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Research paper

Stimulation of IgY responses in gene gun immunized laying hens by combined administration of vector DNA coding for the target antigen Botulinum toxin A1 and for avian cytokine adjuvants

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ABSTRACT

DNA immunization is a convenient and effective way of inducing a specific antibody response. In mammals, co-administration of vectors encoding immunostimulatory cytokines can enhance the humoral response resulting in elevated antibody titers. We therefore set out to investigate the effect using avian interleukin 1β (IL-1β) and avian interleukin 6 (IL-6) as genetic adjuvants when immunizing laying hens. A BoNT A1 holotoxoid DNA immunogen carrying two inactivating mutations was evaluated for its ability to induce a specific and sustained IgY antibody response. Both the holotoxoid and the cytokine sequences were codon-optimized. In vitro, the proteins were efficiently expressed in transfected HEK 293T cells and the cytokines were secreted into the culture supernatants. Whereas eggs from hens immunized via gene gun using a prime boost strategy showed no differences in their total IgY content, the specific αBoNT A1 response was slightly elevated up to 1.4× by the IL-1β adjuvant vector and increased by 3.8× by the IL-6 vector. Finally, although hens receiving the IL-1β adjuvant had laying capacities above the average, hens receiving the IL-6 adjuvant experienced laying problems.

1. Introduction

Inspired by a pioneering study by Paul Ehrlich (Ehrlich, 1892), Klemperer immunized laying hens with increasing doses of virulent Tetanus-Bouillon-Culture to test whether protective antibodies exist not only in the blood but also in the egg (Klemperer, 1893). An extract of yolk from eggs laid by these hens had a protective effect if mice were treated before receiving a lethal dose of Tetanus-Bouillon-Culture. This demonstrated that neutralizing antibodies were also transferred to the egg yolk as a form of "maternal immunity". However, these findings were largely ignored by the field of antibody production and it was not until the second half of the last century that publications dealing with the generation and use of specific avian egg yolk immunoglobulins began to sporadically appear (e.g. Losch et al., 1986; Polson et al., 1980; Vieira et al., 1984). Increasing public concern about the use of animals in the laboratory has now brought the potential of avian antibodies back into focus (Lian et al., 2011). The production and extraction of polyclonal antibodies in chicken egg yolks have been termed 'IgY-technology' (Staak, 1996; Schade and Hlinak, 1996). In addition to animal protection aspects of non-invasive extraction from egg yolk, the avian antibody IgY ('Y' now commonly indicating an origin in yolk) has several other advantages, in particular those resulting from the phylogenetic distance of the avian immune-system from that of mammals. Characteristics such as affinity, titer and specificity can be significantly different between mammalian and avian antibodies, even when mammals and chickens are

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immunized identically (e.g. Gerl et al., 1996; Danielpour and Roberts 1995; Rosol et al., 1993; De Ceuninck et al., 2001). IgYs neither activate mammalian complement nor cross-react with Fc receptors, mammalian rheumatoid factor or mouse anti-human antibodies (Larsson et al., 1991; Larsson et al., 1993). Calzado et al. produced an IgY-based monospecific Coombs reagent devoid of natural hetero-agglutinins (Gutierrez Calzado et al., 2003). One final important advantage is the high yield of IgY obtainable from one hen. With a normal annual laying capacity of approximately 325 eggs, it is possible to obtain around 20 g total IgY from each hen per year (Pauly et al., 2009a).

During the last decade, a growing body of IgY literature has been published concerning its use in immunological assays (Pauly et al., 2009a, b; Matheis and Schade, 2011) as well as in veterinary and human medicine as a therapeutic or prophylactic against a number of diseases (Vega et al., 2011; Sarker et al., 2001; Suzuki et al., 2004; Horie et al., 2004; Chakhtoura et al., 2008; Lee et al., 2002; Ibrahim el et al., 2008; Roe et al., 2002; Nomura et al., 2005; Liou et al., 2010; Hirai et al., 2010; Yokoyama et al., 1992; Nilsson et al., 2008; Nilsson et al., 2007; Kovacs-Nolan et al., 2005; Kovacs-Nolan and Mine, 2004; Schade et al., 2005; Narat, 2003).

In addition to the classical protein antigen/adjuvant approach to immunize chickens for antibody production (see Leenaars et al., 1999 and Schade et al., 2000 for reviews), the use of DNA coding for the protein of interest has undergone a renaissance due to advances in ballistic delivery technologies. 'Gene guns' have now made plasmid vector immunization of mammals and birds increasingly straightforward and efficient (Lian et al., 2011). One of the key benefits of using genes for immunization is their manufacture using recombinant DNA technology: the hazardous or toxic materials produced using conventional methods can be avoided. As no pathogens are involved, DNA can be completely processed under low biosafety conditions until the point of immunization. In addition, the use of plasmid DNA allows precise immunization with a construct coding for a single protein rather than the heterogeneous mixture of potentially impure proteins that commonly constitutes inactivated or purified antigen preparations. Indeed, following removal of bacterial endotoxins, the DNA can be considered free of immunogenic impurities, thereby minimizing the induction of unspecific serum effects.

Furthermore, the ability to tweak coding sequences by introducing minor mutations is a powerful tool to modulate antibody specificity. Finally, high levels of antigen production can be achieved by optimizing the coding sequence to match the codon use of the target species, a modification that, depending on the gene, can increase expression levels by a factor of up to 800 (Hohn, 2004; George et al., 2011).

In principal, there are two common routes for immunizing chickens with plasmid DNA. Customary intramuscular injection requires high doses of DNA to achieve robust transfection and sufficient antigen production in the immunized organism (Cho et al., 2004; Kazimierczuk et al., 2005; Nikbakht Brujeni et al., 2011). In contrast, low doses of DNA applied via gene gun can efficiently induce high antibody titers against the antigen encoded (Witkowski et al., 2009). Advances in our understanding of avian cytokines allowed the identification of several possible adjuvant candidates. Genetic cytokine adjuvants are known to enhance the efficacy of DNA immunization (Mahdavi et al., 2011), but as far as we know, there has been only one study using a plasmid coding for chicken IL-6 to amplify the immune response to the target antigen (Cho et al., 2004). As the aim of our present study is to produce specific IgY as a basis for rapid detection systems, the BoNT A1 antigen was selected for immunization and the adjuvant effect of using IL-6 was compared to classical immunization using antigen alone. In addition, the total IgY (tIgY) yield as well as the laying capacity of the immunized hens was monitored.

2. Material and methods

2.1. Animals

23-week-old chickens (Lohmann brown and Lohmann selected Leghorn [LSL], Spreenhagener Vermehrungsbetrieb für Legehennen GmbH, Bestensee, Germany) were kept separated on the floor under free-range conditions (each a brown and white hen together) at the Research Institutions of Experimental Medicine of the Charité-Universitätsmedizin in Berlin, Germany and the Dept. of Experimental Toxicology and ZEBET (Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch at the BfR) of the Federal Institute for Risk Assessment, Berlin, Germany. Food (ssniff Legehühner-Zucht 1 and 2; ssniff Spezialitäten GmbH, Soest, Germany) and water were available ad libitum. The chickens started laying between 23 and 25 weeks of age and eggs were collected daily, marked and stored at 4 °C until further processing. Chickens were maintained in accordance with current regulations and the guidelines of local authorities (Berlin, No. H0069/03).

2.2. Generation of expression vectors for immunization

BoNT A1 expression constructs suitable for immunization were based on the published open reading frame NC_009495 (NCBI Ref Seq. Database) and codon-optimized according to general avian codon usage (Hanke et al., 2009). R363A and Y365F mutations were inserted to generate detoxified proteins (Pier et al., 2008) and the resulting sequence of 3891 kb length (CDS) was synthesized in vitro by Geneart (Regensburg, Germany) before cloning into the pTH expression vector (Hanke et al., 1998) using the HindIII and BamHI restriction sites to give the immunization vector pTH-BoNT-A1-RYM-V5. Synthesis and cloning of the codon-optimized avian cytokine expression vectors were performed with a similar procedure using the 804 bp ranging coding sequence of avian IL-1β (Y15006/NCBI Ref Seq. Database) and an insert of 726 bp encoding IL-6 (AJ309540/NCBI Ref Seq. Database) for codon optimization. All sequences and cloning steps were confirmed by Sanger sequencing.

2.3. Transfections, immunoprecipitation and Western blot assay

In order to validate protein expression and cytokine secretion, HEK 293T cells were transfected with the immunization vectors using the Polyfect Reagent (Qiagen, Germany) and cell lysates (BoNT A1) or supernatants (IL-1β and IL-6) were analyzed 24 h later for the presence of the relevant proteins. For immunoprecipitation, 1 ml of culture supernatant