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Effects of pidotimod soluble powder and immune enhancement of Newcastle disease vaccine in chickens

Shaoqi Qu[a,b], Cunchun Dai[a,b], Mei Qiu[a,b], Ruili Zhang[a,b], Chunyuan Wang[a,b], Liangliang Cui[a,b], Zhihui Hao[a,b,*]

[a] Agricultural Bio-pharmaceutical Laboratory, Qingdao Agricultural University, Qingdao 266109, China
[b] National-Local Joint Engineering Laboratory of Agricultural Bio-Pharmaceutical Technology, Qingdao 266109, China

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ABSTRACT

The aims of this study were to prepare pidotimod (PDM) soluble powder and to investigate the immune enhancement properties of PDM in chickens vaccinated with Newcastle disease virus vaccine. In vivo experiment, 360 6-day-old chickens were averagely divided into 6 groups. The chickens, except blank control (BC) group, were vaccinated with Newcastle disease vaccine (NDV). At the same time of the vaccination, the chickens in three PDM groups were given water with PDM for 5 days, respectively, with the PDM at low, medium and high concentrations (0.25 g/L, 0.5 g/L, 1 g/L), in control drug group was treated with 0.2 ml/PDM dose *via* drinking water, in vaccination control (VC) and BC group, with equal volume physiological saline, once a day for five successive days. On days 14, 21 and 28 after the vaccination, the growth performance, the lymphocyte proliferation, serum antibody titer, the CD4/CD8 cell ratios and interleukin-2 (IL-2) and interferon-gamma (IFN-γ) were measured. The results showed that PDM at suitable dose could significantly promote growth performance, lymphocyte proliferation, enhance serum antibody titer, CD4/CD8 cell ratios and improve serum IL-2 and IFN-γ concentrations. It indicated that PDM could significantly improve the immune efficacy of Newcastle disease vaccine using doses of 0.5 g/L, these results are consistent with the drug acting as an immunopotentiator.

1. Introduction

Pidotimod ((*R*)-3-[(*S*)-(5-oxo-2-pyrrolidinyl) carbonyl]-thiazolidine-4-carboxylic acid or PDM) represents a new class of biological response modifiers. It enhances immunological activity of both innate and adaptive immune systems [1,2]. *In vivo* studies in animals and humans have demonstrated that PDM has immunoprotective activities [3,4]. This drug also enhances murine macrophage polarization and improves their function in vitro [5]. Moreover, PDM can stimulate human mucosal dendritic cells to release large amounts of pro-inflammatory molecules to drive lymphocyte proliferation and differentiation. This facilitated activation of the innate immune system and promoted a systemic immune response [6].

PDM promotes strong and specific humoral and cellular immune responses to *Toxoplasma gondii* challenge when co-administered with UV-attenuated *T. gondii* [7]. PDM is in use as an experimental biological response modifier, it increases mitogen-induced proliferation and augments the secretion of IL-2 and IFN-γ growth [8,9], but the immune effect of PDM on chickens has been scarcely investigated. In the current study, the effects of PDM on chicken blood lymphocytes proliferation in vitro and the immune response of NDV in vivo were investigated. We performed growth performance of PDM in chicken after oral administration, and serum IL-2 and IFN-γ concentrations were improved. The growth performance indicated that the drug is safely absorbed with no obvious adverse effects. In addition, we demonstrated that PDM influences the immunoprotective effects against Newcastle Disease Virus after vaccination. The purpose of this study access the probability of adjuvant activity of PDM, obtain the optimal dosage and offer theoretical evidence for use as a new immunologic adjuvant.

2. Experimental

2.1. Reagents

PDM soluble powder was obtained from Qilu Animal Health (Jinan, China). Transfer factor oral solution was the product of Shandong Xinde (Zhucheng, China). PDM (≥98.5%) was obtained from Zhejiang Xianju Pharmaceutical (Taizhou, China) and the PDM reference standard

* Corresponding author at: Agricultural Bio-pharmaceutical Laboratory, Qingdao Agricultural University, Qingdao 266109, China.
E-mail address: abplab@126.com (Z. Hao).

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(99.9%) from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Lymphocyte separation solution (Ficoll–Hypaque), ρ: 1.077 ± 0.002, was from Tianjin Hao Yang Biological, (Tianjin, China). Concanavalin A (ConA, Sigma), was dissolved at 2.5 gm L^{-1} with RPMI-1640 supplemented with benzylpenicillin 100 IU/ml, streptomycin 100 IU/ml and 10% fetal bovine serum (Gibco). Analytical HPLC grade acetonitrile was purchased from the Tedia Company and HPLC grade formic acid from Tianjin Kemiou (Tianjin, China). All other reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of PDM soluble powder

Crude PDM powder was first passed through an 80 mesh sieve and 101.0 g of the sieved product was thoroughly mixed with 899.0 g D-glucose. This mixture was then passed through a 40 mesh sieve according to the standard procedure ourlined in the Chinese Veterinary Pharmacopoeia [8].

2.3. Vaccine and virus

Newcastle Disease vaccine (LaSota strain, No. BS20100201) was obtained from the Beijing Veterinary Biological Medicine factory. The Chinese Veterinary Institute supplied Newcastle Disease virus (NDV, F48E9 strain).

2.4. Animals

One-day-old broiler chickens purchased from Beijing Aibo Yijia Poultry were housed in wire cages (90cm × 50cm × 40 cm) in an air-conditioned room at 37 °C.The chickens were exposed to light for 20 h per day at the beginning of the pretrial period. Afterwards, the temperature was gradually decreased to 25 °C and the light was adjusted to 12 h per day, and these conditions were kept constant throughout the remainder of the study.

2.5. Experimental design

A total of 360, one-day-old AA broiler chickens were randomly divided into six groups: blank control, drug control group (Transfer factor oral solution, 0.2 ml/body), vaccine control group, low (0.25 g/L), medium (0.5 g/L) and high (1.0 g/L) PDM dosage groups (Groups 1–6 respectively), each with three replicates. At six days of age, the test drug group was given water with PDM for 5 days. At the seventh day of age, eye drops or intranasal sprays vaccinated all groups with the exception of the blank controls. The control drug group was treated with 0.2 ml/PDM dose *via* drinking water. The blank control group was not given drug nor immunized. At the age of thirty-five days, ten chickens were randomly selected from each group except for the vaccine control group. The virus amount was 10^6 EID50(median egg infectious doses), and the incidence and mortality were observed within 7 days after challenge.

2.6. Sample collection and assays

2.6.1. Growth performance assay

All chickens in each group were weighed at 14, 21, 28 and 35 days of age, and the feed consumption was measured at 14–21, 22–28 and 29–35 days of age. The mean body weight of each group was calculated and the feed/meat ratio was calculated as: Feed ratio (kg/kg) = total feed consumption/total weight gain.

2.6.2. Serum assays

Fifteen blood samples were randomly selected from 14, 21 and 28 day-old chickens\from each group. Antibody titers were determined by hemagglutination as previously described [11]. Briefly, twofold serial dilution of serum samples with 50 μl physiological saline was made in a 96-well V-shaped bottom microtiter plate. 50 μl of Newcastle Disease virus antigen (4 HA units) was added into all the wells except for the controls. Serum dilutions ranged from 1:2 to 1:2048. The plate was shaken for 1 min and incubated 10 min at 25 °C. A 50 μl aliquot of 1% rooster erythrocyte suspension was added into each well, shaken, and incubated for 15 min at 25 °C. 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.

Five blood samples were randomly selected from chickens at 14, 21 and 28 days of age to determine the hematocrit. Heparinized blood samples were also used to assess proliferation of peripheral lymphocyte (5 chickens per group, 3 ml per chicken). The blood was diluted with an equal volume of Hanks' solution and 3 ml lymphocyte separation solution. After 15 min of centrifugation at 2000 rpm, a white cloud-like lymphocyte band was collected. This was washed twice with RPMI 1640 media without fetal bovine serum followed by a 5 min centrifugation at 1500 rpm. The resulting cell pellet was suspended to 5×10^6 cells mL^{-1} with RPMI 1640 media and incubated in 96-well culture plates at 80 μl per well, each sample seeded 4 wells. Then another 20 μl of ConA was added into per well (RPMI-1640 as blank control). The plates were incubated at 37 °C for 44 h in a controlled atmosphere of 5% CO_2. 20 μl of MTT (5 g mL^{-1}) was added into each well, and the plates were incubated for another 4 h and then centrifuged at 1000*g* for 10 min at room temperature. The supernatant was carefully removed and 100 μl of DMSO were added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance at a wavelength of 570 nm (A570 value) of lymphocyte in each well was measured using a universal microplate reader (Model ELx800, BioTek Instruments, Inc.). The mean A570 values were used as the indicator of peripheral lymphocyte proliferation [9].

Five blood samples were randomly selected from 14, 21 and 28 day-old chickens\from each group. The levels of IL-2 and IFN-γ in the serum samples were determined using the corresponding ELISA kits (Nanjing Jiancheng, China) according to the manufactures' instruction.

CD4/CD8 cell ratios and Newcastle Disease-specific CD4/CD8 dynamic changes were measured by flow cytometry using venous blood from 5 chickens in each experimental group at 14, 21 and 28 days of age. lymphocytes were isolated and cultured for 24 h in the presence of inactivated NDV (1 mol) and cells were collected and stained with fluorescent-conjugated antibodies against chicken CD3, CD4 and CD8. The ratio of CD4 to CD8 T-cells was determined by flow cytometry using a FACS Calibur instrument (Becton, Dickinson and Company, USA). Gates were set at forward and side angle light scatter to exclude dead cells and debris.

Macrophage phagocytosis of recombinant *Escherichia coli* expressing yellow fluorescence was measured using peripheral blood lymphocyte according to a previously described protocol [10]. Cells were incubated in a 24-well plate at 10^6 cells/well in RPMI 1640 medium containing 10% (v/v) fetal bovine serum at 37 °C and 5% CO_2 for 48 h. *E. coli* cells were then added and phagocytosis was observed using an inverted fluorescence microscope (Olympus Corporation).

2.7. Statistical analysis

All of the experiments were conducted in triplicate and all the data are presented as the mean ± SD., analyzed using SPSS for Windows version 17.0 (SPSS Inc, Chicago, IL). Differences between means were considered significant at $P < 0.05$.