Immunospecific Antibody Concentration in Egg Yolk of Chickens Orally Immunised with Varying Doses of Bovine Serum Albumin and the Mucosal Adjuvant, RhinoVax®, using Different Immunization Regimes

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Summary

Antibody harvested from eggs of immunised chickens, IgY, has proven to be a non-invasive alternative to antibodies purified from serum of mammals. Taking the non-invasive concept further, the development of oral immunization techniques combined with IgY harvest from chicken eggs may subsequently eliminate all regulated procedures from polyclonal antibody production. In the present study, we report the effects of varying the temporal administration mode of the antigen (immunogen) comparing dosing on three consecutive days with dosing on five consecutive days, and of incorporating a mucosal adjuvant. Two antigen doses were compared: 30 mg bovine serum albumin (BSA) and 300 mg BSA, with and without the mucosal adjuvant, RhinoVax®, administered to laying chickens. The egg yolk of chickens dosed with BSA in combination with 20% RhinoVax®, contained significantly higher concentrations of immunospecific IgY than did egg yolks of chickens fed with BSA without adjuvant. The most efficient dose in the RhinoVax®-treated groups was 300 mg BSA regardless of whether the chickens were initially immunised daily for three or five days. A 3-day dosing regime with BSA alone also induced immunospecific IgY production. This study confirms that RhinoVax® is an efficient oral adjuvant. It also demonstrates the efficacy of daily immunizations on three or five consecutive days on immunospecific IgY production. The chickens received oral booster immunizations one and two months after the initial immunization. No real effect could be recorded after the second and third immunization, although the study did provide some evidence of memory based on an optimum IgY concentration recorded after the 2nd immunization.

Introduction

There is an increasing interest in the use of chickens for polyclonal antibody production. Antibodies can be harvested from the egg yolk which, from an animal welfare aspect, is anattractive alternative to the use of serum as an antibody source (*Hendriksen & Hau, 2002; Hau & Hendriksen, 2005*). Useful guidelines for avian polyclonal antibody production have been published (*Hau 1998; Leenaars et al, 1999*). Purification of the antibodies from the egg yolk is simple if somewhat time consuming (*Jensenius et al, 1981*) and has continuously been refined (*Svendsen et al, 1995; Bizhanov et al, 2004*).

The interest in chicken egg antibody production is focused on two areas. The first is fuelled by the increasing prevalence of antibiotic-resistant bacteria, which has initiated a search for alternative treatment regimes. Oral administration of specific antibodies, produced by laying hens and present in high

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concentration in the egg yolk, is an attractive approach to establish protective immunity against gastrointestinal pathogens in humans and animals. Immunospecific yolk antibodies have been demonstrated to prevent both bacterial and viral infections and long treatment periods have been tested and found to be associated with no side effects for the patients involved (*Carlander et al, 2000*).

The second area relates to the wish to reduce the number of animals needed for antibody production and to refine the immunisation techniques for animal welfare reasons. With the aim to reduce the number of laboratory animals used for antibody production, it is desirable to replace small mammals with chickens, which are phylogenetically lower ranking than mammals. In addition, the productivity is much higher using egg yolk instead of serum as the antibody source (*Hau, 1988; Florence & Hau, 1994*).

The development of an oral immunization technique may eliminate the invasive antigen administration and completely eliminate regulated procedures from routine antibody production.

In general, the normal healthy GI tract is somehow able to distinguish between safe normal flora, food antigens and dangerous pathogens (*Matzinger, 1994*). Oral immunisation generally results in immunological tolerance (*Strobel & Mowat, 1998; Friedman & Weiner, 1994*), but the use of adjuvants can effectively change this. RhinoVax® is a mucosal adjuvant system that is non-toxic and has been used successfully with a number of antigens in different species (*Hrafnkelsdottir et al, 2005; Jacobsen et al, 1999; Gizurarson et al, 1996*) including man (*Gizurarson et al, 1998*) to stimulate both local and systemic immune responses. This adjuvant consists of a mixture of mono-and diglycerides of caprylic- (C8) and capricacid (C-10) where all alcohol groups are pegylated.

Mayo and co-workers (*2003, 2005b*) demonstrated that RhinoVax® (formerly known as Softigen®) coupled with bovine serum albumin (BSA), induced a systemic IgG response in young chickens as well as a high IgY response in the egg yolk of immunised hens (*Persdotter-Hedlund & Hau, 2001*). Klipper and co-workers (*2000*) reported that the systemic immunospecific IgG response in young chickens immunized daily for 3 to 6 days with BSA alone was better than a single administration.

The present study was designed to compare the effects of protein concentration, temporal mode of administration, presence or absence of an adjuvant and booster effect on the induction of IgY antibody production in egg laying hens.

Materials and Methods

Animals and Husbandry

A total of 45[*] approximately 50% in-bred (40%-60% homozygosity) about 35 weeks old, White Leghorn (Line 43) layers (*Gallus domesticus*) selected for male comb related traits for 15 generations were used in this study. The hens were from GAL AB breeding stock housed at Söderby gård, Uppsala, Sweden. They were kept in single cages (height 65 cm, length 48 cm and depth 41 cm). The ambient temperature varied from 18 to 21 ºC and the light cycle was 8 hours dark and 16 hours light. Food consisted of crushed pellets containing 15% protein (Johan Hansson, Uppsala, Sweden) and water was available *ad libitum.*

Treatment Groups and Immunisation Scheme

The chickens were allocated randomly to nine treatment groups, each consisting of five chickens. In all groups, except the control group, the animals were dosed orally with a solution of bovine serum albumin (BSA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in twice-distilled water. The control animals were given twice-distilled water only, and in those groups receiving adjuvant, 20% RhinoVax® (formerly Softigen® from Sasol Gmbh, Witten, Germany) was administered along with the antigen. The animals in the individual groups received the treatment shown in Table 1.

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[*]One chicken from treatment 3 (HP 5 days) died spontaneously during the study.

Table 1. Treatment regime for the nine groups of 5 chickens each. LP (Low Protein) = 30 mg BSA, HP (High Protein)= 300 mg BSA.

Group	RhinoVax®	Days x volume	Conc. BSA	Total BSA/ treatment
LP 5 days	No	5 x 250 ml	20 mg/ml	25 mg
LP 3 days	No	3 x 500 ml	20 mg/ml	30 mg
HP 5 days	No	5 x 250 ml	200 mg/ml	250 mg
HP 3 days	No	3 x 500 ml	200 mg/ml	300 mg
LP 5d + RV	Yes	5 x 250 ml	20 mg/ml	25 mg
LP 3d + RV	Yes	3 x 500 ml	20 mg/ml	30 mg
HP 5d + RV	Yes	5 x 250 ml	200 mg/ml	250 mg
HP 3d + RV	Yes	3 x 500 ml	200 mg/ml	300 mg
Control	No	5 x 250 ml	No	No

The first booster administration was performed 1 month after the first immunization, using the same protocol as described in Table 1. The second booster was performed 1 month after the first booster, and consisted of a single day dose only, but the volume and concentration were maintained for the different groups according to what they had received previously.

Collection of eggs
Eggs from each chicken were collected every week starting at week 1 (about 3 days after the last immunisation) and every 4 weeks thereafter (i.e. week 4, 9 and 13). All eggs were numbered and kept at 4 °C until analysis.

Antibody extraction from egg yolk
To extract antibodies from egg yolk, a slight modification of the method described by Persdotter-Hedlund and Hau (*2001*) and Svendsen et al (*1995*) was performed. Briefly, the egg yolk was separated from the white, and washed with twice-distilled water. The vittelline membrane was punctured and the yolk was placed in a 50 ml tube. The total weight of yolk was measured, after which the yolk was homogenised mechanically, and a sample of 10 g was retained. Thereafter, 40 ml of twice-distilled water was added, the tube shaken for 20 minutes and the contents frozen at –2ºC until further purifi-cation. The mixture was thawed out at room temperature; 1ml of dichloromethane was added, and the mixture shaken for 20 minutes and centrifuged for 45 minutes at 3000g. The supernatant was collected and concentrated in a dialysis tube (MWCO 12000-14000) in Polyethylenglycol 6000 (Merck Schucardt CHG, Hohenbrunn, Germany) to less than 4 ml. Then volume was adjusted to 4 ml with PBS pH 7.4. Samples were vortexed and kept at 4ºC in 0.1% aqueous sodium azide.

Enzyme linked immunosorbent assay (ELISA) quantification of immunospecific chicken anti-BSA IgY antibodies
Indirect ELISA was used to quantify the immunospecific anti-BSA IgY antibodies in egg yolk, as described elsewhere (*Persdotter-Hedlund & Hau, 2001; Mayo et al, 2005*). BSA protein standard was rabbit HRP conjugated anti IgG (Sigma-Aldrich). The concentration of each sample was measured using arbitrary units as described earlier (*Mayo et al, 2003, 2005*). The inter-assay coefficient of variation was 3.4% and the intra-assay coefficient of variation was 2.5%. The cut-off point chosen to determine whether a sample contained immunospecific IgY or not was based on the absorbance of the control samples ±2 SD.

Statistical analysis

Due to a non-normal distribution of the data, non-parametric statistics was used. To compare between groups, these were combined as "Low protein without RhinoVax (LP)", "High protein without RhinoVax (HP)", "Low protein with RhinoVax (LP+RV)", "High protein with RhinoVax (HP+RV)", and control. The five groups were analysed using the Kruskal-Wallis test. To compare between the 3 and 5 days groups of high protein with RhinoVax, the Mann-Whitney test was used. All analyses were performed with SPSS v. 12.1.01.

Ethics committee approval

The present study was approved by the Regional Ethics Committee in Tierp, Sweden.

Results

Daily oral administration of high doses of BSA (total dose 250 to 300 mg) with RhinoVax® for either three (HP3d+RV) or five consecutive days (HP5d+RV) resulted in high anti-BSA IgY concen- trations. The concentrations were significantly higher in these two groups compared to all other groups (week 1, $p<0.9$; week 2, $p<0.1$; week 3, $p< 0.03$; week 4, $p< 0.005$) (Figure 1).

Groups immunized with lower doses of BSA (25-30 mg total BSA) with RhinoVax® (LP 3d or 5d +RV) also produced anti-BSA IgY antibodies, but the concentrations were within almost the same range as in the groups immunized with BSA alone.

Analysis of individual eggs sampled every 4 weeks showed that all 5 chickens in the HP+ RV (3 day or 5 day) groups responded to the treatment as compared to the LP+RV treated and the BSA alone-treated groups, in which a minority (at most 2 of 5 chickens) responded.

The maximal concentration of immunospecific anti BSA IgY was observed in 8 out of 10 chickens between the 2nd and 3rd immunization, about week 7 to week 8. This observation is depicted graphically in Figure 2 in which data from an average of 5 eggs from 5 individual chickens collected and analysed weekly showed that highest concentrations were
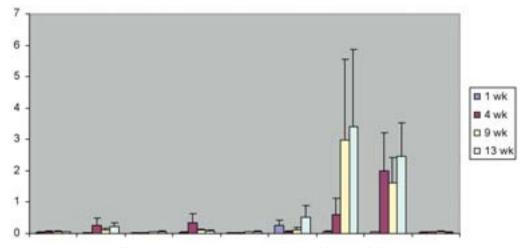

Figure 1. Immunospecific anti-BSA IgY concentrations in the chickens of all treatment groups. Data represents the average of 5 eggs from an individual hen in each treatment group (5 animals each group) analysed each month. Error bars = SEM. LP: Low protein, 20 mg/ml, HP: High protein, 200 mg/ml, 5d: 5 consecutive days of 250 μl; 3d: 3 consecutive days of 500 μl; RV: with adjuvant RhinoVax®.

Weeks: 1 (1st immunisation), week 4 (2nd immunisation), week 9 (3rd immunisation), week 13 (end of experiment).
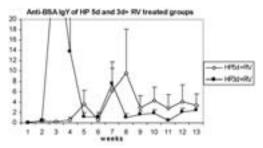
Figure 2. Average anti-BSA IgY titres from 5 eggs of 5 individual chickens analysed weekly.
Each value is an average IgY titre of 5 eggs. Arrows indicate time of immunization.

most often recorded after the 2nd immunization. Although Figure 2 shows a peak on week 3, this peak corresponds to the highest antibody concentration in one single egg sample from one chicken, which responded to the first immunization after which the concentrations continued to decrease throughout the remainder of the period. The other peaks observed in Figure 2 on week 7- 8 were from 4 of 5 chickens in the HP3d+SF group and from 5 of 5 (all) chickens in the HP5d+SF group. Unlike the 2nd immunization (given by continuous 3 or 5 day dosing), the 3rd immunization did not result in a further increase in antibody concentration. When comparing the highest IgY concentrations from 5 egg samples between the HP5d+RV group and the HP3d+RV group there was no significant difference (week 9 ($p = 0.413$); week 13 ($p = 0.602$) analysed by the Mann-Whitney test).

Discussion
New vaccine delivery systems in which antigens have been administered orally have been a challenge with respect to development of adjuvants resulting in an immunopotentiating effect. Effective oral adjuvants have been tested and found efficient. These include biodegradable biospheres and carrier proteins derived from cholera toxin (CT) or *Escherichia coli* heat labile enterotoxin. The adjuvanticity of CT has been found to be correlated to its immunogenicity (*Mayo et al, 2005a*). Similar

positive results have been obtained in animals using glyceride based adjuvants such as RhinoVax® by nasal administration (*Gizurarson et al, 1998; Hrafnkelsdottir et al, 2005*). The present study confirms our previous findings that oral administration of RhinoVax® in conjunction with the antigen has a significant immunopotentiating effect on the production of immunospecific IgY (*Mayo et al, 2005b*). Additionally, the present study demonstrates for the first time, the efficacy of three or five days successive oral immunization of adult chickens on IgY production as compared with a single oral immunisation. Daily dosing for three or five days of large doses of antigen with RhinoVax® induced a good immunospecific IgY response, and the IgY concentrations in groups that received RhinoVax® were at least 10 times higher than the concentrations recorded in the egg yolk from chickens which received antigen without an adjuvant.
RhinoVax®-treated chickens dosed with low doses (LP+RV) produced less IgY than did chickens immunized with higher doses (HP+RV). Interestingly not all immunized chickens responded with production of immunospecific IgY antibodies. The mean values in the LP3d+RV group, for instance, were due to the response of two of four chickens which were high responders as early as the first week after the first immunization (wk 1), whereas the other two chickens in this group did not respond until week 13. The large within-group, between-chicken variation in their immune responses is unfortunate from a practical point of view and confirms previous studies (*Mayo, 2003, 2005b; Persdotter-Hedlund & Hau, 2001*). Mayo and colleagues (*2005b*) reported that some chickens are better responders to either an oral or parenteral immunization than other individuals of the same genetic lineage and that there is a significant difference in immune response between individual lines of chickens. This is, however, not a unique feature of chickens or birds, because a similar inter-individual variation in immune response have been observed in rabbits selected through generations for their high antibody response (*Harboe & Ingild,*

1983).

At first glance of Figures 1 and 2, it may appear as if there is the anticipated booster effect after the 2nd and 3rd immunization of the chickens in the HP3d+RV and 5d+RV groups. However, the increase of IgY observed after the 2nd immunization is not due to a boosting effect but due to the fact that more and more chickens respond to immunization throughout the immunization period.

Each individual chicken would typically exhibit a peak concentration at some point during the course of the immunization and decrease thereafter even after a booster was administered.

There was no maintenance of the achieved IgY concentrations and they did not increase towards a new plateau, as is observed after a classical booster in a conventional subcutaneous or intramuscular immunization protocol.

Although a traditional booster effect was not recorded in the present study, there were indications of immunological memory in the chickens, since each individual responded at different times during the duration of the treatment. Some responded as early as wk 1 after which the IgY concentrations declined, while others did not respond until the last weeks of the treatment. A similar trend was reported by Klipper et al (*2000*) whereby young chickens fed continuously for six days with BSA showed that immunospecific IgG concentrations in circulation peaked at 7 days post-immunization and rapidly declined thereafter while another batch of chickens peaked at 14 days post-immunization and slowly decreased thereafter. Moreover, 28-day continuous antigen feeding resulted in a constant IgG titre, which declined upon cessation of antigen feeding. Finally, when the booster was given, no IgG levels were elevated beyond the primary peak response.

In summary, this is the first reported study on using multiple (three or five) daily oral immunizations, instead of single immunizations, of adult chickens on immunospecific IgY concentration in the egg yolk. The results confirm that RhinoVax® is an effective oral adjuvant. Interestingly, the present study showed that there was no traditional boosting effect on the antibody response associated with subsequent immunizations. Further studies are in progress with the aim to optimise the effect of oral booster immunizations with the eventual goal of approaching an effect similar to subcutaneous boosters in conventional immunization schemes.

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