QTL for natural antibodies in two population laying hens.

M. Siwek^{1*}, A.J. Buitenhuis³, S. J. B. Cornelissen¹, M. G. B. Nieuwland², H.Bovenhuis¹, H.K.Parmentier², R. P. M. Crooijmans¹, M. A. M. Groenen¹, J.J.van der Poel¹

¹Animal Breeding and Genetics Group, ² Adaptation Physiology Group, Wageningem Insitute of Animal Sciences, Wageningen University, P.O. Box 338, NL – 6700AH Wageningen, The Netherlands, ³Danish Institute of Agriculture Sciences, Dept.of Animal Breeding and Genetics, Foulum PO BOX 50, DK 8830 Tjele Denmark.

ABSTRACT

The aim of this study is to identify QTL associated with levels of natural antibodies in two population of laying hens. One experimental (H/L) F2 population originates from a cross (ISA Warren) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to sheep red blood cells (SRBC) at 5 weeks of age. Another experimental (F/P) F2 population was created from a cross of laying hens differing for behavioral traits. For the genotypic analysis, in both populations, Genome Scan has been performed using 174 microsatellite markers, equally distributed over the chicken genome. Total antibody titers to lipopolisacharide (LPS) and lipoteicholic acid (LTA) were determined by ELISA. LPS and LTA represent "homotopes" antigenic determinants of gram negative and gram positive bacteria. Paternal half-sib and line-cross analysis models were used to detect QTL involved in response to these two homotopes. There seem little overlap in QTL between two population and both homotopes.

INTRODUCTION

Immune defense consist of "innate" and "adaptive" immunity. Innate immunity is represented by various natural barriers such as skin, physiological factors such as pH, temperature, oxygene tension, lysozyme and complement and phagocytic cells. The "specific" parts of the innate immune system are formed by natural antibodies (NAb) which are present in non-immunized individuals. NAbs do not require induction of B-cells by on purpose antigenic challenge or mitogenic stimulation. NAb have low binding affinity and a broad specificity repertoire [1, 2]. In mammals, NAb are mainly of the IgM isotype, however, also IgA and IgG have been reported [3, 4] and are probably involved in early clearance of foreign material. The amount of NAbs increases with the age of the individual. The level of NAbs may be enhanced by either polyclonal stimulation by exogenous microbes by initiating responses of auto-reactive B cells or correspond with the secretion of naturally occurring auto – reactive B cell clones. NAbs probably enhance processes of antigen uptake and antigen presentation via dendrites or B cells. It is very likely that NAbs are present in chickens [5]. To date, chicken antibodies binding ovarian antigens [6] and MHC class IV were reported [7,8].

Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) act as homotopes. Homotopes are microbial stimulators of the innate immune system. LTA is shared by gram positive bacteria, whereas LPS is shared by gram negative bacteria. Specific cellular and humoral immune responses depend on the previous activation of the innate immune system. The crucial role of homotopes is polarization of the specific immune system. Homotopes bind to Toll - like receptors (TLR's), receptors of Heat-shock proteins (HsP), complement component C3receptors, and Fcy - receptors on phagocytes and Natural Killer cells which act as pattern recognition receptors (PRR). In general, PRR are expressed on cells of the innate immune system that first encounter pathogens during infection. In mammals, homotopes upon binding to PRR induce expression of different cytokines such as tumor necrosis factor (TNF) - α, IL-6, IL-12, IL-15 and type 1 interferons by Antigen Presenting Cells (APC). LPS induce IL-10 release, and enhance expression of ICAM-1, CD119 (y- interferon receptor) and MHC molecules on epithelial cells [11], whereas LTA induce the release of other cytokines like TNF-α, IL-2, IL-1β, IL-6, IL-12, IL-18, and enhance antibody responses to parentally administered antigen [10, 11, 12, 13]. In general, LPS acts through binding to TLR4 and inducing the release of IL1 or INFy and is associated with TH1 type of inflammatory responses. Whereas LTA acts through binding to TLR2 and induces the release of IL4, IL13 stimulating TH2 type of antibody responses.

The innate immunity is thought to be the initiator of the specific immune response, the current research was focused on the innate/adaptive immunity relation by addressing following questions:

1. Can QTL for Nabs level be detected????

2. when QTL are detected, do they differ for different homotopes and at different age ? To answer these question an experiment was set up in which three points were taken into consideration: 1. Ab responses were measured to two different homotopes (LTA and LPS), which are known to stimulate different pathway of adaptive immune responses and to which high levels of antibody levels are present in chickens [9] 2. Two unrelated chicken populations with different genetic background: one selected for specific antibody responses (H/L population) the second selected for production traits (FP population) were used 3. Abs responses were measured at different ages to establish the age influence on the level of Nabs.

MATERIAL AND METHODS

The first population was the H/L F_2 population that originated from a cross (ISA Warren, medium heavy layers) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to SRBC. Selection was based on the individual antibody titer at 5 d after primary intramuscular immunization with SRBC at 37 d of age [16]. Reciprocal crosses with birds from the 18th generation were made to generate F_1 animals. From the F_1 generation, an intercross was made to produce 672 individuals in 6 hatches of the F_2 experimental population.

The second population was the feather pecking (FP) F_2 population which was created from a cross between two commercial lines of layers as described by Buitenhuis et al. [17]. In brief, reciprocal crosses were made to create F_1 animals. Seven half-sib families were created to obtain 630 F_2 female animals. The F_2 chicks were hatched in 5 hatches at 2 wk intervals. For both populations all birds were housed in brooder cages with free access to water and feed (152 g/kg CP and 2,817 kcal/kg ME). Birds were not beak-trimmed and each individual bird was marked with a wing-band. All birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at hatch, and 2 and 15 d of age, respectively.

Phenotyping of the F₂ H/L and FP Populations.

The Ab levels of LPS and LTA in serum from all the birds were determined by ELISA at 5 wks and 18 wks of age for H/L population and 38 wks of age for FP population. Briefly, 96 well plates were coated with 4 μ g/mL of LPS or 10 μ g/mL LTA. After subsequent washing with tap water, 0.05% Tween, the plates were incubated with serial dilution of serum. Binding of Ab to LPS and LTA antigen was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase. After washing, tetramethylbenzidine and 0.05% H₂O₂ were added and incubated for 10 minutes at room temperature. The reaction was stopped with 2.5N H₂SO₄. Extinctions were measured with Multiscan at wavelength of 450 nm. Titers were expressed as the log₂ values of the highest dilution giving a positive reaction.

Genotyping of the F₂ H/L and FP Populations.

Genomic DNA was isolated using the Gentra Generation Capture PlateTM Kit from the whole blood according to the Capture PlateTM Kit protocol (Gentra Systems, Minneapolis, USA). In total, 208 microsatellite markers were chosen for both populations. For the analysis, only the informative markers for each population were chosen: 170 for H/L population and 180 microsatellite markers for FP population. The genotyping procedure was as already described by Siwek et al. [14, 15]. All genotypes were checked twice. Finally the data was checked for non-Mendelian inheritance using the CRI-Map [18]. All together, 718 animals from generations F_0 (28 individuals), F_1 (18 individuals), and F_2 (672 individuals) of H/L population, and 689 from generations F_0 (24 individuals), F_1 (35 individuals) and F_2 (630 individuals) of FP population were genotyped.

QTL analysis

To estimate the heritabilities for LPS and LTA and their genetic correlations, uni- and bi- variate analyses were performed using an animal model and the ASREML software package [19]. For these analyses the following mixed model was used: $Y = X\beta + Z\mu + e$ where Y is a vector of observations, X is the design matrix for the fixed effects, β is the vector of fixed effects, Z is the design matrix for random effects, u is the random effects with var(u) = $A\sigma_a^2$, and e is the residual with var (e) = $I\sigma_e^2$. The fixed effects for the H/L population are: hatch and sex, and hatch for the FP population.

Statistical analysis.

The analyses were performed separately for both experimental populations. Prior to the QTL analysis, phenotypic data were adjusted for the systematic: hatch (for FP population) or hatch and sex (for H/L population) effects using the PROC GLM Procedure [20]. For the FP population: $Y_{ij} = \mu + H_i + e_{ij}$

For the H/L population: $Y_{ijk} = \mu + H_i + S_j + e_{ijk}$

Where Y_{ij} or Y_{ijk} is the phenotypic value, μ is the grand mean, H_i is the effect of the i_{th} hatch (i = 1, 2,..., 5 for FP population, and i = 1, 2,..., 6 for the H/L population, respectively); S_j is the sex effect of jth individual; and e_{ij} or e_{ijk} represents residual effects. The hatch is referred to a group of individuals hatched at one time.

Regression interval mapping was used for QTL detection. Two different genetic models were used: 1) paternal half-sib analysis [21, 22] and 2) line-cross analysis model [23]. In the paternal half-sib model no assumptions are made concerning the allele frequencies in the founder lines and number of QTL alleles. The F_2 animals are treated as number of unrelated half-sib families using the model:

$Y_{ij} = m_i + b_i p_{ij} + e_{ij}$

where Y_{ij} is the trait score of individual j, originating from sire i; m_i is the average effect for half-sib family i; b_i is the substitution effect for a putative QTL; p_{ij} is the conditional probability for individual j of inheriting the first paternal allele, and e_{ij} is the residual effect.

In the line-cross model, the power of QTL detection depends on the degree of fixation of QTL alleles for the trait of interest in the founder lines. In this model the alternative alleles at the QTL are traced back to the founder lines. At every centi Morgan across the genome the following model is fitted:

 $Y_j = m + ax_{aj} + dx_{dj} + e_j$

where Y_j is the adjusted trait score of animal j, m is the population mean, a and d are the estimated additive and dominant effect of a putative QTL at the given location, x_{aj} is the conditional probability of animal j carrying both alleles from the same line, x_{dj} is the conditional probability of being heterozygous at given location, and e_j is the residual error.

Significance thresholds were determined empirically by permutation [24] 1). chromosome wide which take into account multiple testing on the specific chromosome; 2). suggestive linkage: one false positive is expected in a genome scan and 3). genome-wide significant where 5% risk of false positive is assumed in a genome scan [25]. Data permutation, with at least 10,000 replicates, was used to determine the empirical distribution of the test statistic under the null hypothesis of no QTL associated with the chromosome under study.

RESULTS AND DISCUSSION

Half – sib analysis model.

H/L Population

For the antibody response to LPS at 5 wks of age a suggestive QTL was detected on GGA8 (38cM). For the antibody response to LPS at 18 wks of age a suggestive QTL was detected on GGA3 (106cM).

For the antibody response to LTA at 5 wks of age a suggestive QTL was detected on GGA3 (66cM). A suggestive QTL on GGA10 (56cM) was detected for the antibody response to LTA at 18 wks of age.

FP Population

Two suggestive QTL were detected for the antibody response to LPS at 38 wks of age. A first QTL was detected on GGA8 (42cM). A second QTL was detected on GGA18 (17cM). Two QTL were detected for the antibody response to LTA at 38 wks of age. A significant QTL was detected on GGA3 (276cM). A suggestive QTL was detected on GGA14 (1cM).

Line – cross analysis model

H/L Population

Two suggestive QTL were detected for antibody response to LPS at 5 wks of age. A suggestive QTL was detected on GGA4 (194cM). The second suggestive QTL was detected on GGA12 (76cM). Four QTL were detected for antibody response to LPS at 18 wks of age. A significant QTL was detected on GGA1 (184cM). Three suggestive QTL were detected. A first QTL was detected on GGA4 (207cM), the second QTL was detected on GGA7 (77cM), the third QTL was detected on GGA18 (47cM).

For the antibody response to LTA at 5 wks of age, two suggestive QTL were detected. The first QTL was detected on GGA2 (193 cM), a second QTL was detected on GGA3 (184 cM). A suggestive QTL was detected for antibody response to LTA at 18 wks of age on GGA2 (184 cM).

FP Population

A suggestive QTL was detected on GGA27 (25 cM) for the antibody response to LPS at 38 wks of age. For the antibody response to LTA at 38 wks of age two QTL were detected. A significant QTL was detected on GGA14 (51 cM). A suggestive QTL was detected on GGA7 (78 cM).

In conclusion we suggest that immune responses to both homotopes are under different genetic control. Both populations described in this study were specifically set up for QTL mapping of traits other than Nabs. In this study the pedigree structure is not optimal for the estimation of genetic parameters due to the limited number of animals. This will result in a large standard error on the estimates. Nevertheless, the results presented here give an indication that there is a genetic effect on the regulation of Nabs. In general, the majority of detected QTL are different . Firstly, the QTL are different for the two homotopes LPS and LTA which means the known different nature of LTA and LPS. The suggested different immunomodulatory features of LPS and LTA in opposite directions were already confirmed [32] in the founder lines of current H/L population. Secondly, QTL identified for both homotopes differ at both ages. The exception is a QTL on GGA2 for LTA level which is shared at 5 wks and 18 wks of age what this suggests that some genes related to the immune response/ immune regulation at both ages are located on GGA2. The results of QTL analysis, both models, suggest that line cross analysis model is more powerful in H/L population compared to FP cross. Higher number of QTL under the line cross analysis model was detected for H/L population, compared to FP

cross. This result indicates that selection for specific antibody response (SRBC) influence non specific antibody response (Nabs).

This study on the genetic architecture of NAbs in chicken has provided evidence that the NAb level in chicken has a genetic component and different QTL were detected.

The presented QTL are the first step in the identification of the underlying genes controlling the immune responses in chicken.

ACKNOWLEDGEMENTS

The staff of the experimental farm 'De Haar' is kindly acknowledged for taking good care of hens. This project was funded by the Netherlands Technology Foundation (STW) project WBI 48.

REFERENCES

[1] Casali P, Notkins AL CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol Today 1989; 10:364–8.

[2] Dacie JV Occurrences in normal human sera of incomplete forms of cold autoantibodies. Nature 1950; 166:36.

[3] Boes M Role of natural and immune IgM antibodies in immune responses. Mol. Immunol. 2000; 37:1141-9.

[4] Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, Zinkernagel RM Control of early viral and bacterial distribution and disease by natural antibodies. Science 1999; 286:2156–9

[5] Jalkanen S, Granfors K, Jalkanen M, Toivanen P Immune capacity of the chicken bursectomized at 60 hours of incubation: failure to produce immune, natural, and autoantibodies in spite of immunoglobulin production. Cell Immunol 1983; 80:363–373.

[6] Barua A, and Yoshimura Ovarian autoimmunity in relation to egg production in laying hens. Reproduction. 2001; 121:117-22.

[7] Longenecker BM, Mosmann TR Natural antibodies to chicken MHC antigens are present in mice, rats, humans, alligators and allogeneic chickens. Immunogenetics 1980; 11:293–302.
[8] Neu N, Hala K, Wick G Natural chicken antibodies to red blood cells are mainly directed against the B-G antigen, and their occurrence is independent of spontaneous autoimmune thyroiditis. Immunogenetics 1984; 19:269–77

[9] Parmentier HK, Lammers A, Hoekman JJ, De Vries Reilingh G, Zaanen IT, Savelkoul HF. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. Dev. Comp. Immunol. 2004; Jan 28:39-49.

[10] Maassen CB, van Holten-Neelen C, Balk F, den Bak-Glashouwer MJ, Leer RJ, Laman JD, Boersma WJ, Claassen E Strain-dependent induction of cytokine profiles in the gut by orally administered Lactobacillus strains. Vaccine. 2000; 22:2613-23

[11] Blum S, Alvarez S, Haller D, Perez P, Schiffrin EJ Intestinal microflora and the interaction with immunocompetent cells. Antonie Van Leeuwenhoek. 1999; 76:199-205

[12] Miettinen M, Vuopio-Varkila J, Varkila K Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun. 1996; 64:5403-5

[13] Haller D, Blum S, Bode C, Hammes WP, Schiffrin EJ. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria in vitro: evidence of NK cells as primary targets. Infect Immun. 2000; 68:752-9.

[14] Siwek M, Cornelissen SJB, Nieuwland MGB, Buitenhuis AJ, Bovenhuis H, Crooijmans RPMA, Groenen MAM, de Vries-Reilingh G, Parmentier HK, van der Poel JJ Detection of

QTL for immune response to Sheep Red Blood Cells in laying hens. Anim. Gen. 2003A; 34:422-8. [15] Siwek M, Buitenhuis AJ, Cornelissen SJ, Nieuwland MG, Bovenhuis H, Crooijmans RP, Groenen MA, de Vries-Reilingh G, Parmentier HK, van der Poel JJDetection of different quantitative trait loci for antibody responses to keyhole lympet hemocyanin and Mycobacterium butyricum in two unrelated populations of laying hens. Poult Sci. 2003B; 82:1845-52. [16] Van der Zijpp AJ, Nieuwland MGB Immunological characterization of lines selected for high and low antibody production. 7th European Poultry Conference Paris 1986; 1:211-5. [17] Buitenhuis AJ, Rodenburg TB, van Hierden Y, Siwek M, Cornelissen SJB, Nieuwland MGB, Crooijmans RPMA, Groenen MAM, Koene P, Korte SM, Bovenhuis H, van der Poel JJ Mapping Quantitative Trait Loci Affecting Feather Pecking Behaviour and Stress Response in Laying Hens. Poult.Sci. 2003; 82:1215 -22 [18] Green P, Falls K, Crooks S Documentation for Cri-Map Version 2.4 (3/26/90) Washington University School of Medicine, St. Louis, Missouri. 1990 [19] Gilmour AR, Cullis BR, Welham SJ, Thompson R ASREML Reference Manual, NSW Agriculture, Orange, Australia. 2000 [20] SAS Institute SAS[®] User's Guide: Statistics, Version 5 edition, SAS Institute INC., Cary,NC. 1995 [21] Knott SA, Elsen JM, Halev CS Methods for multiple – marker mapping of quantitative trait loci in half-sib population. Theor. Appl. Genet. 1996; 93:71-80 [22] De Koning DJ, Janss LLG, Rattink AP, Van Oers PAM, De Vries BJ, Groenen MAM, van der Poel JJ, De Groot PN, Brascamp EW, Arendonk JAM Detection of quantitative trait loci for backfat thickness and intramuscular fat content in pigs (Sus suscrofa). Genetics 1999; 152:1679-90 [23] Haley CS, Knott SA, Elsen JM Mapping quantitative trait loci between outbred lines using least squares. Genetics 1994; 136:1195 – 1207 [24] Churchill GA, Doerge RW Empirical threshold values for quantitative trait mapping. Genetics 1994; 138:963-71

[25] Lander ES, Kruglyak L Genetic dissection of complex traits:

Guidelines for interpreting and reporting linkage results. Nat. Genet. 1995; 11:241-

7