IMMUNOLOGY, HEALTH, AND DISEASE

Improvement of Adjuvant Systems to Obtain a Cost-Effective Production of High Levels of Specific IgY

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ABSTRACT Incomplete Freund's adjuvant (IFA) is used as standard adjuvant for the production of specific antibodies. In this study, we evaluated the ability of supplementation of IFA with 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] or C-phosphate-guanosine-oligodeoxynucleotide (CpG-ODN) to enhance the quantity of specific IgY found in the eggs of hyperimmunized laying hens. In this comparative study, the fimbrial adhesin F4 of porcine enterotoxigenic *Escherichia coli* was used as prototype immunogen. Hens of 3 groups received by i.m. injection 20 μg of purified F4 adhesin emulsified with 1 of the following adjuvants: 0.5 mL of IFA alone (F4-IFA group), 0.5 mL of IFA supplemented with 285.6 ng of 1α,25$(OH)_2D_3$ (F4-IFA-D_3 group), or 0.5 mL of IFA supplemented with 10 μg of CpG-ODN (F4-IFA-CpG group). Hens of 2 control groups received PBS or purified F4 alone. Immunization was repeated after 2 and 5 or 7 wk. Eggs were collected at 3- to 4-d intervals from preimmunization to d 79, and whole eggs were tested to measure the quantity of anti-F4 IgY by a standardized indirect ELISA. The quantity of specific anti-F4 IgY present in eggs from immunized hens of the F4-IFA group increased from d 13 to 79, corresponding to the end of the experiment. The values for this group at each time were considered as 100%. Results obtained for the other adjuvants were expressed in relation to this reference method. Supplementation of IFA with 1α,25$(OH)_2D_3$ did not result in any enhancement of the quantity of anti-F4 IgY present in the eggs. On the other hand, supplementation of IFA with CpG-ODN resulted in an enhancement of yield up to 942% of the F4-specific antibody response. Moreover, the use of CpG-ODN is a cost-effective and ethical refinement for the production of specific antibodies, permitting a reduction in the number of immunizations needed. In conclusion, this study provides strong evidence for the use of IFA supplemented with CpG-ODN rather than IFA alone for the production of high levels of specific antibody in laying hens.

Key words: immunoglobulin yolk, Freund's adjuvant, cytosine-phosphate-guanosine-oligodeoxynucleotide, 1α,25-dihydroxyvitamin D_3, F4 (K88)

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INTRODUCTION

Immunoglobulins are widely used for a variety of purposes, such as in diagnostic tests, purification columns, and passive immunotherapy (Kim et al., 1999; Cook et al., 2005). The necessity to produce highly specific Ig in the most cost-effective manner possible is key in both the research and diagnostic fields. Although most domestic animal species have been used to obtain specific Ig, the laying hen has only been considered in the last few decades. The use of the laying hen is an ethical alternative for the production of much specific antibodies at a low cost (Schade et al., 1996). Indeed, IgY produced by hens transit to the egg yolk at high levels (100 to 250 mg per egg; Hatta et al., 1990; Schade et al., 1996; Erhard et al., 2000, CCAC, 2002). Consequently, Ig could be recovered from the egg yolk, avoiding the bleeding of animals. Thus, the method provides a noninvasive and cost-effective way to obtain Ig.

Several aspects of hen immunization, including the inoculation route, have been studied in an effort to improve IgY production and yolk deposition. For instance, Chang et al. (1999) found that i.m. inoculation resulted in a higher quantity of specific antibodies as compared with inoculation by the s.c. route. The most commonly used reference adjuvant eliciting a high specific immune response has been complete Freund's adjuvant (**CFA**), containing heat-killed and dried mycobacteria. However, in recent years, CFA has been less frequently used because of an associated severe inflammation causing necrosis and ulceration of the tissue (Wanke et al., 1996). The most effective substitute found to date is incomplete Freund's adjuvant (**IFA**),

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which is now the adjuvant most commonly used to produce specific Ig. Both IFA and CFA are oil-based adjuvants that create a depot at the injection site, thus protecting the antigen and releasing it at a low rate in the organism and allowing a sustained stimulation of the immune system. However, in IFA, the mycobacterial components were removed to eliminate the tissue necrosis, resulting in an accompanying loss of immunostimulatory effect. Thus, although IFA has been used to date as a substitute for CFA, there remains, nevertheless, the potential for improvement of its immunostimulatory effect.

Supplementation of IFA is 1 way to potentiate its immune stimulation. Previous studies have demonstrated the high potential of 1α,25-dihydroxyvitamin D_3 [**1α,25(OH)$_2$D$_3$**], the active metabolite of vitamin D_3, for the stimulation of the mammalian immune system (Van der Stede et al., 2003). However, the use of 1α,25$(OH)_2D_3$ as an adjuvant has given rise to conflicting results, depending on the animal species examined (Lemire, 1992; Kriesel and Spruance, 1999; Van der Stede et al., 2003; Ivanov et al., 2006). Furthermore, there are no reports of the evaluation of this metabolite as an adjuvant in the avian species.

Oligodeoxynucleotides containing C-phosphate-guanosine (**CpG**) motifs are also a promising adjuvant in mammals (Klinman et al., 1999; Hemmi et al., 2000; Hemmi and Akira, 2005). Ioannou et al. (2002) observed that the use of IFA at reduced doses in combination with CpG-oligodeoxynucleotide (**CpG-ODN**) resulted in attenuation of the tissue damage without compromising the magnitude of the immune response in mice. In the avian species, different in vitro studies have demonstrated that CpG-ODN induces NO production in macrophage cell line HD11, and the recognition of CpG-ODN by avian heterophils in the presence of chicken serum results in their mobilization and induces a dose-dependent heterophil degranulation (He et al., 2005). The biological activity observed with CpG-ODN is motif-dependent, and only a few studies have been done to determine the effective sequences in avian species (Rankin et al., 2001; He et al., 2003; Gomis et al., 2004). He et al. (2003) found the GTCGTT sequence to be the most active CpG motif to stimulate a response in an avian macrophage cell line (HD11) in vitro (He et al., 2003). Nevertheless, there have been no reports on the stimulatory effect of CpG-ODN on the induction of specific egg yolk antibodies in vivo using laying hens.

The aim of this comparative study was to investigate the potential of the adjuvants 1α,25$(OH)_2D_3$ and CpG-ODN to potentiate the immunostimulatory effect of IFA, thus maximizing the production of specific antibodies in the egg yolks of hyperimmunized laying hens.

MATERIALS AND METHODS

Laying Hens

Eighteen 18-wk-old Lohmann White laying hens were obtained from a commercial breeding farm and were housed in cages with mesh floors, 2 hens per cage (Schade et al., 1996), in an environmentally controlled room. The room temperature was maintained at 23.9°C. The hens were subjected to 15 h of artificial light each day (0600 to 2100 h). The hens were fed ad libitum with the commercial diet Natur-Aile (Coopérative Fédérée de Québec, St-Hyacinthe, Canada). Hens were euthanized at the end of the experiment. The experimental procedure conformed to the guidelines of the Canadian Council on Animal Care (CCAC, 2002).

Monitoring of the Laying Hens

The laying hens were monitored daily to determine any secondary effects following the immunization. General appearance, behavior, presence of local inflammation, and any reaction to the injected adjuvant were evaluated on each examination.

Preparation of F4 Immunogen

The major subunit protein FaeG (27.5 kDa) of enterotoxigenic *Escherichia coli* fimbrial adhesin F4 was used as the prototype immunogen. The FaeG protein had been cloned previously and was provided by D. J. Pickard (Kehoe et al., 1981). The protein was produced from *E. coli* strain K12(pMK005), designated EcL 1082, and purified as described by Jacobs and de Graaf, (1985) with some modifications. Briefly, after 5 h of culture, bacteria were centrifuged. The pellet was homogenized twice for 5 min on ice at maximum speed using an X-120 homogenizer (Polyscience, Staufen, Germany), with cooling on ice for 5 min between each homogenization. Clear supernatant was obtained by centrifugation at 12,000 × *g* for 20 min at 4°C and subjected to ammonium sulfate (14% wt/vol) precipitation overnight at 4°C. The proteins were harvested by centrifugation at 12,000 × *g* for 1 h at 4°C and resuspended in 0.1 *M* Tris-HCl, pH 7.8. Salt was removed by dialysis overnight in 0.1 *M* Tris-HCl, pH 7.8. The dialysate was precipitated with 0.1 *M* citric acid at pH 4.0, being the isoelectric point of F4. After a centrifugation for 15 min at 14,000 × *g* at 4°C, the pellet was resuspended in McIlvaine buffer (0.15 *M* K_2HPO_4 and adjusted to pH 6.0 with 0.1 *M* citric acid). The purified F4 migrated as a single band on electrophoresis using a 15% SDS-polyacrylamide gel. The presence of purified F4 protein was confirmed on Western blot using a specific monoclonal antibody against the fimbriae (data not shown). The purified F4 was quantified by the modified Lowry method (Markwell et al., 1978) using BSA as standard. A protein concentration of 1,154 μg/mL was obtained.

Experimental Design

Hens were each immunized i.m. at d 0 with 1 of the following immunogen-adjuvant preparations in 2 sites in the breast muscle when they reached 22 wk of age. The injected volume per site was 0.5 mL, for a total of 1 mL per hen. Immunization was repeated on d 15 and subse-

quently during wk 5 or 7 of the experiment, when specific anti-F4 IgY levels were no longer increasing. Both hens in any 1 cage received the same immunization regimen.

Eggs were collected twice daily from d 0 until the end of the experiment and stored at 4°C until testing by the indirect ELISA, usually on the following day.

Immunogen-Adjuvant Preparations. Four hens were each immunized with 20 μg of purified F4 resuspended in 0.5 mL total of 0.1 *M* PBS at pH 7.4 and emulsified in a 2-way syringe in an equal volume of IFA alone (F4-IFA group), being considered as the reference group. Four hens were each immunized with purified F4 emulsified in IFA supplemented with 285.6 ng of $1\alpha,25(OH)_2D_3$ (F4-IFA-D_3 group). The active metabolite of vitamin D_3, $1\alpha,25(OH)_2D_3$ (Sigma-Aldrich Canada Ltd., Oakville), was dissolved at 1 mg/mL in pure ethanol, aliquoted, and frozen at –20°C until use. An additional 4 hens were each immunized with purified F4 emulsified in IFA supplemented with 10 μg of CpG-ODN (F4-IFA-CpG group). The specific sequence number 2135 of CpG-ODN (5′-TCGTCGTTTGTCGTTTTGTCGTT-3′, BioCorp Inc., Montréal, Canada), previously recognized to activate the immune system of hens in vitro (Rankin et al., 2001), was dissolved at 4.2 mg/mL in distilled water, aliquoted, and frozen at –20°C until use. This nonmethylated sequence has a phosphorothioate backbone that allows it to resist nuclease degradation (Sands et al., 1994). Hence, a relatively small amount of CpG oligonucleotide is required to stimulate the immune system (Sester et al., 2000).

Two hens were immunized with 0.1 *M* PBS at pH 7.4. This was considered to be the negative control group. Four hens, making up the F4 control group, were immunized with 20 μg of purified F4 resuspended in 0.1*M* PBS at pH 7.4. The latter group was used to determine the quantity of specific anti-F4 IgY present in eggs when no adjuvant was used.

Indirect ELISA Test. The entire contents of all eggs belonging to the hens housed in the same cage were pooled and mixed in a Waring blender (Waring Commercial, New Hartford, CT). The antibody response to F4 was measured twice weekly from d 0 until the end of the experiment by indirect ELISA, using 96-well microplates coated with 50 ng of purified F4, as described previously (Girard et al., 2006). The detected absorbance in each sample of the F4-IFA group was considered as 100%. The absorbance values observed at each sampling time for each adjuvant system tested were initially expressed as the mean absorbance in the whole egg contents for the hens of that group. The results were then compared with those of the F4-IFA group at each time and expressed as a percentage. This percentage value represented the increase or decrease observed at each time for each group relative to the standard group (F4-IFA group). Eggs collected before the first immunization were tested by ELISA to demonstrate that no specific anti-F4 IgY was present.

Statistical Test

To compare the immune response to F4 among treatments, areas under the ELISA titration curves were compared using a 1-way nonparametric ANOVA (Tukey-Kramer multiple comparison test). Data were considered different when the probability level was $\alpha \leq 0.05$.

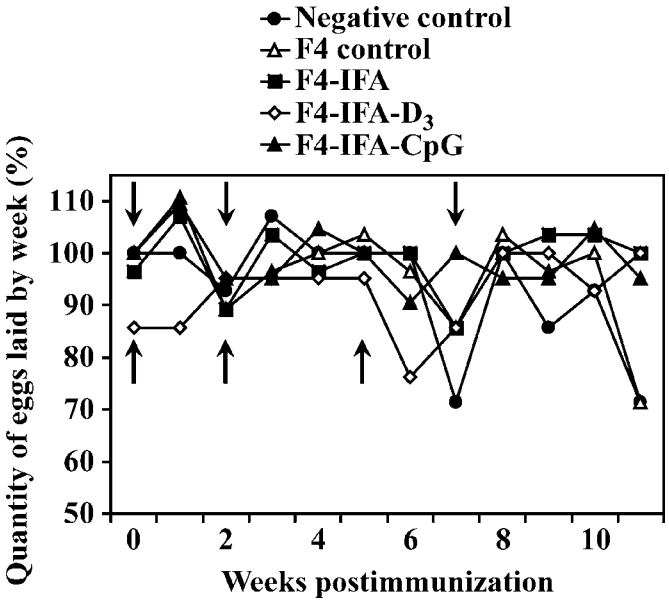

Figure 1. Mean number of eggs laid per week by hens after immunization with the following: enterotoxigenic *Escherichia coli* fimbrial adhesin F4 emulsified in incomplete Freund's adjuvant (F4-IFA); F4 emulsified in incomplete Freund's adjuvant (IFA) supplemented with 1α,25-dihydroxyvitamin D_3 (F4-IFA-D_3); F4 emulsified in IFA supplemented with C-phosphate-guanosine-oligodeoxynucleotide (F4-IFA-CpG); F4 alone (F4 control); or PBS (negative control). A value of 100% corresponds to 7 eggs per week per hen. Immunization days for all groups except F4-IFA-CpG are denoted by arrows pointing up. Arrows pointing down represent the days of immunization for the F4-IFA-CpG group only.

RESULTS AND DISCUSSION

Effect of Immunization with the Different Adjuvants on Overall Health and Egg Yield in the Hens

Hens did not present discomfort or pain in response to the immunization procedure or to any adjuvant used to potentiate the response to the F4 fimbriae. The injection sites were always clean, and no sign of tissue damage, necrosis, or ulceration was observed. Only 1 bird died during the experiment, at d 49 (wk 7). The bird was in the $1\alpha,25(OH)_2D_3$ group. The hen had a severe leg injury with bleeding caused by a "foot trap" in the cage, occurring during night, followed by a reduction in food intake leading to its death. Furthermore, egg yield was monitored to evaluate the possible adverse effect of the adjuvant on the general health or laying capacity of the immunized birds. In general, no significant decrease in egg yield was observed during the experiment, for any of the adjuvants (Figure 1). However, a lower production of eggs was noted for several days after each immunization (e.g., wk 7). This suggests that the decrease in egg laying is due to the stress caused to the animals by the immunization process rather than the negative effect of the adjuvants used.

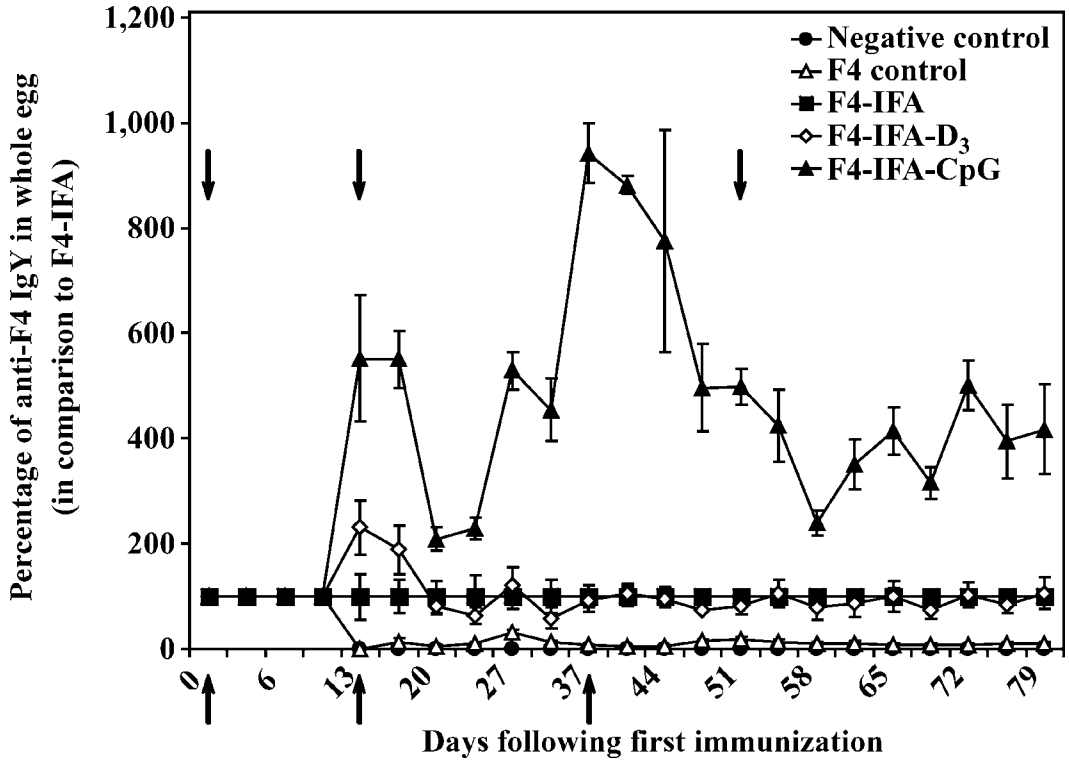

Figure 2. Effect of supplementation of enterotoxigenic *Escherichia coli* fimbrial adhesin F4 emulsified in incomplete Freund's adjuvant (IFA) with C-phosphate-guanosine-oligodeoxynucleotide (F4-IFA-CpG group) or 1α,25-dihydroxyvitamin D_3 (F4-IFA-D_3 group) on specific anti-F4 IgY levels in the eggs of hens at different times after initial immunization. The quantity of specific anti-F4 IgY found by indirect ELISA for each adjuvant system was expressed as a percentage of the quantity of anti-F4 IgY found in whole eggs of the F4-IFA group, which was considered as 100%. Hens of the control groups were immunized with F4 alone (F4 control); or PBS (negative control). Immunization days of all groups except F4-IFA-CpG are denoted by arrows pointing up. Arrows pointing down represent the days of immunization for the F4-IFA-CpG group only. All points represent the mean of duplicate results of ELISA. Error bars represent SE.

Effect of Different Adjuvants on Production of Specific Anti-F4 IgY

No significant difference was observed among individuals with respect to the concentration of specific IgY present for the same adjuvant (data not shown) on indirect ELISA. Consequently, the results for each hen, regardless of cage of origin, were pooled at each sampling time for each adjuvant system tested.

Specific anti-F4 IgY was first detected in hens on d 13 postimmunization. In hens of the F4-IFA group, the mean of anti-F4 IgY deposition in eggs reached a first peak at d 30 postimmunization and stabilized for at least 20 d to reach a second peak at d 58 postimmunization. Because IFA was considered to be the standard adjuvant, the anti-F4 IgY levels of the F4-IFA group at each testing time were set at 100% to facilitate comparison among groups.

When IFA was supplemented with CpG-ODN, an increase of greater than 500% in anti-F4 IgY levels was observed from 13 to 16 and 27 to 51 d after initial immunization, reaching a maximum of 942%, as compared with use of IFA alone (Figure 2). The mean specific anti-F4 IgY concentration for the former was always higher from d 13 until the end of the experiment on d 79, being 480% higher for the duration of the experiment. This difference was significant, as determined by nonparametric ANOVA, the Tukey-Kramer multiple-comparison test (P = 0.001356). High egg levels of specific anti-F4 antibodies were obtained with this adjuvant mix, in spite of the low quantity of F4 protein used for immunization (20 μg) and its small size (27.5 kDa).

In vitro studies have demonstrated the immunostimulatory effect of CpG-ODN sequence number 2135 on avian cells (Rankin et al., 2001; He et al., 2003), including an increase in the number of B cells and their ability to produce antibodies (Krieg et al., 1995, 1999). Such an effect, together with the synergism observed between CpG-ODN and a deposit-forming adjuvant (Ioannou et al., 2002), could explain the high egg levels of specific IgY observed in the present work.

Vleugels et al. (2002) also observed an enhanced specific antibody response in broiler chickens following use of CpG-ODN as an adjuvant for immunization against BSA, although the appearance of this response was delayed. Here, we observed an enhancement in the levels of specific IgY in eggs, although no delay was observed following use of IFA with CpG-ODN as compared with IFA alone.

The hens of the F4-IFA-D_3 group demonstrated a maximum of only 231% of the anti-F4 IgY levels observed in eggs for the F4-IFA group at d 15 following the first immunization. This peak was followed by a rapid decrease until d 23, when levels reached those of birds belonging to the F4-IFA group (Figure 2). Nonparametric

ANOVA showed that the addition of $1\alpha,25(OH)_2D_3$ to IFA did not have any significant effect on the quantity of anti-F4 IgY present in the eggs.

This is the first report on the use of $1\alpha,25(OH)_2D_3$ as an adjuvant in avian species. Hence, we extrapolated the concentration used in this study from that used in pigs (Van der Stede et al., 2003, 2004). However, this concentration did not seem to be sufficient to enhance specific IgY production in birds in the present study. More investigation is needed before using this immunostimulant in birds.

The level of anti-F4 IgY was very low in the F4 control group, corresponding to a maximum of 31% of the quantity present in the F4-IFA group throughout the experiment. This quantity of specific anti-F4 IgY corresponds to the minimal levels detected in eggs throughout the study and represents a maximum of specific antibodies produced when no adjuvant was used. The F4-specific antibodies were not detected for the negative control group throughout the experiment, as expected.

Recently, a novel approach was used for the production of anti-F4 antibodies in laying hens. Cho et al. (2004) immunized birds with a plasmid containing the gene encoding the F4 fimbrial subunit protein FaeG in tandem with the gene for chicken IL-6. This approach resulted in a prolonged deposition of specific antibodies in eggs. However, it required the optimization of dosages and injection times. Moreover, the side effects of immunization with plasmid-encoded chicken IL-6 remain to be determined before its use on a large scale. We believe that the use of CpG-ODN would be more cost-effective and generally acceptable because of the extended time of deposition of anti-F4 IgY in eggs and the fact that this adjuvant system consists of a refinement of a time-proven approach for the production of specific antibodies.

Furthermore, we carried out a longer study of the use of F4-IFA-CpG in commercial farm conditions using 30 Lohmann White hens separated in 5 cages. Hens received only 8 booster immunizations, administered at 5- to 7-wk intervals, after the initial immunization with F4-IFA-CpG, leading to a high rate of anti-F4 IgY deposition in eggs. Data were compared with those obtained in the shorter experiment and demonstrated an enhancement of 344%, in mean values, relative to the F4-IFA group. No adverse effect of immunization on the health of the birds or on the egg yield was observed during 1 complete year. Moreover, the mortality rate was lower for the immunized hens than for the other nonimmunized birds on the farm (data not shown). Thus, our results suggest that CpG-ODN can be used safely in commercial conditions for the immunization of laying hens on a larger scale. In addition, we demonstrated that there was no difference in the concentrations of anti-F4 antibody, as determined by indirect ELISA, in eggs stored for 2 wk at 10 to 12°C in a commercial farm refrigerator or at 4°C. This further underlines the suitability of carrying out large-scale egg harvesting from immunized hens housed in commercial farm conditions.

Cost-Effectiveness

Our findings show clearly that the use of CpG-ODN in the immunization protocol can result in an increase by as much as 480% of the concentration of specific antibody present in eggs, for a very small increase in immunization cost. Taking into consideration the cost of the synthesis of the CpG-ODN sequence and the quantity of specific antibodies produced, the use of CpG-ODN to supplement IFA translates to a cost-effective refinement for the production of specific antibodies using laying hens. Only 10% of IgY in the egg is specific for the antigen injected, following immunization of chickens (Neural Notes, 1996). Considering the increase of 480% in specific IgY following immunization with CpG-ODN, a recovery of about 96 mg of specific IgY per egg may be expected. This corresponds to about 25,000 mg of specific IgY for an entire year (considering a conservative hypothesis of 5 eggs per week). Overall, a greater amount of specific IgY can be produced for a lower cost and in less time when IFA is supplemented with CpG-ODN. These criteria are of primary importance if large amounts of antibodies are required rapidly.

In conclusion, the enhancement of immune stimulation against F4 by supplementation of IFA with CpG-ODN resulted in a large and stable augmentation in the levels of specific IgY present in eggs. The immune-stimulating properties of this mixture, associated with a reduction in the cost of immunization, make for an attractive adjuvant formulation for the production of antibodies in laying hens.

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