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Antigen-dependent effects of divergent selective breeding based on natural antibodies on specific humoral immune responses in chickens

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ABSTRACT

NAb are defined as antigen binding antibodies present without a known previous exposure to this antigen. NAb are suggested to enhance specific antibody (SpAb) responses, but consequences of different NAb levels on immunization are largely unknown. Layer chickens were divergently selected and bred for keyhole limpet hemocyanin (KLH)-binding NAb titers, resulting in a High line and a Low line. In this study, we investigated: (1) the relation of NAb levels with SpAb titers; and (2) the effect of immunization on NAb titers. The 50 highest females of the High line and the 50 lowest females of the Low line of generation 2 were intramuscularly immunized at 33 weeks of age with 1 mL phosphate buffered saline (PBS) containing one of four treatments: (1) negative control (no antigen), (2) 500 μg KLH, (3) 100 μg avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), or (4) 250 μg human serum albumin (HuSA). IgM and IgG titers of NAb and SpAb in plasma were determined prior to immunization and weekly for 5 weeks post immunization by indirect ELISA. In addition, antibody affinity was investigated. No differences in SpAb and NAb response against KLH and PPD were observed as a consequence of different NAb titers, but increased and prolonged SpAb and NAb titer responses against HuSA were observed for the High line compared to the Low line. Different natural antibody titers did not impair SpAb dynamics and SpAb affinity. NAb titers were not, or for only short-term, affected by immunization. We show here that NAb may enhance SpAb responses, but that this effect is antigen-dependent. We hypothesize that NAb play a role in general disease resistance through enhancement of the humoral adaptive immune response.

1. Introduction

Vaccine development and vaccination management successfully contributed to control of high-impact infectious diseases. However, economic losses in poultry production are still considerable [1]. In combination, with new challenges on use of medication and housing conditions [2,3], poultry with a higher general disease resistance is needed. Selective breeding for increased general disease resistance might provide a feasible strategy to reduce disease sensitivity.

Natural antibodies (NAb) are defined as antigen binding antibodies present in individuals without a (known) previous exposure to this antigen [4]. NAb play an essential role in both the innate and adaptive immunity [5,6]. NAb are part of the first line of defense against all types of pathogens (e.g. viruses, bacteria, parasites) in a wide variety of species (e.g. [7–9]). One of the described working mechanisms of NAb is enhancement of antibody responses [5,10]. Due to low antibody affinity and polyspecificity [10], NAb might provide a non-antigen-dependent protection to diseases. Previous studies showed that lower mortality of layer chickens was associated to higher NAb levels binding keyhole limpet hemocyanin (KLH) [11–13]. In addition, NAb are heritable [14]. Selective breeding for NAb could therefore be a feasible strategy in breeding for improving general disease resistance in chicken.

A white layer chicken population was divergently selected, and bred for high and low KLH-binding NAb titers at 16 weeks of age for two generations. This study aimed to investigate: (1) the relation of NAb levels with humoral adaptive immune responses, e.g. specific antibody (SpAb) titers; and (2) the effect of immunization on NAb titers. The 50 highest females of the High line, and the 50 lowest females of the Low line from the second generation of selection were intramuscularly (i.m.) immunized at 33 weeks of age with one of four treatments: (1) negative control (phosphate buffered saline; PBS), (2) the antigen of the selection criterion; KLH,

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(3) a putative T helper 1 (Th1) cell-stimulating antigen; avian tuberculin purified protein derivative of *Mycobacterium avium* (PPD), or (4) a putative Th2 cell-stimulating antigen; human serum albumin (HuSA). IgM and IgG titers in plasma were determined weekly for 5 weeks post i.m. immunization (p.i.) by indirect ELISA. In addition, antibody affinity was investigated. The results are also discussed in light of selection for KLH-binding NAb.

2. Materials and methods

A detailed description can be found in Supplementary Material (SM) Detailed Materials & Methods.

2.1. Ethics statement

The selection and immunization experiments were approved according to Dutch law by the "Dierexperimentencommissie" (Animal Experiment Committee) of Wageningen University (Experiment Codes *Selection*: 2,012,105 & 2,013,091; Experiment Code *Immunization*: 2,014,008).

2.2. Study population

The unselected base population and NAb selection process are described in SM Detailed Material & Methods.

Around 32 weeks of age, 50 High line females with the highest total KLH-binding immunoglobulins (IgT) titer at 16 weeks of age and 50 Low line females with the lowest KLH-binding IgT titer at 16 weeks of age of generation 2 (selected as parents for breeding generation 3) were group housed in 4 pens (3 × 4 m). The females were given 1.5 weeks of acclimatization to the environment. Female within a line were randomly assigned to a pen, resulting in 12 or 13 females per line per pen. Females within a line within a pen were randomly assigned to a treatment, resulting in 3 or 4 females per treatment per line per pen.

2.3. Immunization

Around 33 weeks of age (day 0), all females were i.m. immunized with one of four treatments in 1 mL PBS (randomly assigned within line): 1) only PBS (12 chickens/line), 2) 500 μg KLH (product number H7017, Sigma-Aldrich, St. Louis, MO, USA) (13 chickens/line), 3) 100 μg PPD (Avian Tuberculin PPD 2500, containing 25.00 I.U./ml with 0.5% phenol (w/v), Institute for Animal Science and Health, Lelystad, the Netherlands) (13 chickens/line), 4) 250 μg HuSA (product number A8763, Sigma-Aldrich) (12 chickens/line). Per thigh, 0.5 mL was injected. At day 0 (prior to immunization) and day 7, 14, 21, 28, and 35p.i., plasma samples were collected, and stored at −20 °C until use.

NAb optical density values (OD) were measured for IgT, IgM, and IgG binding KLH at 16 weeks of age, and IgT, IgM, and IgG binding KLH, PPD, and HuSA around 33 weeks of age (at day 0, prior to immunization). SpAb and NAb OD were measured for IgM and IgG binding KLH, PPD, and HuSA at each of the five time points p.i.

2.4. Antibody titers

NAb OD were determined in individual plasma samples by an indirect two-step ELISA as described by [15] with the following additions for PPD-binding NAb OD and HuSA-binding NAb OD: (1) Plates were coated with 16.67 μg/mL PPD for PPD-binding NAb OD or with 4 μg/mL HuSA for HuSA-binding NAb OD. (2) Test dilutions were 1:30, 1:90, 1:270, and 1:810 for HuSA-binding IgG OD. All antibody test dilutions were 1:40, 1:160, 1:640, and 1:2560.

SpAb OD were determined as described for NAb OD, with the following changes: (1) Test dilutions were 1:400, 1:1600, 1:6400, and 1:25,600 for KLH-binding IgM and IgG OD, and HuSA-binding IgG OD, and were 1:500, 1:2,500, 1:12,500, and 1:62,500 for HuSA-binding IgM OD. (2) PPD-binding SpAb OD were determined with the ELISA procedure for determining NAb OD.

Antibody titers were calculated as described by [15,16].

2.5. Antibody affinity estimation

As far as we know, this is the first time antibody affinity has been estimated with this method. More (background) information can be found in SM Antibody Affinity Estimation.

Antibody affinity of a plasma sample was estimated on the OD of all dilutions above the background threshold (with a minimum of two OD above the threshold). Antibody affinity was estimated for NAb on day 0 (prior to immunization), and NAb or SpAb on day 35.

Antibody affinity was estimated by fitting a log-log regression model through the OD values for each sample separately:

$$OD' = \beta_0 + \beta_1 * \log_2\ (dilution)$$

where OD' are the natural logarithms of the OD values of the test dilutions of one sample, β_0 is the intercept, β_1 is the coefficient, and $\log_2$(dilution) is the $\log_2$-value of the test dilutions. The β_1-value represent the antibody affinity, where a higher β_1-value represents a lower antibody affinity. The antibody affinity was estimated with the "=LOGEST()"-function in Microsoft® Excel® 2010 v14.0.7180.5002.

2.6. Statistical analyses

A mixed linear model was used for analyzing NAb titers at 16 weeks of age consisted only of a line effect. This model was extended with a plate effect for analyzing NAb titers at 33 weeks of age (day 0, prior to immunization). The residuals of this extended model were used for estimating Pearson correlation coefficients between (iso)types binding different antigens.

A mixed linear model with repeated observations was used for analyzing SpAb titers, NAb titers, and antibody affinity during the experimental period, which consisted of a line effect, a day effect, a treatment effect (only for NAb titers), all combinations of their interaction effects, and a plate effect.

All statistical analyses were done in SAS v9.3. Significance was declared for P-values ≤ 0.05. The exact number of observations per trait (and statistical model) per time point are shown in Supplementary Material Table 1. In all analyses, the plate effect also accounts for possible differences between pens, because plate and pen were confounded.

3. Results

The number of observations and mean (and SD) per trait per line per time point are shown in Supplementary Material Table 1 and Supplementary Material Table 2.

Descriptive statistics and differences of NAb titers at 16 and 33 weeks of age are shown in Table 1. All KLH-binding, PPD-binding, and HuSA-binding NAb at 16 and 33 weeks of age (day 0, prior to immunization) were significantly higher in the High line compared to the Low line. Titer differences ranged between 1.3 titer points and 4.4 titer points.

Correlation coefficients within IgT and IgG NAb titer residuals were weak and not significant. Correlation coefficients within