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Perspective

Age and genetic selection affect auto-immune profiles of chickens

Henk K. Parmentier *, Elmer Harms, Aart Lammers, Mike G.B. Nieuwland

Adaptation Physiology Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

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ABSTRACT

Specificity, antibody isotype distribution and levels, of natural autoantibodies (NAAb) may be potential informative parameters for immune mediated natural disease resistance, immune modulation, and maintenance of physiological homeostasis. In a previous study we detected IgM and IgG antibodies to liver antigens in plasma from 1 year old chickens. Auto-immune profiles directed towards liver antigens differed between chicken lines divergently selected for specific antibody responses to sheep red blood cells. In the present study we measured the presence and typed levels and antibody isotypes (IgG and IgM) of NAAb binding the 'auto-antigen' complex chicken liver cell lysate (CLL) in plasma samples obtained from chickens at 5 weeks and at 1-year of age, respectively, by quantitative western blotting.

Extensive staining patterns of plasma antibodies binding CLL were found for both isotypes and at both ages in all birds. At both ages, IgM and IgG bound similar numbers of CLL antigens, which remained almost constant for IgM, whereas the number of IgG stained bands in time was enhanced. Significant differences of binding patterns of NAAb (stained antigen fragments of CLL and staining intensity) were detected between the three different chicken lines at both ages and between both ages, and lines could be clustered on the basis of their auto-antibody profile. The present results indicate that analysis of the plasma NAAb repertoire of poultry like in mammals could provide a way of distinguishing differences of immune competence (as reflected by the selection criterion of antibody responses) between individuals and lines, and could provide tools to select individual birds for health and other traits. The age-dependency of the auto-immune profile suggest that such profiles may also reflect immune maturation, which should be taken into account when relating an auto-immune profile with other traits.

1. Introduction

Natural antibodies (NAb) are defined as antibodies present in normal healthy animals under the absence of (previous) deliberate antigenic stimulation or infection (Avrameas, 1991; Baumgarth et al., 2005). Many mammalian NAb were found to bind self antigens (Avrameas, 1991; Lutz et al., 2009; Nagele et al., 2013). These so-called natural auto antibodies (NAAb) are thought to inactivate cytokines, mask auto-antigens, and clear obsolete or damaged cells and metabolic waste (neo-epitopes) as part of anti-tumour surveillance or maintenance of homeostasis (Lutz et al., 2009; Ochsenbein et al., 1999). NAAb are always present in the body albeit lower levels are found in young individuals, but levels may increase with age. In man and mice, quantitative (Western) immunoblotting (Lacroix-Desmazes et al., 1995, 1999; Mouthon et al., 1996; Stahl et al., 2000), or proteomics (Madi et al., 2009; Nagele et al., 2013) were used to analyse the NAAb repertoire of individuals and inbred strains to various tissues like liver, kidney, brain and muscle, or auto-antigen chips. In general, the binding repertoire and levels of IgM binding auto-antigens increased during aging and remained stable later on, resulting in corresponding repertoires between individuals (Stahl et al., 2000). IgM patterns appeared to evolve without exogenous stimulation (Haury et al., 1997), confirming the notion that they are not formed randomly (Hooijkaas et al., 1984). Auto-IgG patterns contained the same bands as IgM with the addition of more specific bands. Since auto-IgG patterns were not expanding with age and remained stable at a young age (Stahl et al., 2000) it was suggested that auto-IgG profiles could represent an 'antibody fingerprinting' of an individual (Francoeur, 1988). Different auto-antibody binding profiles in different murine inbred strains (Nobrega et al., 1993) showed a genetic component underlying the NAAb repertoire. Recently, it was proposed that IgG autoantibodies function as an adaptive mechanism to clear cellular debris (Nagele et al., 2013).

Earlier we reported both IgM and IgG auto-reactivity to chicken liver lysate (CLL) in plasma obtained from 1 year old chickens (De Jong et al., 2013). The auto-antibody profiles were different between chicken lines that were divergently selected for specific agglutination responses to sheep red blood cells (SRBC) or phenotypically

Abbreviations: CLL, chicken-liver-cell-lysate; NAAb, natural (auto-) antibodies.

* Corresponding author at: Wageningen University, Department of Animal Sciences, Adaptation Physiology Group, P.O. Box 338, 6700 AH Wageningen, The Netherlands. Tel.: +31317483120; fax: +31317485006.
E-mail address: Henk.Parmentier@WUR.nl (H.K. Parmentier).

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selected for high or low natural antibody (NAb) levels to keyhole limpet hemocyanin (KLH). The former indicated a genetic component underlying the auto-antibody repertoire, whereas the latter suggested a relation between NAb levels binding KLH and the autoimmune repertoire. In addition, regardless of genotype or phenotype, birds were individually characterized by their IgG auto-immune repertoire. In the present study we determined the presence and levels of antibody isotypes IgM and IgG in plasma of chickens from the two lines that were divergently selected for high (H line) or low (L line) specific antibody agglutination titres to SRBC at 5 days after subcutaneous immunization with SRBC at 5 weeks of age, next to a random bred control (C line). From the same individual birds plasma samples were obtained at 5 weeks of age prior to immunization with SRBC and at 1 year of age. The selection lines used in the present study differed in almost every aspect of specific and innate immune responsiveness. Higher NAb and NAAb levels were found at all ages in the H line as compared with the C and L lines (Parmentier et al., 2004). The purpose of our study was twofold, first we tested and compared line differences in binding profiles of the isotypes IgM and IgG to the auto-antigen CLL both at a young age and later on. Second we correlated and grouped the staining patterns of IgM and IgG antibodies at both ages per line using principal component analysis. Our data suggest first that groups (in the current study genotypes) at both ages or individual birds at both ages can be characterized by auto-antibody profiles, and second that autoimmune profiles are affected by aging as has been indicated for man previously (Madi et al., 2009, 2011; Nagele et al., 2013). This offers opportunities to relate auto-antibody profiles not only with genotypes and phenotypic traits such as immune responses, disease resistance and (metabolic) disorders in chickens at a given age, but also to study individual immune maturation as well.

2. Materials and methods

2.1. Plasma samples

Plasma was obtained at 5 weeks of age prior to immunization with SRBC, and at 1 year of age from 45 ISA-Brown medium heavy laying hens. Birds were kept under normal housing conditions with free roaming. The hens were from three different lines, which were either divergently bred during 29 generations for high (H line) or low (L line) primary (agglutinating) antibody responses at day 5 after primary intramuscular immunization with SRBC at 37 days of age, or a random bred control (C line) resembling the original parental stock. These lines represented genotypic high (H), genotypic low (L) hens, and genotypic control (C) hens. Average anti-SRBC agglutination (2log dilution) titres at 5 days after immunization with SRBC at 5 weeks of age were 13, 6, and 0 for the H, C, and L lines, respectively. Anti-SRBC agglutination titres prior to immunization with SRBC were 0 in all lines.

2.2. Reagents

Chicken liver cell lysate (CLL) was made by freezing 0.5 g liver tissue from a 5 week old C line hen in liquid nitrogen. The liver was lysed with 5 ml lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 2% Glycerol, pH 7.4) with addition of 2.5 ml protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The mixtures were centrifuged for 15 min (13,500 *g*) after which the supernatant containing CLL was kept at –20 °C until use.

2.3. Natural auto-antibody profiles

Western blot analysis was used for the determination of natural (auto-) antibody binding profiles to CLL in plasma of 45 hens obtained at 5 weeks and at 1 year of age, respectively. First, CLL was diluted 1:30 and incubated 1:1 v/v with sample buffer (1:20 β-mercapthoethanol and Laemmli sample buffer (BIORAD, Hercules, CA, USA)) at 95 °C for 5 min. One aliquot was made and used for all the experiments to ensure the same quality of the sample. The CLL was separated on molecular weight in a Miniprotean Tetra cell (BIORAD) with 12.5% SDS-PAGE (sodium dodecyl sulphate-polyacrylamid gel electrophoresis) under reducing (β-mercapthoethanol) conditions. Molecular weight markers consisted of Precision Plus Dual Color Protein™ Standards (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa (BIORAD)).

After separation on molecular weight, fragments were blotted to a polyvinylidene fluoride membrane (PVDF, BIORAD) with a semi-dry transfer gel blotting system (Trans-blot SD, BIORAD) for 10 min at 10 V (2.5 A) for two blots simultaneously. Blots were incubated overnight at 4 °C in blotting buffer (PBS, 5% normal horse serum, and 0.05% Tween), or for 1 h on the rocking table at room temperature.

Blots were incubated with plasma samples in a 16 lane miniblotter (Immunetics, Boston, MA, USA). Each lane was filled with 200 μL 1:40 diluted plasma or dilution fluid (PBS, 0.5% normal horse serum and 0.05 % Tween) to fill up empty lanes (negative control). As a positive control, one lane of normal rooster plasma was added. The miniblotter rotated for 1 h at room temperature. Thereafter, the blots were rinsed with dilution fluid five times for 10 minutes on the suspension mixer. Next, the second antibody (conjugate) was added to the blots and incubated for 1 h on the suspension mixer. The following conjugates to detect NAAb isotypes were used: IgM: 1:500 diluted rabbit anti-chicken IgM coupled to PO, and IgG: 1:500 diluted rabbit anti-chicken IgG_{fc} coupled to PO all from Nordic, Tilburg, The Netherlands). After washing, bands were visualized with the Opti-4CN substrate kit (BIORAD) according to manufacturer's instructions.

2.4. Analyses

To analyse the number of bands and the staining intensities, CLL blots were scanned with a flatbed scanner and saved as a tif file. The TotalLab v2006 software program (NonLinear Dynamics) was used, with a minimum slope of 75 and a linear log curve for identification of stained fragments, measurement of pixel intensities, calculations and graphs. Bands between 10 and 100 kDa were considered reliable, and fragments outside this range were not analysed. To detect significant differences of the number of CLL fragments stained, and the extinction values between the binding profiles of the plasma antibodies from the three chicken lines to CLL, after decoding of the samples, a one-way ANOVA for line was performed separately on the number of stained fragments and the extinction values of the fragments for both ages. In addition a two-way ANOVA was done to test the effect of line and age and their interaction using the bird nested within line option. Analyses were done with SAS 9.2 (proc GLM) (SAS Institute, 1990). The level of significance used was $P < 0.05$. Mean differences between lines (contrasts) were tested with Bonferroni's test. Clustering of lines on binding profiles of the two antibody isotypes to CLL was done by principal component analysis (PCA) on log-transformed extinction data using the CANOCO package for Windows (Leps and Smilauer, 2003).

3. Results

3.1. Western blot profiles

Western blot profiles were generated for the two isotypes IgM and IgG using plasma of all birds at both ages. In Fig. 1 representative blots are shown for each of these isotypes, where each similar numbered lane represents an individual bird at a given age.