBV on Sepadex TM200 10/300. Elution with 0.1 M ammonium formate buffer, pH 4.5. Determination of main components were compared with standard proteins by the optical density at 280 nm (A) and SDS-PAGE analysis (B). SDS-PAGE is 4 – 20% gradient gel. B was stained with Coomassie blue R-250. Lane 1, sigma molecular weight standard; lane 2, whole BBV.

Results

BBV purification

Proteins from BBV were separated by size exclusion gel chromatography (Fig. 1A). The determination of main components of BBV was resulted by comparison with honeybee venom and bumblebee (*Megabombus fervidus*) venom protein standards (sigma, USA). Identification of whole BBV was performed by SDS-PAGE (Fig. 1B). The comparison between peaks of venom of *Megabombus fervidus* standards (not shown) and whole AVB showed that all major venom protein and peptides were found in similar to compositions but a little difference proportions. But, that of honeybee venom was not similar to compositions.

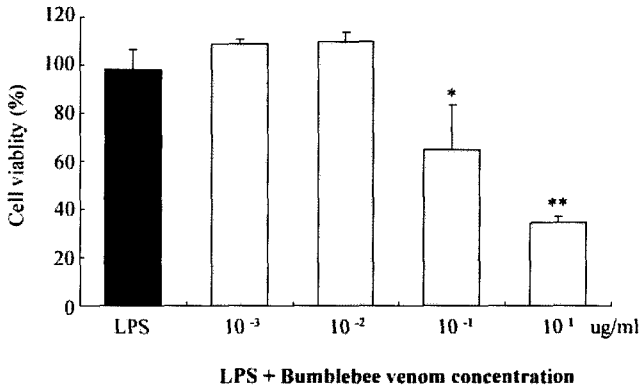

Fig. 2. Cell viability (%) after 24 h of incubation with BBV at the respective concentrations. RAW 264.7 cells were treated with LPS (100 ng/ml) or BBV either alone or in combination. Viability was evaluated by MTT assays at 24 hrs after treatment. Values are given as percent (mean ± S.D.).
$^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's t-test.

MTT assay

Twelve hours incubation with LPS (100 ng/ml) and BBV at the variable concentrations produced the respective viabilities relative to that of the normal group (not treated with LPS and BBV) in the RAW 264.7 cell line cultures (Fig. 2). In the control group, which was treated with LPS only, the viability was 97.9 ± 8.3% (mean ± S.D.). Percent viability with 1 μg/ml BBV was significantly decreased different from that of the normal group.

NO generation analysis

In order to observe the effects of bumble bee venom on the generation of NO, the RAW 264.7 cells were stimulated with LPS and treated with BBV. Thereafter, the levels of nitrite, an index of NO formation, were measured. The effects of BBV on the NO generation by LPS are shown in Fig. 3. BBV dose-dependently reduced the concentration of nitrite. In the normal group, which was not treated with LPS and BBV, the nitrate level was 3.2 ± 0.3 μM, whereas that of the control group, which was treated with LPS only, was 81 ± 0.3 μM. The nitrate levels of the 1, 10 and 100 ng/ml BBV treated groups, which were treated with LPS and BBV, were 56 ± 0.4 μM, 41 ± 3.7 μM and 14 ± 6.9 μM, respectively (Fig. 3).

TNF-α and IL-6 measurement assay

The cells were treated with LPS alone or with various concentrations of BBV for 20 hrs. RAW 264.7 cells activated with LPS secrete TNF-α and IL-6, an important proinflammatory mediator (Fig. 4 and 5). Production of TNF-α was significantly decreased by BBV dose-dependently (Fig. 4). BBV, at the concentration of at 100 ng/ml,

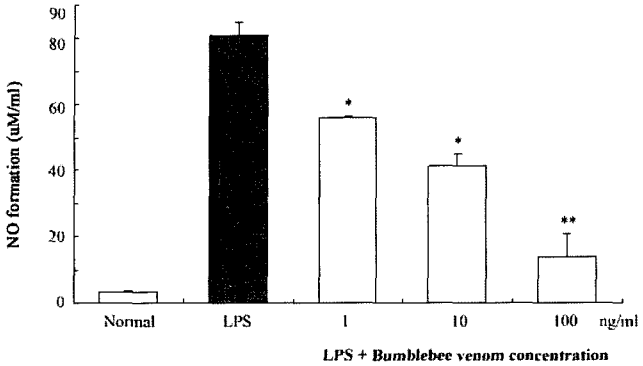

Fig. 3. Inhibitory effect of the BBV on LPS-induced NO production in RAW 264.7 cells were incubated for 22 hrs with LPS (100 ng/ml) in the presence or absence of indicated concentration of BBV. Accumulated nitrite in the culture medium was determined by the Griess reaction. The values are mean ± S.D. of three independent experiments.
$*p < 0.05$ and $**p < 0.01$ by Student's t-test.

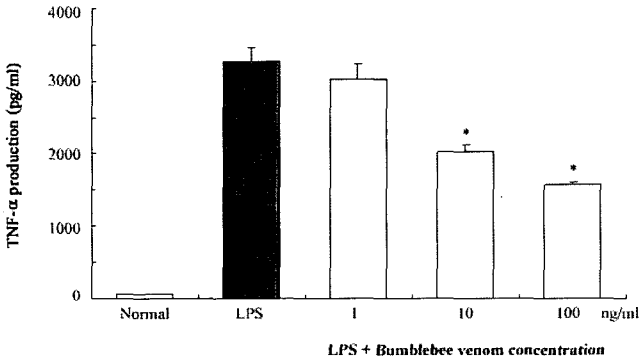

Fig. 4. BBV inhibited LPS (100 ng/ml)-induced TNF-α production from RAW 264.7 cells. BBV was added to the cell culture with final concentration of 1 – 100 ng/ml. 22 hrs culture supernatants were assayed for TNF-α by an ELISA kit. The values are mean ± S.D. of three independent experiments.
$*p < 0.05$ by Student's t-test.

inhibited more on the LPS-inducted IL-6 production from RAW 264.7 cells (Fig. 5).

Discussion

Acute inflammation can cause septic shock, rheumatoid arthritis or other disease in human and experimental animals (Higgs *et al.*, 1984). It was been reported that the excessive production of NO, TNF-α and IL-6 could cause various inflammatory diseases (Higgs *et al.*, 1984; Vince *et al.*, 2005). In the present study, exposure of macrophages to LPS for several hours was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This LPS induced NO production was inhibited significantly by 100 ng/ml of BBV. The level of NO in RAW 264.7 was reduced by BBV treatment in a dose-dependent manner. It indicates that BBV directly or indirectly suppresses the formation. In relation with cytokines, we evaluated TNF-α and IL-6, those cytokines are correlated with activations of macrophages. Cytokines, such as TNF-α and IL-6 have been clearly involved in the inflammatory process (Gorman *et al.*, 2002; Trikha *et al.*, 2003). Recently, anti-TNF therapy has defined a molecular target and new approach for treating inflammatory disorders. BBV inhibited TNF-α and IL-6 production to a lesser degree in LPS stimulated RAW 264.7 macrophages. It is supposed that the decrease of NO, TNF-α and IL-6 by BBV dose of 100 ng/ml is caused by a few possible reasons. But, it must not be influenced by toxicity to cells. As we documented above, the viability of RAW264.7 cells were assessed to be above 95% by MTT method at the sample concentrations for NO, TNF-α and IL-6 assay. Further investigation is necessary to clarify the mechanisms of NO, TNF-α and IL-6 inhibition by BBV.

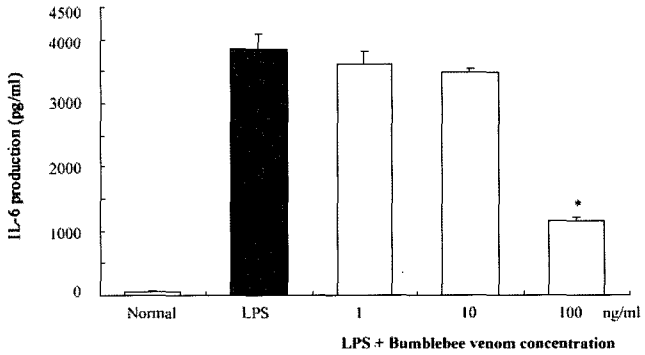

Fig. 5. BBV inhibited LPS (100 ng/ml)-induced IL-6 production from RAW 264.7 cells. BBV was added to the cell culture with final concentration of 1 – 100 ng/ml. 22 hrs culture supernatants were assayed for TNF-α by an ELISA kit. The values are mean ± S.D. of three independent experiments. $*p < 0.005$ by Student's t-test.

The BBV was analyzed by HPLC and SDS-PAGE in this study. However, peaks of the principal components have been identified. Further investigation is needed to clarify unknown constituents, which may be more active than BBV itself. Studies on the isolation and characterization of the active chemical constituents are in progress.

In conclusion, we have demonstrated that BBV acts as an NO inhibitor of macrophages activation by LPS. Furthermore, BBV inhibited the production of TNF-α and IL-6. These finding suggests that BBV can be useful as a

potential immunotherapeutic agent, which is potentially applicable for inflammation.

References

Argiolas, A. and J. J. Pisano (1985) Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J. Biol. Chem.* **260**, 1437-1444.

Billingham, M. E. J., J. Morley, J. M. Hanson, R. A. Shipolini and C. A. Vernon (1973) An anti-inflammatory peptide from bee venom. *Nature* **245**, 163-164.

Choi, S. H., S. K. Cho, S. S. Kang, C. S. Bae, Y. H. Bai, S. H. Lee and S. C. Pak (2003) Effect of apitherapy in piglets with preweaning diarrhea. *Am. J. Chin. Med.* **31**, 321-326.

Dotimas, E. M. and R. C. Hider (1987) Honeybee venom. *Bee World* **68**, 51-70.

Faris-Eisner, R., M. P. Sherman, E. Aeberhard and G. Chaudhuri (1994) Nitric Oxide is an important mediator for tumoricidal activity in vivo. *Proc. Natl. Acad. Sci.* **91**, 9407-9411.

Gorman, J. D., K. E. Sack and J. C. Davis, Jr. (2002) Treatment of ankylosing spondylitis by inhibition of tumor necrosis factor alpha. *N. Engl. J. Med.* **346**, 1349-1356.

Habermann, E. (1972) Bee and wasp venoms: the biochemistry and pharmacology of their peptides and enzymes are reviewd. *Science* **177**, 314-322.

Higgs, G. A., S. Moncada and J. R. Vane (1984) Eicosanoids in inflammation. *Ann. Clin. Res.* **16**, 287-299.

Hirai, Y., T. Yasuhara, T. Nakajima, M. Fujino and C. Kitada (1979) New mast cell degranulating peptide mastoparan in the venom of *Vespula lewisii*. *Chem. Pharm. Bull.* **27**, 1942-1944.

Hugues, M., G. Romey, D. Duval, J. P. Vincent and M. Lazdunski (1982) Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells-voltage-clamp and biochemical-channel in neuroblastoma cells-voltage-clamp and biochemical-characterization of the toxin receptor. *Proc. Natl. Acad. Sci.* **79**, 1308-1312.

Kim, K. S., U. S. Choi, S. D. Lee, K. H. Kim, Y. C. Chung, K. K. Park, Y. C. Lee and C. H. Kim (2005) Effects of bee venom on aromatase expression and activity in leukaemic FLG 29. 1 and primary osteoblastic cells. *J. Ethnopharmacol.* **99**, 245-252.

Kondo, Y., F. Yakano and H. Hojo (1993) Inhibitory effect of bisbenzylisoquinoline alkaloids on nitrite oxide production in activated macrophages. *Biochem. Pharmacol.* **46**, 1887-1892.

Lang, F., J. M. Robert, P. Boucrot, I. Welin and J. Y. Petit (1995) New anti-inflammatory compounds that inhibit tumor necrosis factor production: Probable interaction with protein kinase C activation. *J. Pharmacol. Exp. Ther.* **275**, 171-176.

Manogue, K. R., J. H. Van Denventer and A. Cerami (1992) Tumor necrosis factor-α or cachectin. Academic Press.

Orsolic, N., L. Sver, S. Verstovsek, S. Terzic and I. Basic (2003) Inhibition of mammary carcinoma cell proliferation in vitro and tumor growth in vivo by bee venom. *Toxicon* **41**, 861-870.

Park, E., M. R. Auinn, C. Wright and G. B. Schuller-Levis (1993) Taurine chloramines inhibits the synthesis of nitric oxide production and the release of tumor necrosis factor in activated RAW 264.7 cells. *J. Leukoc Biol.* **54**, 119-124.

Piek, T. (1986) Venoms of the Hymenoptera. Academic Press, London.

Piek, T. (1991) Neurotoxic kinins from wasp and ant venoms. *Toxicon.* **29**, 139-149.

Schimmer, B. P. and K. L. Parker (2001) Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In *The pharmacological basis of therapeutics*. Hardman, J. G., L. E. Limbird and A. Goodman Gilman, pp. 1649-1677. McGraw-Hill, New York

Stuehr, D. J., H. J. Cho, N. S. Kwon, M. Weise and C. F. Nathan (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci.* **88**, 7773-7777.

Trikha, M., R. Corringham, B. Klein and J. F. Rossi (2003) Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: A review of the rationale and clinical evidence. *Clin Cancer Res.* **9**, 4653-4665.

Vince, V., M. Brelen, J. Delbeke and I. Colin (2005) Anti-TNF-α reduces the inflammatory reaction associated with cuff electrode implantation around the sciatic nerve. *J. Neuroimmunol.* **165**, 121-128.