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Immune response of AA broilers to IBV H120 vaccine and sodium new houttuyfonate

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A B S T R A C T

In this report, 120 healthy one-day-old AA broilers were divided into six groups. Groups 1–4 received 100, 200, 400 and 800 mg/L of sodium new houttuyfonate (SNH) with IB vaccine H120 respectively. Group 5 received PBS and H120 and group 6 IL-2 and H120. The chickens were inoculated at 7 and 14 days of age. On 0, 7, 14, 21, 28 and 35 post first vaccination, the dynamic changes of peripheral lymphocyte proliferation, cytokine assays and serum antibody titers were assayed respectively by MTT method, ELISA and hemagglutination inhibition assay (HI). The results showed that sodium new houttuyfonate significantly raised IB antibody titer in the chickens and also markedly promoted lymphocyte proliferation. The serum levels of IFN-γ and IL-4 in groups 1–4 were higher than those in groups 5 and 6. Hence, the immunologic enhancement of SNH was slightly superior to that of IL-2 adjuvant. Following challenge with IBV, chickens inoculated with SNH showed fewer and less severe clinical signs, lower death rate and less kidney pathology, as compared to those of the control groups. It indicated that SNH could enhance immune responses and increase protection against virulent IBV challenge in chickens.

1. Introduction

Infectious diseases of animals, especially viral, often cause great losses in the domestic animal industries. Infectious bronchitis (IB) is an acute and highly contagious respiratory disease of chickens, and is still a major health problem in the chicken industry. Infectious bronchitis virus (IBV), an enveloped coronavirus containing an unsegmented, single-stranded, positive-sense RNA genome, is one of the primary causes of respiratory disease in domestic fowl. Infection with IBV reduces the performance of broilers and causes drops in egg production and egg quality (Bijlenga et al., 2004; Cavanagh and Naqi, 2003; Cavanagh, 2003). There are problems with vaccines against infectious bronchitis applied in the field. Live attenuated vaccine may spread to unvaccinated flocks (Farsang et al., 2002); inactivated vaccine does not elicit an immune response against coronavirus (Yang et al., 2004; Stohlman et al., 1992), and severe side effects and long-lasting local response may occurs after inoculation.

Clinical practice has indicated that the application of vaccine with adjuvant or immunopotentiator could improve efficacy. However, chemical adjuvants (e.g. aluminium hydroxide (Al(OH)₃), aluminium phosphate (AlPO₄), and mineral oil) commonly cause side effects, such as strong local reaction and carcinogenesis. Sometimes a chemical adjuvant may fail to enhance the immunogenicity of a weakly antigenic vaccine (Xie, 1995; Gong and Wu, 1996; Sun, 1998). Therefore, there is a need to assess an immunopotentiator that is both safe and efficacious.

Sodium new houttuyfonate (SNH, sodium lauroyl-α-hydroxyethyl sulfonate) is a novel antimicrobial medicine that has been recently developed (the chemical structure is shown in Fig. 1). Previous studies showed that houttuyfonate homologues (HOU-Cn) had immunoregulatory activities and typical adjuvanticity, and could improve the immune ability of mice and inhibit the growth of Staphylococcus aureus, Bacillus subtilis and pneumococci (Ye et al., 2006; Wang et al., 2002, 2004). It was reported that the immunoregulatory and antibacterial activity of SNH were higher and stronger than that of sodium houttuyfonate (Yuan et al., 2004a; Ye et al., 2005; Yang et al., 2007).

Because of its potential pharmaceutical effects on health, the purpose of this study was to evaluate the immune enhancement properties of SNH as a new type of immunopotentiator.

2. Materials and methods

2.1. Snh

SNH was obtained from Jiangsu Jichun Pharmaceutical Co., Ltd.
2.2. Preparation of vaccines

IB disease vaccine virus H120 strain (HA titer 2 \times 10^8) was supplied by Jiangsu Provincial Academy of Agricultural Science. After safety was tested by injecting the virus into 11-day-old SPF chicken embryos, the virus and SNH were mixed (V/V = 1:3), and four inocula, containing 100 mg/L (group 1), 200 mg/L (group 2), 400 mg/L (group 3) and 800 mg/L (group 4) of SNH, were prepared. Two additional inocula, PBS and H120 (group 5, negative control) and IL-2 and H120 (group 6, positive control), were prepared. All vaccines contained the same antigen content.

2.3. Reagents

Filtered (0.22 \mu m) RPMI 1640 media (Gibco) supplemented with benzylpenicillin (100 IU/ml), streptomycin (100 IU/ml) and 10% fetal bovine serum (Sijiqing, Zhejiang province) was used for culturing the cells. The same formula, minus bovine serum, was used for washing and diluting the mitogen. ConA (Sigma), as the adherent lymphocytes separation media. After 20 min centrifugation at 800 g, the white cloud-like lymphocyte band was collected and washed twice with RPMI 1640 media without fetal bovine serum. The resulting pellet was re-suspended to 5 \times 10^6/ml in RPMI 1640 media with fetal bovine serum and incubated in 96-well culture plates (Nunclon), 80 \mu l/well, then another 20 \mu l/well of the ConA (four wells) or RPMI 1640 with 10% fetal bovine serum (four wells) was added and each sample seeded eight wells. After 44 h of incubation at 37 \degree C in a humid atmosphere of 5% CO2 (Revco, Co., USA), 20 \mu l/well of MTT was added, and the plates re-incubated for 4 h. The plates were centrifuged (10 min at 1000g, room temperature) and the supernatant carefully discarded. Hundred microlitre/well of DMSO was added and the plates shaken for 5 min to dissolve the formazan crystals. The absorbance of cell in each well was measured by microtiter enzyme-linked immunosorbant assay reader (Model DG-3022, East China Vacuum Tube Manufacturer) at a wave length of 570 nm and the stimulation index (SI) was calculated. The stimulation index was determined from the formula: stimulation index (SI) = experimental OD/negative OD (Barta et al., 1992; Maheswaran and Thies, 1975).

2.5.2. Cytokine assays

To determine the immune activity of SNH, the levels of IFN-\gamma and IL-4 in the vaccinated animals were examined. For the detection of IFN-\gamma and IL-4 production in the chickens, sera were obtained at 0, 7, 14, 21, 28 and 35 dpi and determined quantitatively using commercially available cytokine ELISA Kits (chicken IFN-\gamma/IL-4 kits, sensitivity: 1 pg/ml, Adlitteram Diagnostic Laboratories (ADL), USA) as per protocol.

2.5.3. Serum HI antibody assay in chicken

Blood samples (3 ml per chick) were drawn from the main brachial vein into Eppendorf tubes and allowed to clot at 37 \degree C for 2 h. Serum was separated by centrifugation and inactivated (56 \degree C, 30 min) for use. Briefly, two-fold serial dilutions of serum were made in a 96-well, V-shaped bottom microtiter plate containing 25 \mu l of PBS in all wells. 25 \mu l of IBV antigen (without purification, diluted to 4 HA units with PBS) was added to all the wells except the last row, which served as a control. Serum dilutions ranged from 1:2 to 1:2048. The antigen–serum mixture was incubated for 10 min at 37 \degree C. Twenty five microliter of a 1% rooster erythrocyte suspension was added to each well and the plates were re-incubated for 30 min. A positive serum, a negative serum, erythrocytes, and antigen were also included as controls. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal log2 values of the highest dilution that displayed HI.

2.6. Challenge

All chickens were challenged with 100 EID of the IBV M41 strain in 0.3 ml by intramuscular injection (i.m.) at 49 days, and were examined daily for 2 weeks for clinical signs such as coughing, sneezing, ataxia, dyspnoea or death. Dead chicks were necropsied to confirm the death due to IBV infection.

2.7. Statistical analysis

Data are expressed as the mean ± S.D. Duncan’s multiple range test was used to determine the difference among every adjuvant and control groups. Differences between means were considered significant at p < 0.05.
3. Results

3.1. The dynamic changes of peripheral lymphocyte proliferation responses

The dynamic changes of stimulation index (SI) value are listed in Fig. 2. On Day 0 after primary vaccination, the values of groups 1–4 were larger than those of IL-2 and PBS groups. At 7 dpi, the SI values of 800 mg/L group were significantly higher than those for the PBS or IL-2 groups (p < 0.05). At 14 dpi, the values for Group 1 were higher than those for Groups 2, 3 and 4; however, there was no significant difference between these groups. At 21, 28 and 35 dpi, the values of 200 mg/L group were significantly larger than those of IL-2 or PBS groups (p < 0.05). At 21, 28 and 35 dpi, the values of 200 mg/L group were significantly larger than those of 100, 400 and 800 mg/L groups (p < 0.05).

3.2. Cytokine responses

The ELISA kits were employed to detect the production of IFN-γ and IL-4 in sera. The results showed that the mean concentration of IFN-γ was higher in groups 1–4 compared to groups 5 and 6. The 800 mg/L group was significantly higher than the PBS or IL-2 group at 7 and 14 dpi (p < 0.05), but there was no significant difference between the 100, 200 and 400 mg/L groups. At 21 and 28 dpi, the 200 mg/L group was significantly higher than the PBS or IL-2 (p < 0.05), but there was no significant difference between other experimental groups (Fig. 3A). At 35 dpi, high levels of the experimental groups were observed comparing to the PBS or IL-2, but there was no significant difference (P > 0.05).

Similarly, there was almost the same change in IL-4 between groups 1–4 and group 6 after vaccination, but only at 7 and 14 dpi. Including 800, 400 and 200 mg/L groups at 14, 21 and 28 dpi a significantly higher level of IL-4 was observed compared to that in the PBS or IL-2 groups (groups 5 and 6) (p < 0.05), whereas there was no significant difference between groups 1, 5 and 6 (p > 0.05) (Fig. 3B). The results indicated SNH could promote both Th1-type and Th2-type cytokine responses, and there was a dose-dependant effect.

3.3. The dynamic changes of serum antibody titer

The dynamic changes of HI antibody titer are listed in Table 1. On day 28 after primary vaccination, the antibody titers of groups 1–4 were higher than those of groups 5 and 6. At 14 and 21 dpi, group 4 was significantly higher than the PBS or IL-2 group (p < 0.05). Except for groups 1 and 3, the titers of groups 2 and 4 at 21 dpi were significantly higher than those of groups 5 and 6 (p < 0.05). At 28 and 35 dpi, in comparison of other groups, the titer of group 2 was significantly larger (p < 0.05).

3.4. Clinical evaluations

Chickens started to show clinical signs or die from viral infection on day 8 after challenge, but deaths occurred only in group 5. The chickens immunized with PBS were not protected and developed coughing, nasal discharge and dyspnea. The morbidity and mortality of the PBS immunized group 5 were 100% (10/10) and 80% (8/10), respectively, after challenge with IBV M41 stain (Fig. 4). Chickens in groups 1–4 had lower morbidity, mortality and pathological changes when compared to those of the PBS group 5, but the difference was insignificant. Groups 1–4 showed clinical signs such as depression, anorexia, a shrinking neck and fluffed down feathers, but no chicken died except for those in groups 1 and 6. This suggests that SNH confers resistance against a virulent IBV challenge.

4. Discussion

The use of houttuynonate as an adjuvant has been accompanied by side effects (e.g., anaphylactic shock, systemic anaphylaxis, dyspnea), and so its clinical use has been limited. Recent pharmacological studies have shown that SNH and houttuynonate have similar pharmacological properties, with a variety of biological functions, in addition to antibacterial, diuretic, antitussive, but also play a strong role in immune regulation. In addition SNH is safer and has fewer side effects than houttuynonate (Jiang et al., 2006; Zhu et al., 2008a; Yuan et al., 2004b). However, its application in the poultry industry has seldom been reported. In this study, with chicken IBV H120 strain live attenuated vaccine in chickens, by measuring peripheral blood lymphocyte proliferation and serum cytokines and antibody titers, the humoral and cellular immunity induced with SNH was researched. The results indicated SNH could enhance immune function in a dose-dependent manner. The results show that SNH played an important role in protecting against challenge by virulent virus.

It is well known that in both mouse and human systems, the Th1 and Th2 paradigm has emerged as a critical concept governing cellular and humoral immune responses. IFN-γ and IL-4 are prototype Th1 and Th2 cytokines, respectively, whose antagonistic actions against each other have been widely reported (Mosman and Sad, 1996). They reciprocally regulate not only Th1 and Th2 cell survival and subsequent differentiation, but also activation and differentiation of B cells as well as monocytes (Constant and Bottomly, 1997; Snapper and Paul, 1987; Cao et al., 1989). Specific target molecules that are counter-regulated by IFN-γ and IL-4 play...
important roles in mediating Th1 or Th2 immune responses. Serum levels of the two cytokines increased, and this effect shows a certain degree of dose–effect relationship, especially obvious with the 200 mg/L dose. Therefore it was important to determine the

![Fig. 3. Serum concentrations (pg/ml) of IFN-γ (A) and IL-4 (B) in the serum. Samples (n = 5) were examined by using commercially available chicken cytokine ELISA kits. An asterisk indicates the concentration of cytokine is significant (p < 0.05). Data were shown as mean ± S.D. Error bars represent group standard deviations. Arrows (↑) indicate time of initial immunization and boost.](image)

![Fig. 4. Morbidity and mortality ratio of each group after challenging with IBV M41 strains.](image)

**Table 1**
The dynamic variation of antibody titers in chicken (log2).

| Groups         | dpi | 0     | 7     | 14    | 21    | 28    | 35    |
|---------------|-----|-------|-------|-------|-------|-------|-------|
| Group 1 (100 mg/L) |     | 3.2 ± 0.3a | 4.1 ± 0.7a | 4.9 ± 0.2a | 6.7 ± 0.3a | 7.0 ± 0.3a | 5.3 ± 0.1a |
| Group 2 (200 mg/L) |     | 3.5 ± 0.4a | 4.4 ± 0.6a | 5.3 ± 0.4a | 7.7 ± 0.6b | 9.1 ± 0.6b | 8.3 ± 0.3b |
| Group 3 (400 mg/L) |     | 3.3 ± 0.5a | 4.9 ± 0.4a | 5.7 ± 0.4a | 6.5 ± 0.4a | 7.0 ± 0.4a | 5.2 ± 0.4a |
| Group 4 (800 mg/L) |     | 3.4 ± 0.3a | 5.1 ± 0.5a | 6.7 ± 0.7b | 7.0 ± 0.7b | 7.3 ± 0.3a | 5.1 ± 0.7a |
| Group 5 (PBS)    |     | 3.5 ± 0.4a | 3.8 ± 0.1a | 4.2 ± 0.4a | 5.1 ± 0.3a | 6.3 ± 0.1a | 4.7 ± 0.4a |
| Group 6 (IL-2)   |     | 3.6 ± 0.4a | 4.4 ± 0.7a | 5.1 ± 0.3a | 5.9 ± 0.3a | 6.7 ± 0.7a | 5.7 ± 0.5a |

Data within a column with different letters differ significantly (p < 0.05); the same letters means no significance.
appropriate dose of SNH to take advantage of the body’s immune system. At the same time, T lymphocyte-mediated cellular immunity was also researched. The results show that SNH can significantly enhance the immune function of chicken cells by promoting lymphocyte proliferation. So, SNH improved cellular immunity in comparison with PBS or IL-2.

HI tests are mostly used to monitor antibody levels induced by IBV live attenuated vaccine. Therefore, the HI titer was considered the key factor in the appraisal of the vaccine adjuvant in this study. Although the maternal antibody IBV titers influence the results, the final result indicated no influence. The co-administration of antigen and SNH increased HI titers, and the titers of the SNH groups were significantly higher than those of the PBS or IL groups, which confirmed that SNH could improve antibody formation. Thus, it qualifies as a new-type immunopotentiator.

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