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Activation of Murine Macrophage Cell Line RAW 264.7 by Korean Propolis

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Monocytes and macrophages play a major role in defense mechanism of the host response to tumor, in part through the secretion of several potent products and macrophage cytokines. Monocytes and tissue macrophages produce at least two groups of protein mediators of inflammation, interleukin 1 (IL-1) and tumor necrosis factor (TNF). Recent studies emphasizes that TNF and IL-1 modulate the inflammatory function of endothelial cells, leukocytes, and fibroblasts. In this study, our work is directed toward studying the *in vitro* effects of Korean propolis on the ability to induce cellular and secretory responses in murine macrophage cell line, RAW 264.7. It was found that Water Extract of Korean Propolis (WEP) could activate macrophages by producing cytokines. The production of the macrophage cytokines, IL-1 and TNF-α, by RAW 264.7 treated with WEP was examined from 2.5 μg/ml up to 25 μg/ml with dose dependent manner. Nitric oxide (NO) production was also increased when cells were exposed to combination of LPS and WEP from 2.5 μg/ml up to 25 μg/ml. At high dose of WEP (50 to 100 μg/ml) used to prescribe for anti-inflammatory and analgesic medicine showed inhibition of NO production in LPS-stimulated macrophage. Besides cytokine production, NO release, surface molecule expression and cell morphologic antigen expression were increased in response to the stimulation by WEP. These results suggested WEP may function through macrophage activation.

Key words: Macrophage activation, Inflammation, Cytokines, Korean Propolis

INTRODUCTION

Propolis is a resinous, sticky, and varnish-like hive product collected by honeybees, *Apis mellifera* (Apidae). It consists of secretions and/or exudations from parts of plants and buds, mixed by honeybees with their wax to seal cracks and crevices of their hives.

Propolis, also known as bee glue, is used in folk medicine as early as 300 B.C.. In traditional Chinese and Korean medicine, beehives is so-called No-Bong-Bang instead of propolis (Volpert and Elstner, 1991). Generally, it is stated that propolis contains 30% wax, 55% resins and balsams, 10% ethereal oils and approximately 5% pollen. Furthermore, many constituents have been identified, such as flavonoid aglycones, phenolic acids and their derivatives. It is thought that a compound among them, caffeic acid phenyl ester could induce contact dermatitis. The proportion of these constituents depends on the place and time that propolis is collected (Bankova *et al.*, 1983; Miyataka *et al.*, 1997).

Propolis and its constituents have been reported for several biological properties such as anticancer (Khayyal *et al.*, 1992), antioxidant (Matsushige *et al.*, 1996), anti-inflammatory (Khayyal *et al.*,1979), antibiotic (Kujumgiev *et al.*, 1975), antiviral (Gama, 1993), antihepatotoxic (Prusotam *et al.*, 1996) and immunostimulating activities (Arvouet *et al.*, 1993).

Macrophage activation is normally mediated by a wide variety of mechanisms. These include exposure to cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2). They also can be activated by exposure to bacteria, bacterial products and particulates (Adams and Hamilton, 1984). Macrophages can destroy tumor cells after being treated with both recombinant IFN-γ and bacterial LPS suggesting that at least two stimuli are required for complete activa-

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tion (Drysdale *et al.*, 1988). It has been shown that each molecules interact with specific high affinity receptors located on the macrophage plasma membrane (Lorsbach *et al.*, 1993).

Mononuclear phagocytes have also emerged as cells central to the inflammation sites. Although these cells release a plethora of inflammatory mediators, two of the more important cytokines, interleukin (IL-1) and the tumor necrosis factor (TNF), appear to play an important role in the immune processes. Both IL-1 and TNF have a profound effect on tissue remodeling, repair, and inflamrnation by coordinating the activities of many other cells, including endothelial cells, granulocytes, osteoclasts, fibroblasts, hematopoietic cells, and lymphoid cells. An important concept emerging from these studies is that cytokines play a role in the homeostasis of cells and tissue under certain physiological conditions. Inflammation processes and the response to injury are associated with augmented releases of these protein mediators and toxic or life-threatening syndromes, e.g., cachexia, shock, etc, result from the exaggerated and prolonged release (Linna and Ian, 1996).

As of todays research, LPS was used as a positive control for macrophage activation. Macrophage activation was expressed as increased cell size, cytoplasmic spreading, increased nitric oxide (NO) production, increased cytokine release, and increased expression of some adhesion molecules and Fc receptors (FcRs).

The aim of this paper was to examine the effects of WEP on macrophage functions, NO production, cytokine release, and expression of some adhesion molecules.

MATERIALS AND METHODS

Water Extract Propolis (WEP)

Propolis was obtained from Chonghak Beekeeping Farm in the area of Namyangju city in Kyunggi Province, which was collected from may to august in 1997. One kilogram of the crude mass of propolis was frozen in a deep freezer at -20°C, squashed into small pieces and ground into powder by a grinder. It was extracted with water at a proportion of one to five (propolis to water) for two hours with stirring while the temperature was kept less than 50°C, was extracted with water using the Lee's method (1988). It was filtered by Whatman #4 paper, and evaporated to dryness under vacuum by Freeze dryer (OPR-FDUM-305, Operon Eng. Co.), which resulted in 32.5 grams of brownish powered propolis (Choi 1998). Various concentrations of the test compounds dissolved in distilled water.

Animals

Eight-week old male BALB/c mice (26-28 g) were purchased from Daehan Biolink. They were maintained in plastic cages under conventional conditions at the laboratory animal center in Sahmyook University.

Splenocyte preparation

The mice were sacrificed by cervical dislocation under sterile conditions. The splenocytes were prepared from the spleens using a method described previously (Cho *et al.*, 1998). Briefly, the splenocytes were released by teasing into a RPMI1640 medium supplemented with 20 μM HEPES buffer. After removing the red blood cells with an ACK buffer (pH 7.4), the splenocytes were washed 3 times in Ca^{2+}-Mg^{2+} free Hanks balanced salt solution and resuspended in RPMI 1640 with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. The total concentration of cells was to 5×10^6 cells/ml.

Cell culture

The mouse macrophages cell line (RAW 264.7), the IL-1 dependent cell line (D10), the TNF-α sensitive cell line (WEHI-164) were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle Medium (DMEM), which was supplemented with a high glucose, L-glutamine, 110 μg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1%(v/v) penicillin (10000 U/ml)/streptomycin (10000 U/ml) (P/S). The LPS (*Escherichia coli*, 0127: B8 Westphal type) was purchased from the Sigma Chemical Co. (St. Louis, MO. USA). The recombinant murine IL-1β (specific activity $1\text{-}2\times 10^8$ U/mg) was purchased from R&D Systems (Oxon, UK).

Total lymphocyte proliferation assay

The spleen cells were treated with LPS (50 μg/ml), ConA (10 μg/ml), LPS/WEP (5, 10, 20, 50, 100 μg/ml), ConA/WEP (5, 10, 20, 50, 100 μg/ml), all which were treated with CM-10. They were instilled into each well 2×10^5 cells/200 μl and were cultured for 3 days in a CO_2 incubator (37°C, 5.5% CO_2). XTT (a cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit II, Promega, WI, USA) were used to measure the proliferation on spleen cells. Subsequently 50 μl XTT reagent were added to each well, which were then cultured under the same conditions for 3 hours and measured by an ELISA reader at 490 nm.

Cytokines bioassays

The RAW 264.7 cells were cultured in DMEM with 10% FBS in 24-well-flat plates at a density of 5×10^5 cells/well. The cells were treated with various concentrations (0, 2.5, 5, 12.5, 25, 50, 100 μg/ml) of WEP and LPS 100 ng/ at 37 °C for 48 hours in humidified air with 5.5% CO_2, then the supernatants were collected for cytokines bioassays.

IL-1 assay: In order to measure the IL-1 activity, the ability of the stimulated monocyte supematants to cause proliferation of the IL-1 dependent T-cell line was assessed. An example was the conalbumin-spectific D10-G4.1 murine

T-cell clone (ATCC), which requires IL-1β (1 u/ml) as well as specific antigens for growth. The IL-1 production assay was carried out according to the Manual of Clinical Laboratory Immunology (Noel *et al.*, 1991). The cells with IL-1β and those without in different culture supernatants of WEP (5, 10, 15, 20, 25 μl) were prepared as the treated groups. 8 wells per each group were used and 100 μl of the cell suspension (4×10^4 cells/well) were added to each well and incubated in humidified air with 5.5% CO_2 for 48 hours 37°C. Subsequently, 50 μl of the XTT reagent was added to each well, cultured under the same conditions for 3 hours. They were then measured by an ELISA reader at 490 nm.

TNF-α assay: To measure the TNF activity, the supernatants of stimulated monocytes was used that it can lyse a highly TNF-sensitive murine fibrosarcoma cell line, WEHI-164-JD (ATCC) (Djeu *et al.*, 1988). The TNF-α production assay was carried out according to the Manual of Clinical Laboratory Immunology (Noel *et al.*, 1991). The WEHI-164 cells were prepared in different culture supernatants of the WEP (5, 10, 15, 20, 25 μl) treated groups. 8 wells per were used and 100 μl of the WEHI-164 (2×10^4 cells/well) cells were added each well. The plates were then incubated in humidified air with 5.5% CO_2 for 48 hours at 37°C. Subsequently, 50 μl of the XTT reagent was added to each well, cultured under the same conditions for 3 hours and measured by an ELISA reader at 490 nm.

Western blot analysis

The RAW cells were washed with phosphate buffered saline and lysed by boiling with a lysis buffer (1% SDS, 1.0 mM sodium vanadate, 10 mM Tris-Cl buffer, pH 7.4) for 5 min. 23 μg protein from the cell lysates was applied to 15% SDS-polyacrylamide gels. Proteins were electophoretically transferred to nitrocellulose (NC) membrane and the Western Blot Kit was used to detect antibody-antigen reactivity (LumiGLO System, KPL, U. S. A.).

Nitric oxide assay

Flat-bottomed 96 well, LPS (10 ng/ml), LPS/WEP (2.5, 5, 10 20, 50, 100 μg/ml), Media only (DMEM-10), RAW 264.7 cell line, and Griess reagent (stock-I : 0.2% naphylendia HCl, stock-II : 2% sulfanilamide in 5% H_2PO_4) were used as the materials in this study. NO production was carried out according to the method of reported by Stuehr and Nathan (1989). The following: LPS, cells only (1×10^6 cells/ml) LPS/WEP (6.25, 12.5, 25, 50, 100 μg/ml), were prepared as the treated groups. 6 wells per each group were used and 200 μl of the cells (1×10^6 cells/ml) was added to each well. The plates were incubated overnight and 100 μg from the surface of each well was transferred into new plate and incubated with 100 μl of Griess reagent, and then measured by an ELISA reader at 540 nm. Standard calibration curves were prepared using sodium nitrite as the standard.

Cell staining

To determine the effects of WEP on the macrophage morphology, the cells were cultured in sterile glass-slide chambers at a density of 1000 cells/well for 48 hours. The culture medium was removed, and the cells were treated with either LPS (100 ng/ml), LPS/WEP (2.5, 5, 10, 20, 40 μg/ml) only for 2 days. Following the treatment, the culture supernatant was removed. The cells were fixed and stained in a Diff-quick Solution (Baxter, Houston, TX).

Flow cytometry

Surface molecule expression: The RAW (3×10^5 cells/ml) cells were cultured in DMEM for 2 days. The media were changed and the cells were then incubated in the presence of either LPS (100 ng/ml), LPS/WEP (2.5, 5, 10, 25, 50 μg/ml) or the medium for 2 days. The treated cells were then scraped into PBS-0.1% sodium azide with 1% FBS (PBS-washing buffer, pH 7.2) and washed 2 times in a washing buffer at 4°C. Before staining the cells with FITC (Fluorescein isothiocyanate)-monoclonal antibodies, the cell surface Fc receptors were blocked by incubating the cells with 20 μg/10^6 cells of purified anti-CD-16 for 30 min at 4°C. The residual antibodies were removed by washing. Anti-IA^b-FITC, Anti-CD40-PE (Phycoerythrin), Anti-CD54-FITC were then added and the cells were stored at 4°C for 30 min. The cells stained with mouse IgG-FITC served as the control for nonspecific binding. The cells were then washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2). Flow cytometry analysis was performed using an EPICS V analyzer (Coulter, Hialeah, EL). The fluorescence intensity was determined on the 10,000 cells from each sample using logarithmic amplification.

Cell cycle analysis: The RAW cells were cultured in DMEM for 2 days. The media were changed and the cells were then incubated in the presence of either LPS (100 ng/ml), LPS/WEP (5, 10, 50 μg/mml)or medium for 2 days, harvest cells and prepare single cell suspension in buffer, and washed cells 2 times in PBS and resuspend at 1~2× 10^6 cells/ml. The pellet was resuspended in 0.5 ml/10^6 cells of buffer containing 200 μg/ml of propidium iodide (Sigma, St. Louis, Mo) and 50 μg/ml of RNase (Puregene, Minneapolis, MN) followed by incubation for 15 min at 4 °C. Labeled cells were analyzed by using a FACS can flow cytometer affixed with a doublet discrimination module to include only single cells in the cell cycle analysis. Data were analyzed using CellQuest software (Crissman and Steinkamp, 1973).

Statistical analysis

Nitrite and cytokines production is expressed as a means

± SD of 2 to 6 independent experiments. The statistical significance was estimated using by Student-t tests.

RESULTS

Effect of WEP on splenocytes proliferation

In this assay, Con A was added to the splenocytes at a concentration of 10 μg/ml. WEP alone induced minimal lymphocyte proliferation (data not shown), Con A or LPS stimulated more lymphocyte proliferation, and much greater proliferation was observed by the combined action of WEP and Con A or LPS. The number of total lymphocytes increased with WEP concentrations from 0 to 100 μg/ml, as shown in Fig. 1. The proliferation of the T lymphocytes treated by Con A was increased at a 10-100 μg/ml compared to Con A (Fig. 2). Seeing that WEP accelerates the increment of the total lymphocytes, we may assume that WEP is immunostimulatory.

Direct cytokines production in response to WEP

To determine whether WEP (2.5, 5, 10, 50, 100 μg/ml) had a direct effect on cytokines production, a biological assessment of TNF-α activation was measured using the macrophage cell line. As shown in Fig. 3, 4, WEP increased TNF-α and IL-1 productions in the presence of LPS (100 ng/ml) in a dose dependent manner. IL-1 and TNF-α are the important proinflammatory cytokines which are mainly produced by monaytes and macrophages (Vileek and Lee, 1991). Western blot analysis (Fig. 5.A) showed that IL-1 production occurred after WEP treatment with the LPS. But It was different compared with TNF-α. Fig. 5. B showed that macrophages activated by LPS in the presence of WEP (2.5, 5 μg/ml) contained induced amounts of TNF-α protein compared with the WEP 50 μg/ml group. But WEP alone did not appeared by western blot analysis (data not shown).

Effect of WEP on NO production in murine macrophage cell line, RAW 264.7

As shown in Fig. 6, the macrophages did not release NO in response to the medium. In this study, LPS was

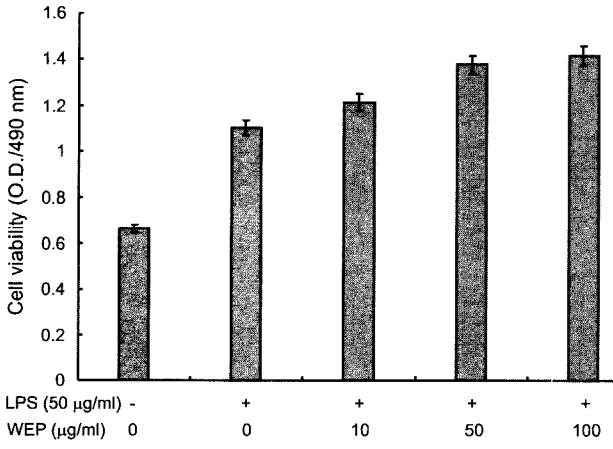

Fig. 1. Proliferation effect of WEP on LPS-treated total lymphocytes from the spleen of BALB/c mice. The cells were purified from the spleen. The cells were treated with WEP and LPS at a concentrations of 50 μg/ml LPS and various WEP concentrations (0, 5, 10, 50, 100 μg/ml) and incubated for 3 days at 37°C and 5.4% CO_2. After adding 50 μl of the XTT labeling mixture. They were then incubated for 3 hours at 37°C in 5.4% CO_2. The absorbance was read using an ELISA reader at 490 nm.

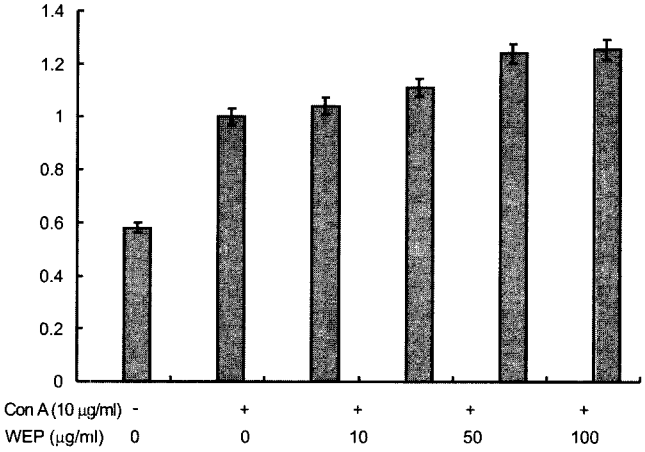

Fig. 2. Effect of WEP on Concanavallin A (Con A : 2 μg/ml)-stimulated spleen cells. Treatnet various WEP concentrations (0, 10, 20, 50, 100 μg/ml) with the Con A stimulated spleen cells. Using 8 wells per group, the cells were stimulated for 3 days at 37°C in 5.4% CO_2. After adding 50 μl of the XTT labeling mixture. They were then incubated for 3 hours at 37°C and in 5.4% CO_2. The absorbance was read using an ELISA reader at 490 nm.

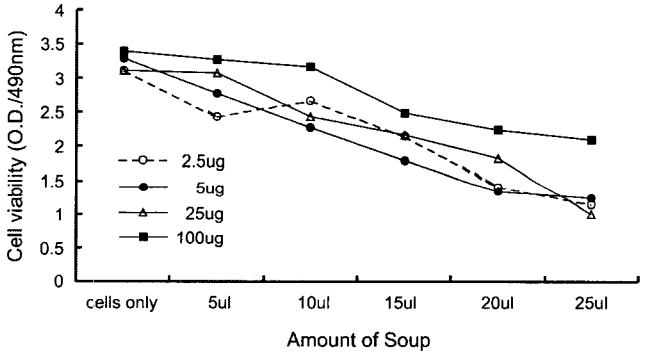

Fig. 3. LPS-induced TNF-α production by WEP in the mouse macrophage cell line. Effect of increasing concentration of WEP (2.5, 5, 12.5, 25, 50, 100 μg/ml) on LPS-induced TNF-α production. The RAW 264.7 cells (5×10^5 cells/ml) were cultured with the indicated WEP doses (2.5, 5, 12.5, 25, 50, 100 μg/ml) for 48 hours in the presence of 100 ng/ml LPS. The TNF-α bioassay was measured in the TNF-α sensitive cell line, WEHI-164, by their proliferation. The supernatant with the LPS-induced TNF-α production in the RAW cell was added to the TNF-α sensitive cell line, WEHI-164. The results are reported as a mean± S.D. of three independent experiments.

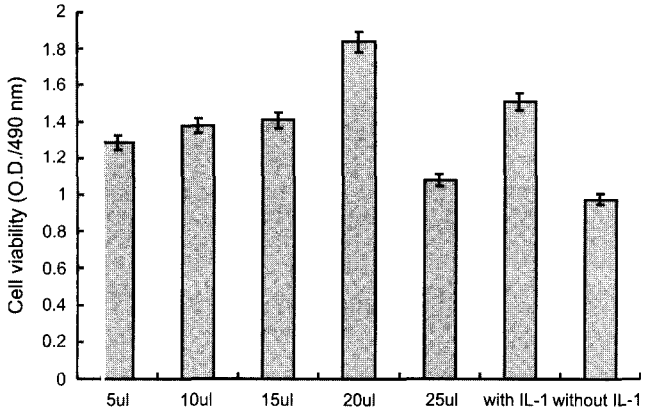

Fig. 4. Effect of increasing concentration of WEP (25 μg/ml) on LPS-induced IL-1β production. The RAW 264.7 cells (5×10^5 cells/ml) were cultured with the indicated WEP dose WEP (25 μg/ml) for 48 hours in the presence of 100 ng/ml LPS. The IL-1β bioassay was measured in the IL-1β dependent cell line, D10-S, by their proliferation. The supernatant with the LPS-induced IL-1β production in the RAW cell was added to the D 10-S. The amounts of IL-1β in the culture supernatants were determined by ELISA Reader at 490 nm. The results are reported as a mean± S.D. of three independent experiments.

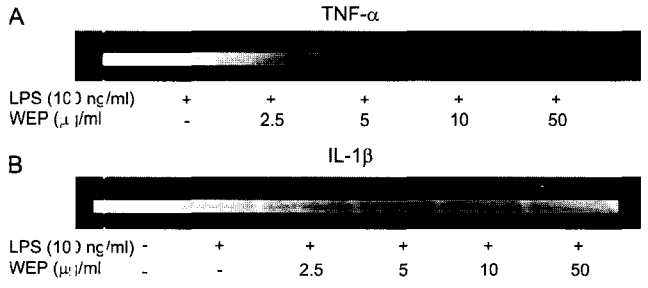

Fig. 5. Western Blot Analysis of the TNF-α in lysates of RAW 264.7 cells (2×μg Protein/Lane). The untreated cells and the cells incubated with LPS (100 ng/ml) alone and in combination with either WEP (2.5, 5, 10, 50 μg/ml) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with A) a mouse monoclonal anti-IL-1-β antibodies, B) a mouse monoclonal anti-TNF-α antibodies.

used as a positive control for macrophage activation. In the LPS (10 ng/ml) stimulated RAW 264.7 cell culture system, NO production was increased by i-NOS. When various WEP concentrations (2.5, 5, 10, 50, 100 μg/ml) were added to the culture media at the time (40 hours) cell stimulation, NO production was increased. Significantly, more NO was produced when the cells were exposed to a combination of LPS and WEP (2.5 μg/ml up to 25 μg/ml). From 50 to 100 μg/ml, a dose dependent decrease was observed (Fig. 6). These results suggest that WEP may function, at least in part, through the macrophage activation in the host defense response.

Effect of WEP and LPS on macrophage morphology

Normal RAW cells, when cultured in medium alone, tend

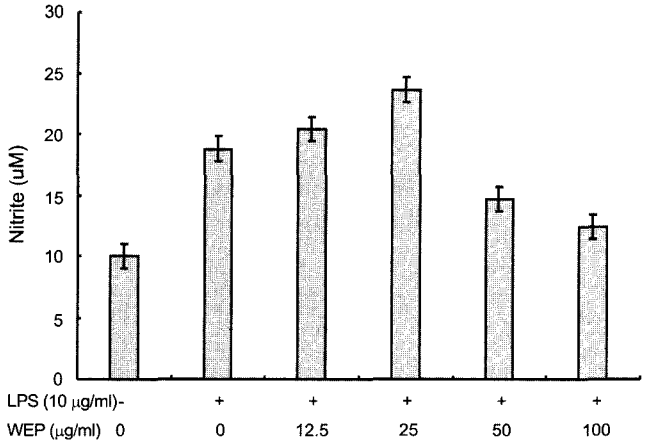

Fig. 6. Effects of WEP on NO production in the LPS (lipopolysaccharide)-stimulated RAW 264.7 cells. The cultures were incubated with 10 μg/ml of LPS in the presence of different WEP concentrations (12.5, 25, 50, 100 μg/ml). 6 wells per group were used and 200 μl of the cells were added to each well. The plates were then incubated overnight and 100 from the surface of each well was transferred into a new plate. The accumulated nitrite concentration were measured overnight after stimulation used a Griess reagent for 10 minutes at room temperature and read using an ELISA reader at 540 nm. NO production was determined by the accumulation of nitrite using the method reported Stuehr and Nathan (1989). The results are reported as a mean± S.D. of three independent experiments.

to be round. None appeared to spread over the surface (Fig. 7A). Fig. 8 shows that the cells treated with LPS/WEP (5 μg/ml) (Fig. 7B) are larger and rougher than those exposed to either LPS/WEP (10 μg/ml) (Fig. 7C) or LPS/WEP (20 μg/ml), LPS/WEP (40 μg/ml) (Fig. 7D). These results suggested that cells treated with LPS/WEP were larger and rougher than those exposed to either media or LPS alone.

Effect of WEP on surface molecule expression

Since the adhesion molecules play an important role in the macrophage activation process. The RAW cell surface expression of I-A^b, CD40, ICAM-1 (CD54) was examined by flow cytometry (Fig. 8). The intercellular adhesion molecules (ICAMs) ICAM-1, ICAM-2, and ICAM-3 are cell-surface ligands for the leukocyte integrins. They are crucial in the binding of lymphocytes and other leukocytes to certain cells, including antigen-presenting cells and endothelial cells. They are members of the immunoglobulin superfamily. B-cell growth is triggered in part by the binding of CD40 ligand, expressed on activated helper T cells, to CD40 on the B-cell surface. The number of MHC class molecules, B7 molecules, and CD40 and TNF receptors on the macrophage surface increases, making the cell both more effective in presenting antigen to fresh T cells, which may thereby be recruited as effector cells, and more responsive to CD40 ligand. Increased I-A^b, CD40, ICAM-1

(CD54) expression occurred when cells were treated with WEP. However, the expression of CD40 on cells treated with LPS/WEP (2.5 μg/ml) was decreased than in media.

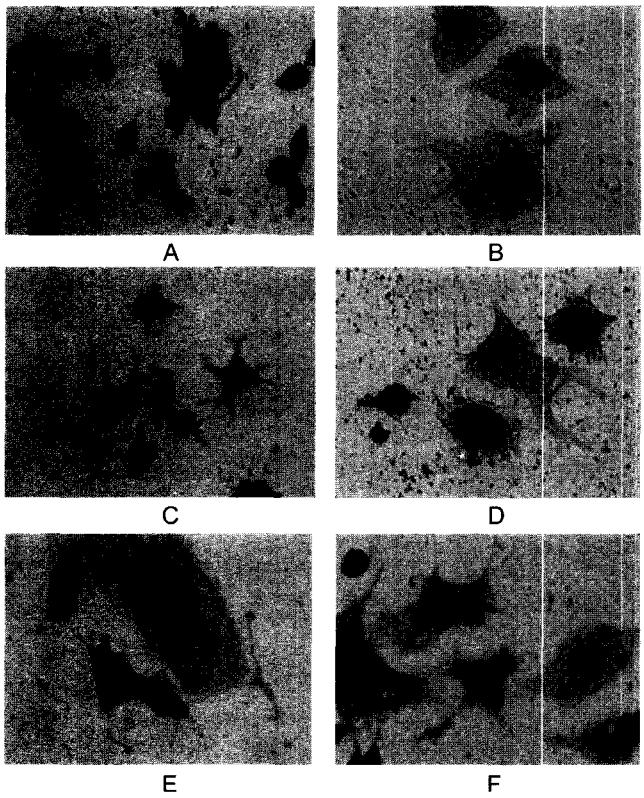

Fig. 7. Macrophage morphological changes in response to WEP. The RAW cells were cultured on cover slips in the presence of different concentration of the medium (A), LPS (100 ng/ml: B), LPS/WEP (5 μg/ml)(C), LPS/WEP (10 μg/ml) (D), LPS/WEP (20 μg/ml) (E), LPS/WEP (40 μg/ml) (E) and for 48 hours. The cells were fixed and stained in Diff-quick Solution.

Effect of PWE on cell cycle

The repetitive growth and division of cells is called the cell cycle. The cell cycle is often divided into 5 phases. There are: G1 (for "gap 1"), a period of growth before DNA replication; S (for "synapsis"), as DNA is replicated; G2 (for "gap 2"), a period of growth following DNA replication; M (for "mitosis"), the period of cell-division. Following mitosis, the daughter cells may re-enter the G1 phase, or proceed to a 5th phase called "G0" where growth and replication stops. Cells in G0 are said to be "quiescent". G0 cells may eventually re-enter G1 or perhaps die. This kind of assay is widely used in the study of cancer cells. In Medicine, it is used to classify some cancers with respect to prognosis, which may impact upon the treatment or therapy selected by the physician. And, in research, it is often used to evaluate the effects of drugs on the growth and division of cells. Although the DNA content of the cells did not change from WEP 5 μg/ml to 50 μg/ml, nearly all cells had side scatter values corresponding to the cell sizes of G_2-M-phase cells (Table 1).

Table 1. Effect of WEP on cell cycle distribution in RAW 264.7 cell line

	% of cells		
	G1	S	G2/M
Cells only	29.6	64.9	5.6
LPS only	28.2	64.9	6.8
WEP 5 μg/ml	27.3	67.7	5.0
WEP 10 μg/ml	31.6	64.9	3.5
WEP 50 μg/ml	30.6	65.8	3.6

RAW 264.7 cell line was treated with WEP and DNA flow cytometric cell cycle analysis was performed comparing untreated control cells with treated in WEP.

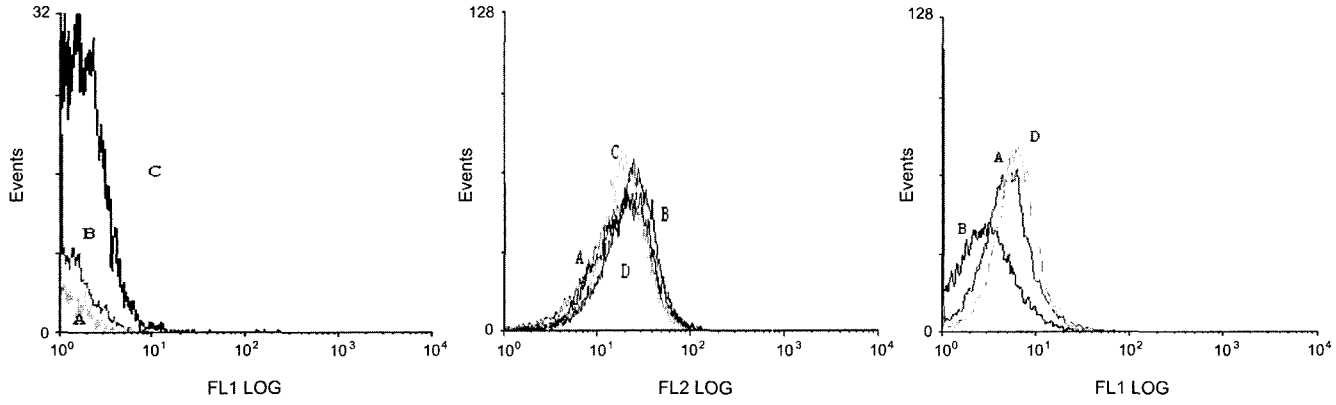

Fig. 8. Expression of the co-stimulator molecules ICAM-1 (CD54), CD40, IA^b expression. The RAW cells were cultured in presence of medium (A), LPS (100 ng/ml: E), LPS/WEP (2.5 μg/ml) (B), LPS/WEP (25 μg/ml) (C), LPS/WEP (50 μg/ml) (D) for 48 hours. The surface CD40, CD54, IA^b molecules were labeled with either anti-CD54-FITC, anti-CD40-PE, anti-IA^bFITC and the were cells stained using anti-v β8.1+8.2-FITC, anti-v β2-PE, anti-v β2-FITC, which served as an isotype control for the nonspecific binding (data not shown). The X-axis shows the fluorescence density. The Y-axis shows the relative cell numbers. The shaded curve denotes the background fluorescence.

DISCUSSION

Activated macrophages fuse their lysosomes more efficiently to phagosomes, exposing intracellular or recently ingested extracellular microbes to a variety of microbicidal lysosomal enzymes. Activated macrophages also produce oxygen radicals and nitric oxide (NO), both of which have potent antimicrobial activity, in addition to synthesizing antimicrobial peptides and proteases that can be released to attack extracellular parasites. Additional changes in activated macrophages assist in amplifying the immune response. The number of MHC class molecules, B7 molecules, and CD40 and TNF receptors on the macrophage surface increases, which cause the cell to be more effective at presenting an antigen to fresh T cells. These may therefore be recruited as effector cells, and be more responsive to both a CD40 ligand and to TNF-α.

NO is a multi-functional mediator which plays an important role in regulating various biological function *in vivo*. NO production by iNOS in macrophages is essential for the defense mechanisms against microorganisms and tumor cells (Faris *et al.*, 1994; MacMicking *et al.*, 1995). The NO is produced by iNOS, and mice that have had the gene for iNOS knocked out are highly susceptible to infections with several intracellular pathogens (Charles *et al.*, 1999) NO exhibits cytoprotective properties to some cells such as hepatocytes and endothelial cells (Kim *et al.*, 1997) However, its excessive production in inflammation is thought to be a causative factor for septic shock, cellular injury and carcinogenesis (Wolfe and Dasta, 1995). Therefore, the dysregulation of NO production is causatively related to the pathogenesis of various diseases which include inflammation, cancer, immunological disorders and vascular diseases. The inhibitory effect on NO production from activated macrophages has been demonstrated in some crude drugs with anti-inflammatory properties. WEP, which inhibits iNOS expression, may have potential for treating endotoxemia and the inflammation that accompanies NO overproduction.

Most effector T cells express members of the TNF protein family as cell surface molecules. The most important TNF family proteins in the T-cell effector function are TNF-α and TNF-β (which can also be produced as secreted molecules), the Fas ligand and the CD40 ligand, the latter two always being associated with the cell surface. TNF-α is produced by T_H1 cells, some T_H2 cells, and cytotoxic T cells in both a soluble and membrane-associated form, and can also deliver activating signals to macrophages. Some members of the family of TNF receptors can stimulate apoptosis (Charles *et al.*, 1999). T_H2 cells express B-cell-activating effector molecules, whereas T_H1 cells express effectors molecules that activate macrophages. TNF-α production was dependent on the WEP dose.

WEP was found stimulate macrophages to release TNF-α, IL-1β. NO production, cell morphological changes and surface molecule expression were largely unaffected by WEP alone. However, these effects could be potentiated by LPS addition. Therefore, these results demonstrate the ability of WEP to activate RAW cell both directly for cytokine production and indirectly for NO production and stimulate the expression of some of the surface molecules.

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