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Immunology

Zymosan A enhances humoral immune responses to soluble protein in chickens

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ABSTRACT. Vaccination is the most effective method for controlling the infectious diseases that threaten the poultry industry worldwide. The use of adjuvants or immunostimulants is often necessary to improve vaccine efficacy, particularly for vaccines based on recombinant protein or inactivated pathogens. The adjuvant effects of zymosan A on antigen-specific antibody production were investigated in chickens. First, the optimal adjuvant dose of zymosan A was determined. Chicks were immunized with dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) at a dosage of 2 mg/kg body weight (BW) with or without zymosan A (at a dosage of 0.5 mg/kg BW) co-administration at 4, 5 and 6 weeks of age. Different routes of immunization (oral, intranasal (i.n.), intraocular (i.o.), subcutaneous (s.c.), intramuscular (i.m.) and intraperitoneal (i.p.) were tested. Anti-DNP IgY and IgA concentrations in serum samples from all chicks were measured by an enzyme-linked immunosorbent assay. The results revealed that co-administration of zymosan A with DNP-KLH significantly increased anti-DNP IgY concentrations in chicks immunized by the oral and s.c. routes of administration when compared with control groups. In addition, co-administration of zymosan A with DNP-KLH significantly increased anti-DNP IgA concentrations in chicks immunized by the oral, i.o. and s.c. routes compared with control groups. In conclusion, zymosan A is a useful immune-potentiator adjuvant in chickens, and its co-administration with vaccine antigens enhances humoral immune responses.

KEY WORDS: adjuvant, chick, IgA, oral, zymosan A

The modern poultry industry depends greatly on vaccines to protect against a wide range of infectious microorganisms. The new generation of subunit vaccines have a defined composition of either purified or recombinant microbial protein antigens, but are often linked with lower immunogenicity and require the use of adjuvants to induce potent and persistent immune responses [25]. Adjuvants are molecules, compounds or macromolecular complexes that increase the magnitude and/or duration of the immune response to the vaccine antigen [60]. Vaccine adjuvants are divided into two main groups: (1) vaccine delivery vehicles and (2) immune potentiators [45]. They facilitate the initiation of an immune response to a co-administered antigen by either increasing the normal immune initiation processes or by altering the physical properties of the injected antigen to become more available to the immune system [38]. Immune potentiators induce abundant amounts of specific antibody and large populations of memory B and T lymphocytes through activation of innate immune receptors on antigen-presenting cells, which take up and present antigens to T cells [3, 5, 47].

Zymosan A, the insoluble polysaccharide component of the cell wall of *Saccharomyces cerevisiae*, contains 50–57% 1→3-β-glucans [16]. Zymosan can activate macrophages, monocytes and leukocytes in mice [51, 59, 65], resulting in the stimulated secretion of inflammatory products including tumor necrosis factor (TNF)-α, interleukin-8, hydrogen peroxide and arachidonic acid [10, 14, 42, 44, 57]. The interaction of zymosan A with macrophages is generally considered to be the first step in the initiation of an immune response. Zymosan may signal through several receptors and among the postulated receptors for non-opsonized zymosan are the mannose receptor, the Toll-like receptors (TLRs), scavenger receptors, the glucan receptor and the C-type lectin and mannan receptor SIGNR1, found on resident macrophages [9, 11, 18]. Both TLR2 and TLR6 are required for the activation of nuclear factor kappa-B (NF-κB) and the production of inflammatory cytokines, such as TNF-α, by zymosan particles [29]. Glucan receptors play an important role in mediating the binding of zymosan to macrophages [55]. The glucan receptor dectin-1 is found on macrophages, neutrophils and dendritic cells [26, 48, 49, 58, 62] and on recognizing its ligand, it activates

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several signaling pathways to promote innate immune responses through the activation of phagocytosis, reactive oxygen species production, and induction of proinflammatory mediators [1, 8, 9, 24, 27, 61]. Furthermore, dectin-1 expression enhances TLR-mediated activation of NF-κB by zymosan, and in macrophages and dendritic cells, dectin-1 and TLRs are synergistic in mediating production of cytokines such as interleukin-12 and TNF-α. In addition, dectin-1 triggers production of reactive oxygen species, an inflammatory response that is primed by TLR activation [21]. Thus, the present study was carried out to investigate whether zymosan A has an adjuvant or modulatory effect on antigen-specific antibody if co-administered with vaccine antigen via different routes of immunization in chickens.

MATERIALS AND METHODS

Animals

White leghorn chicks were used in this study. Chicks were bred in our animal facilities and were provided with food and chlorinated water *ad libitum* in accordance with the Guidelines for Animal Experiments of Hiroshima University.

Antibodies

HRP-labeled goat anti-chicken IgY heavy and light chain (Bethyl Inc., Montgomery, TX, U.S.A.) and HRP-labeled goat anti-chicken IgA (Bethyl Inc.) were used.

Antigen preparation

A classical hapten-carrier protein antigen, dinitrophenylated keyhole limpet hemocyanin (DNP-KLH), was prepared as described previously [20, 22]. Briefly, in a clean, dry and dark container, 200 mg of K_2CO_3 was dissolved in 6 m*l* of distilled water. This was then placed on a magnetic stirrer, after which 200 mg of KLH (Calbiochem Behring Co., Darmstadt, Germany) was slowly added, and the solution was left to stand at room temperature. At the same time, 200 mg of 2,4-dintrobenzene sulfonic acid sodium salt (DNBS) (Eastman Kodak Co., San Diego, CA, U.S.A.) was dissolved in 4 m*l* of distilled water. DNBS solution was added to the KLH solution. The mixture was then stirred in the dark at room temperature for 18 to 24 hr, and dialyzed against PBS at 4°C until reaching a zero absorbance value at 360 nm against PBS. Finally, the mixture was passed through a 0.22-μm filter and the protein content of this antigen was determined based on the absorbance value measured at 280 nm. The conjugation ratio of hapten to protein was determined as described previously [20, 22]. The final product was DNP_{32}-KLH, and the antigen was kept in a refrigerator at 4°C until use. Dinitrophenylated bovine serum albumin (DNP_{28}-BSA) was prepared in the same manner.

Immunization of chicks

Chicks were immunized with DNP-KLH (2 mg/kg BW) alone or with the optimal adjuvant dose of zymosan A (0.5 mg/kg BW) via six different routes. This optimal dose of zymosan A was determined by using different doses of zymosan A with the immunizing antigen (data not shown). Sixty chicks were divided into six groups. Each group was subdivided into groups A and B (five chicks per group). Chicks in all A groups were immunized with DNP-KLH co-administered with zymosan A. Chicks in all B groups were immunized with DNP-KLH alone. All groups were immunized three times at 4, 5 and 6 weeks of age. The first group was immunized with DNP-KLH by the intramuscular (i.m.) route. The second group was immunized with DNP-KLH by the intraperitoneal (i.p.) route. The third group was immunized with DNP-KLH by the subcutaneous (s.c.) route. The fourth group was immunized with DNP-KLH by the intraocular (i.o.) route. The fifth group was immunized with DNP-KLH by the intranasal (i.n.) route. The sixth group was immunized with DNP-KLH by the oral route.

Collection of blood

Blood samples were collected every week from each chick from the wing vein using a 1-m*l* syringe with a 27 G needle. Serum was separated from the clotted blood by centrifugation at 10,000 ×*g* for 5 min, and was stored at −80°C until use.

ELISA

Concentrations of anti-DNP IgY and anti-DNP IgA antibodies were measured by ELISA as described previously [63]. Briefly, ELISA plates (NUNC, Roskilde, Denmark) were coated overnight with 55 μl of DNP-BSA solution (50 μg/m*l*), followed by a 2-hr blocking period at 37°C with 350 μl of 25% Block Ace (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan) in PBS. Four-fold serum dilutions were then added and the plates were incubated for 1 hr at 37°C. Each plate contained negative and positive control samples for measuring the concentrations of anti-DNP antibodies. Following incubation, plates were washed five times with PBS-Tween, and were then treated with diluted HRP-labeled goat anti-chicken IgY heavy and light chain (Bethyl Inc.) (1/3,000, diluted in 10% Block Ace in PBS) or with diluted HRP-labeled goat anti-chicken IgA (Bethyl Inc.) (1/3,000, diluted in 10% Block Ace in PBS) for 1 hr. Plates were then washed five times with PBS-Tween, and the substrate solution was then added to the plate; plates were left for 10–20 min in the dark until the appearance of yellow color, and the reaction was stopped using 2 N H_2SO_4. Finally, the optical density was measured at 490 nm using a micro-plate reader (BIO-RAD Model 680; Bio-Rad, Tokyo, Japan). Plates containing dilution buffer instead of sample acted as negative controls, and standard purified anti-DNP IgY antibodies (1 mg/m*l*) acted as positive controls. Concentrations of serum anti-DNP IgY antibody were measured after conversion of the ELISA data into mg/m*l* using standard anti-DNP IgY antibody samples of known concentration. Concentrations of anti-DNP IgA antibody were measured using half the plateau dilution units as described previously [19].

Statistical analysis

The mean anti-DNP antibody titers of chick sera from different groups were compared using the Student's *t*-test. All values were expressed as means ± SD and were considered to be significant at $P<0.05$, and highly significant at $P<0.005$.

RESULTS

Effects of zymosan A on antigen-specific IgY responses in chicks immunized with DNP-KLH via different administration routes

Anti-DNP IgY concentrations in serum samples were measured by ELISA. Firstly, one week after the first (Fig. 1A), second (Fig. 1B) and third (Fig. 1C) immunizations, anti-DNP IgY responses of the chicks immunized with DNP-KLH co-administered with zymosan A by the s.c. route were significantly higher when compared with the chicks immunized with DNP-KLH alone ($P<0.05$). In addition, one week after the third immunization, anti-DNP IgY responses of the chicks immunized with DNP-KLH co-administered with zymosan A by the oral route were significantly higher than those of chicks immunized with DNP-KLH alone ($P<0.05$) (Fig. 1C). However, anti-DNP IgY responses in chicks immunized with DNP-KLH co-administered with zymosan A by the i.m. route were significantly lower than those in chicks immunized with DNP-KLH alone ($P<0.05$) (Fig. 1C). Furthermore, anti-DNP IgY responses in chicks immunized with DNP-KLH by the i.p., i.o. or i.n. routes in the presence of zymosan were not significantly different from those in chicks immunized with DNP-KLH alone ($P>0.05$) (Fig. 1).

Effects of zymosan A on antigen specific IgA responses in chicks immunized with DNP-KLH via different administration routes

Anti-DNP IgA concentrations in serum samples from all chicks were measured by ELISA. One week after the first (Fig. 2A), second (Fig. 2B) and third (Fig. 2C) immunizations, anti-DNP IgA responses in chicks immunized with DNP-KLH co-administered with zymosan A by the s.c., i.o. and oral routes were significantly higher than those in chicks immunized with DNP-KLH alone ($P<0.05$). However, anti-DNP IgA responses in chicks immunized with DNP-KLH by the i.m., i.p. or i.n. routes in the presence of zymosan A were not significantly different from those in chicks immunized with DNP-KLH alone ($P>0.05$) (Fig. 2).

DISCUSSION

Vaccination has become one of the most effective strategies for prevention of infectious diseases in the poultry industry worldwide. Adjuvants constitute essential components of human and animal vaccines. Recent studies have confirmed that β-glucans can function as potent adjuvants to stimulate innate and adaptive immune responses [34, 35, 56]. Zymosan A, a cell wall extract from *S. cerevisiae* that is composed mainly of β-glucan, stimulates macrophages to produce cytokines and chemokines to recruit further immune cells to the site of infection, which in turn trigger adaptive immunity [12, 52, 57, 64]. Zymosan probably acts as a PAMP and is recognized by appropriate cell surface receptors, inducing both innate and adaptive immune responses. A number of such receptors have been identified, including dectin-1, complement receptor 3, scavenger receptors and the TLRs [9]. After a cell-surface TLR2 or TLR6 recognizes zymosan, a signal is passed to the cell. This results in an increase in NF-κB, which activates the genes that encode the cytokines, interleukin-1, interleukin-6 and TNF-α [52, 57]. Gantner *et al*. [21] reported that zymosan enhances TLR2-mediated NF-κB activation on co-expression of TLR2 and dectin-1. In addition, zymosan is able to induce maturation of dendritic cells *in vitro* and to stimulate the production of interleukin-2 [23], providing evidence for a link between the innate and adaptive immune responses.

The potential adjuvant effect of zymosan A in chicks was investigated. Chicks were immunized with an optimal dose of DNP-KLH co-administered with different doses of zymosan A. The results revealed that 0.5 mg/kg BW was the optimal dosage of zymosan A for enhancing anti-DNP antibodies (data not shown). Thus, co-administration of zymosan A with DNP-KLH could enhance anti-DNP responses and may be a useful immune-potentiator adjuvant in chickens.

At present, the intensive raising of large numbers of chickens is of great economic importance. The scale of the poultry population necessitates routine vaccination against known poultry pathogens. Effective vaccination is dependent on proper vaccine administration. Therefore, the aim of our second experiment was to determine the most suitable route of administration of vaccine antigens, particularly those requiring immune-potentiator adjuvants in chickens.

Interestingly, co-administration of zymosan A with DNP-KLH significantly increased anti-DNP IgY responses in chicks immunized via the s.c. or oral routes when compared with chicks immunized with antigen alone via the same routes. Furthermore, chicks immunized with DNP-KLH by the s.c., i.o. or oral routes in the presence of zymosan A showed a significant increase in anti-DNP IgA responses compared with chicks immunized with DNP-KLH alone. The route of inoculation is critical for effective immunization and antibody production in mammals and birds [7, 17, 31, 33, 39, 50].

Different routes of administration bring an immunogen into contact with different local lymphoid cell populations. Oral, intranasal and intraocular immunizations introduce antigens into mouth, nostrils and eyes, respectively, and the antigens are delivered to mucosa-associated lymphoid tissue, nasal-associated lymphoid tissue, and conjunctiva-associated lymphoid tissue, respectively. On the other hand, subcutaneous, intramuscular and intraperitoneal immunizations introduce antigens into the fatty hypodermis layer beneath the skin, muscle and peritoneal cavity, respectively, and the antigens delivered to regional lymph nodes and spleen. As a consequence, this may result in different processing of the antigen and thus varying effects on the immune response observed. Basically, the adjuvants can provoke local inflammation and draw large numbers of immune cells to the site of

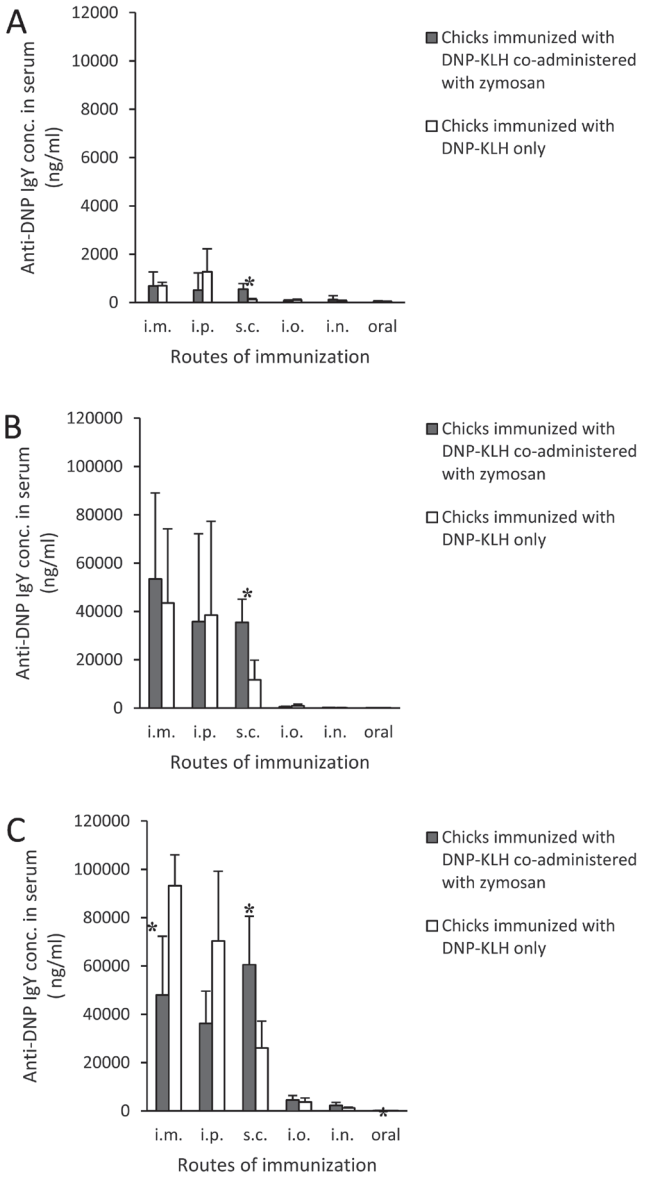

Fig. 1. Effects of zymosan A on anti-DNP IgY responses in chicks immunized with DNP-KLH via different administration routes. One week after the first (A), second (B) and third (C) immunizations, anti-DNP IgY responses in chicks immunized with DNP-KLH co-administered with zymosan A by the s.c. route were significantly higher than those in chicks immunized with DNP-KLH alone (P<0.05). In addition, one week after the third immunization, anti-DNP IgY responses in chicks immunized with DNP-KLH co-administered with zymosan A by the oral route were significantly higher than those in chicks immunized with DNP-KLH alone (P<0.05) (C). However, anti-DNP IgY responses in chicks immunized with DNP-KLH co-administered with zymosan A by the i.m. route were significantly lower than those in chicks immunized with DNP-KLH alone (P<0.05) (C).

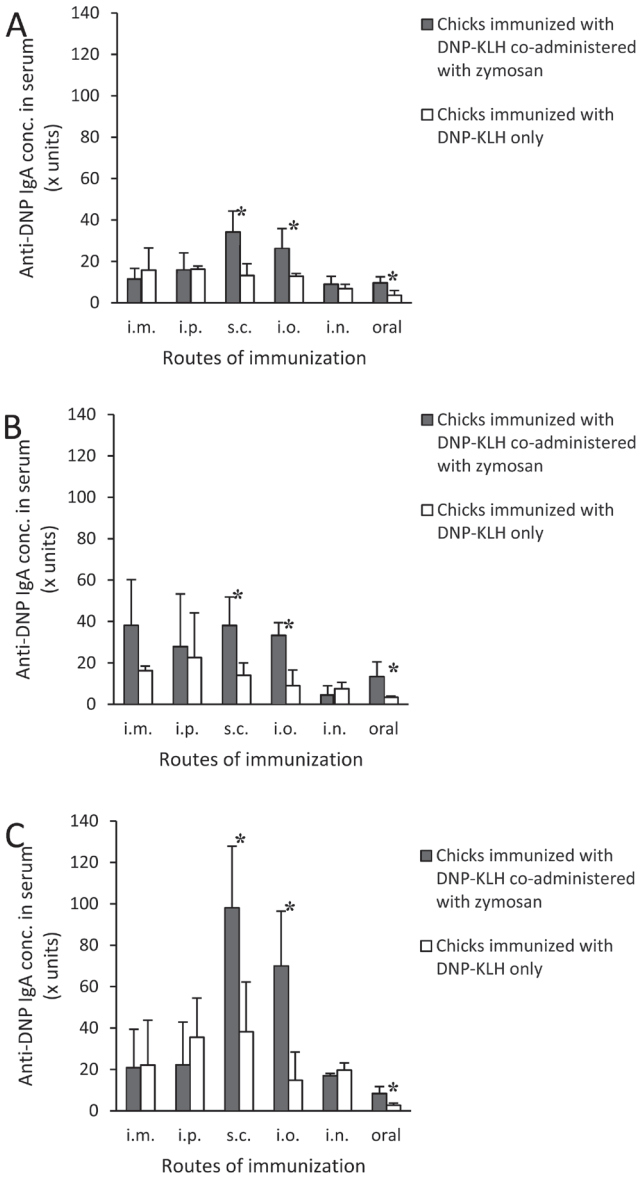

Fig. 2. Effects of zymosan A on anti-DNP IgA responses in chicks immunized with DNP-KLH via different administration routes. One week after the first (A), second (B) and third (C) immunizations, anti-DNP IgA responses in chicks immunized with DNP-KLH co-administered with zymosan A by the s.c., i.o. and oral routes were significantly higher than those in chicks immunized with DNP-KLH alone (P<0.05). However, anti-DNP IgA responses in chicks immunized with DNP-KLH by the i.m., i.p. or i.n. routes in the presence of zymosan A were not significantly different from those in chicks immunized with DNP-KLH alone (P>0.05).

injection, where they can interact with the antigen. Zymosan A may trigger different degrees of inflammatory reaction according to the site of injection and in turn, lead to different immune responses.

In the present study, co-administration of zymosan A with DNP-KLH significantly decreased anti-DNP IgY responses in chicks immunized via the i.m. route when compared with chicks immunized with antigen alone via the same routes. Vaccine adjuvants administered to the muscle have a central role in inducing transient inflammation at the delivery site that promotes immune cell

recruitment and activation [36]. The magnitude of the host response to an inflammatory insult in any given anatomical location is the net result of the action of local pro-inflammatory mediators and the opposing action of endogenous anti-inflammatory mediators. Repeated co-administration of zymosan with antigen every week in our experiment may lead to failure of the initial inflammatory insult clearance or resolution. Iqbal *et al.* [30], stated that failure to clear the initial inflammatory insult or failure of inflammation resolution leads to continuous escalation of leukocyte recruitment, leukocyte activation (locally and systemically) and unrestrained inflammatory cytokine production, often leading to irreversible tissue remodeling, tissue damage, and loss of function.

Intraperitoneal injection of zymosan has been widely used as a self-resolving model of acute innate inflammation that peaks within a few hours and is cleared within 48 to 72 hr [13] or took more than 3 weeks [41]. Injection of the TLR2 ligand zymosan into the peritoneal cavity leads to an inflammatory reaction and immediate recruitment of innate immune cells to the site of inflammation. The Inflammatory reaction induced by zymosan A may be failed to resolve due to repeated co-administration with antigen every week in our study. Failure to resolve this reaction can result in chronic inflammation. Chronic inflammation continues for longer time and is characterized by continuing leukocyte recruitment and leukocyte activation and unrestrained inflammatory cytokine production, often leading to irreversible tissue alteration [30].

Using TLR ligands, it has been shown that antigens associated with their ligands can produce exceptionally high antibody levels and rapid immune responses [37, 54]. TLRs are expressed by various cell types. Most importantly, they are expressed on sentinel cells located on or near the surface of the body. These include macrophages, mast cells and dendritic cells (DCs), as well as eosinophils and the epithelial cells that line the respiratory and intestinal tracts. Different routes of immunization can lead to differential antigen presentation by specific DC subsets. For example, dermal DCs and Langerhans cells play distinctive roles in adaptive immunity [32]. DCs have an immature phenotype in peripheral tissues, specialized for antigen uptake, but upon recognition of foreign material migrate to the lymph node T cell paracortex where they arrive as mature cells, expressing costimulatory molecules and having lost the capacity to take up antigens [28, 53]. The response of DCs to foreign antigens is part of the innate immune response, and by providing a link between antigen recognition and antigen processing for presentation to naïve T cells, these cells bridge innate and adaptive immunity [6]. In addition, mast cells are scattered throughout the body in connective tissue, under mucosal surfaces, in the skin, and around nerves. They are found in high numbers at sites in the body exposed to potential invaders such as under the skin or in the intestine.

Previously, zymosan was demonstrated to enhance the humoral and cellular immune response to a DNA vaccine against HIV-1 in adult mice through complement activation [4]. In addition, zymosan may be an effective adjuvant in future mucosal vaccines against respiratory syncytial virus infection by stimulating TLR2-mediated activation of innate immunity [15, 40]. Zymosan has been shown to enhance the immune response to intranasal influenza immunization with poly (I:C) in adult mice through a synergistic increase in cytokine production by DCs, involving the co-activation of poly (I:C)-stimulated TLR3-mediated signaling pathways and zymosan-stimulated TLR2-mediated signaling pathways [2]. Zymosan-mediated TLR2 signaling may also be useful for developing a neonatal vaccine adjuvant [43]. On the other hand, intratracheal challenge with Zymosan-A for 5 consecutive days prior primary and secondary immunization with human serum albumin (HuSA) (subcutaneously administered) did not significantly affect antibody responses to HuSA in laying hens [46]. Hence, to our knowledge, the present study is the first study to investigate the adjuvant effects of zymosan on vaccine immunization if co-administered with the vaccine antigen via different routes of immunization in chickens.

In conclusion, the results of the present study revealed that co-administration of zymosan A with protein antigen strongly enhances its immune response in chickens, depending on the route of administration. Therefore, the route of administration should be carefully selected before carrying out clinical trials to optimize the immune response.

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