Proanthocyanidins (PA) are naturally occurring compounds that are widely found in fruits, vegetables, nuts, seeds, flowers, and bark (Bagchi et al., 2000; Rapport and Lockwood, 2001). Proanthocyanidins belong to the category known as condensed tannins, 1 of 2 main plant tannin categories (Bruyne et al., 1999). Proanthocyanidins are polyphenolic natural products composed of flavan-3-ol subunits linked mainly through C4–C8 (or C6) bonds (Kennedy and Taylor, 2003). Proanthocyanidins are potent free radical scavengers (Prior and Gu, 2005; Ku and Mun, 2008a), antibacterial agents (Shan et al., 2007), and effective enzyme inhibitors (Stevens et al., 2002; Ku and Mun, 2008b). They also exhibit vasodilatory, antiallergic, antiinflammatory (Torras et al., 2005), cardioprotective (Kohama et al., 2004), immune-stimulating, antiviral, and estrogenic activities (Bagchi et al., 2000). Researchers have focused on the potent antioxidant capacity and possible human health protective effects of PA, with an aim of reducing the risk for chronic diseases such as cancers and cardiovascular diseases (Schmidt et al., 2006). However, few reports are related to the immunomodulatory effects of PA. Phenolic compounds such as PA stimulate or suppress the immune system due to the presence of numerous hydroxyl groups in their structures. These groups affect enzyme or electron-transfer systems, resulting in immunomodulation of specific responses, particularly phagocytosis (Manosroi et al., 2003).

Pinus radiata bark is an important source of PA (Ku and Mun, 2007). We previously reported the immunomodulatory effects of a PA-rich extract (PAE) from P. radiata bark in specific-pathogen-free White Leghorn chickens (Park et al., 2011). The objective of this study was to evaluate the immunomodulatory effects of PAE by dosing period in Korean native chickens.

**MATERIALS AND METHODS**

**Birds**

Two hundred 1-d-old Korean native chickens were used to determine the immunomodulatory effects of PAE. The chickens were wing-banded individually and reared under uniform management care in an isolator. They were brooded initially at 31 to 33°C during the first 5 d, followed by a weekly 2 to 3°C reduction in temperature until the temperature reached 22 to 23°C. The chickens had free access to water and feed.

**Experimental Design**

For experiment 1, chickens were divided into 5 groups, 4 that received PAE and 1 that received none. Groups 1...
to 4 (n = 10 per group) were administered 1.25, 2.5, 5, or 10 mg of PAE/kg of BW, respectively, once daily by oral gavage. Group 5 (n = 10) received an equal volume of normal saline on the same schedule and served as the control. All chickens were administered PAE or saline for 5 wk. Body weight was checked after 30 d. Peripheral blood mononuclear cell (PBMC) proliferation was determined at the age of 2 and 5 wk. Splenocyte and thymocyte proliferation was checked at the age of 5 wk.

The chickens were divided into 6 groups of A, B, C, D, E, and F (n = 25 per group) for experiment 2. Group A was not administered PAE as the control. Group B was administered PAE until the age of 2 wk, group C was administered PAE until the age of 4 wk, group D was administered PAE until the age of 6 wk, group E was administered PAE until the age of 8 wk, and group F was administered PAE until the age of 10 wk. The PAE was administered through drinking water at a concentration of 50 mg/L. All chickens were raised until the age of 10 wk. Proliferation of PBMC, splenocytes, and thymocytes was determined every 2 wk (n = 5/group).

Isolation of PBMC, Splenocytes, and Thymocytes

Whole blood was collected via wing-vein puncture into an EDTA-containing tube to isolate PBMC. The blood was layered on 1077 Histopaque (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1,400 × g at room temperature for 25 min. The PBMC were collected from the gradient interface, and the plasma suspension was combined and washed 3 times with Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich). Spleens and thymuses were isolated. The tissues were kept separate and were dissociated between the frosted ends of 2 microscope slides, and erythrocytes were lysed in red blood cell lysis buffer for 5 min at room temperature and centrifuged at 504 × g at room temperature for 5 min. The splenocytes and thymocytes were washed twice with DMEM.

Proliferation Assay

Each isolated splenocyte and thymocyte cell fraction was cultured alone or with 10 μg/mL of concanavalin A (Con A; Sigma-Aldrich) or 1 μg/mL of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) at a density of 10⁶ cells/mL to test whether PAE promoted or inhibited PBMC proliferation and the interaction of mitogen and PAE on proliferation. The cells were cultured at 37°C in 5% CO₂ and DMEM containing 10% fetal bovine serum (Sigma-Aldrich) and antibiotics for 48 h. Cell proliferation was determined by the 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay (Buttke et al., 1993), using a CellTiter 96 Aqueous Non-radioactive Cell Proliferation assay kit (Promega, Madison, WI). Absorbance was measured using a microplate reader at 490 nm.

Analysis of T and B Cells by Flow Cytometry

Splenocytes were isolated from Korean native chickens as described above. The T cells were identified by staining the cells with FITC-conjugated anti-chicken CD4 and R-PE-conjugated anti-chicken CD8α (Southern Biotech, Birmingham, AL). The B cells were identified by staining the cells with R-PE-conjugated anti-chicken Bu-1 (Southern Biotech). Splenocytes (1 × 10⁶ cells) were washed twice with wash buffer (PBS containing 0.1% NaN₃), and the samples were incubated with the conjugated antibodies for 30 min at 4°C. Each sample was resuspended in 0.5 mL of fixative solution (PBS containing 2% formaldehyde and 0.05% NaN₃) and analyzed with a BD FACs Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA) was used to analyze the flow cytometry data.

Statistical Analysis

All data were analyzed with SPSS 12.0 statistical software (SPSS, Chicago, IL). Data are expressed as mean ± SD. Statistical differences were examined independently using Student’s t-test and Pearson correlation test. A P-value <0.05 was considered significant.

RESULTS

BW of Korean Native Chickens

Body weights were obtained at 30 d. No significant difference in weight was observed at 30 d of age between the control (763 ± 52.69 g) and the groups administered 1.25, 2.5, 5, or 10 mg/kg of PAE (821 ± 123.99, 818 ± 86.12, 897 ± 162.84, and 825 ± 42.90 g, respectively). Therefore, PAE had no effect on growth of Korean native chickens.

Effect of PAE on PBMC Proliferation

In experiment 1, PAE was orally administered to Korean native chickens at doses of 1.25, 2.5, 5, or 10 mg/kg of BW per day for 5 wk. In vitro, to test whether PAE promoted or inhibited mitogen-stimulated PBMC proliferation, PBMC were isolated and cultured alone or with 10 μg/mL of Con A or 1 μg/mL of LPS. After 2 wk of administration, mitogen-stimulated PBMC proliferation in chickens administered 1.25, 2.5, 5, or 10 mg/kg of PAE was significantly higher than that in control chickens (Figure 1A). However, mitogen-stimulated PBMC proliferation in the PAE-administered chickens was similar to that of control chickens after 5 wk of administration (Figure 1D).
In experiment 2, PAE was administered through drinking water at a concentration of 50 mg/L, and mitogen-stimulated PBMC proliferation was checked every 2 wk. Mitogen-stimulated PBMC proliferation in the chickens administered PAE for 6 wk was continuously higher than that in the other groups (Figure 2A). However, mitogen-stimulated PBMC proliferation in the chickens administered PAE for >6 wk did not increase further.

**Flow Cytometry Analysis**

In experiment 2, the T and B cell populations were analyzed in splenocytes by flow cytometry. We found significantly increasing cell populations for which CD4+CD8− (Th cells) and Bu-1+ (B cells) protein was expressed on the cell surface of CD4+CD8− (Th cells) and Bu-1+ (B cells) in the chickens administered PAE for >4 wk (Tables 1 and 2). However, the CD4−CD8+ cell population was similar to that in the control group (Table 1). These results indicate that PAE might have an immunomodulatory role in cellular and humoral immunity.

**DISCUSSION**

Proanthocyanidins are natural polyphenolic compounds that are widely distributed in many plants and have long been used as human dietary supplements. The present results provide evidence that PAE from *P. radiata* bark exhibits immunomodulatory activities in chickens.

After 2 wk of administration with 1.25, 2.5, 5, or 10 mg/kg of PAE, PBMC proliferation in Korean native chickens was significantly higher than that in control chickens. However, PBMC proliferation in the PAE-administered chickens was similar to that of the control chickens after 5 wk of administration. These results indicate that PAE enhanced the early events of PBMC proliferation. Additionally, the proliferation of splenocytes and thymocytes in the PAE-administered chickens was significantly higher after 5 wk than that in...
control chickens. Certain compounds enhance immunocyte proliferation, suggesting an important role in immune function (Gupta et al., 2006). The evaluation of

Figure 2. Proanthocyanidin-rich extract (PAE) continuously promotes peripheral blood mononuclear cell (PBMC), splenocyte, and thymocyte proliferation during the supplementation period. Cells were cultured in triplicate wells without the stimulants in a 5% CO₂ atmosphere at 37°C for 48 h, and cell proliferation was determined by the MTS assay. Cell proliferation in the chickens administered PAE for 6 wk was continuously higher than that in the other groups. (A) Not administered PAE as a control; (B) administered PAE until the age of 2 wk; (C) administered PAE until the age of 4 wk; (D) administered PAE until the age of 6 wk; (E) administered PAE until the age of 8 wk; and (F) administered PAE until the age of 10 wk. *P < 0.05, **P < 0.005.

Table 1. The T cell population in proanthocyanidin-rich extract (PAE)-administered chickens in experiment 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4+/CD8− (%)</th>
<th>CD4−/CD8+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>23.36 ± 0.58</td>
<td>24.22 ± 0.90</td>
</tr>
<tr>
<td>B</td>
<td>23.48 ± 0.56</td>
<td>24.28 ± 0.66</td>
</tr>
<tr>
<td>C</td>
<td>23.58 ± 0.67</td>
<td>27.60 ± 0.72**</td>
</tr>
<tr>
<td>D</td>
<td>23.50 ± 0.65</td>
<td>27.71 ± 0.98*</td>
</tr>
<tr>
<td>E</td>
<td>23.69 ± 0.47</td>
<td>28.70 ± 1.69*</td>
</tr>
<tr>
<td>F</td>
<td>23.69 ± 0.47</td>
<td>28.96 ± 1.69</td>
</tr>
</tbody>
</table>

The percentage of CD4+/CD8− T cells increased in PAE-administered chickens. Group A was the control; group B was administered PAE until the age of 2 wk; group C was administered PAE until the age of 4 wk; group D was administered PAE until the age of 6 wk; group E was administered PAE until the age of 8 wk; and group F was administered PAE until the age of 10 wk. *P < 0.05, **P < 0.005.
substances that either promote or inhibit immunocyte proliferation is crucial to study immunomodulation and for drug discovery. Synergy effect of mitogen-stimulated cells (PBMC, splenocyte, and thymocyte) proliferation was noticed in administration of PAE compared with nonadministration of PAE. Although mitogen-stimulated cell proliferation was increased with administration of PAE compared with nonadministration of PAE, mitogen-stimulated cell proliferation was slightly decreased without stimulants (PAE alone) under administration of PAE. From the results, PAE enhanced the mitogen-stimulated cell proliferation than stimulated cells alone, but mitogen did not enhance cell proliferation in administration of PAE. These phenomena seem to indicate that different pathways enhanced the cell proliferation. In spleen and thymocyte, different responses might be caused by the different immune cell composition of each tissue. It was known that B220+ B cells were higher than CD3+ T cells in spleen and T cell precursor and thymocyte exist in the thymus (Abbas et al., 2011). Concanavalin A is a plant mitogen and is known for its ability to stimulate mouse T-cell subsets, giving rise to 4 functionally distinct T cell populations, including precursors to suppressor T-cell (Dwyer and Johnson, 1981). Lipopolysaccharide from gram-negative bacteria is commonly used in B cell mitogen, which may directly activate B cells, regardless of their antigenic specificity (Janossy et al., 1976). The PAE might stimulate various immune cell proliferation differently from LPS or Con A. Therefore, PAE may have enhanced both cellular and humoral immunity in Korean native chickens. We verified the immunomodulatory effect in Korean native chickens. Proliferation of PBMC, splenocytes, and thymocytes in the chickens administered PAE for 6 wk was continuously higher than that in the other groups. However, proliferation of PBMC, splenocytes, and thymocytes in chickens administered PAE for >6 wk did not increase further (Figure 2), indicating that PAE maintains monocyte proliferation after 6 wk in Korean native chickens.

The number and ratio of 2 main lymphocyte T subsets (CD4+ cells or T helpers, and CD8+ cells or T cytotoxic cells) have been recognized as meaningful parameters to evaluate the balanced state of immunomodulation and homeostatic responses of the intrinsic immune system (Dhur et al., 1991). In our study, the CD4+CD8− (Th cells) and Bu-1+ (B cells) cell populations were significantly upregulated after 4 wk in PAE-administered chickens, suggesting that PAE stimulated cellular and humoral immunity. The incorporation of grape seed PAE into the diet significantly reduces mortality and improves broiler chicken performance after an *Eimeria tenella* infection (Wang et al., 2008). The PA isolated from *Rhododendron spiciferum* leaves exhibits immunomodulatory activities on the mouse immune system in vitro and enhances both cellular and humoral immunity (Liu et al., 2010). The present results reflect those of the latter study. However, to provide more conclusive data, plans are underway to evaluate the immunomodulatory effects of pure PA, oligomeric PA, and monomeric polyphenols isolated from PAE.

In conclusion, our results demonstrated that PAE stimulated proliferation of PBMC, splenocytes, and thymocytes and increased CD4+CD8− (Th cells) and B cell populations. These results indicate that the immunomodulatory effects of PAE from *P. radiata* bark differ by dosing period in chickens.

### ACKNOWLEDGMENTS

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