Bursopentine as a Novel Immunoadjuvant Enhances both Humoral and Cell-Mediated Immune Responses to Inactivated H9N2 Avian Influenza Virus in Chickens

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There is an urgent need for identification of a new adjuvant capable of selectively promoting an efficient immune response for use with vaccines and especially subunit vaccines. Our previous study showed that Bursopentine (BP5) is a novel immunomodulatory peptide and has the ability to significantly stimulate an antigen-specific immune response in mice. In this study, the potential adjuvant activities of BP5 were examined in chickens by co-injection of BP5 and an inactivated avian influenza virus (AIV) (A/Duck/Jiangsu/NJ08/05 [AIV H9N2 subtype]). The results suggested that BP5 markedly elevated serum hemagglutination inhibition (HI) titers and antigen-specific antibody (anti-HA) IgG levels, induced both Th1 (interleukin 2 [IL-2] and gamma interferon [IFN-γ]) and Th2 (IL-4)-type cytokines, promoted the proliferation of peripheral blood lymphocytes, and increased populations of CD3+ T cells and their subsets CD4+ CD45+ CD8+ CD28+ T cells. Furthermore, a virus challenge experiment revealed that BP5 contributes to protection against homologous avian influenza virus challenge by reducing viral replication in chicken lungs. This study indicates that the combination of inactivated AIVs and BP5 gives a strong immune response at both the humoral and cellular levels and implies that BP5 is a novel immunoadjuvant suitable for vaccine design.

The immune-promoting activity of any given vaccination strategy is set not only by the presence of the relevant antigenic components in the vaccine formulation but also by the complement of suitable adjuvants (9, 20). When incorporated into a vaccine formulation, a suitable adjuvant acts to accelerate, extend, or enhance the magnitude of a specific immune response to the vaccine antigen (6). Strategies for improving current vaccines have emphasized making currently available vaccines more efficacious by developing a better adjuvant, especially for inactivated viral and subunit vaccines.

Bursopentine (BP5: with an amino acid sequence of Cys-Lys-Asp-Val-Tyr) is a novel immunomodulatory peptide isolated from chicken bursa of Fabricius (19). As it has the ability to significantly stimulate antigen-specific immune responses at both the humoral and cellular levels in mice immunized with inactivated avian influenza viruses (AIVs) (19), its potential adjuvant activities were assessed in chickens in this study by using a model antigen of an inactivated AIV, A/Duck/Jiangsu/NJ08/05 (AIV H9N2 subtype).

In many countries, H9N2 AIVs are an enormous economic burden on the commercial poultry industry when they cause signs of mild respiratory disease and a reduction in egg production. In April 1999, two World Health Organization reference laboratories independently confirmed the isolation of avian influenza A (H9N2) viruses for the first time in humans (39). An increased risk of direct transmission of these viruses to humans is possible (21, 25, 29). Inactivated vaccines have been used to control AIV infection, but the best protection against AIV infection remains effective vaccination. Previously, it has been shown that inactivated vaccines elicit strong humoral responses, and it is commonly accepted that no adequate mucosal or cellular immunity is achieved (37). However, cellular immunity is essential for virus clearance at the end stage of many viral infections (4). Adjuvants are able to improve the quantity and quality of innate immune responses by enhancing their speed and duration and by inducing adequate adaptive immunity (31). In the current study, BP5 was used as an adjuvant for our AIV vaccination strategy to provide an effective way to prevent and control H9N2 AIV infection.

The effect of BP5 on humoral and cell-mediated immune responses induced by inactivated AIV vaccination was evaluated in 1-week-old specific-pathogen-free White Leghorn chickens. Humoral immunity was measured by detection of antigen-specific antibody titer and antihemagglutinin (anti-HA) IgG responses using the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA), respectively. Cell-mediated immunity was evaluated by detection...
The AIV hemagglutination titer of the inoculated allantoic fluid was 1:210, H9N2 virus strain JS-1 (A/Chicken/Jiangsu/JS-1/2002) was isolated and kept in the Jiangsu Academy of Agricultural Sciences (Nanjing, China). Avian influenza virus replication in chicken lungs. Experiments were assayed to evaluate the protection of activated AIV vaccine administered with BP5 against homologous avian influenza virus replication in chicken lungs.

**Materials and Methods**

**Preparation of BP5.** Synthetic BP5 was purchased from Shanghai Biotech Bioscience and Technology Co., Ltd. (People’s Republic of China). The sequence of the synthetic peptide was confirmed by electrospray ionization tandem mass spectrometry (ESI-MS/MS), and the purity of the synthetic peptide was >98% by reversed-phase high-performance liquid chromatography (RP-HPLC).

**Viruses and chicken.** 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). Avian influenza H9N2 virus strain JS-1 (A/Chicken/Jiangsu/JS-1/2002) was isolated and kept in our own laboratory. AIVs were cultured in the allantoic sacs of chicken embryos. The AIV hemagglutination titer of the inoculated allantoic fluid was 1:2,000 corresponding to 10^{-5} 50% tissue culture infective doses (TCID_{50})/ml. The A/Duck/Jiangsu/NJ08/05 AIV was inactivated with 0.025% formaldehyde for 72 h at 4°C. Its efficacy was tested by three blind virus passages in specific-pathogen-free (SPF) eggs (19, 38), and the inactivated AIV was used as a vaccine antigen for the following experiments.

One-week-old SPF White Leghorn chickens from Qian Yuan hao Co., Ltd. (Nanjing, China), were obtained as fertilized eggs, hatched, and maintained in an isolation facility at the Poultry Research Institute (Nanjing, China). All groups of chickens were housed, handled, and immunized in accordance with the guidelines and with the approval of the local institutional animal experiment committee.

**Vaccination of chickens.** SPF White Leghorn chickens were randomly divided into six experimental groups of 18 chickens each and intramuscularly immunized two times on days 0 and 14 with (i) a mixture of 400 μl AIV (A/Duck/Jiangsu/NJ08/05, 10^{-7} TCID_{50}/ml) and 100 μl phosphate-buffered saline (PBS), (ii) a mixture of 400 μl AIV (A/Duck/Jiangsu/NJ08/05, 10^{-7} TCID_{50}/ml) and 25, 5, or 1 mg BP5 in 100 μl PBS/kg body weight, (iii) 400 μl commercially inactivated AIV/H9N2 vaccine (an oil-formulated vaccine obtained from Qian Yuan hao Co., Ltd., Nanjing, China [10^{-7} TCID_{50}/ml]) plus 100 μl PBS as a positive control, or (iv) 500 μl PBS as a negative control (Table 1).

**HI assay.** On days 14 and 28 postimmunization, serum hemagglutination inhibition (HI) antibody titers of each group of chickens were evaluated with an HI test based on Hirst’s principle (10). The serum was diluted 10-fold with saline before an additional 2-fold dilution with PBS was made. Standard avian influenza antigen (Harbin Veterinary Research Institute, China) with 4 hemagglutination units was then added to each diluted serum sample and mixed for approximately 15 min. An equal volume of 0.5% chicken red blood cells was added to the virus-serum mixture and incubated for 30 to 60 min before the results were read. The HI titers were defined as the highest serum dilution capable of preventing hemagglutination.

**Estimation of antigen-specific antibodies (IgG).** Sera from chickens were collected on days 14 and 28 postimmunization. Specific hemagglutinin (anti-HA) IgG of chicken sera was analyzed by ELISA. Briefly, ELISA plates were coated with a purified prokaryote-expressed recombinant JS-1 (A/Chicken/Jiangsu/JS-1/2002, H9N2 AIV) HA protein (preserved in our laboratory, 10 μg/ml) (40). Serially diluted chicken sera were then incubated for 2 h at room temperature, followed by a 1-h incubation with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (GenScript Co., Ltd., China). Titers at half-maximal optical densities (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.

**Cytokine assays.** One day 28 postimmunization, the serum levels of Th1-type cytokines (IL-2 and IFN-γ) in chickens were determined with commercial ELISA kits (Cusabio Biotech), whereas Th2-type cytokine (IL-4) was measured by another commercial ELISA kit (R&D Systems, United Kingdom). The procedure followed the manufacturer’s instructions.

**Lymphocyte proliferation assay and immunophenotyping assay.** To detect changes in cellular immunity, a peripheral blood lymphocyte proliferation assay and an immunophenotyping assay were performed. Fourteen days after the second immunization (day 28), the blood samples were collected for lymphocyte separation. Peripheral blood lymphocytes were separated as described previously, with some modification (11, 28). The cell suspension from the blood was layered on Ficoll-Paque lymphocyte separation medium by density gradient centrifugation. Peripheral blood lymphocytes were obtained from the interface and washed twice with Hanks’ balanced salt solution. After centrifugation, the final pellet was resuspended in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum at a concentration of 2 × 10^6 cells/ml.

The peripheral blood lymphocyte proliferation assay was performed using a modified MTT method as described previously (13, 22). Briefly, the peripheral blood lymphocytes (2 × 10^6 cells/ml) were dispersed and incubated in 96-well flat-bottomed microtiter plates (80 μl/well). Another 20 μl of concanavalin A (ConA; 10 μg/ml, positive control), the recombinant JS-1 (A/Chicken/Jiangsu/JS-1/2002, H9N2 AIV) HA protein (10 μg/ml, specific antigen stimulation), or RPMI 1640 medium without antigen (negative control) was added to each well, and each sample was seeded in four wells. After 44 h of incubation at 39.5°C in a 5% CO2 incubator, 20 μl of MTT (dissolved in PBS, 5 mg/ml) (Sigma) was added to each well and the incubation was continued for another 4 h. Then 100 μl of dimethyl sulphoxide (DMSO) was added, and incubation was continued for an additional 4 h before measurement of OD at 750 nm (OD_{750}) using an ELISA reader (Bio-Tek Instruments, VT). Cell viability is expressed as the percentage of the OD_{750} of cells treated with the control OD_{750} of the control sample.

Flow cytometric analysis of peripheral blood lymphocytes was carried out as previously described (30). Peripheral blood lymphocytes (2 × 10^6 cells/ml) were made complex with the monoclonal antibody phycoerythrin (PE)-labeled anti-chicken CD3+ and then with PE-labeled anti-chicken CD4+ and fluorescein isothiocyanate (FITC)-conjugated anti-chicken CD8+ (Southern Biotechnology) for 1 h at 4°C. PE- and FITC-conjugated isotype controls were also included. Cells were analyzed by fluorescence-activated cell sorting (BD Biosciences).

**Virus challenge experiment.** Two weeks after the second vaccination, all chickens were intranasally challenged with 2 × 10^{7} TCID_{50} of avian influenza H9N2 virus strain JS-1 (A/Chicken/Jiangsu/JS-1/2002) in 0.1 ml. Lungs were collected from six chickens from each group at 1, 3, and 5 days after virus challenge (Table 1). All lung samples were stored at −80°C. Viral copy numbers in lungs were determined by using real-time PCR. An RNasey RNA extraction kit (Invitrogen, Norway) was used to prepare total RNA from the lung samples. The RNA was reverse transcribed to cDNA by using the reverse transcription system from Promega (Germany). A 2-μl portion of cDNA was used to amplify the HA gene by real-time PCR using one pair of PCR primers: HA2, forward, 5'-CTACCTGT TGGGAGGAAGAGAATGGT-3'; HA-reverse, 5'-TGGGGCTTCTGAATAGGGTGA-3'. PCR primers were designed based on the HA gene sequence of avian influenza H9N2 virus strain JS-1 (A/Chicken/Jiangsu/JS-1/2002) in GenBank (accession no. AY364228). The amplification was performed by using SYBR green (ABI, Warrington, United Kingdom) according to the method described previously (24), with some modifications. The standard curve for real-time PCR quantification was constructed using the HA gene in the vector pET32a-HA (H9N2), a gift from Qisheng Zheng (Institute of Veterinary Science, Jiangsu Academy of Agricultural Sciences). The pretreatment of the re-
action mixture was carried out at 94°C for 10 min, and then the mixture was subjected to 40 cycles of amplification at 95°C for 15 s and at 60°C for 30 s.

Statistical analysis. Antibody titers, cytokine levels, percentages reflecting lymphocyte proliferation, percentages of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ cells in the peripheral blood, and numbers of viral copies in chicken lungs were recorded as means ± standard deviations (SD). Bonferroni correction multiple-comparison tests were used to evaluate any differences between groups. Differences between means were considered significant at a P of <0.05 or a P of <0.01.

**RESULTS**

**BP5 stimulates significant antigen-specific immune responses.** To test antigen-specific immune responses to immunization, chickens were immunized two times with a mixture of BP5 and inactivated avian influenza viruses (AIVs) or a commercial AIV (H9N2) vaccine (positive controls) or with PBS (negative control). Chickens coimmunized with inactivated AIVs and BP5 produced significantly higher hemagglutination inhibition (HI) antibody titers (Fig. 1A) (after priming with 25 and 5 mg/kg [P < 0.05 {†}] and boosting with 25 and 5 mg/kg [P < 0.05 {#}] and 1 mg/kg [P < 0.01 {##}]) than those immunized with inactivated AIVs alone. Compared to chickens immunized with the commercial H9N2 AIV vaccine (with a combination of oil and water [oil/water] as an adjuvant), chickens coadministered inactivated AIVs and BP5 also produced significantly higher HI (Fig. 1A) and IgG (Fig. 1B) antibody titers (after priming with 1 mg/kg [P < 0.05 {‡}] and boosting with 5 mg/kg and 1 mg/kg [P < 0.05 {§}]).

**BP5 increases the production of both Th1- and Th2-type cytokines.** We then tested the levels of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines upon coimmunization with inactivated AIV and BP5 in chickens. Compared with restimulation with inactivated AIVs alone, coimmunization with inactivated AIVs and BP5 remarkably increased the levels of both Th1-type (IL-2 and IFN-γ [P < 0.05]) and Th2-type (IL-4 [P < 0.01]) cytokines in chickens, whereas only Th1-type cytokines increased with commercially inactivated H9N2 AIV vaccine restimulation (Fig. 2). BP5 significantly enhances peripheral blood lymphocyte proliferation. To investigate the effects of BP5 on peripheral lymphocyte proliferation, we collected peripheral lymphocytes from chickens treated with different dosages of BP5 coadministered with inactivated AIV and treated them with recombinant IS-1 (A/Chicken/Jiangsu/IS-1/2002, H9N2 AIV) HA protein in vitro. When chickens were immunized with inactivated AIVs and BP5, a significant proliferative response was observed (Fig. 3; *, P < 0.05, compared with chickens immunized with the inactivated AIVs alone; †, P < 0.01, compared with chickens immunized with PBS; ‡, P < 0.01, compared with chickens immunized with the commercially prepared H9N2 AIV vaccine with oil/water as an adjuvant). The data showed that chickens immunized with a combination of BP5 and inactivated AIVs also induce the highest AIV-specific cellular proliferation, in addition to the humoral responses described above.
BP5 stimulates both CD4⁺ and CD8⁺ T cells. The percentages of overall CD3⁺ T cells and their subsets (CD4⁺ T cells [CD3⁺ CD4⁺] and CD8⁺ T cells [CD3⁺ CD8⁺]) in the peripheral blood lymphocyte populations were significantly increased in the chickens immunized with a mixture of inactivated AIV and BP5 (5 mg/kg, P < 0.05; 1 mg/kg, P < 0.01) compared with those in chickens immunized with inactivated AIV alone (Table 2). However, CD8⁺ T cells were only modestly affected by administration of the commercial H9N2 AIV vaccine. This indicated that BP5 has an adjuvant activity in that it promotes the AIV vaccine by stimulating not only CD4⁺ T cell proliferation but also CD8⁺ T cell proliferation.

BP5 significantly promotes immune protection against H9N2 AIV challenge. To verify that a killed vaccine in combination with BP5 can provide better protection against H9N2 AIV infection, we applied a real-time PCR assay using SYBR green 1 for detection of AIV copies in the lungs of chickens on days 1, 3, and 5 after H9N2 AIV challenge. In the assay, the dissolution curve showed that the HA primer had a good specificity, and the standard curve results showed that the amplification efficiency of the HA primer, which could be used for detection of virus in lung samples, was 99.89% (data not shown). As shown in Table 3, numbers of lung viral copies were significantly reduced in the chickens coimmunized with inactivated AIVs and BP5 compared to those in the chickens immunized with inactivated AIV alone on days 1, 3, and 5 after H9N2 AIV challenge (25 mg/kg and 5 mg/kg, P < 0.05; 1 mg/kg, P < 0.01). Compared to the number of lung viral copies in the chickens immunized with the commercial H9N2 AIV vaccine (with oil/water as an adjuvant), lung viral copies were also reduced significantly in the chickens coadministered inactivated AIVs and BP5 (1 mg/kg, P < 0.05) (Table 3).

DISCUSSION

Many adjuvant approaches have been evaluated for use in vaccines. However, since most of the adjuvants used in conjunction with antigen have unacceptable levels of side effects, such as toxicity and adverse site reactions, only a few of them are used clinically (26, 35). Aluminum-based mineral salts (aluminum adjuvant; alum) have commonly been used in many veterinary and human vaccines because of their safety (1), but they induce antibody production weakly and are poor at eliciting cell-mediated immune responses (3), which are significant drawbacks for their use in vaccines against intracellular parasites and some viruses. The oil-based adjuvants, which are common in veterinary vaccines, in contrast, are limited by their induction of side effects and adverse site reactions (5, 18, 34). Thus, research to find new and optimal adjuvant candidates for vaccine formulations has been described in many publications. In some of these publications, research on some small peptide immunostimulants used for vaccine adjuvant strategies has also been reported (2, 8, 36).

In our previous study, we isolated and purified a novel bursa pentapeptide, BP5, which was capable of enhancing antigen-specific humoral and cell-mediated immune responses in mice (19). In the present study, we found that a simple mixture of inactivated H9N2 AIVs and BP5 also enhanced humoral and cell-mediated immune responses in chickens. When coinjected with the model antigen (an inactivated avian influenza virus [AIV], A/Duck/Jiangsu/NJ08/05 [AIV H9N2 subtype]), BP5

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**TABLE 2. Flow cytometric analysis of CD3⁺ T cells and their subsets CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells from the peripheral blood lymphocytes of immunized chickens**

| Treatment          | % of peripheral blood lymphocytes of type: |
|--------------------|-------------------------------------------|
|                    | CD3⁺ | CD3⁺ CD4⁺ | CD3⁺ CD8⁺ |
| PBS                | 35.15 ± 2.14 | 14.36 ± 1.89 | 9.22 ± 1.51 |
| AIVs               | 45.99 ± 1.23 | 20.87 ± 2.13 | 13.68 ± 2.12 |
| AIVs + 25 mg/kg BP5| 48.78 ± 2.86 | 20.54 ± 1.38 | 16.87 ± 1.89 |
| AIVs + 5 mg/kg BP5 | 58.82 ± 2.48 | 26.89 ± 1.56* | 22.67 ± 1.78† |
| AIVs + 1 mg/kg BP5 | 61.46 ± 1.61**† | 30.44 ± 2.24** | 25.01 ± 1.53**† |
| H9N2 AIV vaccine   | 56.87 ± 2.31* | 28.57 ± 1.79** | 16.51 ± 1.35 |

* Chickens were sacrificed on day 28 after first immunization, and the peripheral blood lymphocytes were collected for immunophenotyping. The data presented are means ± SD of results from four replicates. *, P < 0.05, and ***, P < 0.01, compared with chickens immunized with the inactivated AIVs alone. †, P < 0.05, compared to chickens immunized with the commercial H9N2 AIV vaccine.

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**DISCUSSION**

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induced higher levels of antigen-specific hemagglutination inhibition (HI) antibody titers and antigen-specific HA antibody (IgG) titers in chickens than were induced in chickens immunized with inactivated avian influenza virus alone. Furthermore, chickens coadministered inactivated AIVs and proper concentrations of BP5 (used as an adjuvant) produced significantly higher HI and IgG antibody titers than chickens immunized with the commercial H9N2 AIV vaccine (prepared with oil/water as an adjuvant). In some literature, it has been reported that a single administration of commercial H9N2 AIV vaccine in oil emulsion induced higher HI antibody titers (about 9 log$_2$) 3 weeks after vaccination than the control (16), whereas in other literature, it has been reported that oil adjuvant H9N2 AIV vaccine produced HI antibody titers that were less than 6 log$_2$ 2 weeks after the first vaccination, less than 7.0 log$_2$ 3 weeks after the first vaccination, and less than 8.0 log$_2$ 3 weeks after the second vaccination (17). It is well known that various factors, like source of erythrocytes, type of diluent, incubation temperature, and incubation period, affect hemagglutination activity, and thereby, they affect the HI titers (12). In view of this, the data for HI antibody titers obtained from this study are generally consistent with the data reported by Lee et al. (17). Although the HI antibody titers induced by the commercial AIV vaccine and by BP5 adjuvant-inactivated AIVs were not very high in this study, BP5 adjuvant-inactivated AIVs induced higher HI antibody titers than oil adjuvant commercial AIV vaccine. This suggested that BP5 has an effective adjuvant activity in vaccines that enhances antigen-specific humoral immune responses.

In addition to humoral responses, cellular immunity plays an important role in fighting influenza virus infections (14). In this study, cell-mediated immunity was evaluated in vaccinated chickens through cytokine analysis and in vitro proliferation assay of peripheral blood splenocytes pre- and postimmunization. Currently, special attention is being given to adjuvants capable of efficiently promoting a Th1-type immune response, which is considered the best correlate of a protective immune response to infections (32). However, the most powerful Th1-promoting adjuvants exhibit some toxicity, which limits their clinical use (27). The most remarkable finding reported in the present study is the demonstration that BP5, coadministered with inactivated AIVs, represents an unexpectedly powerful adjuvant, not only inducing the production of Th1-type cytokines (IL-2 and IFN-γ) but also inducing the production of Th2-type cytokines (IL-4). Moreover, in vivo/ex vivo, using MTT incorporation to measure cell proliferation and flow cytometric analysis to measure immunophenotyping of T lymphocytes, significant increases in peripheral blood lymphocyte proliferation and in the sizes of CD3$^+$ T cell populations, including CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ T cell populations, were found in chickens coadministered inactivated AIVs and BP5. In contrast, although the levels of cytokines in sera and the levels of peripheral blood lymphocyte proliferation and CD3$^+$ T cell populations were increased in chickens immunized with a commercial, inactivated AIV vaccine (prepared with oil/water as an adjuvant), levels of only Th1-type cytokines increased, and the CD8$^+$ T cells were only modestly affected. These results indicate that BP5 has the potential to affect cell-mediated responses and balance Th1- and Th2-type immune responses when used as an adjuvant.

To further evaluate the influence of BP5 as an adjuvant on the immunity protection provided by inactivated AIVs against avian influenza virus infection, chickens were challenged intranasally with avian influenza H9N2 virus strain JS-1 (A/Chicken/ Jiangsu/JS-1/2002) on day 28 after they had been coimmunized with inactivated AIVs and BP5. At 2 days postchallenge, the nonvaccinated chickens that received the challenge virus were mildly depressed. No other clinical signs were observed in that group or any of the other groups, which is typical of low-pathogenicity AIVs in chickens (15, 33). At 5 days postchallenge, only the nonvaccinated challenged group had mild, grossly detectable lesions in both the respiratory and gastrointestinal tract. As JS-1 H9N2 virus is a low-pathogenicity avian influenza virus and all challenged chickens survived the infections, we used SYBR green 1-based real-time PCR to assess the extent of virus infection, monitoring the protection level of inactivated AIVs after they were coadministrated with BP5. We detected the challenge virus in the lungs of the challenged chickens on days 1, 3, and 5. Our data indicated that viral replication (viral shedding) occurred and that virus shedding could be more efficiently blocked or reduced after a homologous vaccine was coadministered with BP5, which was used to vaccinate chickens against the challenge virus. This result suggests that BP5 has the potential to be used in vaccine formulations to provide improved protection against H9N2 AIV infection in poultry.

Several small peptides have been synthesized in an effort to discover an idealized peptide sequence with significant immunological adjuvant activity (7, 8). Our previous study revealed that B lymphocyte proliferation induced by BP5 is mediated by reactive oxygen species generated from thiol auto-oxidation of Cys in BP5 (19). We presume that Cys plays an important role in the immune functions of BP5. Thus, analogs of BP5, such as Gly-Lys-Asp-Val-Tyr, Ala-Lys-Asp-Val-Tyr, and Glu-Lys-Asp-Val-Tyr, were also synthesized and used to evaluate their immune activities in mice and chickens. In the assays, no significant immune adjuvant activities of these peptides were detected (data not shown). This suggests that the specific immune inducer properties of BP5 are associated with its special amino acid sequence. Further research on the relationship between the structure and the immune activity of BP5 will contribute new insights into the mechanisms of adjuvant activity and may lead to the development of a practical application in vaccine design. Further studies are also needed to further compare the effects of BP5 and other adjuvants.

In summary, we demonstrated that BP5 enhanced the avian influenza virus-specific cell-mediated and humoral immune responses induced by inactivated AIVs. Furthermore, intramuscular immunization with a mixture of inactivated AIVs and BP5 enhanced protection against a homologous avian influenza virus challenge by reducing viral replication in chicken lungs. This study indicates that BP5 possesses adjuvant activities and that it may be used as a new experimental reagent for immuno-adjuvant uses.

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