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ORIGINAL ARTICLE

Immunomodulatory activities of a new pentapeptide (Bursopentin) from the chicken bursa of Fabricius

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Abstract The bursa of Fabricius (BF) is a central immune organ in birds, and some peptides from chicken BF have demonstrated important immune functions. Here, a new 626.27 Da pentapeptide, Bursopentin (BP5, Cys-Lys-Arg-Val-Tyr) was isolated and purified by reverse-phase high-performance liquid chromatography. In this study, we examined the effects of BP5 on antigen-specific immune response in BALB/c mice sensitized with inactivated avian influenza virus (AIV) [A/Duck/Jiangsu/NJ08/05 (AIV H9N2 subtype)]. The results suggested that BP5 enhanced anti-hemagglutinin antibody (IgG, the isotypes IgG1 and IgG2a) production, induced both of Th1- (IL-2 and IFN-γ) and Th2-type (IL-4 and -10) cytokines, increased proliferations of splenic lymphocyte subsets CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+) and B cells, and enhanced cytotoxic T-lymphocyte activity of the activated splenocytes against NIH3T3 cells. The effects of BP5 on the proliferation of isolated T- and/or B-cell populations of BALB/c mice were assessed, and the data suggested that BP5 promoted spleen lymphocyte proliferation by activating B cells directly and T cells indirectly. Further analysis revealed that B-lymphocyte proliferation induced by BP5 is mediated by reactive oxygen species generated from thiol auto-oxidation of BP5. Furthermore, our data indicated that protein kinase C, mitogen-activated protein kinase, and nuclear factor kappa B are involved in the signal transductions during the BP5-induced B lymphocyte proliferation. This study indicates that BP5 could be a potential immunomodulator for future immuno-pharmacological use.

Keywords Peptide · Immunomodulation · Lymphocyte proliferation · Reactive oxygen species

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Introduction

The bursa of Fabricius (BF), a primary immune organ unique to birds, is the site of early B-lymphocyte proliferation and differentiation (Cooper et al. 1966; Glick et al. 1956; Lydyard et al. 1976). The discovery of this anatomical site in chickens provided the first evidence of a humoral immune system in these animals (Cooper et al. 1966; Glick et al. 1956; Mueller et al. 1962). Studies with BF extracts showed that they contain multiple biologically active factors. For example, BF extracts from 2- to 3-month-old Peking ducks were shown to increase the number of plaque-forming cells (PFCs) in normal or

cyclophosphamide-treated mouse spleens and increased E-rosette formation of swine thymus cells (Zheng and Gao 1991), and a high dose of chicken BF extract could enhance blastogenic responses to T-cell mitogens (Murthy and Ragland 1992). These observations suggested that there are elements in the BF that could enhance or modulate the immune system. Indeed, several soluble factors isolated from BF extracts, such as bursin and bursal antisteroidogenic peptide (BASP) have been shown to play a role in B-cell proliferation and differentiation (Lassila et al. 1989; Schat and Kaiser 1997). Bursin, a tripeptide (Lys-His-Gly) hormone isolated from chicken BF, can induce B-cell differentiation (Audhya et al. 1986), while BASP is required for synchronization of B-cell division during embryogenesis and neonatal life (Caldwell et al. 1999; García-Espinosa et al. 2002; Moore et al. 2003). In addition, bursopeptide-1 (Tyr-Glu-Glu) and bursopeptide-2 (Trp-Thr-Ala-Glu-Glu-Lys-Gln-Leu) isolated from BF also play roles in B-cell differentiation. Bursopeptide-1 could stimulate B-cell differentiation antigen expression in lymphocytes from patients with burns, whereas bursopeptide-2 stimulated the expression of differentiation antigens on T cells, B cells, and natural killer cells (Tsepelev and Tsepelev 2003). There may be additional unknown soluble factors from BF extracts that play roles in immunity.

In this study, we isolated and purified a new pentapeptide, "Bursopentin" (BP5), from chicken BF by reverse-phase high-performance liquid chromatography (RP-HPLC). In vivo, enzyme-linked immunosorbent assays (ELISA) were performed to evaluate the effect of BP5 on antigen-specific antibodies production in avian influenza virus (AIV)-immunized BALB/c mice. In vivo/ex vivo assays were taken to examine the effect of BP5 on Th1/Th2 cytokine production by cultured splenocytes derived from immunized mice. Immunophenotyping assay and cytotoxic T lymphocyte (CTL) assay were also taken to investigate the effect of BP5 on lymphocyte subpopulations and on CTL activity in cells derived from mouse spleens upon restimulation. In vitro assays were performed with T- and/or B cells separately purified from mice splenocytes to evaluate the direct or indirect effects of BP5 on proliferation of these cells. It was reported that some thiol compounds such as glutathione (Ishii et al. 1987) and L-cystine (Iwata et al. 1994) participate in the regulation of lymphocyte proliferation. This thiol-dependent regulation is complicated by the presence of reactive oxygen species (ROS) signaling, and the ROS is generated by thiol (–SH) auto-oxidation. Therefore, we investigated the potential relationships between BP5 activation, ROS generation, and B-lymphocyte proliferation. It has been observed previously that ROS are involved in the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and nuclear factor kappa B (NF-κB) in murine splenic B lymphocyte (Zhang et al. 2001). Therefore, we also investigated whether there is an effect of PKC, MAPK, and NF-κB on BP5-induced B lymphocyte proliferation.

Materials and methods

Bursal sample preparation

Fresh bursas were collected from 4- to 6-week-old chickens from the Shanghai abattoir and frozen at −20°C prior to extraction. Bursa tissues (500 g) were homogenized with ultra-pure water and then frozen and thawed three times. Homogenized material was centrifuged twice at 12,000*g* for 30 min at 4°C. Supernatants were ultrafiltered, and fractions with MW lower than 1,000 Da were collected and lyophilized. This lyophilized material was stored at −20°C for further analysis.

Peptide purification

The lyophilized bursa extracts were solubilized in ultra-pure water at 1 mg/ml. The pH was adjusted to 2–3 with 10% trifluoroacetic acid (TFA, Sigma), and the samples were centrifuged at 7,000*g* for 10 min at 4°C. Chromatographic analysis was performed on the supernatant by RP-HPLC with a Zorbax C-18 (ODS) column (150 mm × 4.6 mm, Agilent) using a linear gradient of acetonitrile (5–18%, EMD Chemicals) containing 0.1% TFA at a flow rate of 1 ml/min for 20 min at 30°C. The elution was monitored at 280 nm with a UV–Visible diode-array detector (UV-DAD, Agilent). The fraction of the peak with a retention time of 14.098 min was collected and then lyophilized. A yield of 4.82 mg BP5 was obtained.

Determination of the BP5 amino acid sequence

The BP5 amino acid sequence was analyzed using Edman degradation analysis on the PROCISE® cLC Sequence system (Applied Biosystems Co.). MS and tandem mass MS/MS experiments were performed on a Q-TOF mass spectrometer (Micromass Co., Manchester, UK) equipped with a nano-ESI source. After obtaining the amino acid sequence, the peptide was synthesized by Shanghai Biotech Bioscience and Technology Co., Ltd (People's Republic of China). The purity of the isolated BP5 and the synthetic peptide was >98% by RP-HPLC, and the sequence of the synthetic BP5 was confirmed by electrospray ionization tandem mass spectrometry (ESI–MS/MS). To rule out lipopolysaccharide (LPS) contamination, both peptides were tested using the E-Toxate Limulus LPS detection kit (Sigma Chemical Co.), which is sensitive to 0.05–0.1 endotoxin units/ml according to the manufacturer's

instructions. If the peptide preparations showed a negative result with this test, they were considered uncontaminated, and only those preparations were used.

Animals and cell lines

Female BALB/c mice (4–6 weeks old) were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). All groups of animals were handled in accordance with the approval of the local institutional animal experiments committee. NIH3T3 cells were purchased from the cell bank of the Chinese Academy of Sciences.

Evaluating the immunomodulatory activities of BP5 in vivo/ex vivo

Model antigens

AIV A/Duck/Jiangsu/NJ08/05 (AIV/H9N2 subtype) was provided by the Institute of Animal Husbandry and Veterinary Medicine, Jiangsu Academy of Agricultural Sciences (Nanjing, China). AIV was cultured in the allantoic sac of chicken embryos. The AIV hemagglutination titer of the inoculated allantoic fluid was $1{:}2^{10}$ corresponding to 10^7 $TCID_{50}$/0.1 ml. The AIV was inactivated with 0.025% formaldehyde for 72 h at 4°C, and the efficacy was tested by three blind transfer assays. Oil formulated vaccine of inactivated AIV/H9N2 was obtained from Qian Yuan hao Co., Ltd (Nanjing, China).

Sensitization protocols

Mice were randomly divided into six experimental groups of ten mice each and subcutaneously immunized three times on days 0, 14, and 28 with 0.1 ml AIV (10^7 $TCID_{50}$/0.1 ml) alone, a mixture of 0.1 ml inactivated AIV plus 25, 5 and 1 mg/kg body weight of BP5, 0.1 ml commercially inactivated AIV/H9N2 vaccine (10^7 $TCID_{50}$/0.1 ml) as a positive control, or 0.1 ml PBS as a negative control. On the following day, the mice were killed and their spleens harvested.

Estimation of antigen-specific antibodies by ELISA

Sera from mice were collected on days 7, 21, and 35 post-immunization. Specific anti-hemagglutinin (HA) IgG and the isotypes IgG1 and IgG2a of mice sera were analyzed by ELISA (Zheng et al. 2005). Briefly, ELISA plates were coated with a purified prokaryotic-expressed recombinant JS-1 (A/Chicken/Jiangsu/JS-1/2002, H9N2 AIV) HA protein (preserved in our laboratory, 10 μg/ml). Serially diluted mice sera were then incubated for 2 h at room temperature, followed by a 1-h incubation with HRP-conjugated goat anti-mouse IgG (Bo Shi De Corporation, Wuhan, China) or HRP-conjugated goat anti-mouse IgG1 and IgG2a (Sigma). Titers at half maximal OD were determined by linear interpolation between the measured points neighboring the half maximal OD. Linear interpolation was calculated using the logarithm of the titer values. Each serum titration was repeated in triplicate.

Estimation of IL-2, IFN-γ, IL-4, and IL-10 by ELISA

On the seventh day after final immunization, mice were killed by cervical dislocation; their spleens were aseptically removed and cell suspensions made by passing them through a sterile cell strainer (Becton-Dickinson, USA). The cell suspensions were washed twice in RPMI-1640 medium supplemented with 10% FBS, and adjusted to 1×10^7 cells/ml. The cells were plated at 2 ml/well in a 24-well cell culture cluster and challenged with inactivated AIV (10^7 PFU). The samples or PBS were added to the 24-well culture cluster at 37°C in triplicate in a CO_2 incubator for 3 days. After 3 days of incubation, the amount of several cytokines (IL-2, IFN-γ, IL-4, and IL-10) in the culture supernatants after incubation was measured by ELISA according to the manufacturer's instruction (eBioscience Corporation, USA).

Immunophenotyping assay

A single cell suspension of spleen was prepared on the seventh day after final immunization as mentioned earlier. For immunophenotypic analyses, an aliquot of splenic lymphocytes cells (2×10^6 cells/ml) was incubated with anti-CD3/FITC and anti-CD4/PE, anti-CD8/PE, anti-CD19/PE or Pan-NK/PE (BD Pharmingen) antibodies in PBS/1% FBS for 30 min, and washed three times with PBS. Samples were analyzed by fluorescence-activated cell sorting (BD Biosciences).

Cytotoxic T lymphocyte assays

The CTL activity of the activated splenocytes against NIH3T3 cells was determined in a CTL assay. Effecter cells for cytotoxic assays were prepared as previously described with some modification (Bennink et al. 1986; Yewdell et al. 1985). Splenic lymphocytes were isolated on the seventh day after final immunization and were adjusted to 1×10^7 cells/5 ml in serum-free RPMI-1640 media (Invitrogen). Splenic lymphocytes from syngeneic non-immunized mice were adjusted to 5×10^6 cells/100 μl and were then infected with H9 subtype virus A/chicken/Jiangsu/JS-1/2002 (10^7 PFU) for 2 h at 37°C, washed three times, incubated in 5 ml RPMI-1640 culture media for 3 h,

and finally gamma irradiated at 3,000 Rads. Next, an equal volume of splenic lymphocytes (5 ml) from immunized mice and splenic lymphocytes infected with H9 subtype virus A/chicken/Jiangsu/JS-1/2002 (preserved in our laboratory) from non-immunized mice were mixed and incubated for 6 days at 37°C, 5% CO_2 to induce virus-specific CTLs (effecter cells). Then the virus-specific CTLs were harvested and adjusted to 1×10^7 cells/ml in 10% FBS RPMI-1640 media for CTL assays.

NIH3T3 target cells (1×10^6 cells/ml) were prepared by Cytotoxic×96 Non-radioactive Cytotoxicity Assay kit (Promega, Charbonnières-les-bains, France). The concentrations of effector cells (lymphocytes) and target cells (NIH3T3 cells) were adjusted to 100:1 and 50:1 with media. CTL assays were performed in quadruplet and the percentage of lysed target cells was determined by OD_{570} according to the manufacturer's instructions.

Characterizing the immunomodulatory functions of BP5 in vitro

Preparation of cells

Lymphocytes were obtained from spleens of male BALB/c mice. T- and B cells were purified as described previously (Akashi et al. 1998; Evans et al. 2000; Seddigheh et al. 1999). Briefly, single-cell suspensions of spleen were centrifuged, and after lysis of red blood cells, were incubated for 2 h at 37°C to allow adherence of macrophages. Non-adherent cells were collected and incubated in Petri dishes. T cells were purified by the passage of macrophage-depleted spleen cells over a nylon-wool column (nylon-wool non-adherent cells). B cells (T-depleted spleen cells) were isolated by complement-mediated T-cell lysis using anti-mouse Thy1.2 antibody (Abcam, USA) plus rabbit complement treatment. The purity of the T cells and B cells were 85 and 94%, respectively, by FACS analysis. Only cell preparations with ≥95% viability by trypan blue exclusion were used.

Lymphocyte proliferation

Proliferation responses in total splenic lymphocytes, purified B, and/or T cells from BALB/c mice were performed as previously described with modifications (Sopori et al. 1990). Briefly, in a final volume of 0.2 ml of RPMI-1640 medium containing 5% fetal calf serum, whole splenic lymphocyte preparations (5×10^6 cells/well), purified B cells (2×10^6 cells/well), purified T cells (2×10^6 cells/well), and a mixed population of B and T cells were cultured in quadruplets in flat-bottomed 96-well microtiter plates in the presence and absence of LPS/phytohemagglutinin (PHA) with BP5. Plates were incubated at 37°C, in 5% CO_2. After 48 h, cultures were labeled with 1.0 μCi of [^{3}H]thymidine per well and, after 24 h, cells were harvested onto glass fiber filters. After drying at 80°C for 2 h, the radioactivity was determined by liquid scintillation counting (Beckman, USA) and presented as counts/min.

Measurement of intracellular ROS generation

Intracellular ROS was measured as described previously with some modifications (Yamaji-Kegan et al. 2006). BP5 (0.2–25.0 μg/ml) was added to the purified B cells (2×10^6 cells/well) together with 5 μM DCF-DA (Invitrogen), a reliable fluorogenic marker for ROS. LPS (0.5 μg/ml)-treated cultures were chosen as a positive control to show mitogen-induced B-cell proliferation. Phorbol 12-myristate 13-acetate (1 μM, Sigma-Aldrich) was used as a positive control for ROS induction. All samples were incubated at 37°C and 5% CO_2 for 1 h. The fluorescence intensity was measured by a microplate reader using a 485/52-nm filter set. The conversion of DCF-DA to DCF was determined by reference to a DCF standard (Sigma-Aldrich), and it was calculated as nmol/2×10^6 cells (well)/1 h.

Inhibition of ROS scavengers and specific inhibitors of PKC, MAPK, and NF-κB

B lymphocytes (2×10^6 cells/well) were pretreated for 30 min with catalase (a scavenger of H_2O_2) at 250 mg/ml or 1% DMSO (an OH· scavenger) or SOD 500 U/ml (an O_2^- scavenger) or 5 μM calphostin C (inhibitors of PKC) or 10 μM SB203580 (inhibitors of p38 MAPK) or 5 μM Bay 117082 (inhibitors of NF-κB), and then stimulated with BP5 (5.0 μg/ml) for 72 h. Then the proliferation of B lymphocytes was determined by [^{3}H]thymidine incorporation as above.

Statistical analysis

The data were recorded as mean ± standard deviation (SD). Biochemical and physiological parameters were analyzed statistically using one-way analysis of variance (ANOVA) followed by Dunnet-*t*-test using SPSS statistical software to evaluate variations between groups. $P < 0.05$ was considered significant, and $P < 0.01$ was highly significant.

Results

Isolation of a new peptide (BP5) from chicken BF

Small peptides from the chicken BF have important biological functions, such as enhancing immune responses (Audhya et al. 1986; Murthy and Ragland 1992) and

anti-infection responses (Tsepelev and Tsepelev 2003), and anti-uremia (Abiko and Sekino 1995). We performed RP-HPLC using chicken BF and identified a novel peptide in the fraction with the elution peak at 14.098 min (Fig. 1a) and sequence of X-Lys-Asp-Val-Tyr (X, any amino acid), as determined by Edman degradation analysis. This amino acid sequence was incomplete due to the absence of the Cys residue peak in the standard amino residue spectra. Further analysis by mass spectrometry (MS) and tandem mass spectrometry (MS/MS) using ESI-Q-TOF–MS resulted in characterization of the peptide termed Bursopentin-5 (BP5) with a sequence of Cys-Lys-Asp-Val-Tyr (Fig. 1b) and a calculated molecular weight of 626.27 Da.

BP5 stimulates significant antigen-specific immune response in vivo/ex vivo

Serum IgG and the isotypes IgG1 and IgG2a were measured in an ELISA to evaluate the combined effects of BP5 on humoral and cellular immune responses to immunization. Mice immunized with inactivated AIV and BP5 produced significantly higher anti-HA antibody (IgG) titers after the first boost (day 21) and second boost (day 35) (5 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$) than those immunized with inactivated AIV alone (Fig. 2a). Following immunization with the commercially inactivated AIV vaccine alone, there was an increase in IgG1 (after first and second boost, $P < 0.05$) (Fig. 2b), but only a minor increase in IgG2a (Fig. 2c) compared with immunization with inactivated AIV alone. In contrast, BP5 significantly enhanced both the levels of IgG1 [after first boost (5 and 1 mg/kg, $P < 0.05$) and second boost (5 and 1 mg/kg, $P < 0.01$)] (Fig. 2b) and IgG2a [after prime and first boost (5 and 1 mg/kg, $P < 0.05$), and second boost (5 and 1 mg/kg, $P < 0.01$)] (Fig. 2c).

We tested the levels of Th1- (IL-2 and IFN-γ) and Th2-type (IL-4 and -10) cytokines (Mosmann and Coffman 1989) produced by splenocytes. Compared with re-stimulation

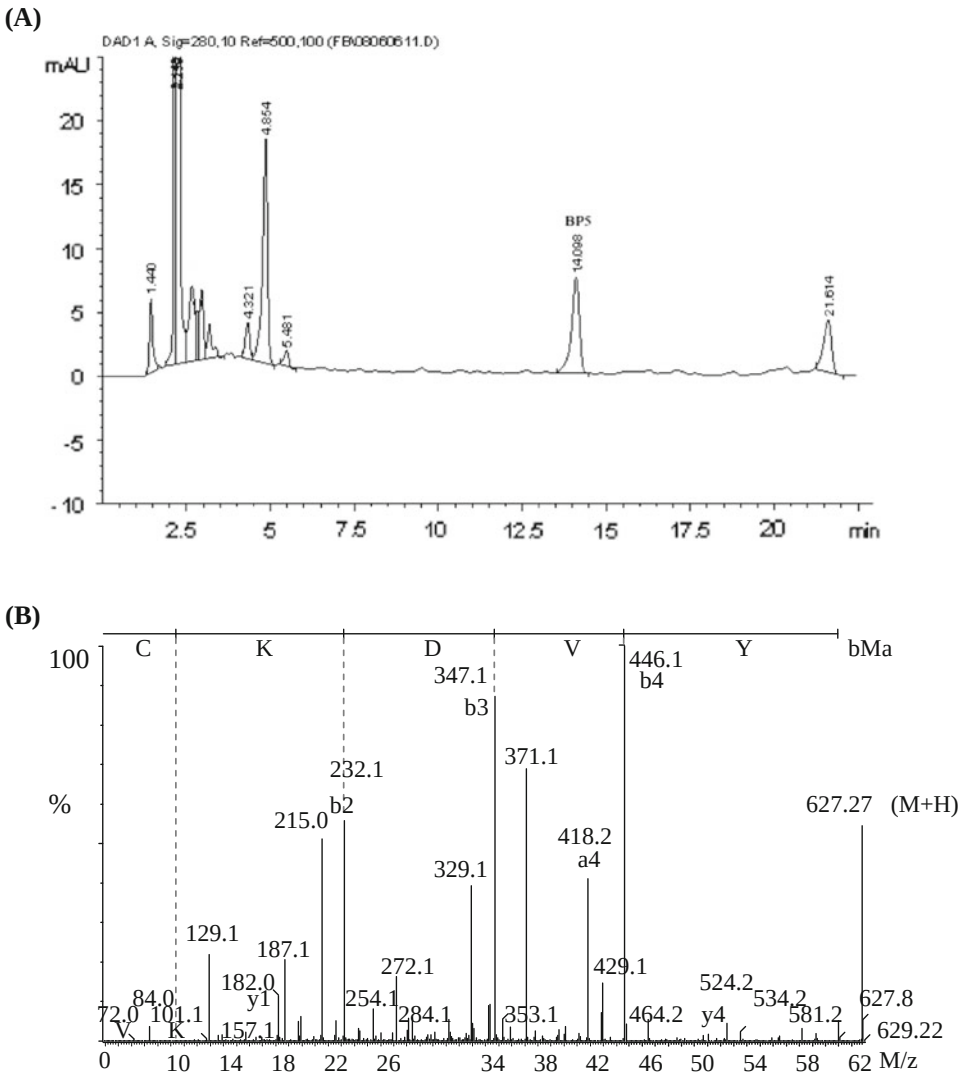

Fig. 1 Identification of a new pentapeptide (BP5). **a** Isolation and purification of BP5 by RP-HPLC. The retention time of BP5 is 14.098 min. Elution was performed with the linear gradient of acetonitrile (5–18%) containing 0.1% TFA at a flow rate of 1 ml/min, at 30°C for 20 min. **b** Determination of the amino acid sequence of BP5. MS/MS experiments were performed on a Q-TOF–MS equipped with a nano-ESI source. From the analysis by MS/MS, the amino acid sequence of the native peptide is Cys-Lys-Asp-Val-Tyr with a molecular weight of 626.27 Da

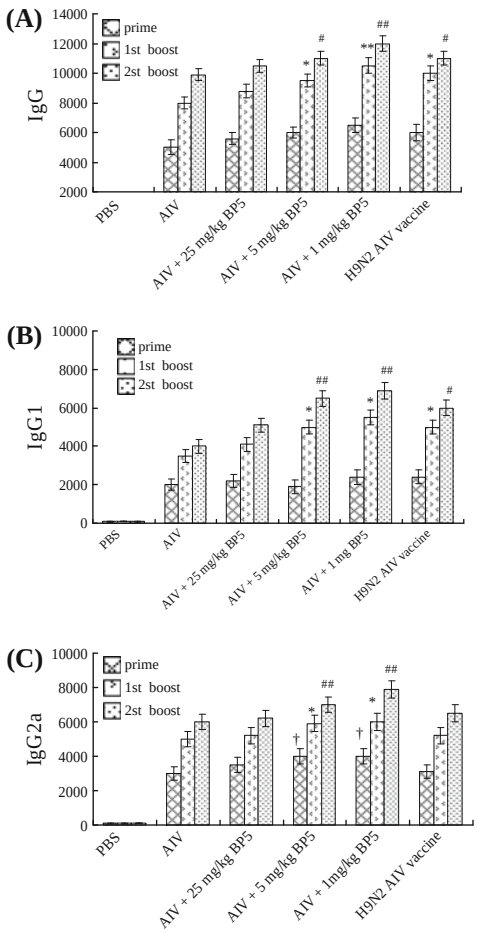

Fig. 2 BP5 stimulated significant antigen-specific humoral and cell-mediated immune response. Mice were immunized three times and the sera were collected on days 7, 21, and 35 post-immunization. Mice-specific anti-HA antibody (IgG, the isotypes IgG1 and IgG2a) were analyzed by ELISA in triplicate. **a–c** BP5 enhanced the levels of anti-HA antibody IgG, IgG1, and IgG2a induced by inactivated AIV. Anti-HA antibody IgG, IgG1, and IgG2a titers expressed as half-maximum titers ± SD. $^{\dagger}P < 0.05$ (prime); $^{*}P < 0.05$, $^{**}P < 0.01$ (first boost); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$(second boost), compared with the inactivated AIV alone

with inactivated AIV alone, inactivated AIV and BP5 remarkably increased both Th1- and Th2-type cytokines, whereas only Th1-type cytokines increased with commercially inactivated AIV re-stimulation (Table 1). We conclude that BP5 is capable of enhancing T cells and T-cell-mediated immune responses, and balancing Th1 and Th2 responses.

The percentages of CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+) in the splenic lymphocyte populations were significantly increased in the mice immunized with a mixture of inactivated AIV and BP5 (5 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$) than in mice immunized with inactivated AIV alone (Table 2). And the results in Table 2 also indicate that BP5 displayed significant stimulation of B cell proliferation (5 and 1 mg/kg, $P < 0.05$). However, a significant stimulation of NK cells was not seen in spleen.

To further evaluate the effect of BP5 on the cell-mediated immune response, AIV-infected cell-specific CTL activity was examined. We found that BP5 was capable of enhancing CTL activity induced by inactivated AIV (Fig. 3). Consistent with the effects of BP5 on cytokine production, the highest CTL activity was detected at the lowest dose of BP5 (5 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$). The results above indicate that BP5 induces potent antigen-specific immune responses in mice.

BP5 significantly stimulates proliferation of mice splenic lymphocytes in vitro

The effect of BP5 on lymphocyte proliferation in mice were tested by culturing splenic lymphocyte with BP5 (0.2–25.0 μg/ml) in the presence and absence of lymphocyte mitogens. The powerful B- and T-lymphocyte stimulants LPS and PHA were used as positive controls. BP5 (0.2–10 μg/ml) caused a significantly enhanced splenic lymphocyte proliferation indicated by [^{3}H]thymidine incorporation (Fig. 4a). In addition, BP5 (1–10 μg/ml) also potentiated LPS (0.5 μg/ml) or PHA (2.5 μg/ml) induced B- or T-cells splenic lymphocyte proliferation, compared with LPS or PHA alone (Fig. 4b). However, the proliferative responses decreased significantly when the BP5 concentration exceeded 25.0 μg/ml.

BP5 increases B-cell proliferation directly and increases T-cell proliferation indirectly

To avoid secondary B- and T-lymphocyte signals interfering with each other in the cell proliferation assays, B and T cells in the total splenocytes were isolated to further characterize whether this peptide affected purified B-cell or T-cell proliferation or both. BP5 (5.0 μg/ml) stimulated B-cell but not T-cell proliferation (Fig. 5), and the B-cell-mitogenic effect was comparable to that seen with unfractionated splenocytes. T cells appeared to be completely unaffected by BP5 in the absence of PHA (T-cell mitogen). The mixed B and T cells had a much higher proliferation rate than that of either purified B or T cells with 5.0 μg/ml of BP5, suggesting an interaction between these cell populations. T cells may be activated by secondary signaling from stimulated B cells, leading to T-cell proliferation. These results suggest that BP5 exerted a direct stimulatory effect on B cells, and this immunostimulating effect of BP5 may lead to subsequent T-cell proliferation.

Table 1 Effect of different doses of BP5 and inactivated AIV on Th1/Th2 cytokine productions in splenocytes

Treatment	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
PBS	29.47 ± 10.65	69.87 ± 9.95	43.65 ± 10.75	31.17 ± 9.56
AIV	74.43 ± 11.58	90.03 ± 20.86	84.34 ± 15.95	47.24 ± 5.88
AIV + high dose of BP5	85.67 ± 16.47	105.38 ± 19.68	95.34 ± 14.81	49.73 ± 11.63
AIV + medium dose of BP5	92.36 ± 19.56*	119.34 ± 20.58*	115.22 ± 19.45*	68.91 ± 10.86*
AIV + low dose of BP5	111.68 ± 18.98**	138.79 ± 26.86**	120.54 ± 18.11**	90.58 ± 18.56*
Commercial AIV Vaccine	96.45 ± 19.56*	114.08 ± 19.63*	100.78 ± 20.11	45.47 ± 11.87

Cytokine release was measured by using sandwich ELISA method using ELISA kits from eBioscience Corporation. Compared with re-stimulation with inactivated AIV alone, a low dose or a medium dose of BP5 remarkably increased both Th1-(IL-2 and IFN-γ) and Th2-type (IL-4 and -10) cytokines in inactivated AIV immunized mice, whereas only Th1-type cytokines increased with commercially inactivated AIV re-stimulation. The data presented are mean ± SD values of four replicates

PBS Phosphate buffer solution, *AIV* avian influenza virus

* $P < 0.05$, ** $P < 0.01$, compared with the inactivated AIV alone

Table 2 Flow cytometry analysis of lymphocyte subsets from spleen of immunized mice

Treatment	CD3+CD4+	CD3+CD8+	CD19	Pan-NK
PBS	23.15 ± 2.14	15.37 ± 1.89	8.84 ± 1.33	3.66 ± 0.53
AIV	37.09 ± 1.22	16.90 ± 2.13	12.51 ± 1.45	3.99 ± 0.44
AIV + high dose of BP5	40.49 ± 2.16	21.43 ± 1.38	13.93 ± 1.87	3.04 ± 0.41
AIV + medium dose of BP5	43.80 ± 2.37*	24.22 ± 1.56*	18.64 ± 2.11*	3.68 ± 0.32
AIV + low dose of BP5	47.35 ± 1.75**	25.03 ± 2.12**	18.82 ± 1.37*	3.56 ± 0.36
Commercial AIV vaccine	47.59 ± 2.23**	19.63 ± 1.86	18.66 ± 2.02*	2.98 ± 0.44

Mice were killed on day 35 (second boost), and the spleen lymphocytes were collected for immunophenotyping. A low dose or a medium dose of BP5 displayed significant stimulation of B cell (CD19) proliferation and significantly increased the percentages of CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+) in the splenic lymphocyte populations. Values represent the relative lymphocytes population (%) ± SD, $n = 4$

PBS Phosphate buffer solution, *AIV* avian influenza virus

* $P < 0.05$, ** $P < 0.01$, compared with mice immunized with the inactivated AIV alone

BP5-induced B-lymphocyte proliferation is mediated by reactive oxygen species

To investigate whether BP5 could induce intracellular ROS production, the ROS levels in the B-cell cultures were measured using the redox-sensitive fluorescent dye carboxy-2′,7′-dichlorofluorescein diacetate (DCF-DA). The intracellularly hydrolyzed DCF-DA, the active probe, which further becomes processed to the green fluorescent oxidation product DCF, can be quantified by a microplate reader with a 485/52-nm filter set. The conversion of DCF-DA to DCF is dependent on intracellular production of ROS (Schmidt et al. 1995). Here, BP5 at 0.2–10 μg/ml could significantly enhance ROS production in the B-cells cultures compared with ROS in the no BP5 treated cultures (Fig. 6a). However, when BP5 exceeded 25 μg/ml, no significant increase of intracellular ROS was observed.

For further evidence of oxygen radicals mediated by BP5-induced B-lymphocyte proliferation, we pre-treated cells for 30 min with ROS scavengers of catalase, DMSO or SOD, and then stimulated with BP5 (5.0 μg/ml) for 72 h. The proliferation of BP5-induced B lymphocytes was greatly depressed as compared with the cultures without ROS scavengers (Fig. 6b). As a positive control, LPS-induced B-lymphocyte proliferation was also inhibited by these antioxidants.

PKC, p38 MAPK, and NF-κB pathways are involved in the BP5-induced B lymphocyte proliferation

In order to assess the role played by different signal transduction pathways in BP5-induced B lymphocyte proliferation, the cells were pretreated for 30 min with the PKC inhibitor calphostin C, p38 MAPK inhibitor SB203580, and NF-κB inhibitor Bay 117082 before stimulation with BP5. As shown in Fig. 7, all inhibitors of PKC, p38 MAPK, and NF-κB significantly depressed BP5-induced B lymphocyte proliferation.

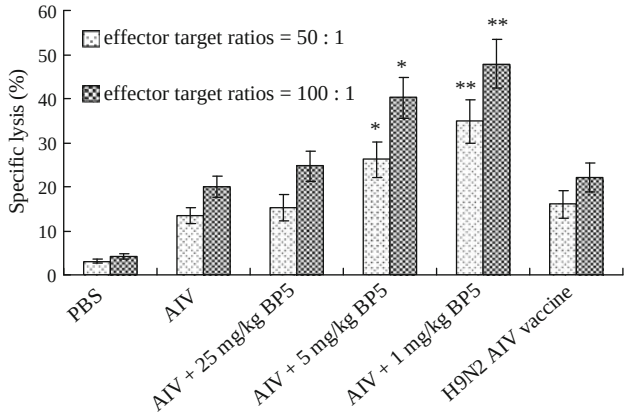

Fig. 3 Co-treatment of BP5 and inactivated AIV stimulated virus-specific CTL responses. Splenic lymphocytes from immunized mice on day 35 (second boost) and splenic lymphocytes infected with avian influenza virus (AIV) from non-immunized mice were co-cultured to induce virus-specific CTLs for cytotoxic T lymphocytes assays. The percentage of lysis was determined by using CytoTox 96® Non-Radioactive Cytotoxicity Assay performed in quadruplet. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with mice immunized with the inactivated AIV alone

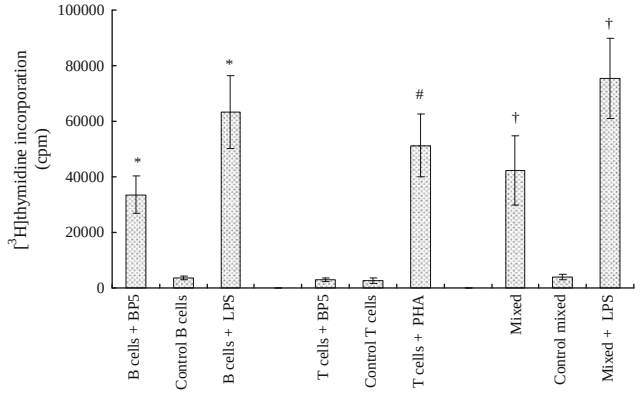

Fig. 5 BP5 increases B-cell proliferation directly and increases T cells proliferation indirectly. Purified B cells (2×10^6 cells/well), purified T cells (2×10^6 cells/well), and a mixed population of T and B cells (mixed) were incubated with BP5 (5 μg/ml) or LPS (0.5 μg/ml), BP5 (5 μg/ml) or PHA (2.5 μg/ml) and BP5 (5 μg/ml), or LPS (0.5 μg/ml), respectively, in complete RPMI-1640 medium. Negative controls were cells incubated in the absence of BP5. Data are the mean ± SD from four separate experiments. $^{*}P < 0.05$, compared with control B cells. $^{\#}P < 0.05$, compared with control T cells. $^{\dagger}P < 0.05$, compared with control mixed cells

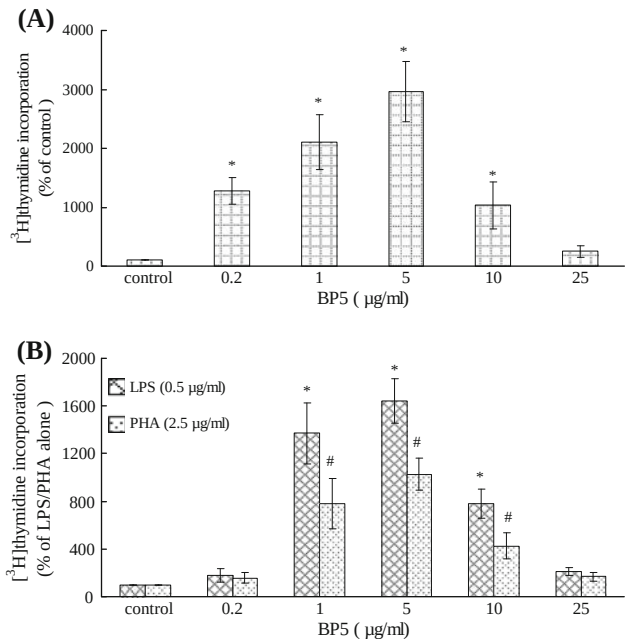

Fig. 4 BP5 significantly stimulates splenic lymphocytes proliferation. **a** Effects of BP5 on splenic lymphocytes proliferation. Splenic lymphocytes were incubated with BP5 (0.2–25.0 μg/ml) for 72 h. Cell proliferation was assayed by [^{3}H]thymidine incorporation. Results are expressed as mean ± SD from four separate experiments. $^{*}P < 0.05$, compared with control. **b** Effects of BP5 on mitogen-induced splenic lymphocyte proliferation. Lymphocytes were cultured with BP5 by adding either LPS (0.5 μg/ml) or PHA (2.5 μg/ml). Proliferative response was evaluated by pulsing the culture with [^{3}H]thymidine. Data are the mean ± SD from four separate experiments. $^{*}P < 0.05$, compared with LPS alone. $^{\#}P < 0.05$, compared with PHA alone

Discussion

The BF plays a pivotal role in the immune system and has been intensively investigated. To date, bursin and BASP are the two most well-known BF cytokines that have been implicated in B-cell development and differentiation (Garcia-Espinosa et al. 2008). In addition to the role of the BF cytokines in establishing humoral immunity, several lines of evidence have prompted investigators to explore novel functions of bursal factors. First, BF chicken extracts enhance chicken peripheral blood lymphocyte blastogenic responses to PHA (Schat and Kaiser 1997). Second, bursopeptide-2 stimulates expression of differentiation antigens on T and B cells and natural killer cells (Tsepelev and Tsepelev 2003). Third, chicken BF extracts have anti-steroidogenic activity in avian and mammalian cells in vitro and suppress mitogen-stimulated DNA synthesis in chicken BF cells (Garcia-Espinosa et al. 2008). Thus, the biological function of BF factors is far from clear, and unveiling novel activities may provide important clues for pharmacological manipulations in the future.

Here, we isolated and purified a novel bursa pentapeptide (BP5) by RP-HPLC. In our study, mice treated with a mixture of BP5 and inactivated AIV produced high levels of IgG2a antibodies together with IgG1 antibodies. This suggests that BP5 has effects on the antibody response and on the function of immune cells, as IgG1 is believed to indicate a humoral immune response, whereas IgG2a is indicative of a cellular response (Hauge et al. 2007). We examined immunostimulatory properties of BP5 on

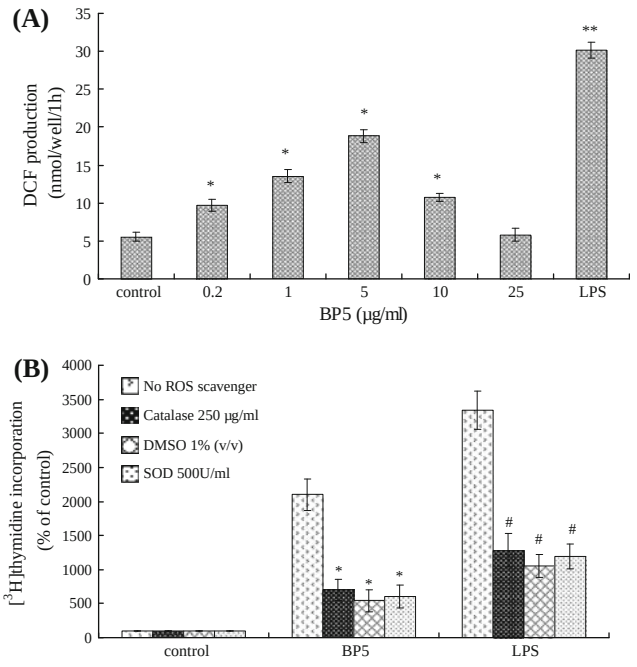

Fig. 6 BP5-induced B-lymphocyte proliferation is mediated by reactive oxygen species. **a** Effect of BP5 on DCF formation in B lymphocytes. B cells were incubated with BP5 (0.2–25.0 μg/ml) or LPS (0.5 μg/ml) and then treated 5 μM carboxy-2′,7′-dichlorofluorescein diacetate (DCF-DA) for 30 min. Phorbol 12-myristate 13-acetate (1 μM) was used as a positive control for ROS production. ROS levels were expressed as DCF production (nmol per well/25 min) ($n = 4 \pm$ SD, $^{**}P < 0.01$ compared with control). **b** Inhibitory effects of ROS scavengers on BP5-induced B-lymphocyte proliferation. Cells were pretreated with ROS scavengers, catalase 250 mg/ml, 1% DMSO or SOD 500 U/ml for 30 min and then incubated with BP5 (5.0 μg/ml) or LPS (0.5 μg/ml) for 72 h. Cell proliferation was evaluated by [^{3}H]thymidine incorporation. Results were expressed as mean ± SD from five separate experiments. $^{*}P < 0.05$, compared with BP5 alone; $^{\#}P < 0.05$, compared with LPS alone

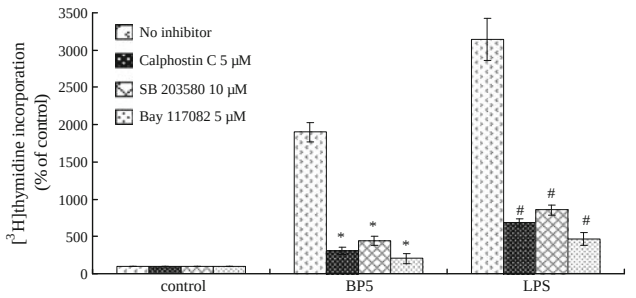

Fig. 7 Roles of PKC, p38 MAPK, and NF-κB in BP5-induced B lymphocyte proliferation. Cells were pretreated with calphostin C (an inhibitor of PKC) or SB203580 (an inhibitor of p38 MAPK) or Bay 117082 (an inhibitor of NF-κB) for 30 min, then stimulated with BP5 (5.0 μg/ml) or LPS (0.5 μg/ml, as a positive control). Cell proliferation was assayed by [^{3}H]thymidine incorporation. Data were expressed as mean ± SD of the percentage of [^{3}H]thymidine incorporation of control from five separate experiments. $^{*}P < 0.05$, compared with BP5 alone; $^{\#}P < 0.05$, compared with LPS alone

immune cells and tried to delineate the type of immune response by measuring the secretary cytokines produced by splenocytes of mice co-immunized with inactivated AIV and BP5. It is generally accepted that CD4+ T cells are subpopulations containing two cell types (Th1 and Th2), based on their different patterns of cytokine secretion (Mosmann and Coffman 1989). Th1 cells secrete IL-2, IFN-γ, and IL-12. Th2 cells produce IL-4, -5, and -10. IL-2, a key cytokine secreted from activated lymphocytes can trigger a variety of immune cells such as lymphocytes and macrophages (Chong et al. 2005). IFN-γ is produced by stimulated T cells and has important immunomodulatory effects (Farrar and Schreiber 1993). IL-4 can promote B-cell differentiation and enhance the production of antibodies by sensitized B cells (Belardelli 1995). IL-10 has been described as a stimulating factor for CD8+ CTL (Santin et al. 2000). In the present study, productions of IL-2, IFN-γ, IL-4, and -10 were all increased by BP5 in activated splenocytes, indicating Th1- and Th2-type of immunostimulation of BP5. Moreover, populations of CD4+ and CD8+ T cells, B cells and activity of CTL induced by inactivated AIV were also elevated by BP5. These results suggest that BP5 acts as a multi-functional modulator in immunized mice.

It is difficult to wholly evaluate immune responses in vivo. However, feasible in vitro assays to screen the lymphoproliferation activity of peptides or some immunomodulators are documented. The level of lymphocyte proliferation is a significant method for measuring cell immunity (Lafuente et al. 2003). In the present experiment, BP5 at a suitable concentration (0.2–10 μg/ml) significantly enhanced total splenic lymphocyte proliferation. However, knowledge of which specific cell subpopulations are stimulated by BP5 is important for its future use in immuno-pharmacology. We showed that BP5 exerted stimulatory effects specifically on B cells and not T cells. However, 5 μg/ml of BP5 induced much higher proliferation rates in mixed B and T cells than in isolated purified B and T cells, suggesting an interaction between these two populations. Since B lymphocytes may act as antigen-presenting cells for CD4+ T-cell priming (Constant 1999), we assumed that BP5 exerted a direct stimulatory effect on B cells and subsequently delivered a secondary signal to activate and induce T-cell proliferation. Considering BP5 stimulated both Th1 and Th2 cytokine production in activated splenic lymphocytes, we believed that BP5 increased B-cell proliferation directly and increased T-cell proliferation indirectly by co-stimulating B cells.

During immune cell activation, there is an incremental increase in free radical production, which in turn supports the normal functions of activated immune cells (Gambera et al. 2007). To verify the mechanism that ROS acts as a mediator in BP5-induced B lymphocyte proliferation, the

production of intracellular ROS was measured by DCF-DA. In this study, BP5 at 0.2–10 μg/ml significantly enhanced productions of intracellular hydrogen peroxide (H_2O_2), hydroxyl ion (OH·), and superoxide anion (O_2^-) in B-cells cultures. However, antioxidants attenuated BP5-induced B-lymphocyte proliferation. These results suggested that the production of intracellular ROS is involved in the effect of BP5-induced B-lymphocyte proliferation.

PKC plays a critical role in signal transduction pathways leading to a variety of cellular functions, such as cell growth and differentiation (Zhang et al. 2001). The MAPKs are serine/threonine kinases that transduce signals from the plasma membrane to the cell nucleus. They play a critical role in controlling cell survival, proliferation, and differentiation (Chang and Karin 2001). At least four types of MAP kinase express in mammalian cells: Jun amino-terminal kinases (JNK), p38 MAPK, extracellular regulated kinase (ERK), and ERK5. JNK and p38 MAPK pathways are primarily activated by stress stimuli and are linked to the induction of differentiation and apoptosis, whereas the ERK1/2 group is activated mainly by mitogenic stimuli, and it has been linked to cell survival. NF-κB activation is redox sensitive and is induced through PKC- and MAPK-dependent pathways (Hoshi et al. 2000; Yerneni et al. 1999). ROS has been demonstrated effect on NF-κB translocation (Blackwell et al. 1996). The increased ROS production generated by BP5 may be partially responsible for the NF-κB activation. The present study demonstrated that BP5-induced B cell proliferation was highly sensitive to inhibitors of PKC, p38 MAPK, and NF-κB, suggesting the involvement of PKC, p38 MAPK, and NF-κB signal transduction pathways in BP5 activation.

Several small peptides have been used in clinical applications. Thymopentin has been used to treat various diseases, including primary and secondary immune deficiencies and autoimmune disorders (Singh et al. 1998), and cancer (Sundal 1992). Bursin has been patented for the treatment of hypogammaglobulinemia (Audhya et al. 1988). Additionally, glutathione, a well-known tripeptide (Glu-Cys-Gly) ubiquitously distributed in most living cells (Sies 1999), has immune-stimulating and antioxidant activities (Dröge and Breitkreutz 2000) and plays a role in immunity against chronic diseases (Julius et al. 1994). Since BP5 has immunomodulating activities, it may be considered a promising immune enhancer for immuno-pharmacological use.

The dosage of BP5 used in our animal experiments is from 1 to 25 mg/kg body weight and the concentration of BP5 used in our cell cultures is from 0.2 to 25 μg/ml, respectively. In fact, physiological concentrations of many cytokines are quite low. In physiological concentrations of pg/ml of plasma, cytokines mediate a series of metabolic and biochemical changes in responding cells (Aguillon et al. 2001). In contrast to hormones, cytokine activities are often purely local and, consequently, their active physiological concentration is hard to define and the local concentrations of cytokines might be much greater than expected (Kaplan 1996). Further researches on the effective concentrations of BP5 in B cell activation will contribute new insights into the mechanisms of immunomodulation and may lead to the development of practical application in chickens or other animals, or even humans.

In summary, we demonstrated that a novel 626.27 Da pentapeptide displays strong immuno-stimulating activity by promoting B-cell proliferation directly and T-cell proliferation indirectly. Furthermore, this study provides some insights into the potential mechanisms involved in the effects of ROS and signal transduction pathways of PKC, p38 MAPK, and NF-κB on BP5-induced B-cell proliferation. This study indicates that BP5 may be used as a new experimental reagent for immuno-adjuvant or immuno-pharmacological uses.

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