

The Chicken Egg: A “Nature’s Miracle”

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Abstract

The avian egg has great importance because is an important part of the human consumption. This paper provides the rationale for the use of eggs in immunology and opens new horizons for future endeavours including the study of its strong immune system that protect the embryo. Chickens were vaccinated with several immunogens including viral and bacterial proteins and the titre of antibodies produced was assessed by several immunological techniques including staphylococcal protein-A (SpA) affinity chromatography, Enzyme-Linked Immunosorbent Assays (ELISAs) and dot blot analysis. This study proved that immunizations of laying hens or oral administration of hyper-immune eggs produced an effective immune response against the viral and bacterial antigens used to protect the embryo and it makes a novel contribution in the field of the immunoglobulin Y (IgY) technology. The anti-SpA antibodies are important because they can be diagnostically used or employed in the therapy of *S. aureus* infections as antimicrobials. The anti-HIV antibody developed can be used as a chemical reagent in immunoassays to diagnose HIV infections.

Keywords: Immunogens, Elisa, Avian Eggs

Introduction

Eggs are laid by animals of different species, including birds. The avian eggs consist of an egg shell, egg yolk and egg white and it constitutes the embryo from which will develop a chick. From day zero the avian egg contains a powerful immune system comprised by antibodies against each foreign agent to which her mother (laying hen) has been in contact with. The egg yolk possesses high titre of immunoglobulin Y (IgY), which is equivalent, to immunoglobulin G (IgG) in mammalian species, and the egg white contains immunoglobulin M (IgM) and immunoglobulin A (IgA). The aim of this study is to provide evidences of the effectiveness of the avian embryo's immune system against microorganisms by immunizing laying hens with several viral and bacterial proteins.

Methodology

Raising titres of anti-SpA antibodies in egg whites

Six healthy layer chickens (brown Leghorn) were immunized with Staphylococcal protein A (SpA) with 0.5 mg of SpA in 0.5 ml Complete Freund's Adjuvant (CFA) on day 0, and 0.25 mg of the same antigen in incomplete Freund's adjuvant (IFA) on days 14, 28. The antibodies were purified by SpA-affinity chromatography [1] and their titres in the egg white were investigated by an enzyme linked immunosorbent assay (ELISA) as described below [2].

Purification of anti-SpA antibodies in eggs and chick sera by affinity chromatography

A commercial protein-A antibody purification system, PURE-1A (Sigma-Aldrich) was used to purify anti-SpA antibodies from the egg yolk, the egg white of hyper-immune eggs, and the sera of the chicks

fed hyper-immune and non-hyper-immune eggs. The instructions of the manufacturer were followed in performing this procedure.

ELISA for anti-SpA antibodies in egg whites

The 96 well polystyrene microplates (U-shaped bottom) were coated with 500 ng of SpA (Sigma-Aldrich) in coating buffer for 4 h at 37°C. The microplates were washed four times with PBS-Tween-20 and blocked with 3% non-fat milk in PBS, 25 µl/well, 1h, RT. The microplates were washed four times again. Samples were added 1:10 dilutions of egg whites. After incubation for 1h at RT the microplates were washed four times and 50 µl of peroxidase-labelled protein-A diluted 1:3000 (Sigma-Aldrich) was added. The microplates were then incubated for 1h at RT, washed four times. Tetramethylbenzidine (TMB) solution (50 µl) was used. After a further incubation of 15 min in the dark, the reaction was stopped with 3M H₂SO₄ and read in a microplate reader at 450 nm.

Production of anti-HIV gp41 antibodies in egg yolk

Two healthy brown Leghorn layer hens, aged approximately 6 months, were injected intramuscularly at multiple sites on the breast with a HIV conjugated vaccine containing peptide 579-601 from HIV glycoprotein 41 (gp41) [3]. The IgY was separated by the method of Polson, 1990 [4] and the titre was tested by ELISA [3] and confirmed by dot blot analysis [5].

Method of Polson for IgY isolation

The IgY fraction was isolated from the egg yolks of immunized birds by the chloroform-polyethylene glycol (PEG) method as follows: the eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 in phosphate buffered saline (PBS), pH 7.4. To 1/3 of the egg yolk mixture an equal volume of chloroform was added, the mixture was then shaken and centrifuged for 30 min (1000×g, RT). The supernatant was decanted and mixed with PEG

6000 (12%, w/v), stirred and incubated for 30 min (RT). The mixture was then centrifuged as previously described. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/6 of the original volume of the egg yolk and dialyzed against 1L of PBS (pH: 7.4 for 24 h at 4°C). The IgY was removed from the dialysis tubing. IgY concentration was determined by the Bradford method. IgY samples were stored at -20°C

ELISA for anti-HIV antibodies in egg yolks

The 96 well polystyrene microplates (U shaped bottom) were coated with 100 ng of 579 601 of the HIV gp41 in coating buffer for 4 h at 37°C. The microplates were washed 4 times with PBS Tween 20 and blocked with 3% non-fat milk in PBS, 50 µl/well, 1h at room temperature (RT). The microplates were washed 4 times again. Samples were added (50 µl of WSF). After incubation for 1h at RT the microplates were washed 4 times and 50 µl of peroxidase labelled anti IgY conjugate diluted 1:30,000 was added. The microplates were then incubated for 1h at RT, washed 4 times. Tetramethylbenzidine (TMB) solution (50 µl) was added to each well. After a further incubation of 15 min in the dark, the reaction was stopped and read in a microplate reader at 450 nm. The cutoff value was calculated from the mean optical density (OD) of the negative control plus 0.25. The cut off points of ELISAs for the detection of anti peptide (579 601) was 0.423.

Raising anti-SpA antibodies in the egg yolks

Two healthy layer chickens (brown Leghorn), aged 6 months, were injected intramuscularly at multiple sites on the breast with 0.5 mg of SpA in 0.5 ml complete Freund's adjuvant (CFA) on day 0, and 0.25 mg of the same antigen in incomplete Freund's adjuvant (IFA) on days 14, 28 and 42 [6]. The eggs were collected post-immunization and stored.

Oral immunization of chicks with anti-SpA hyper-immune eggs

Hyper-immune eggs with a titre of 1: 2048 were fed every day to six chicks aged zero and another six chicks were fed non-hyper-immune egg solutions. At the end of the feeding program (4 weeks) blood samples were taken. The antibody titre were purified by affinity chromatography and measured by indirect ELISA as described above [6].

Dot-blot analysis for the detection of anti-SpA (in egg yolk and egg whites) and anti-HIV antibodies (in egg yolk)

Briefly, 2 µl of 1mg/ml of each isolated antibody protein sample were dotted onto nitrocellulose paper placed in a Bio- Dot SF apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The membrane was blocked with 5 µL/well of fetal bovine serum with 1% Tris buffer saline. Then 5 µL of a commercial conjugate (peroxidase-labelled HIV proteins; Murex Diagnostics, Norcross, USA) was added and allowed to drain by gravity or instead peroxidase-labelled SpA 5 µg/µL (for detection of anti-SpA antibodies). Then the substrate 3,3', 5,5'-tetramethylbenzidine (Sigma Chemical Co., St Louis, MO, USA) was added and the mixtures were incubated for 20 min. The reaction was stopped by washing the wells with distilled water under a vacuum. The membrane was left to dry for the final colour visualization reading using previously described method.

Results and Discussion

As shown in Figure 1 high titres of antibodies (anti-SpA and anti-HIV gp41 Abs) were present in eggs from laying hens immunized with Staphylococcal protein A and fragment 759-601 from HIV gp41. The production of these antibodies demonstrated that both viral and bacterial proteins were highly immunogenic and the immunization route was efficient for the antigenic delivery. Both egg yolk and egg white showed that the humoral immune response in chickens was effective and as expected driven to protect the embryo from day zero. We have demonstrated that the hyper-immune egg yolk in hens can be analogue to human breast milk or cow colostrums, when it is fed to chicks developed specific immune responses (antibodies) that protected them from infectious diseases [6]. The production of chicken antibodies, known as IgY technology has proved efficient as anti-microbial and in the production of reagents (including primary and secondary antibodies) to be used in ELISA, Western blotting, radioimmunoassay and dot blot analysis. The administration of hyper-immune eggs to chicks in this study demonstrated that the egg can be considered as an oral vaccine. In general it has the capacity to induce mucosal immunity, which involves strong humoral and cellular reactions [2].

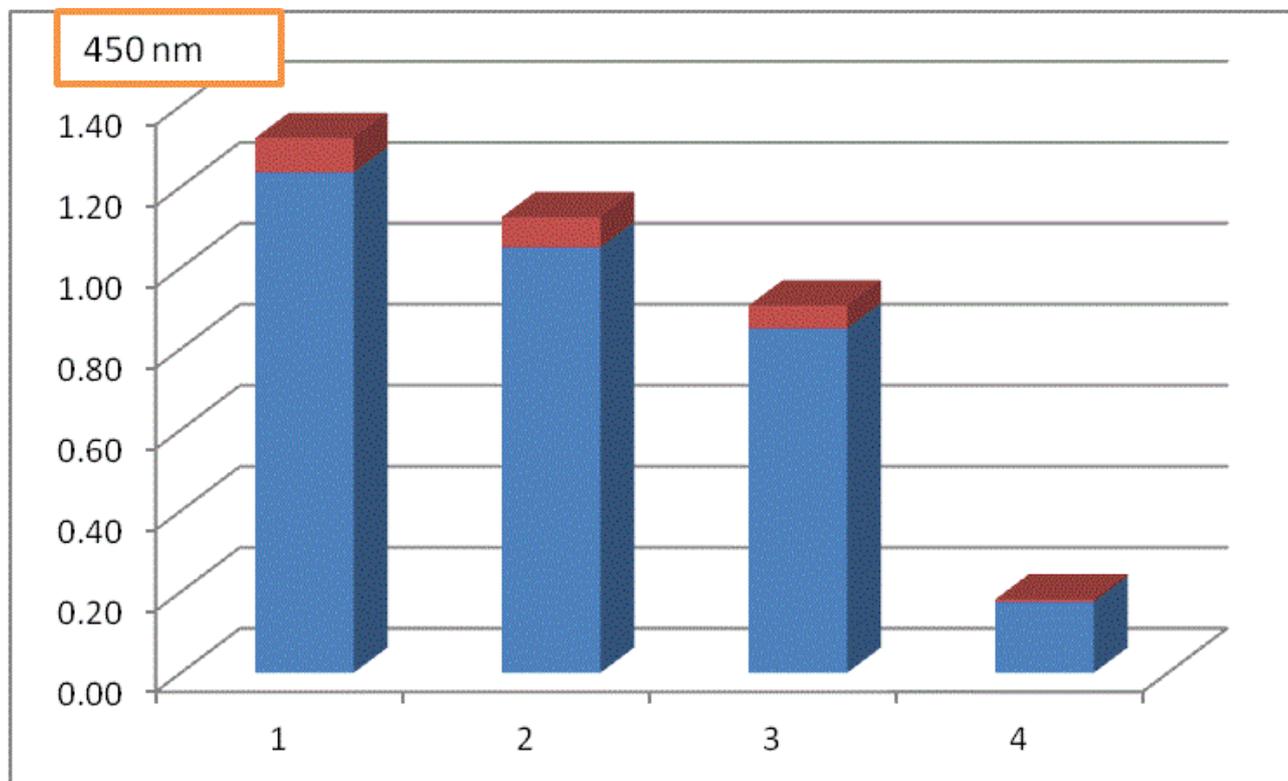


Figure 1: Mean of optical density (blue)/standard deviation (brown) of (1): anti-HIV antibody in the egg, (2): anti-SpA antibody in eggs, (3) anti-SpA antibody in plasma, (4) Non-hyperimmune egg samples that represented chickens that were not immunized and instead were injected with 0.9% normal saline. Previous results were confirmed by dot blot analysis. It was estimated that between 10%-25% was the amount of specific antibody yielded post-vaccination. Furthermore there was a significant difference ($p < 0.01$) between the mean optical absorbance of immunized birds versus non-immunized chickens that backed up the results of the immunogenicity studies.

Two new methods were reported for the IgY purification from the egg yolk. They used an effective delipidation solution and ammonium sulphate to precipitate the IgY [7-8]. Several methods have been described for the IgY isolation/purification. The water dilution method (WD) was compared with three other methods, namely polyethylene glycol (PEG), dextran sulphate (DS) and xanthan gum (Xan), in terms of yield, purity, ease of use, potential scaling up and immunoactivity of IgY. The WD method gave the highest yield, followed by DS, Xan and PEG methods in that order. 9.8 mg IgY/ml egg yolk was routinely obtained from the WD method compared to 4.9 mg IgY/ml egg yolk with the popular PEG method [9].

Several examples are cited in the literature that demonstrates that immunization of layer hens with bacterial and viral proteins produce specific hyper-immune eggs that neutralize the original antigen. For example You et al (2014) reported the use of a recombinant enterotoxigenic Escherichia coli (ETEC) fusion enterotoxin protein that expressed the haptens STa and STb after immunization of layer hens induced their corresponding neutralizing antibodies [10]. Specific IgY against Rabbit hemorrhagic disease virus (RHDV) promoted rabbit protection against RHDV infection [11]. Chicken egg yolk antibody (IgY) controlled *Solobacterium moorei* under in vitro and in vivo conditions [12]. It was also reported the **effectiveness of egg yolk immunoglobulin (IgY) against periodontal disease-causing *Fusobacterium nucleatum*** [13].

IgY antibodies has been used as reagents in immunodiagnosis or detection of chemicals such as haptens and micromolecules, for example florfenicol amine (FFA) residues [14], cancer antigen 15-3, which is an important breast tumour marker [15] and the **detection of isoprothiolane in food, soil, and water samples by enzyme-immunosorbent assay using avian IgY molecules** [16]. A novel method based on IgY for identification and profiling circulating antigens in sera of *Schistosoma japonicum* infected patients was also reported [17]. Parks S et al (2012) recorded the development of an ELISA methods based on IgY technology for the detection of *Cronobacter mutytjensii* in food [18]. These various examples suggested the versatile use of avian eggs. Sun H et al (2013) reported that the regression analysis showed simple linear regression between IgY levels in hen serum, yolk and offspring serum, suggesting that total IgY levels could be used as an index for chicken fitness [19]. The ELISA and dot blot analysis used in this research could be further improved to detect anti-HIV antibodies in human given their high sensitivity (95% for ELISA and 97% for dot blot analysis) and specificity (98% for both ELISA and dot blot analysis).

For the isolation of egg yolk IgY is compulsory the removal of lipids, which can be found in the egg yolk in high concentrations. Several techniques have been reported for the lipid separation, including use of caprylic acid, and water dilution method. Livetins are egg yolk proteins that coexist with IgY, which is in extremely high

amounts. For the isolation of IgY from livetins several techniques have been used including alcoholic and salt precipitation followed by centrifugation [20]. In this investigation we used the Polson method (1990) [4], which proved to be efficient in the IgY purification and produced a high protein yield that was demonstrated by ELISA.

Production of antibodies in chickens is advantageous since large amount of antibodies can be isolated from the hyper-immune eggs at low cost and since chickens are able to produce antibodies in response to any protein including those with low immunogenicity. In literature is reported the development of a network of antibodies to bovine serum albumin (BSA) in eggs from chickens immunized with BSA [21]. In addition, the egg can be considered a complete nutrient as it provides several proteins with antimicrobial properties such as lysozyme [22], and antioxidants such as phosvitin [23]. The use of hyper immune eggs as oral vaccine is advantageous for the large amount of antibodies in response to almost any antigen, the reduction of antigenic variation, low cost, reduced toxicity and danger encountered with the use of live vaccines. Immunoglobulin (Ig)-Y is the major antibody produced by birds and have advantages compared with mammalian IgG because there is no activation of mammalian complement system, no cross-reactivity with HAMA (human anti mouse antibody), and no interference caused by rheumatoid factors or human blood group antigens [24].

Conclusion

This study proved that immunizations of laying hens or oral administration of hyper-immune eggs produced an effective immune response against the viral and bacterial antigens used to protect the embryo and it makes a novel contribution in the field of the IgY technology. The anti-SpA antibodies are important because they can be diagnostically used or employ in the therapy of *S. aureus* infections as antimicrobials. The anti-HIV antibody developed can be used as a chemical reagent in immunoassays to diagnose HIV infections.

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