Inhibition of Major Histocompatibility Complex (MHC)-Restricted Presentation of Exogenous Antigen in Dendritic Cells by Korean Propolis Components

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ABSTRACT

Background: Dendritic cells (DCs) play a critical role not only in the initiation of immune responses, but also in the induction of immune tolerance. In an effort to regulate immune responses through the modulation of antigen presenting cell (APC) function of DCs, we searched for and characterized APC function modulators from natural products. **Methods:** DCs were cultured in the presence of propolis components, WP and CP, and then examined for their ability to present exogenous antigen in association with major histocompatibility complexes (MHC). **Results:** WP and CP inhibited class I MHC-restricted presentation of exogenous antigen (cross-presentation) in a DC cell line, DC2.4 cells, and DCs generated from bone marrow cells with GM-CSF and IL-4. The inhibitory activity of WP and CP appeared to be due not only to inhibition of phagocytic activity of DCs, but also to suppression of expression of MHC molecules on DCs. We also examined the effects of WP and CP on T cells. Interestingly, WP and CP increased IL-2 production from T cells. **Conclusion:** These results demonstrate that WP and CP inhibit MHC-restricted presentation of exogenous antigen through down-regulation of phagocytic activity and suppression of expression of MHC molecules on DCs. **(Immune Network 2005;5(3):150-156)**

Key Words: Propolis, dendritic cell, major histocompatibility complex, antigen presentation

Introduction

Protein must be processed to small peptides in order to be presented either on MHC class II molecules for activation of CD4 T cells or MHC class I molecules for activation of CD8 T cells. Exogenous antigens (Ags) are normally processed and presented on class II MHC molecules. Recent studies documented that exogenous antigens can enter the class I MHC presentation pathway, a process termed cross presentation (1-3). Subsequently, it has shown that the cross presentation pathway is an obligatory mechanism in several situations. A strict requirement of cross presentation has been demonstrated in the induction of cytotoxic T lymphocytes (CTL) responses to graft tissues, tumor cells and viruses that infect only non-hematopoietic cell (4-7). The important physiological function of cross-presentation in viral infections became clear when cross-presentation is necessary and sufficient to initiate CTL responses to viruses that do not infect professional APC (pAPC)(8,9). However, little is known about the mechanisms underlying this process. Current knowledge of the processing of viral Ags into MHC class I-associated ligands is based almost completely on *in vitro* studies using nonprofessional antigen presenting cells (APCs)(10-14). APCs have the unique ability to acquire Ag from exogenous sources and present them on their own MHC class I molecules. This ability of APC to present exogenous Ags can be readily observed *in vitro* by co-culturing dendritic cell (DC) with many different antigenic formulations (10,15). There is enough evidence that, among the APC able to present the antigen, DC have

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the unique property to activate antigen-specific, naive T cells *in vitro* and *in vivo*, and are essential on control of the immune response that function by surveying peripheral tissues for Ag (16-20). DC can readily capture Ag from dead and dying cells for presentation to MHC class I-restricted CTL (17,21). Since cross presentation emerges to be required for the effective generation of CTL responses, it is interesting to see whether modulation of cross presentation capability could be a feasible way of immunosuppression on the cross presentation capability of DC (22-26).

In this study, we tested whether components of propolis, WP and CP, affected the capacity of DC to present Ags to CD8 T cells showing that DC cross-present a model Ag of OVA supplied exogenously and wished to determine whether presentation of exogenous OVA could be inhibited by WP and CP, which have been used for the regulator of immune response as a traditional medicine remedy. Our data show that, *in vitro*, the WP and CP affect the presentation of DC, selectively down-regulate the cross presentation of MHC molecules to CD8 T cell line, B3Z. Therefore, these results demonstrate that WP and CP inhibited class I MHC-restricted presentation of exogenous antigen (cross-presentation) in both DC cell line, DC2.4 cells, and DCs generated from bone marrow cells with GM-CSF and IL-4. The inhibitory activity of WP and CP appeared to be due to not only inhibition of phagocytic activity of DCs, but also suppression of expression of MHC molecules on DCs. We also showed the effects of WP and CP on T cells. Interestingly, WP and CP increased IL-2 production from T cells.

Materials and Methods

Animals. Five to 8-week-old C57BL/6 (H-2^b) and BLAB/c mice were used. The mice were maintained in pathogen-free conditions in animal facilities at University of Sahmyook (Seoul, South Korea).

Preparation of propolis components (WP and CP). Propolis was obtained from Chunghak Beekeeping Farm in the area Gyeonggi province, which was collected from May to August in 1997. One kilogram of the crude mass of propolis was frozen in a freezer at $-20^{\circ}C$, squashed in to small pieces and ground into powder by a grinder. It was, then, extracted with water at a proportion of one to five (propolis to water) for two hours with stirring while the temperature was kept lower than $50^{\circ}C$. Immediately, the solution was filtered by 2 pieces of Whatman #2 paper, frozen at $-70^{\circ}C$ and processed using freeze- drying machine, which resulted in 32.5 grams of brownish powdered propolis WP (water extract of Korean propolis)(24). Chloroform layer of WP was obtained after further isolation and harvested 1.885 grams (CP).

Cell lines and reagents. A DC cell line, DC2.4, was obtained from Dana-Farber Cancer Institute, Boston, MA, USA (27). T cell hybridomas, B3Z86/90.14 (B3Z) and DOBW were kindly provided by Dr. Nilabh Shastri (University of California, Berkeley, CA)(28) and by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH)(29), respectively. A B cell hybridoma, 25-D1.16 clone 21, was kindly provided by Dr. Jonathan W. Yewdell (National Institutes of Health, Bethesda, MD)(30).

Cell culture. DC2.4 cells were obtained from bone marrow cells infected with a retrovirus encoding *myc* and *raf* by using supernatant from NIH J2 Leuk cells (3). Cells were cultured in Dulbecco's modified Eagle Medium (DMEM), which was supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10,000 U/ml)/streptomycin (10,000 U/ml)(P/S), which were purchased from Sigma Chemical Co. (St. Louis, MO. USA). B3Z, a T cell hybridoma, which recognizes K^b with peptide 257~264 (SIINFEKL) of OVA (31). B3Z contains a DNA construct coding for the *lacZ* gene, under the control of the IL-2 regulatory elements. Upon activation of B3Z through the TCR, the *lacZ* gene is expressed, allowing determination of the activation of the T cell hybrid through colorimetric assays.

Generation of bone marrow derived dendritic cells. BM derived-DCs were generated by using mouse rmGM-CSF and rmIL-4 (22). Briefly, total BM cells obtained from femurs of BALB/c or C57BL/6 mouse were cultured in a 6 well plate (5×10^6/well) in a culture medium supplemented with 80 ng/ml rmGM-CSF (PeproTech) and 40 ng/ml rmIL-4 (PeproTech). At days 4 and 5 from the initiation of the culture, nonadherent cells were discarded by replacing the culture medium with fresh medium containing the cytokines after gentle shaking. DCs were harvested at day 7.

B3Z T hybridoma activation assays. Activation of B3Z cells was measured by *lacZ* activity. Briefly, 4×10^4 DC2.4 cells/well of 96-well plate were treated with different samples in 5.5% CO_2 for 2 hrs at $37^{\circ}C$, and then particulate OVA was added for another 2 hrs. The cells were washed with phosphate buffered saline (PBS, pH7.2) and then incubated with 1×10^5 B3Z cells/well of 96-well plate to evaluate antigen presentation. After 4 hrs, the supernatant was removed, and the cells were lysed by a lysis buffer (0.1% Triton $\times$ -100, 250 mM Tris, pH 8.0) and keep in $-70^{\circ}C$ for 10 min. The plates were thawed at room temperature for 10 min and added 50 μl of PBS containing 0.5% BSA. Overlaid with 100 μl of substrate (1 mg/ml of Chlorophenolred-β-D-galactopyranoside in β-galactosidase buffer: 60 mM sodium dibasic phosphate buffer (pH 8.0), 1 mM magnesium sulfate,

10 mM KCl, 50 mM β-mercaptoethanol. The plates were incubated in 5.5% CO_2 for 12 hrs at 37°C and were measured by an ELISA reader at 580 nm (Vmax, Molecular Devices, and USA).

MHC class II-restricted presentation assay. DCs treated with mitomycin C were added to 96-well microtiter plate (4×10^4/well), incubated for 2 hrs at 37°C, and then added with different amounts of OVA microspheres and WP and CP. After 2 hrs incubation at 37°C, unphagocytized OVA-microspheres and WP and CP were removed by suction. The plate was then washed twice with 300 μl/well of pre-warmed DMEM, and added with OVA-specific CD4 T (1×10^5/well) isolated from spleen of DO11.10 mouse (23). After 24 hrs incubation at 37°C, the plate was centrifuged at 1,800 rpm, and the culture supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit (BD Biosciences).

Phagocytosis assay. DC2.4 cells (1×10^6/well) were incubated with the presence or absence of WP (100 μg/ml) or CP (5.8 μg/ml) for 2 hrs at 37°C, and then added with microspheres containing OVA-FITC for 2 hrs incubation at 37°C. Treated cells were then scraped into PBS-0.1% sodium azide with 1% FBS (PBS-washing buffer, pH 7.2) and washed twice in washing buffer at 4°C and then analyzed in a FACS. We tested both DCs cell line, DC2.4, and BM-derived DCs. Control represents DCs treated with neither p-OVA nor samples. Cells were then washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2). Flow cytometry analysis was performed on an EPICS V analyzer (Coulter, Hialeah, EL). Fluorescence intensity was determined on 50,000 cells from each sample using logarithmic amplification.

Antibodies and flow cytometry. DC2.4 (1×10^6 cells/ml) cells were cultured in DMEM for 2 hours. The media were changed and the cells were incubated in the presence of either WP (100 μg/ml), CP (5.8 μg/ml) or

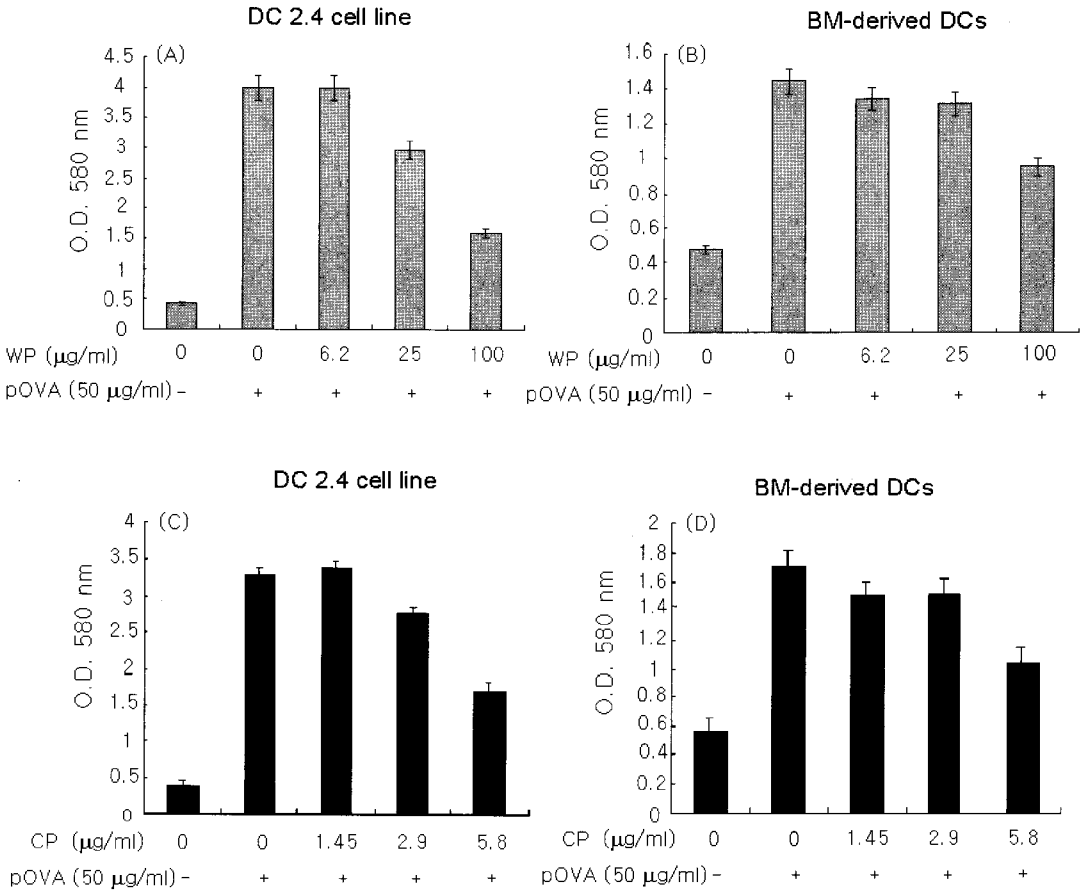

Figure. 1. DC2.4 cells were incubated with the amount of microencapsulated OVA co-cultured with a T cell hybridoma, B3Z cells. The amount of β-galactosidase expressed in B3Z cells were determined by an ELISA reader. These results are representative of more than ten experiments. An indicated amount of WP (6.2, 25, 100 μg/ml, (A), (B)) or CP (1.45, 2.9, 5.8 μg/ml, (C), (D)) were added to cultures of DC2.4 cells (A, C) or BM-derived DCs (B, D) together with OVA-microspheres. Each data point represents the mean $\pm$SD of values obtained from four experiments.

medium for 2 hours. Treated cells were then scraped into PBS-0.1% sodium azide with 1% FBS (PBS-washing buffer, pH 7.2) and washed twice in washing buffer at 4°C. Before cells were stained with FITC (Fluorescein isothiocyanate)-monoclonal antibody, the cell surface Fc receptors were blocked by incubating cells with 20 μg/10^6 cells of purified anti-CD-16 for 30 min at 4°C. The residual antibody was removed by washing. The monoclonal antibody, anti-H2-K^b (clone AF6-88.5), and an isotype-matched control antibody were purchased from BD Biosciences. Anti-H2-K^b and Anti-I-Ab-FITC were then added and cells were kept at 4°C for 30 min. The monoclonal antibody, 25-D1.16, was purified from ascitic fluid collected from mice injected with 25-D1.16 clone 21 cell, and then digested with pepsin to obtain F $(ab')_2$ fragments. FITC-labeled anti-mouse IgG1 antibody was purchased from BD Biosciences. Soup of 25-D1.16 were then added and cells were kept at 4°C for 30 min. Cells stained using mouse IgG-FITC served as a control for nonspecific binding. Cells were then washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2). Flow cytometry analysis was performed on an EPICS V analyzer (Coulter, Hialeah, EL). Fluorescence intensity was determined on 50,000 cells from each sample using logarithmic amplification.

Results

WP and CP inhibit cross-presentation of exogenous antigen in both DC2.4 cells and BM-derived DCs. We examined the effects of propolis (WP or CP) on the MHC-restricted presentation of exogenous antigen. Treatment of DC2.4 cell line or BM-DCs with WP (25~100 μg/ml: Fig. 1A, 1B) or CP (1.8~5.8 μg/ml: Fig. 1C, 1D) resulted in a population of cells with an inhibited ability to present particulate OVA. The cross-presentation inhibitory activity was also examined in normal DCs generated from BM cells of C57BL/6 mouse. Since cross presentation emerges to be required for the effective generation of CTL responses, it is interesting to see whether modulation of cross presentation capability could be a feasible way of suppressing the cross presentation capabilityof DCs.

CP inhibits phagocytic activity of DCs. To test whether the antigen presentation-inhibitory activity of WP and

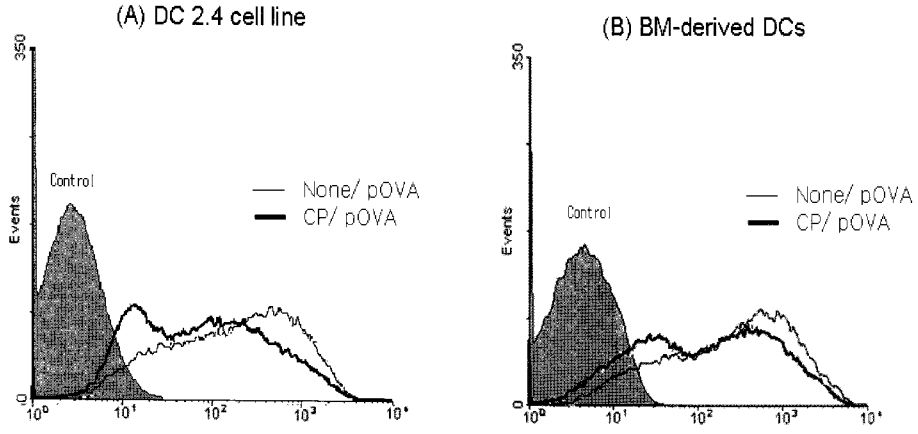

Figure. 2. DC2.4 cells were incubated with microspheres containing OVA-FITC in the presence or absence of CP (5.8 μg/ml) and then analyzed in a FACS. We tested both DCs cell line, DC2.4 (A) and BM-derived DCs (B). Control represents DCs treated with neither p-OVA nor samples.

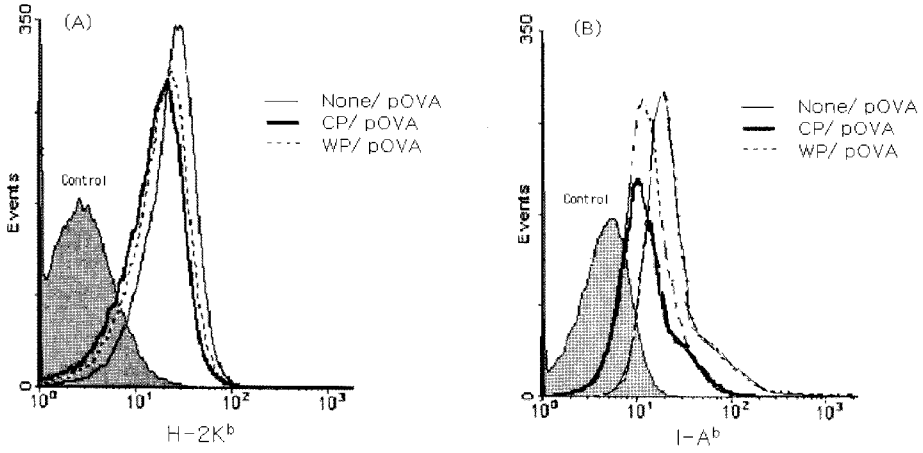

Figure. 3. DC2.4 cells were incubated without OVA-microspheres in the presence of WP (100 μg/ml) or CP (5.8 μg/ ml) and the expression level of H-2K^b molecules (A) and I-A^b complexes (B) were determined by monoclonal antibodies. Control represents DCs treated with neither p-OVA nor samples.

CP was due to inhibition of phagocytic activity, DC2.4 cells were incubated with microspheres containing both OVA and FITC, washed, and then harvested by gentle pipetting after cooling on ice. Flow cytometric analysis of the harvested cells showed that CP inhibit phagocytic activity of cell line DC2.4 and bone marrow derived DCs generated *in vitro* DCs (WP data not shown)(Fig. 2).

WP and CP decrease total expression of H-2K^b and I-A^b molecules. To prove that WP and CP inhibit an extracellular event of the antigen processing pathways, DC2.4 cells were incubated with different concentrations of WP or CP, washed, anti-H2-K^b and anti-I-A^b-FITC were added and cells were kept on ice for 20 min. Fluorescence intensity was determined on 50,000cells from each sample using logarithmic amplification. As shown in Fig. 3, WP and CP inhibited presentation of total expression of H-2 K^b and I-A^b molecules.

CP inhibits the expression peptide-H-2K^b Complexes. We examined whether CP inhibited the expression of OVA-specific class I MHC molecules. DC2.4 cells were incubated with CP (5.8 μg/ml) for 2 hrs in the presence of OVA-microspheres, washed, and then the expression level of SIINFEKL-H-2K^b complexes was determined by F(ab')$_2$ fragment of SIINFEKL-H-2K^b-specific monoclonal antibody, 25-D1.16. We found that CP inhibited the expression of SIINFEKL-H-2Kb complexes (Fig. 4)(WP data not shown).

IL-2 production was increased on class II MHC presentation of exogenous antigen by WP and CP. For class II MHC-restricted presentation assays, DCs generated from BM cells of BALB/c mouse (H-2^d) were incubated with OVA-microspheres, and the amount of OVA peptide-class II MHC complexes was measured by OVA-specific CD4 T cell line, DOBW, originated from spleen of DO11.10 mouse (H-2^d). Exogenous antigens are normally processed and presented on class II MHC molecules. This result showed that WP and CP did not block the classical exogenous pathway (Fig. 5B). Because we also examined the effects of tacrolimus (FK506) on the class II MHC-restricted presentation of exogenous OVA in DCs generated from BM cells of BALB/c mouse. As shown in Fig. 5A, FK506 inhibited class II MHC-restricted presentation of exogenous OVA (23), but Interestingly, WP and CP increased IL-2 production from T cells. WP and CP may have a different mechanism of cross presentation pathways since WP and CP could enhance

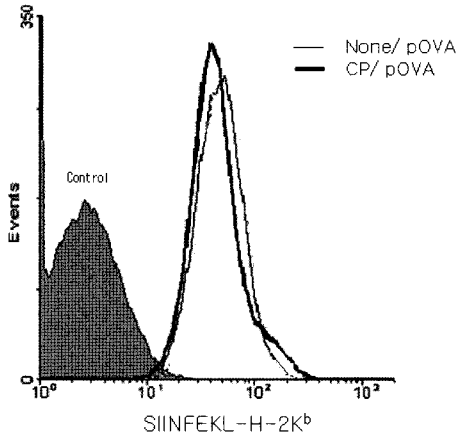

Figure. 4. DC2.4 cells were incubated with microspheres containing OVA in the presence of CP (5.8 μg/ml) and the expression level of SIINFEKL-H-2K^b complexes was determined by monoclonal antibodies. Control represents DCs treated with neither p-OVA nor samples.

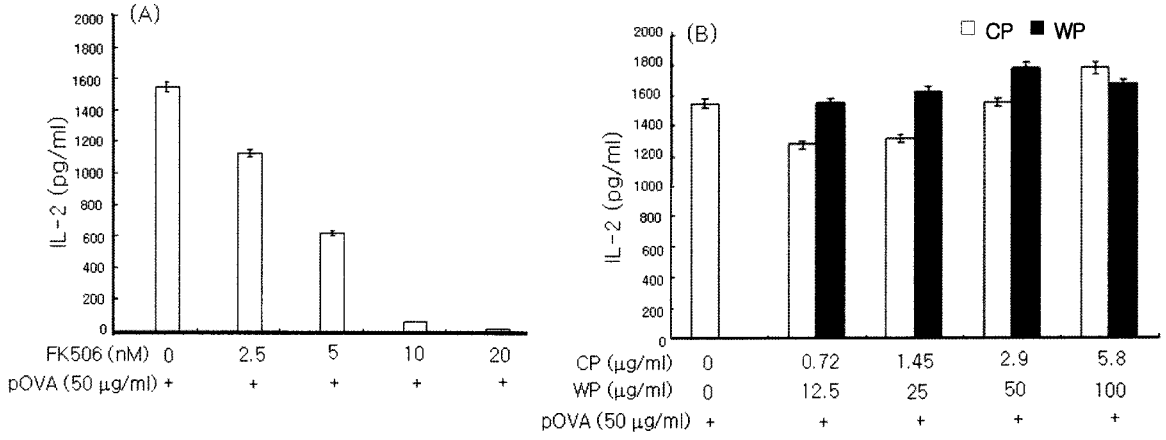

Figure. 5. Mitomycin C-treated DCs (generated from BM cells of BALB/c mouse) were incubated with OVA-microspheres and then added with CD4 T cells isolated from spleen of DO11.10 mouse. Supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit. Values represent mean±SD of triple samples and are representative of five experiments. We used FK506 as positive control (A) and WP or CP (B).

IL-2 production with class II MHC presentation of exogenous antigen while these components inhibited action of cross presentation.

Discussion

In vivo and *in vitro*, professional antigen-presenting cells (APCs), in particular dendritic cells (DCs), are capable of processing cell-associated antigens for presentation on major histocompatibility complex (MHC) class I molecules (14). *In vitro*, several forms of antigen can access the exogenous pathway for cross-presentation, apparently through phagocytic or nonphagocytic mechanisms (32). Antigens such as particulate antigens, immune complexes, and heat shock proteins (HSPs) have been shown to be loaded onto MHC class I molecules in fashions either independent of or dependent on proteasome degradation, transport into the ER via TAP, and MHC class I peptide loading in the ER (33).

The present study demonstrates that components of propolis, CP and WP, decreased class I MHC-restricted presentation of exogenous antigen (cross presentation) in both DC cell line, DC2.4 and DCs generated with GM-CSF and IL-4 from mouse BM cells. The inhibitory activity of WP and CP appeared to be due to not only inhibition of phagocytic activity of DCs, but also suppression of expression of MHC molecules on DCs. We also examined the effects of WP and CP on T cells. Interestingly, WP and CP increased IL-2 production from T cells. CP and WP appeared to enhance class II MHC-restricted presentation of exogenous antigen in DCs generated from BM cells of BALB/c mouse by producing IL-2.

Our study of murine DCs reveals many unique features of cross-presentation in vitro. Cross-presentation was inhibited in the presence of WP or CP, consistent with the interpretation that internalization and possibly processing of OVA. In all of the experiments described in the present study, DCs were exposed with WP and CP only for 2 hrs in the presence of OVA-microspheres. Thus, it is obvious that the inhibitory activity of WP and CP on MHC-restricted antigen processing is initiated right after exposure to these materials. The concentration of WP and CP inhibiting MHC-restricted antigen processing did not effect of cytotoxic dose. Expression of H-$2K^b$ and I-A^b was somewhat lower in presence WP and CP. DCs can internalize exogenous antigens via different mechanisms such as clathrin-mediated endocytosis, fluid-phase endocytosis, macropinocytosis, or phagocytosis (34). To evaluate if uptake via macropinocytosis or phagocytosis is involved in the cross-presentation of cell-associated OVA by DCs, both WP and CP clearly inhibited these processes at different temperature, 37°C or 0°C respectively.

These results demonstrates that WP and CP can regulate class I MHC-restricted presentation of exogenous antigen through downregulation of phagocytosis and total expression of MHC class I and MHC class II molecules in both cell line DC2.4 and bone marrow derived DCs generated *in vitro*. Further studies will be required to characterize the role of WP and CP in the intracellular processing events of antigens processing and expression pathways.

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