RhinoVax® is an Efficient Adjuvant in Oral Immunisation of Young Chickens and Cholera Toxin B is an Effective Oral Primer in Subcutaneous Immunisation with Freund’s Incomplete Adjuvant

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Abstract. Forty-five approximately 50% in-bred 14-day-old White Leghorn female chickens (Gallus domesticus) originating from 11 hens were distributed into 5 treatment groups containing one sister in each treatment group. Phase 1 involved oral administration of an antigen, Bovine Serum Albumin (BSA), in combination with various adjuvant preparations, either Cholera Toxin B-subunit (CTB) and/or RhinoVax® (RV). A positive control group received BSA emulsified in Freund’s Incomplete Adjuvant (FIA) by subcutaneous injection. All chickens responded with immunospecific IgA, IgM and IgG antibodies in their circulation. Classical parenteral immunisation with FIA was generally the most potent mode of antigen administration. The highest immunospecific IgG concentrations recorded in the orally-immunised chickens were in the group immunised with 20% RV as the adjuvant. The concentration in this group was approximately 5 times lower than that recorded in the FIA group. For practical egg yolk polyclonal antibody production purposes, the oral regime using 20% RV as adjuvant seems an attractive alternative to the more invasive technique of injecting the antigen in FIA emulsions. In Phase 2 all chickens were subjected to traditional subcutaneous immunisation with a new antigen, human IgG emulsified in FIA. The two groups of chickens that had received CTB orally during Phase 1 responded with significantly higher immunospecific antibody concentrations than did the other chickens, indicating that oral administration of CTB prior to traditional parenteral immunisation may have a priming effect on the humoral immune system. The immunospecific antibody response varied between the 11 families of chickens. There was no correlation between familial responsiveness to oral and subcutaneous immunisations. Families that were high responders to oral immunisation were not high responders to parenteral immunisation and vice versa.

Development of non-invasive production methods for polyclonal antibody production is attractive for animal welfare reasons as well as for practical reasons because the production ceases to be a regulated procedure subject to laboratory animal legislation. Studies dating back more than 20 years demonstrated that IgG antibodies could be harvested in generous quantities from the egg yolk of immunised chickens (1). Replacing mammals with egg-laying hens for antibody production thus eliminates the need for repeated blood sampling of the animals. Chicken IgG (transovarian IgG is often termed IgY) differs somewhat from mammalian IgA with respect to a number of physico-chemical properties and immunochemical assays often have to be modified slightly (2), but functional properties, as for example avidity, are similar to that of rabbit antibodies (3).

Administration of an antigen, often in combination with an adjuvant, by the traditional routes, multiple intradermal, subcutaneous or intramuscular injections, causes a certain degree of discomfort to the chickens associated with capture, handling, restraint and the injections themselves. For some time it has thus been an ambition to develop reasonably efficient oral immunisation protocols by testing antigens mixed with commercially available adjuvants to produce an acceptable systemic antibody response. We have tested a number of adjuvants and demonstrated that oral

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administration of antigens (human IgG) mixed with adjuvants (Cholera Toxin B-subunit (CTB) or RhinoVax® (RV)) to egg-laying hens can indeed induce a systemic immune response (4). From studies of mammals, CTB is known to be a potent oral adjuvant (5-7) and intranasal, intra-intestinal and oral administration of CTB to chickens has been reported to potentiate the humoral immune response (8-10). RV is a pegylated mono/di-glyceride, which has been used successfully as a nasally-administered adjuvant in clinical trials in animals and humans resulting in a systemic IgG response (11, 12). These studies illustrate the potential of oral immunisation as a non-invasive alternative mode of antigen administration. Combined with the harvest of antibodies from chicken eggs, oral immunisation may completely eliminate discomfort from animals used for polyclonal antibody production.

The aims of the present study were to: i) optimise oral immunisation protocols focusing on CTB and RV as adjuvants, ii) gain insight into the genetic contribution to the between animal variation in immune response, and iii) test if chicken responsiveness to oral immunisation is correlated with responsiveness to traditional (subcutaneous) immunisation.

Materials and Methods

Animals and husbandry. A total of 45 (one chicken died spontaneously on day 3 after arrival at the animal house) approximately 50% in-bred (40%-60% homozygosity), one-day-old White Leghorn female chickens (Gallus domesticus) from Lövsta Poultry Facility of the Swedish Agricultural University, Uppsala, Sweden, were acclimatised for two weeks. The chickens originated from 11 hens (A, B, C, D, E, F, G, H, I, J and K), which were mated with the same cock. Five chickens from each hen (A to E) were distributed among the 5 treatment groups so that there was one sister in each treatment group. Chickens from hens which had less than 5 offspring (F to K) were distributed evenly among the treatment groups to make a total of 9 chickens per treatment group from 9 different hens (9 blocks). Each chicken was individually marked with a numbered aluminium tag clipped to its wing. They were group-housed on the floor in a pen with wood shavings (Tapvei, Oy, Kaavi, Finland) as bedding, in a temperature gradient from 25ÆC to 35ÆC in the pen for the first week after hatching and 25ÆC for later rearing, with 8 hours dark, and 16 hours artificial light cycle. Food granules (batch # 55102, Tapvei, Oy, Kaavi, Finland) as bedding, in a temperature were group-housed on the floor in a pen with wood shavings were available. Odal Lantmän, Uppsala, Sweden) free of animal protein and tap and 16 hours artificial light cycle. Food granules (batch # 55102, Tapvei, Oy, Kaavi, Finland) as bedding, in a temperature

The immunisation protocol consisted of two Phases, 1 and 2. Phase 1 involved administration of an antigen, Bovine Serum Albumin (BSA), in combination with various adjuvant preparations, either Cholera Toxin B- subunit (CTB) and/or RhinoVax® (RV), given by oral route, except for the treatment of the positive control group which received Freund’s Incomplete Adjuvant (FIA) by subcutaneous (s.c.) injection.

There were 5 treatment groups with 9 chickens each in group 1 (BSA+20% RV), group 2 (BSA+CTB), group 3 (BSA+CTB+RV) and group 5 (100% RV), and 8 chickens in group 4 (BSA+FIA). The immunogen mixture was administered by gavage to the pharynx at 200 Ìl/chicken. All chickens were immunised on days 0, 14 and 28. They were 15 days old when first immunised (day 0). The treatment groups were:

Group 1: 2 mg BSA and 20% RV. RV (Softigen® from Sasol Gmbh, Witten, Germany) was added to a concentration of 1:5 (v:v) in a BSA (Sigma, Sweden, Cat. No. A-4505) solution (10mg/ml phosphate-buffered saline (PBS), pH 8.0).

Group 2: BSA and CTB glutaraldehyde-conjugated. BSA and CTB (Sigma, Cat. No. C9930) were conjugated (1:1 molar ratio) with glutaraldehyde. The protocol was based on van der Heijden and co-workers (13). Briefly, CTB (containing ≤0.5% Cholera Toxin A-subunit) and BSA dissolved in PBS (pH 8.0) were mixed, after which glutaraldehyde was slowly added to a concentration of 15 mM. The mixture was gently stirred for one hour at room temperature. The reaction was stopped by adding glycerine to reach a glycin concentration of at least 60 mM. The mixture was dialysed against PBS overnight at room temperature.

Group 3: BSA and CTB and 20% RV glutaraldehyde-conjugated. BSA and CTB (1:1 molar ratio) and 20% RV were conjugated with glutaraldehyde as described in Group 2.

Group 4: 1 mg BSA with FIA (positive control). A solution of 10 mg BSA dissolved in 1 ml PBS was emulsified in 1 ml of FIA (Sigma). Chickens were immunised s.c. with 200 Ìl on days 0, 14 and 28.

Group 5: 2 mg BSA and 100% RV. RV was added to a concentration of 1:1 (v:v) in a BSA solution (10mg/ml PBS, pH 8.0).

In Phase 2, another antigen, human IgG emulsified with FIA, was administered by s.c. injection to all chickens in all treatment groups of Phase 1, 14 days after the last oral immunisation, i.e. 42 days after the first immunisation was given (42 dpi). On the last blood sampling in Phase 1, at 42 dpi, Phase 2 was initiated. Each chicken in every treatment group was immunised s.c. (4 sites on the breast with 200µl of 200µg human IgG emulsified in FIA). Injections were given on days 0 and 14.

Collection of blood and serum samples. All chickens were bled (using a 23-G needle on a 2.5 ml syringe) from the wing vein (1.0 ml) before every oral or s.c. immunisation. Blood sampling was done every fortnight, at the same time when immunisation treatments were given, i.e. for Phase 1 on days 14, 28 and 42, and for Phase 2, on days 14 and 28 after the initial s.c. injection (dpsi). Serum samples were collected from centrifuged clotted blood and stored at −20°C until analysis. On the final day of sampling, the chickens were exsanguinated by cardiac puncture during anaesthesia with 400 µl Ketamine (Ketalar, Park-Davis/Warner Lambert, Sweden) (50mg/ml) and 200 µl Xylazine (Rompun Vet, Bayer, Sweden) (20mg/ml), injected intramuscularly.

Enzyme-linked immunosorbent assay (ELISA) quantification of immunospecific chicken anti-BSA IgG, IgM and IgA antibodies. Indirect ELISA was used to quantify the immunospecific anti-BSA IgG antibody in chicken serum. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µg/ml BSA protein standard (Sigma Cat. # P-0834) in carbonate buffer (pH 9.6, 100 µl/well) and incubated overnight at 4°C in a moist chamber. The plates were
washed four times in PBS-Tween-20 (pH 7.4) in between each step. After coating and washing, the plates were incubated with the chicken serum samples diluted 1:100. As standard series, a pool of serum from another batch of chickens previously immunised with BSA emulsified in FIA (n=4) was diluted 2-fold starting at 1:2,000 to 1:512,000, for IgG; 1:4 to 1:4,096 for IgM; and 1:64 to 1:65,536 for IgA. The standard serum pool was defined as containing 100 arbitrary units (AU)/ml. The IgG antibody was detected with horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (1:10,000, Sigma); for IgM antibody, HRP-conjugated goat anti-chicken IgM (1:1000, Nordic Immunology Lab). An OPD-substrate (100 µl/well, Kem-En-Tec, Copenhagen, Denmark) was used for colour development. Treated plates were incubated for 15 minutes in the dark at room temperature to react, after which the reaction was stopped with 1 M H₂SO₄ (150 µl/well). The plates were read in an ELISA reader (Multiskan RC, Labsystems, Sweden) at 492 nm.

Figure 1. Mean serum anti-BSA IgA concentration. BSA antigen mixed w/ adjuvants (FIA, Freund’s Incomplete Adjuvant; CTB, Cholera Toxin B-subunit; RV, RhinoVax). Error bars = SEM; n=9

Figure 2. Mean serum anti-BSA IgM concentration. BSA antigen mixed w/ adjuvants (FIA, Freund’s Incomplete Adjuvant; CTB, Cholera Toxin B-subunit; RV, RhinoVax). Error bars = SEM; n=9

Since we have experienced unspecific binding to BSA which cannot be eliminated by various blocking protocols (14), only values with OD readings > mean of serum from saline-treated control chickens (n=4) + 1 standard deviation were considered positive. The
sensitivity of the assay based on controls was 0.094 AU/ml for IgG, 13.65 AU/ml for IgM and 2.15 AU/ml for IgA. The inter-assay coefficient of variation for IgG was 3.4%, 4.7% for IgM and 5.8% for IgA. The intra-assay coefficients of variation for IgG, IgM and IgA were 2.5%, 4.5% and 5.1%, respectively.

ELISA quantification of chicken anti-human immunoglobulin-G IgG. Microtitre plates were coated with 1 mg/ml human IgG (Sigma) in carbonate buffer (pH 9.6, 100 μl/well) and incubated overnight at 4°C in a moist chamber. The plates were washed four times in PBS-Tween-20 (pH 7.4) in between each step. The plates were incubated for 1 hour at room temperature (RT) with 1% L-tryptophan (Sigma), washed and incubated for 1 hour with the chicken serum samples (diluted to fix the standard curve). After washing, the plates were blocked with 1:500 rabbit anti-human IgG (Sigma) for 1 hour at room temperature. IgY purified from egg yolk of chickens s.c. immunised with human IgG emulsified with FIA was used as standard (4). The IgY standard preparation was diluted 2-fold starting at 1:1,040 to 1:104,000. The standard serum pool was defined to contain 100 AU/ml. The IgG antibody was detected with HRP-conjugated rabbit anti-chicken IgG (1:10,000, Sigma). An OPD-substrate (100 μl/well, Kem-En-Tec, Copenhagen, Denmark) was used for colour development. Treated plates were incubated for 15 minutes in the dark at room temperature to react after which the reaction was stopped with 1 M H₂SO₄ (150 μl/well). The plates were read in an ELISA reader (Multiskan RC) at 492 nm. Inter- and intra-assays coefficient variations were 1.35% and 1.51%, respectively.

Statistical analyses. Data were analysed by ANOVA and correlations with Excel (Microsoft) software programme. P values <0.05 were considered significant.

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

Results

Immunospecific IgA response to oral immunisation with BSA (Phase 1). The mean anti-BSA IgA serum concentrations in the chickens of the five treatment groups are shown in Figure 1. The chickens of group 4 (FIA+BSA) had significantly higher concentrations than the chickens in groups 1, 2 and 5 (20% RV, CTB or 100% RV). No antibody response was detectable 14 dpi in the chickens in group 1 (20% RV), while a low response was seen in the chickens of the other groups at this time point. The concentrations recorded at 28 dpi and 42 dpi were significantly higher than those at 14 dpi.

Immunospecific IgM response to oral immunisation with BSA (Phase 1). The mean anti-BSA IgM serum concentrations in the chickens of the five treatment groups are shown in Figure 2. The chickens in the FIA group had significantly higher concentrations compared to the chickens of the orally-treated groups, except for the chickens in the CTB+RV group. The concentrations increased during the study period, except for the chickens in groups 4 (FIA) and 5 (100% RV) in which the concentrations peaked at 28 dpi.

Immunospecific IgG response to oral immunisation with BSA (Phase 1). The mean anti-BSA IgG serum concentrations in the chickens of the five treatment groups are shown in Figure 3. Among the chickens of the four oral treatment groups, those of group 1 (20% RV) had a significantly higher anti-BSA IgG response compared to the other chickens. The chickens in group 4 (FIA) had significantly higher concentrations than did any of the orally-immunised chickens.
The difference in mean serum anti-BSA IgG concentration for the chickens immunised with 20% RV+BSA was significant between 14 dpi and 28 dpi and between 14 dpi and 42 dpi. There was, however, no difference in concentrations between 28 dpi and 42 dpi (Figure 3).

Immunospecific chicken IgG response to subsequent immunisation with human IgG emulsified in FIA (Phase 2). The mean anti-human IgG concentrations in the chickens of the five treatment groups following subsequent s.c. immunisation with human IgG are shown in Figure 4. The concentrations increased from 14 dpi to 28 dpi in all chickens. The concentrations of the chickens which had received CTB in Phase 1 (groups 2 and 3) were significantly higher than the concentrations of the chickens in the other groups.

Family variation in antibody response in Phases 1 and 2. Each treatment group contained a sister from the offspring of 11 Mayo et al: RhinoVax® and Cholera Toxin B in Immunisation
hens (A-K) and a significant difference between families with regard to immunospecific antibody concentration following immunisation was recorded (Figures 5 and 6). With respect to declining mean immune response after oral immunisation, the families had the following order: H,B,K,A,C,J,E,D,I,G,F (Figure 5) and after classical immunisation the order was A,G,C,F,B,H,K,J,D,I,E (Figure 6).

**Body weight gain.** There were no significant differences in body weight gain during the study period between treatment groups.

**Discussion**

In the search for effective oral adjuvants potentiating a humoral immune response in chickens a steadily increasing number of candidate adjuvants have been tested. Sodium fluoride and Quillaja saponin both exhibit some adjuvanticity (15-17), and RV and CTB both stimulate the humoral immune system when administered with the antigen orally (4, 14). RV has the advantage of being completely non-toxic and approved for use in the human (European Pharmacopoeia 01/2002:1443 p. 1497).

We have recently found that even very young chickens (14 days old) respond with an immunospecific immune response to oral immunisation (14), and in Phase 1 of the present study, all chickens responded with an immunospecific IgA response against BSA. Of the orally-immunised chickens, the ones in group 3 immunised with CTB+RV responded with the highest concentrations comparable to the concentrations recorded in the group 4 chickens immunised s.c. with FIA. The combination of RV with CTB thus seems to have a synergistic effect on the immunospecific IgA response. A similar effect was seen with respect to the immunospecific IgM response. All chickens responded well and chickens immunised with the RV-CTB combination exhibited the highest immunospecific IgM anti-BSA concentrations as compared to the other orally-treated animals. Moreover, the mean IgA and IgM concentrations for the RV+CTB-treated chickens were similar to those of the chickens immunised s.c. with FIA (group 4), which had significantly higher concentrations than the chickens in groups 1, 2 and 5.

For practical polyclonal antibody production purposes IgG is the important immunoglobulin class, because it is transferred from the circulation of the chicken into the egg yolk, after which it is termed IgY. Twenty % RV was the most efficient oral adjuvant in the present study and the concentrations of the chickens immunised with this adjuvant were significantly higher than the concentrations recorded in the other orally-immunised treatment groups. The dose of RV also has an influence on the resulting antibody concentration. In a previous study (14), we used lower doses of RV and observed lower concentrations than when using the present dose.

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**Figure 6.** Mean serum anti-human IgG IgG concentration, grouped per family; each family contains one chicken from each treatment group. Error bars = SEM; n=5
Twenty % RV was more potent than 100% RV, probably because water is a necessary factor for keeping the correct three-dimensional structure when the antigen (BSA) is presented for the antigen-presenting cells. The chemical structure of RV is very similar to the asylglycerides that are found as breakdown products in inflamed tissue; these compounds stimulate the macrophages through the Fc receptor (but not through C3b) and affect the production of arachidonic acid. They are found to augment the antigen presentation cells and dendritic cells and stimulate the cytokine production between macrophages and B- and T-cells (18, 19).

Classical immunisation with s.c. administration of the antigen emulsified in FIA was approximately 5 times more potent than oral immunisation with 20% RV. From a scientific point of view this may seem disappointing, but the practical application aspects are encouraging. Traditional immunisation with administration of the antigen-adjuvant mixture through injections should be under the auspices of laboratory animal legislation and regulations. By contrast, oral immunisation methods, when combined with the use of eggs as the antibody source, are non-invasive and not different from other agricultural procedures. As a consequence, the chickens do not have to be housed in facilities approved for housing laboratory animals, and there is no requirement for personal and project licenses and staff with specialist competence in laboratory animal science.

In Phase 2 of this study, all chickens in the five groups produced immunospecific IgG antibodies when parenterally-injected with another antigen – human IgG. The chickens in groups 2 and 3, which had received CTB orally in Phase 1, produced significantly higher immunospecific IgG concentrations than did the chickens in the other groups. This indicates that CTB seems to act as a priming agent in animals subsequently immunised by traditional s.c. administration of antigen and FIA. This confirms the findings in mammals of Hornquist and Lycke (20), who reported that oral priming with keyhole limpet hemocyanin (KLH) administered with CTB followed by parenteral immunisation with KLH+CTB induced an increase in primed KLH-specific T-cells compared with non-primed animals. In contrast, if chickens are primed with antigen alone, they may respond with a lower serum IgG response following subsequent parenteral immunisation (15).

The mammalian antibody response to most antigens seems to be genetically determined and under polygenic control involving histocompatibility antigens (21,22). Outbred stocks like rabbits, although selected through generations for high antibody response, have been documented to exhibit a remarkable inter-individual variation in their immune response (23). The immune response in chickens has also been demonstrated to be subject to genetic variation (24-25) and there was a clear and significant between family variation in humoral immune response in the present study. We expected that those individuals belonging to a family who responded well to an oral treatment would also have a good response to a subsequent s.c. treatment. However, this was not the case. In spite of the small scale of the present study, this indicates that chicken lines selected for a good humoral immune response to parenteral immunisation may not necessarily respond equally well to oral immunisation and vice versa.

In summary, all orally-immunised chickens produced immunospecific IgA, IgM and IgG antibodies. Compared with the chickens classically immunised with the antigen emulsified in FIA and injected s.c., the orally-immunised chickens’ immunospecific IgG response was approximately five times lower. For practical production purposes, oral immunisation may be an attractive alternative to parenteral immunisation because it is non-invasive and thus not subject to laboratory animal legislation and regulation. The genetic control of the humoral immune response seems to be dependent on the mode of antigen administration.

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References


