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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 immunostimmulatory 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 1.4x by the IL-1β adjuvant vector and increased by 3.8x 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 et al. 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. Lösch et al. 1986, Polson et al. 1980, Viereira 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 has been termed 'IgY-technology' (Staak in 1995, 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 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 (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 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 Leenars 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, 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 3,891 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 was 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 hours later for the presence of the relevant proteins. For immunoprecipitation, 1 ml of culture supernatant was incubated under rotation with 25 µl of a V5-specific-agarose bead slurry (Life technologies Ltd., Germany) for 3 hours at 4°C. The agarose beads were pelleted by

centrifugation and rinsed three times with washing buffer. The beads were then resuspended in protein gel loading buffer, denatured by heating at 95°C for 10 minutes and subjected to SDS-PAGE. IL-1β-V5 and IL-6-V5 were detected by Western blot using V5-specific HRPO-conjugated antibody (Life technologies Ltd., Germany). BoNT A1 was detected using the αBoNT A1 heavy chain monoclonal antibody clone A1688 (Pauly et al. 2009b) diluted at 250 ng/ml Blocking buffer followed by an HRPO-conjugated α-mouse polyclonal serum (AbD Serotec, Germany). As positive control, purified BoNT/A1 (Metabiologics, Madison, WI, USA) has been used in parallel and inactivated by SDS-treatment prior to loading onto the gel.

2.4 DNA immunization by gene gun

Plasmid coated gold particles were prepared according to the manufacturers recommendations (BioRad). Hens were immobilized horizontally to expose the breast muscle regions of the apteria-area selected for immunization, with any remaining plumage being removed by hand to avoid interference with the trajectory of the gold particles. The gene gun was set up to give a helium gas discharge of 300 psi and positioned near the skin at the standard proximity (as determined by a spacer with a circular contact area of approximately 2 cm^2 mounted on the barrel). Four isolated immunization 'shots' (a total of 4 µg DNA) were applied to each animal, two on the left and two on the right of the breast muscle. To maintain levels of total DNA, empty pTH vector was included for animals (H96 / H97) not receiving a cytokine. The whole procedure took five minutes or less and hens appeared to be only minimally affected. Booster immunizations were repeated regularly every 3-4 weeks to give a total of 15 immunizations.

2.5 Egg sampling and IgY preparation

Each third or fourth egg was sampled and stored at 4^{o}C until further processing. Eggs were processed no later than three months after collection as longer periods of storage can result in yolk skins rupturing when rolled on filter paper (Pauly et al. 2011). Total IgY (tIgY) was purified using a method adapted from Polson et al. (Polson et al. 1980). Briefly, egg yolk was diluted 1:2 in sterile phosphate-buffered saline (PBS, pH 7.4, Roche, Germany) and 3.5% (w/v) polyethylene glycol 6000 (PEG 6000, Roth, Germany) was added to eliminate lipids and lipoproteins. After gentle shaking followed by centrifugation (10,000 × g for 20 min at 4^{o}C), the supernatant was decanted, solid PEG 6000 was added to a final concentration of 12% and the mixture was again centrifuged. The precipitate was then dissolved in 10 ml PBS,

PEG was added to 12% and the suspension centrifuged once more. Finally, the precipitate was dissolved in 1.2 ml PBS, transferred into a QuixSep Micro Dialysis device (Roth, Germany) and dialyzed against PBS at 4°C to give a final volume of around 2 ml. Adsorption at 280 nm was measured photometrically and protein content calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for tIgY. Previous observations showed extraordinary stability of IgY stored over extensive periods (years) at various temperatures between +4°C and -24°C without loss of activity (Schade et al., 2000). Generated preparations were stored in aliquots at optimal conditions of -24°C.

2.6 Use of BoNT A1-specific IgY in an antigen-capture ELISA

Nunc Maxisorp 96 well assay plates (Nunc GmbH, Germany) were coated overnight at 4°C with 50 µl of the BoNT A1-specific mAb A1688 (Pauly et al. 2009b) at a concentration of 10 µg/ml in PBS and excess antibody was then removed by washing 3 times with PBS-T (PBS + 0.1% Tween 20 [Sigma-Aldrich Chemie GmbH, Germany]). Wells were next blocked with 200 µl blocking buffer (PBS-T + 2% fat-free skimmed milk powder (TSI GmBH, Germany) for 1h at 37°C before rinsing three times with PBS-T. Wells were then incubated with 50 µl of an antigen (500 ng/ml E. *coli* derived BoNT A1_RYM in PBS) for 2h at 37°C before removing unbound antigen by washing with PBS-T. An individual IgY sample prepared from eggs collected at two-week intervals was diluted 1:100 in blocking buffer, 50 µl were added to the wells in duplicate and the plates incubated at 37°C for 1h. Excess antibody was then removed by washing and wells incubated with 50 µl of a 1:30,000 dilution of horseradish peroxidase (HRPO)-conjugated rabbit anti-IgY antiserum (Sigma Aldrich, Germany) for 1h at room temperature. Finally, wells were washed 5 times as described previously and incubated for 30 min with o-phenylenediamine containing H_2O_2-substrate solution (1 mg/ml OPD (Sigma Aldrich Chemie GmbH, Germany) in PBS). The reaction was terminated by adding 25 µl of 1 M H_2SO_4 and absorbance (A) was estimated at λ=495/620. Values were expressed as absorbance with immune samples minus the absorbance with preimmune IgY samples. For comparison, BoNT A1-specific IgY induced by administration of immobilized BoNT A1 protein was included in the ELISA (AC29, Pauly et al. 2009b).

2.7 Statistical analyses

Statistical analyses (type indicated individually) were performed using the GraphPad Prism program (Version 3).

3. Results

3.1 Construction and validation of vectors for DNA immunization with BoNT A1 antigen and adjuvant cytokines

A full-length BoNT A1 reading frame coding for the 150 kDa protein served as the basis of the immunization vector. A short C-terminal V5-tag was added to facilitate detection and two point mutations causing amino acid substitutions R363A and Y365F were introduced to eliminate toxicity of the mature protein. Although abolishing protease activity, these substitutions conserve the structural integrity and antigenicity of the protein and should therefore not hinder the induction of conformation dependent and independent antibodies (Pier et al. 2008). In order to maximize protein expression, the BoNT A1 gene was synthesized in a codon-optimized form and cloned under control of the CMV immediate early promoter into pTH, a plasmid vector optimized for DNA immunization (Hanke et al. 1998). The resulting pTH-BoNT-A1-RYM-V5 expression vector was transfected into HEK 293T cells and expression of the toxoid in cell lysates checked by Western blot. As shown in Fig. 1A a protein of 150 kDa, the expected size of the single-chain holotoxoid, was detected using an αBoNT A1 heavy chain monoclonal antibody (clone A1688, Pauly et al. 2009b). The size of the eukaryotic holotoxin was therefore similar to that produced in E. coli (Pier et al. 2008). Furthermore, the antibody also detected a 100 kDa heavy chain present in inactivated, processed toxin as previously reported by Pier et al. (2008), indicating specificity for the BoNT A1 heavy chain (Fig. 1A). As positive control, clostridial di-chain BoNT/A1 was used, which is reduced under the experimental conditions into the 100 kDa heavy chain and the 50 kDa light chain, of which the former is detected by the monoclonal antibody A1688 (Fig. 1A) These results demonstrate that a full-length holotoxoid is efficiently expressed in cells transfected with the pTH-BoNT-A1-RYM-V5 vector.

One of the major goals of the study was to investigate the effects of avian IL-1β and IL-6 when used as DNA adjuvants. Codon-optimized versions of these cytokine genes carrying a C-terminal V5-tag were therefore synthesized and cloned into the pTH vector. Following transfection of HEK 293T cells, proteins of the expected sizes (35 kDa for IL-1β-V5 and 27 kDa for the IL-6-V5) were precipitated from supernatants and detected by Western blotting via the V5-tag (Figs. 1B and 1C). Specificity was confirmed by the fact that such proteins were not precipitated from cells transfected with the empty pTH vector. Both cytokines, which are known to enhance B-cell maturation and antibody production in birds (Weining et al. 1998, Nishimichi et al. 2004), were therefore efficiently expressed and secreted, making the vectors potentially suitable for use as adjuvants.

3.2 Immunization scheme and monitoring of laying capacity

Animals were immunized by an initial priming with DNA followed by booster shots at intervals of 3-4 weeks. These intervals were chosen on the basis of previous mouse experiments performed to determine an optimal prime-boost regimen (data not shown). Each group of hens consisted of one Lohmann brown and one Lohmann selected Leghorn (white) to exclude any breed-specific influences.

A laying hen has normally a capacity of 5-7 eggs per week for a period of around 72 weeks, after which a decrease in laying capacity usually occurs. As shown in Fig. 2, laying capacities were not affected by the gene gun procedure itself since rates remained stable on the days following immunization. Whereas hens immunized with BoNT A1 (96 and 97) showed a regular laying capacity, the laying capacity of those treated with IL-1β in addition to BoNT A1 (hens 100 and 101) was exceptionally prolonged and at a very high level, with both constantly producing between 5 and 7 eggs per week over the whole observation period (Fig. 2A). Indeed, as shown in figure 2B, the difference in the mean laying capacities of the two groups was statistically highly significant (Mann Whitney test, $P<0.0001$). Hens 98/99 (treated with BoNT A1 plus IL-6) were not included in this analysis because hen 98 had "laying problems" from the beginning (Fig. 2A).

3.3 Total IgY-monitoring

The pattern of total IgY (tIgY) varied according to the individual hen. Although in general, tIgY in all hens oscillated considerably (Fig. 3A), the trend for hen 96 showed a statistically significant increase in tIgY over the course of the study (Fig. 3B). Such a clear trend was not discernible for hens 99, 100 and 101, probably because of the shorter observation period. Hen 99 was unusual in having a strong transient increase in tIgY between the middle of November and December (Fig. 3A). The mean tIgY values also varied from hen to hen (Fig. 3C), although the often significant differences did not appear to be an outcome of the type of immunization. Hen 101 has somewhat less IgY per egg compared with the eggs of other hens ($P<0.005$, Mann Whitney test) The other differences in tIgY are also quite significant (hen 96 to hen 97, $P<0.0001$; hen 97 to hen 99, $P<0.0001$; hen 99 to hen 100, $P<0.0001$, Mann Whitney test). However, these differences are largely individual variations and do not reflect differences related to the received immunizations, since for example the eggs of hen 100 receiving the IL-1β adjuvant contain a significantly higher amount of tIgY than the eggs of hen101 (Fig. 3C). Thus, the tIgY content is not significantly influenced by the immunizations

performed. Similarly, no differences in tIgY between the brown and white hens were observed.

3.4 Development of anti-BoNT A1 IgY

Production of BoNT A1-specific IgY in the eggs was analyzed by ELISA. In general, hens 96 and 97 (BoNT A1 alone) developed the lowest levels of specific antibody (Fig. 4A). Hen 96 reacted with a gradual increase after the second booster immunization, with the titer persisting at a low level and a new transient peak occurring after the fifth boost. However, the titer then declined and the animal did not respond to further booster shots. Hen 97 showed a similar pattern of antibody development, with a slow transient increase observed after the second and fifth boosts. Hens 98 and 99 (BoNT A1 combined with IL-6) showed a quite different pattern of antibody development (Fig. 4A). Although, due to sporadic laying, only a few data points were obtained for hen 98, this animal already developed specific antibodies after the initial immunization and responded moderately to the forth boost and strongly to the eighth. Hen 99 developed a low transient peak in antibody titer after the first immunization followed by a strong increase after the third and fourth boosts after which antibody levels plateaued for several weeks. The titers then declined gradually to the end of the study interrupted by only minor reactions to the immunizations. Hens 100 and 101 (BoNT A1 combined with IL 1β) showed relatively late but strong responses to the DNA immunizations (Fig. 4A). Hen 100 responded to the third boost with a slow but persistent increase in titer, a dramatic increase following the sixth boost and relatively prolonged peaks following the eight, tenth and twelfth boosts. Hen 101 had an unexpectedly delayed response to gene gun immunization with the no significant development of BoNT A-specific antibodies occurring before the tenth booster immunization. The titer then dropped before rising again in response to the twelfth boost (albeit to a lower level). On average, the BoNT A1-specific immune responses were almost 4 times increased in hens receiving the IL-6 adjuvants compared to animals receiving the immunogen alone (Fig. 4B). Application of the IL 1β showed only a slight difference and an elevation in the specific titer of 1.4x.

4. Discussion

In this present study we generated immunization plasmids coding for the BoNT A1 antigen in toxoid form and codon-optimized for high expression in eukaryotic cells. In addition, analogous immunization vectors expressing the avian cytokines IL-6 and IL-1β were prepared for use as genetic adjuvants. These cytokines are known to affect B-cell maturation and antibody production *in vitro* and *in vivo* (Nishimishi et al. 2005; Weining et al. 1998). Immunoprecipitation and Western blot analyses confirmed protein expression and cytokine secretion from transfected cells. Using the Helios® gene gun, we then carried out immunization studies in hens to compare the different antigen/adjuvant combinations with the classic prime-boost DNA immunization strategy without adjuvant.

The number of studies involving vaccination with plasmid DNA has been increasing steadily in recent decades. Although both the immune responses and the levels of protection induced vary dramatically depending on the animal, the vaccination strategy, the antigen and the vectors used, several studies successfully used vectors coding for immunostimulatory cytokines in combination with the antigen to successfully enhance the immune responses (Chow et al. 1997).

Despite most studies being conducted in mammals, several reports involving DNA immunization of avians (ducks, chickens) have been published (Rollier et al. 2000, Kazimierczuk et al. 2004, Brujeni et al. 2011). Plasmids coding for the target antigen were usually administered intramuscularly, resulting in one case in strong antibody responses to *Helicobacter pylori* urease (Kazimierczuk et al. 2004) and in another, in rather weak responses to bovine interferon-gamma (Brujeni et al. 2011). In contrast, Witkowski and coworkers (2009) achieved high titers of antibodies specific for poxviral proteins by using a gene gun to deliver the plasmids.

In one of the first studies to use interleukin (IL-6) as genetic adjuvant (Cho et al. 2004), the authors immunized chickens intramuscularly with a DNA vector encoding both the K88 fimbrial protein FaeG of *E. coli* and IL-6. Twelve weeks after immunization, the antibody titers in chickens receiving the combination plasmid were significantly higher than in those immunized with the FaeG plasmid alone. Although in our experiments the antigen and adjuvants were encoded on separate vectors, the ensuing levels of specific αBoNT A1 IgY were also enhanced by coexpression of IL-6 as a genetic adjuvant. Despite IL-1β having a weaker impact on the development of specific IgY, titers in IL-1β treated hens was still higher than in the non-adjuvant control group. Similar to our results using IL-6 and IL-1β, it has been recently demonstrated that using genetic adjuvants and antigens in a polycystronic

manner can also lead to a significant enhancement in the adjuvant effect of GM-CSF (Li et al. 2011).

The laying hens used in our study and in recent studies (Pauly et al. 2009a) usually have the capacity to lay 5-7 eggs per week over a period of 72 weeks, after which laying activity decreases continuously and often stops after two years. Surprisingly, hens 100 and 101 maintained a stable laying rate throughout the study period. As the only difference between hens 96/97 and 100/101 was the use of a genetic adjuvant (IL-1β), it is tempting to speculate that IL-1β overexpression improved the laying capacity of the hens. Since IL-1β exerts pleiotropic effects on a wide range of target cells (Weining et al. 1998) such a stimulatory effect on the avian reproductive tract is not inconceivable. However, although interleukins and their corresponding receptors are present in the reproductive tract of hens, very little is known about their functions in this context (Davison et al. 2008).

In contrast, IL-6 expression appeared to be associated with an early cessation of egg production in both hens. Although this may have been caused by the activity of the cytokine, one of the hens (H98) did develop an aberrant anatomy that could have affected its egg production. Despite laying problems, both hens produced the highest titers of αBoNT A1 IgY and the total IgY content of their eggs was comparable to that of other hens in the study. The increased antibody titers were therefore not simply the result of an overall increase in IgY production.

Fluctuations in the levels of tIgY were first observed in 2001 (Schade et al. 2000) and were later studied in more detail (Pauly et al. 2009a). It appears that tIgY is subject to a circaseptan rhythm and multiples thereof. The trend of gradual increasing tIgY for hen 96 is consistent with this and other studies (Pauly et al. 2009a; Le Bris et al. 2005). As expected, there was no difference between the brown and white hens in terms of antibody yields.

In summary, our results demonstrate that DNA immunization by gene gun using a prime and multiple boost regime induced a substantial antibody response to a structurally conserved BoNT A1 toxoid. Co-administration of the avian cytokines IL-1β and especially IL-6 increased mean titers of αBoNT A1 IgY. Although the small size of the study groups (two hens) precludes the drawing of general conclusions, initial evidence suggests that IL-1β overexpression might enhance the frequency and length of the laying period whereas IL-6 might be detrimental. Further experiments involving higher numbers of hens are necessary to further clarify and expand upon these observations.

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Figure Legends

Fig. 1

Expression analysis by Western blot. (A) Evaluation of BoNT A1 expressed in transfected HEK 293T. The left lane contains control BoNT A1-RYM toxoid generated in *E. coli* in a proteolytically uncleaved form (holotoxoid=150 kDa). The second lane contains a lysate of HEK 293T cells transfected with the pTH-BoNT-A1-RYM immunization plasmid showing expression of the non cleaved BoNT holotoxoid (150 kDa). The third lane contains purified active clostridium-derived toxin BoNT A1 (SDS inactivated). The active toxin is cleaved into the BoNT heavy chain (100 kDa) and a 50 kDa light chain. In this case only the heavy chain of BoNT A1 is visualized due to the specificity for the heavy chain of the monoclonal antibody used for detection. The right lane contains a lysate of HEK 293T cells transfected with the empty pTH vector as control. (B) Immunoblot of precipitated IL 1β-V5 from supernatants of cells transfected with the pTH-IL-1β-V5vector or the empty vector control. (C) Immunoblot of IL 6-V5 precipitated from supernatants of transfected HEK 293T cells and the transfected pTH control vector. (B, C) have been developed with an αV5 mAb.

Fig. 2

Comparison of the laying capacities of hens immunized with or without genetic adjuvant. (A) Laying capacities of hens 96-97 immunized with BoNT A1 plasmid only, hens 98-99 receiving BoNT A1 plus IL 6 and hens 100-101 immunized with BoNT A1 plus IL-1β. (B) Mean laying capacities of hens receiving the same immunization. The difference between the laying capacities of hens 96/97 and 100/101 is significant (Mann Whitney test, $P<0.0001$). Due to the laying problems, the mean value for hens 98-99 has not been calculated. The arrows indicate the times of immunizations.

Fig. 3

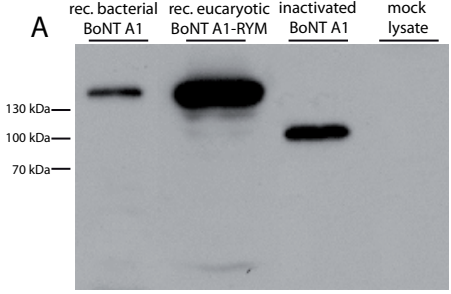
Depiction of the total IgY content per egg yolk. (A) Monitoring of tIgY during the immunization period. Due to early laying problems, the tIgY content for hen 98 was not monitored. (B) Linear regression of the continued increase in tIgY per egg yolk of chicken 96 during the immunization period. The regression coefficient and p-value are given. (C) Mean values and standard deviations of tIgY per hen. (n=number of eggs included in the evaluation)

Fig. 4

Monitoring of the specific αBoNT A1 IgY titers in immunized hens using capture ELISA. (A) Antibody titers in the eggs of each hen immunized with BoNT A1 DNA with and without cytokine adjuvants. A pool of sera (AC29 pool 2) from hens conventionally immunized with BoNT A1 protein was included as positive control. The baseline was set using eggs collected before immunization. Arrows indicate boost immunizations. (B) Mean OD values (495nm/620nm) over the entire study period. The total number of eggs is shown below the columns (A:=Absorbance).

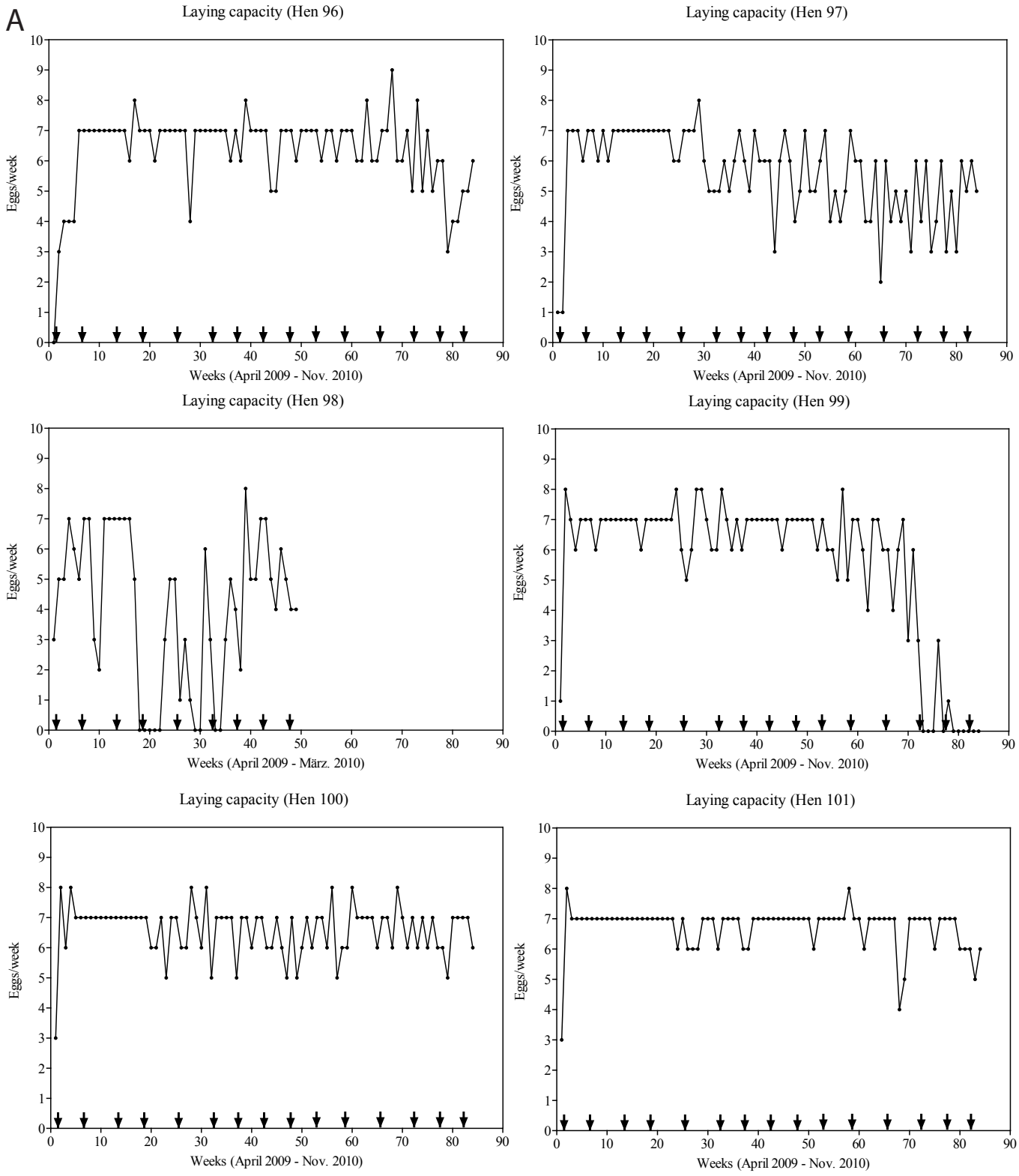
Figure(s)

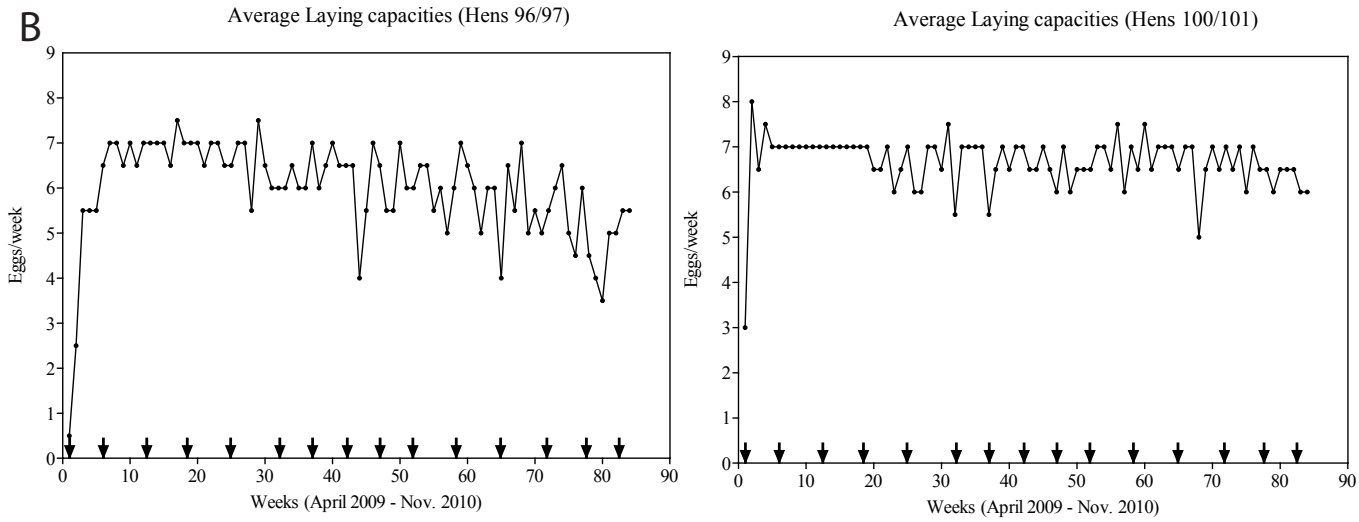
Figure 1



Figure(s)

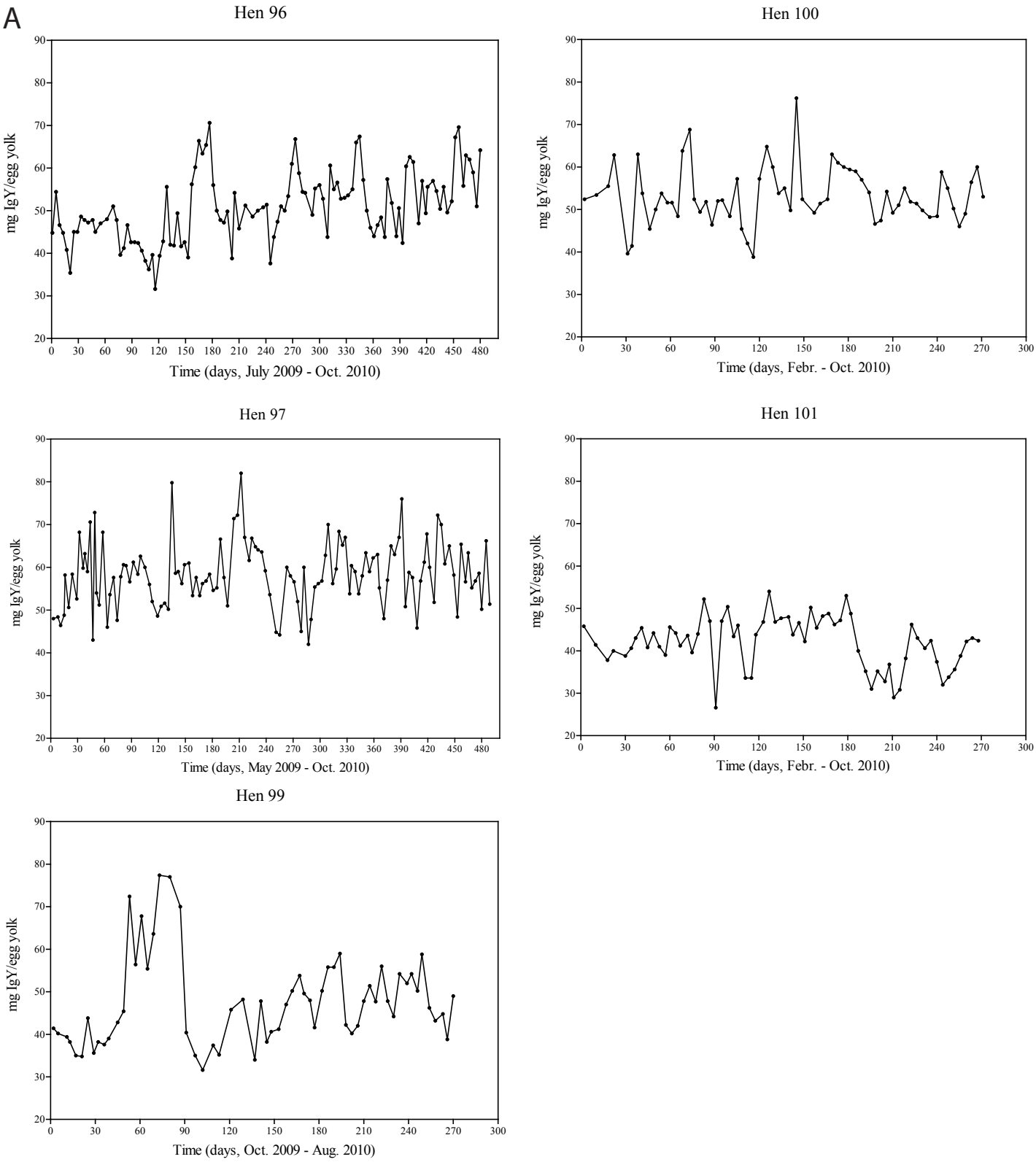
Figure 2





Figure(s)

Figure 3



Figure(s)

Figure 4