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Research paper

Improving adjuvant systems for polyclonal egg yolk antibody (IgY) production in laying hens in terms of productivity and animal welfare

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ABSTRACT

The antibody production in the egg yolks of immunized laying hens is seen as a way of improving animal welfare compared with conventional production by mammals. Immunoglobulin Y (IgY) technology, however, has still to address welfare issues linked to the widespread use of an adjuvant in vaccines. Currently, Freund's adjuvants, complete (FCA) or incomplete (FIA), remain the standard. This study sought to evaluate various approaches used to enhance egg yolk antibody production in terms of both productivity and avian welfare. The outer membrane protein (OMP) of *Salmonella* Typhimurium was used as the prototype antigen. At 20 weeks of age, 56 ISA Brown hens, with specific-*Salmonella*-free status, were divided into seven groups ($n = 8$) and received an initial intramuscular immunization. Hens in the two negative control groups received phosphate buffered saline (PBS) or FIA alone. Hens in the other groups received 80 μg of *Salmonella* OMP emulsified with one of the following adjuvants: 200 μl of FIA alone (T1); 200 μl of FIA supplemented with 8 μg of C-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) (T2); and 280 μl of Montanide ISA 70 VG (T4). Birds in the T3 group received the antigen in emulsion with FIA and were given the tested immunostimulatory component (L-carnitine) via their feed (100 mg/kg). A positive control group (PC) received FCA for the first and final immunizations and FIA for the other boosters. Immunization was repeated after 20, 46, 82 and 221 days. Eggs were collected regularly until 242 days after the first immunization and the anti-*Salmonella* Typhimurium activities in the yolk were determined by ELISA. After 242 days, the birds were euthanized and the injection sites were evaluated for gross and microscopic lesions. Among the tested immunostimulatory approaches, supplementation of FIA with CpG-ODN led to a significant and long-lasting enhancement of the specific antibody response. This treatment was even higher than the positive benchmark using FCA in the first immunization. The study results showed that a clinical examination of injection sites is insufficient for drawing conclusions about the local tolerance of vaccines. Tissue damage was noticeable in all treatment groups. The birds receiving the Montanide adjuvant,

Abbreviations: IgY, immunoglobulin Y; FA, Freund's adjuvants; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; ODN, oligodeoxynucleotide; CpG, C-phosphate-guanosine; OMP, outer membrane protein; dpi, day post-first-immunization; NC1, negative control 1; NC2, negative control 2; PC, positive control; T1, treatment 1; T2, treatment 2; T3, treatment 3; T4, treatment 4; PBS-T, phosphate buffered saline containing 0.1% Tween 20; S, sample.

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however, had fewer and less severe lesions. Given these limited side-effects, Montanide ISA 70 VG could provide the depot effect needed to ensure the immunomodulatory efficiency of CpG-ODN. The association of these two adjuvants could prove a promising alternative to Freund's adjuvants (FA).

1. Introduction

Immunized laying hens can be used to produce large amounts of antibodies (IgY) specific to a broad range of antigens, including bacteria, mold, viruses or parts of these. These antibodies can be used for a variety of purposes, from diagnostic laboratory reagents to immunotherapy and immunoprophylaxis tools in humans and animals (Kovacs-Nolan and Mine, 2012). Compared with mammals, laying hens are economically interesting in terms of polyclonal antibody production because the antibody yields are higher (e.g. 5–6 times more than a rabbit over a 2 weeks period; Narat, 2003) and antibody production lasts longer since a laying hen can be used during the whole laying period and beyond (Trott et al., 2009). This reduces the number of animals and of expensive booster injections needed to obtain the antibodies. This economic advantage is reinforced by the lower cost of feeding and housing laying hens and the existence of cost-effective IgY extraction protocols (Schade et al., 2005). In addition to these economic advantages, animal welfare is improved in that IgY are obtained non-invasively and continuously from the egg, whereas stressful bleeding is required when collecting serum in mammals.

Nevertheless, antibody production from laying hens does need to address some welfare issues. Even the most immunogenic antigens must be administered in combination with an adjuvant that can affect animal welfare. Obviously, adjuvants that give the highest amount of highly specific antibodies are primarily considered and Freund's adjuvants (FA) are therefore usually the first choice. For many years, Freund's complete adjuvant (FCA) has been the gold standard for generating high levels of antibodies in animals. FCA is a mixture of a mineral oil and heat-killed and dried mycobacteria. Like all oil-based adjuvants, it forms a depot at the injection site and slows down the rate of release of the antigen in the vaccinated organism so that a substantial and long-lasting production of antibodies ensues. The main disadvantage of FCA is that it induces severe tissue reactions at injection sites, usually attributed to its mycobacteria component. For this reason, Freund's incomplete adjuvant (FIA) (i.e., FA without mycobacteria) was developed and is now commonly used for all booster injections, with FCA being used only for the first immunization (Marcq et al., 2013). Nevertheless, the FCA/FIA combination is still questionable. The first FCA injection could be enough to induce adverse side-effects (Olbrich et al., 2002), and even FIA can have side-effects (Weeratna et al., 2000). This need to improve both the immunostimulatory and welfare effects of adjuvants, together with the unacceptability of models using FCA, led to the study reported in this paper. The study was designed to evaluate the efficacy and safety of alternative vaccine protocols formulated with the same antigen, but using different adjuvants or immunostimulating components (supplied directly in the vaccine or via the feed). The benchmarks were, on the one hand, the classical protocol (FCA for the first immunization and FIA for subsequent booster injections) and, on the other, the use of FIA for all immunizations.

Oligodeoxynucleotides (ODN) containing C-phosphate-guanosine (CpG) motifs are promising components for enhancing immune response. Many studies have demonstrated their immunostimulatory effect in several species, but there has been limited work on the ability of CpG-ODN to increase the deposition of specific egg yolk antibodies in laying hens (Lévesque et al., 2007; de Paula et al., 2011). So far as we know, no detailed evaluation of CpG-ODN on tissue damage in avian species had been done to date. An alternative commercially available adjuvant, Montanide ISA 70VG (Seppic, Paris, France), was also assessed as a potential replacement for FA. This is also an oil-based adjuvant, claimed to be as efficient as FA and to have fewer side-effects (Fodey et al., 2008; Liu et al., 2011). Again, however, the literature does not provide a complete evaluation of this product, the studies reported so far having been designed to evaluate the protective effect of the obtained vaccine on chickens rather than the efficiency of the immunization of laying hens intended for the massive production of IgY. Finally, the immunostimulatory effect of a feed additive, L-carnitine (β-OH-(γ-N-trimethylamino)-butyrate), was tested. Dietary L-carnitine supplementation has been shown to enhance antigen-specific IgY in vaccinated broilers (Mast et al., 2000). Enhancing the efficiency of vaccines through nutrition deserves attention because it also represents an attractive form of refinement of IgY technology.

Salmonella enterica serovar Typhimurium was used as a model antigen because (1) *Salmonella* is still of economic importance worldwide (Galis et al., 2013) and (2) the production of antibodies against *Salmonella* in egg yolk is a well-controlled protocol in our laboratory (Chalghoumi et al., 2008). We therefore chose to work again with a *Salmonella* antigen for this immunization study. Nevertheless, the optimization strategies developed in this work could be applied to any target antigen.

2. Materials and methods

2.1. Birds and housing

Fifty-six non *Salmonella*-vaccinated, 17-week-old ISA Brown pullets were obtained from a commercial hatchery (De Biest, Kruishoutem, Belgium). Their *Salmonella*-free status was confirmed by bacteriological analysis of cloacal swabs, as described in Annex D to ISO 6579