Improving animal health is a major goal of the animal breeding industry (Soller and Anderson, 1998). Genetic enhancement of the immune response can increase vaccine efficacy and disease resistance (Lamont et al., 2002). Single nucleotide polymorphisms (SNP) may be utilized to improve antibody production by marker assisted selection, if the allele effect associated with antibody response can be estimated (Zhou and Lamont, 2003a).

Candidate genes selected for studying immune response traits may have known physiological functions with immune response or be in regulatory or biochemical pathways affecting immune response (Zhou and Lamont, 2003a). After QTL are mapped to a chromosomal region, positional candidate genes can be identified among the genes mapped to that region or by comparative genomic analysis with regions of conserved synteny in other species (Rothschild and Soller, 1997) For the present study, four candidate genes, natural resistance associated macrophage protein 1 (SLC11A1 or NRAMP), prosaposin (PSAP), interferon Gamma (IFNG), and toll-like receptor 4 (TLR4), were investigated, based upon prior reports of association with immune response in chickens (Zhou et al., 2001; Kramer et al., 2003; Malek et al., 2004).

Prosaposin (PSAP) is a multifunctional protein encoded by a single-copy gene. It contains four domains occurring as
tandem repeats connected by linker sequence (Hazkani-Covo et al., 2002). Prosaposin was a positional candidate because of its proximity to a microsatellite marker that is linked to SE vaccine antibody response and SE bacterial burden; early resistance to SE burden in chicks is associated with a SNP in PSAP (Liu and Lamont, 2003).

Interferon-γ (IFNG) is a pleiotropic cytokine involved in most phases of immune and inflammatory responses, and has been shown to prime heterophil functional activities (Kogut et al., 2005). Associations between IFNG promoter polymorphisms and chicken primary and secondary antibody response to SRBC and Brucella abortus (BA) have been reported (Zhou et al., 2001). A recent report (Ye et al., 2006) demonstrated associations of an IFNG promoter SNP with infectious bursal disease virus vaccine response in broilers.

Toll-like receptors (TLRs) are a family of genes whose proteins are the main sensors utilized by the innate immune system to detect invading pathogens. Werling and Thomas (2003) showed that TLRs are crucial not only in the early phase of infection when innate immunity is important, but also link innate and adaptive immunity through the entire course of the host defense response. The TLR4 in the chicken has been associated with response to SE (Leveque et al., 2003). Dil and Qureshi (2002) demonstrated that TLR4 expression is required for production of lipopolysaccharide-mediated inducible nitric oxide in chicken macrophages. The relative number of TLR4 receptor molecules on the macrophage surface varies between chicken strains, thereby varying the level of LPS binding among strains (Dil and Qureshi, 2002). The goal of the current study was to identify associations of SNP in four genes with antibody response kinetics and body weight in chickens.

**MATERIALS AND METHODS**

Resource population, antigen administration, and sample collection

An F1 population resulted from mating genetically distinct, highly inbred lines.

One sire from each MHC-congenic Fayoumi line (M5.1 and M15.2) was mated with nine dams of the Leghorn G-B1 line. The F2 population resulted from intercrossing between one male and eight females within each F1 line. The F2 population consisted of 71 females from the M5.1 grandsire and 87 from the M15.2 grandsire. The two separate lineages of the F2 population genetically differed only in the MHC alleles contributed by the two MHC-congenic Fayoumi grandsires (Zhou et al., 2001). All tested F2 birds were from one hatch.

Body weights were measured at 2, 6, 12, and 20 wk of age. At 19 and 23 wk of age, F2 chickens were injected intramuscularly with SRBC and BA (Zhou et al., 2001). Blood samples were obtained from each bird pre-immunization, at 7 d after primary immunization, and at 4, 7, 10, 18, 32, and 63 d after secondary immunization. Sera were collected after centrifuging the blood samples and were stored at -20°C until all assays were run simultaneously. The SRBC and BA antibodies were assayed by micro agglutination (Zhou et al., 2001).

**Sequence polymorphism analysis of the SLC11A1, PSAP, IFNG, and TLR4 genes**

Chicken genomic DNA was isolated from venous blood collected in ethylenediaminetetraacetic acid (EDTA). The PCR was performed in a total volume of 25 μl, containing 25 ng of genomic DNA, 5 pmol of each oligonucleotide primer, 2.5 μl of 10× PCR reaction buffer, 1.5 mM MgCl2, 200 μM of each dinucleotide triphosphate and 1 unit of Taq DNA polymerase (Promega Corporation, Madison, WI). Cycle parameters were: 94°C for 3 min, followed by 39 cycles of 93°C for 45 s, optimal annealing temperature for 45 s (Table 1), and 72°C for 35 s, with a final extension step of 10 min at 72°C.

The PCR was carried out with genomic DNA from 6 birds from the highly inbred lines (2 from each line) to produce product to detect sequence polymorphisms. The PCR products were purified using ExoSAP-IT solution.

**Table 1.** Primers used to identify polymorphisms within the natural resistance associated macrophage protein 1 (SLC11A1), prosaposin (PSAP), interferon gamma (IFNG), and toll-like receptor 4 (TLR4) genes

| Gene | Primer sequences | PCR product (bp) | Annealing temperature |
|------|------------------|------------------|----------------------|
| SLC11A1 | Forward: 5’- GCACGATGCCCACCCCTTG-3’ | 642 bp | 64°C |
| | Reverse: 5’- GGGACATTGCTGGCGTCAGT-3’ | | |
| PSAP | Forward: 5’-AGATGGACTTGGGGGAAACT-3’ | 647 bp | 60.1°C |
| | Reverse: 5’-TGGATGTGGCCTAACCTCTT-3’ | | |
| IFNG | Forward: 5’-AGCCGGGTATTGCTATTGTG-3’ | 396 bp | 60°C |
| | Reverse: 5’-GCTGCTATGACCACTGCAAA-3’ | | |
| TLR4 | Forward: 5’-GTGCTGTCTCGAGGTGGTTGTA-3’ | 676 bp | 60°C |
| | Reverse: 5’-GGAGGAAGGGAATCATCAA-3’ | | |
(USB Corporation, Cleveland, Ohio). Nucleotide sequencing was performed by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences (n = 12 per gene fragment, from 6 birds DNA sequenced in both directions) were analyzed using BioEDIT Sequence Alignment Editor Software version 7.0.5.2 (Tom Hall Ibis Therapeutics, Carlsbad, CA). The SNP were selected for analysis at sites where the alleles were shared between Fayoumi sires and different than the alleles in Leghorn dams. The restriction enzyme sites in these sequences were detected by NEBcutter V2.0 (http://tools.neb.com/NEBcutter2).

**Genotyping the F2 population**

The DNA was isolated from F2 blood samples using a Gentra DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). PCR was performed as above. The PCR products were then digested overnight at 37°C with 1 U NlaIII, 55°C with 1 U BstI, 37°C with 1 U HpyCH4III, and 37°C with 1.5 U XbaI, respectively (New England Biolabs, Inc., Beverly, MA). Individual PCR-RFLP fragment sizes were determined based on standard DNA molecular weight markers.

**Statistical analysis**

The analysis of antibody response to SRBC and BA were separately conducted by antigen and by phase (primary and secondary). For the SRBC and BA primary phase, the single time-point measurement taken at 7 d postprimary immunization (Y) was used. Secondary phase parameters of maximum (Ymax) and minimum (Ymin) titers and time needed to achieve maximum (Tmax) and minimum (Tmin) titers were estimated from seven individual time points, from days 4 to 63 after secondary immunization, by using a nonlinear regression model (Weigend et al., 1997). This procedure yielded a total of five antibody kinetic parameters: primary level, and four parameters for secondary level including Ymin, Tmax, Tmin, and BW at 2, 6, 12, and 20 wk). The data were analyzed separately for F2 progeny from the different MHC-congenic grandsires because the MHC haplotype has been found in many previous studies to impact antibody production (Zhou et al., 2001; Zhou and Lamont, 2003b; Malek et al., 2004). Genotypes were designated as Leghorn homozygote (LL), heterozygote (LF), and Fayoumi homozygote (FF). General linear model tests for association between genotype and antibody response parameters were conducted using the JMP program (Sall and Lehman, 1996). While both fixed and random effects are considered in the preliminary statistical model, the insignificant effects and the two-way SNP interaction effects of p≥0.1 were eliminated from each final model. The models were:

\[
\text{M5.1 cross } Y_{ijklm} = \mu + SLC11A1i + PSAPj + IFNGk + TLR4l + SLC11A1i \times TLR4l + SLC11A1i \times IFNGk + e_{ijklm}
\]

\[
\text{M15.2 cross } Y_{ijklm} = \mu + SLC11A1i + PSAPj + IFNGk + TLR4l + TLR4l \times IFNGk + SLC11A1i \times IFNGk + e_{ijklm}
\]

where \( Y_{ijklm} \) was defined as each dependent trait (Y, Ymax, Ymin, Tmax, Tmin, and BW at 2, 6, 12, and 20 wk).

**RESULTS**

**Sequence variation and PCR-RFLP restriction site selection**

For the SLC11A1 gene, a 642 bp product was amplified and sequenced. There were two SNP between the Leghorn and both Fayoumi lines (T/G at base 314, and G/A, at base 434). The T/G SNP at base 314 was used for genotyping the F2 hens. The restriction enzyme NlaIII, which has a unique restriction site at base 309 in the Fayoumi lines, was used to genotype the F2. For the PSAP gene, a 647 bp product was sequenced. There was a SNP between the Leghorn and both Fayoumi lines (G/A at base 425). The restriction enzyme BstI was used to differentiate between Leghorn and Fayoumi lines with a unique restriction site at base 377 for Leghorn line. For the IFNG gene, a 396 bp product was sequenced. There were two SNP between the Leghorn and both Fayoumi lines (C/T at base 172 and A/G at base 214). The C/T SNP at base 172 was used for analysis after digestion with HpyCH4III. This enzyme has a unique restriction site at base 170 in the Fayoumi line. For the TLR4 gene, a 676 bp product was sequenced. There were two SNP between Leghorn and both Fayoumi lines (T/G at base 477 and T/A at base 609). The T/G SNP at base 477 and XbaI, which cut at a restriction site in only the Leghorn line, was used for this analysis.

**Association of gene polymorphisms with antibody response**

The current study tested the effects of one SNP in each of four unlinked genes. To account for the multiple test comparisons, a p value of p<0.0125 (0.05 divided by 4, the number of tested genes) was considered the most relevant threshold for declaring significance of association tests. Additionally, consistent patterns of significance at p<0.05 within any specific variable are taken as supportive of an association.

The IFNG SNP had strong associations with three parameters of antibody production in the F2 hens: Tmin to BA (p<0.004) in the M5.1 lineage, level of primary response to SRBC (p<0.008), and Ymin to BA (p<0.004) in the M15.2 lineage (Table 2). For each of the other three
tested genes, only single instances of significance with antibody traits were p<0.05, and none were p<0.0125. No individual two-way interaction effects were significant at p<0.0125. Pair-wise interactions for variables for which p<0.05 for at least one trait are shown in Table 2.

**Effect of allele on antibody response**

The allelic effects of the IFNG gene SNP on antibody response in both F2 lineages are presented for the 3 traits with associations of significance at p≤0.0125 (Table 3). In the M5.1 lineage, the hens that were homozygous for the Leghorn SNP (IFNG-LL) required a significantly longer time to reach the minimum antibody response to BA than the other two allelic combinations. For the M15.2 lineage, the mean primary antibody response to SRBC of hens with IFNG-LL was significantly higher than the Fayoumi homozygote. The heterozygote hens for the IFNG polymorphism had significantly higher Ymin to BA than both homozygotes.

**Association of the gene polymorphisms with body weight**

The IFNG SNP was highly significantly associated (p<0.005) with BW at 12 wk of age in the M5.1 F2 cross. Additionally, 3 of the 7 remaining tests of association of IFNG polymorphism with BW at various ages in the two lines were p<0.05. No other genes were associated with BW in the F2 hens.

**Effect of allele on body weight**

The allelic effects of IFNG polymorphisms on BW at 2, 6, and 12 wk of age are presented in Table 4. For BW at 2 and 12 wk, F2 hens of the M5.1 cross which inherited the homozygous Fayoumi (IFNG-FF) genotype had significantly lower BW than the other two allelic combinations. For BW at 2 and 6 wk, F2 hens of the M15.2 cross with the homozygous Leghorn (IFNG-LL) genotype had higher BW than the other two allelic combinations.
Resistance to most diseases is likely controlled by several genes. Molecular genetic approaches can be used to investigate polygenic control of immune response and disease resistance, and the resulting understanding of the genetic basis of polygenic control of immune response may be utilized to improve chicken health (Lamont, 1998). In the current study, associations of DNA polymorphisms in four immune-related genes with antibody response kinetics to SRBC and BA, and BW in an F2 population were determined and the major finding was the association of a novel IFNG promoter-region SNP with multiple traits of immune response and with BW in the F2 population.

There were three SNP detected previously in the IFNG promoter region in the same F2 population as used in the current study, (Zhou et al., 2001). The current study analyzed a new region within the IFNG promoter region, to expand knowledge on polymorphisms and effects of SNP in this gene’s promoter. Identifying two additional SNP in the 396 bp sequenced showed the IFNG promoter region to be highly polymorphic, much more than the average of 5 SNP per kb between chicken lines (Wong et al., 2004). Previous studies on the same F2 population demonstrated that a different IFNG promoter SNP than that characterized in the current study was associated with the level of circulating IFNG protein level after both primary and secondary immunization (Zhou et al., 2002). The circulating IFNG protein level was associated with SRBC antibody response traits, suggesting that the expressed protein level may be one mechanism by which IFNG gene polymorphisms modulates the immune response (Zhou et al., 2002). In the same lines as were used to establish the F2 population evaluated in the current study, IFNG mRNA levels were increased in Salmonella enteritidis-infected chicks compared to non-infected chicks, providing additional support for a role of this gene’s expression in immune response (Cheeseman et al., 2006).

Zhou et al. (2001) characterized, on the same F2 population, the effect of a different IFNG SNP than that analyzed in the current study. They found effects on level of primary response to SRBC, and Tmax and Ymax to BA (Zhou et al., 2001), which are different than the effects reported in the current study. The detection of different effects on antibody response of closely located SNP indicates that it is worthwhile to characterize the specific effects of multiple SNP in regions dense with genetic regulatory elements such as promoters.

The effects of the IFNG SNP differed between the two F2 lineages, which were founded by different MHC-congenic sires. Because the only difference between the genetics of the two F2 lineages is expected to be in the MHC contribution of the grandsires, the different effects detected between the F2 lineages suggests an interaction of the IFNG SNP effect with the MHC alleles. This result may occur through the IFNG protein level, which is the main macrophage activating cytokine, by altering the expression of MHC antigens that modulate the immune response (Janeway and Travers, 1997).

A recent study of the effects of an IFNG SNP on various traits in broiler chickens (Ye et al., 2006) demonstrated effects on mortality, and immune-related (infectious bursal disease vaccine antibody) and growth-related (BW at 40 d and feed conversion ratio) traits. Therefore, it was of interest to explore the effect of the IFNG SNP on BW, as well as the antibody traits, in the current study. The IFNG SNP was associated with 12-wk BW in the M5.1 lineage in the current study, with the birds that were homozygous for the Fayoumi allele being significantly lighter than the other two genotype classes. This genotype also had a more rapid immune response, with the Fayoumi homozygotes for the IFNG reaching the Tmin to BA significantly faster than the Leghorn homozygotes. In the Ye et al. (2006) study, the same IFNG SNP allele that was associated with higher BW and lower mortality, was associated with lower antibody response to infectious bursal disease vaccine. Cumulatively, these studies and others (Siegel and Gross, 1980; Martin et al., 1990; Mashaly et al., 2000) indicate a negative association between the effect of gene polymorphisms on BW and their effects on antibody response parameters, perhaps due to the elevation in metabolic rate of the high immune response genotype. Fifty percent of the elevation in metabolic rate during an infection is estimated to be attributed to the energetic cost of the immune protein synthesis (Borel et al., 1998). Negative nitrogen balance is typical during an immune reaction, with inflammatory cytokines and glucocorticoids mediating this energy-dependent proteolytic response in skeletal muscles.
In summary, the current study identified new SNP within the SLC11A1, IFNG, and TLR4 genes and expands previous research on associations of IFNG promoter-region polymorphisms and antibody kinetics. The effect of the IFNG gene polymorphisms on both antibody response kinetics and BW emphasizes the importance of evaluating the effect of genetic variation on multiple phenotypic systems, as negative pleiotropic effects may be deleted.

ACKNOWLEDGMENTS

The authors gratefully acknowledge William Larson and the Poultry Research Center crew at Iowa State University for managing the birds. Huaijun Zhou and Steffen Weigend are thanked for their excellent work on earlier stages of this project. Jason Hasenstein is thanked for his perfect technical support. The author thanks Dr. Susan J. Lamont, for serving as mentor for the author while he conducted this work in her laboratory as a visiting Fulbright scholar at Department of animal science, Iowa State University. Financial support for this project was from Animal Health, Hatch Act, and State of Iowa Funds. The Binational Fulbright Commission provided the scholarship to the author.

REFERENCES

Blackwell, J. M., T. Goswami, C. A. W. Sibthorpe, N. Papo, J. K. White, S. Searle, E. N. Miller, C. S. Peacock, H. Mohammed and M. Ibrahim. 2001. SLC11A1 (formerly NRAMP1) and disease resistance. Cell. Microbiol. 3:773-784.

Cellier, M., A. Belouchi and P. Gros. 1996. Resistance to intracellular infections: comparative genomic analysis of NRAMP. Trends Genet. 12:201-204.

Cheeseman, J. H., M. G. Kaiser, C. Ciraci, P. Kaiser and S. J. Lamont. 2006. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without Salmonella enteritidis infection. Dev. Comp. Immunol. 31: 52-60.

Dil, N. and M. A. Qureshi. 2002. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 84:191-207.

Hazkani-Covo, E., N. Altman, M. Horowitz, and D. Graur. 2002. The evolutionary history of Prosaposin: Two successive tandem-duplication events gave rise to the four saposin domains in vertebrates. J. Mol. Evol. 54:30-34.

Janeway, C. A. and P. Travers. 1997. Immunobiology, The Immune System in Health and Disease, 3rd ed. Current Biology Ltd., London, UK.

Kogut, M. H., L. Rothwell and P. Kaiser. 2005. IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of Salmonella enterica serovar enteritidis. J. Interferon Cytokine Res. 25:73-81.

Kramer, J., M. Malek and S. J. Lamont. 2003. Association of twelve candidate gene polymorphisms and response to challenge with Salmonella enteritidis in poultry. Anim. Genet. 34:339-348.

Lamont, S. J., M. G Kaiser and W. Liu. 2002. Candidate genes for resistance to Salmonella enteritidis. Vet. Immunol. Immunopathol. 87:423-428.

Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredo-Osti, K. Morgan and D. Malo. 2003. Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica Serovar Typhimurium infection in chicken. Infect. Immun. 71:1116-1124.

Liu, W., M. G. Kaiser and S. J. Lamont. 2003. Natural resistance-associated macrophage protein1 gene polymorphisms and response to vaccine against or challenge with Salmonella enteritidis in young chickens. Poult. Sci. 82:259-266.

Liu, W. and S. J. Lamont. 2003. Candidate gene approach: potential association of Capase-1, Inhibitor of Apoptosis Protein-1, and Prosaposin gene polymorphism with response to Salmonella enteritidis challenge or vaccination in young chicks. Anim. Biotechnol. 14:61-76.

Lochmiller, R. L. and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: Just what is the cost of immunity? OIKOS. 88:87-98.

Malek, M., J. R. Hasenstein and S. J. Lamont. 2004. Analysis of chicken TLR4, CD28, MIF, MD-2, and LITAF genes in a Salmonella enteritidis resource population. Poult. Sci. 83:544-549.

Martin, A., E. A. Dunnington, W. B. Gross, W. E. Briesle, R. W. Briesle and P. B. Siegel. 1990. Production traits and alloantingen systems in lines of chickens selected for high or low antibody response to sheep erythrocytes. Poult. Sci. 69:871-878.

Maresh, M. M., M. J. W. Heetkamp, H. K. Parmentier and J. W. Schrama. 2000. Influence of genetic selection for antibody production against sheep red cells on energy metabolism in laying hens. Poult. Sci. 79:519-524.

Rothschild, M. F. and M. Soller. 1997. Candidate gene analysis to detect genes controlling traits of economic importance in domestic livestock. Probe 8:13-20.

Siegell, P. B. and W. B. Gross. 1980. Production and persistence of antibody in chicken to sheep erythrocytes. 1. Directional selection. Poult. Sci. 59:1-5.

Soller, M. and L. Andersson. 1998. Genomic approaches to the improvement of disease resistance in farm animals. Rev. Sci. Tech. 17:329-345.

Wiegem, S., N. Mielzen and S. J. Lamont. 1997. Application of nonlinear regression function to evaluate the kinetics of antibody response to vaccine in chicken lines divergently selected for multitrait immune response. Poult. Sci. 76:1248-1255.

Werling, D. and T. W. Jungi. 2003. Toll-like receptors linking innate and adaptive immune response. Vet. Immunol. Immunopathol. 91:1-12.
Ye, C. Chen, N. Wei, L. Dong, F. Lan, Y. Sun, Z. Yang, Y. Yu, Y. Huang, D. He, Y. Xi, D. Wei, Q. Qi, W. Li, J. Shi, M. Wang, F. Xie, X. Zhang, P. Wang, Y. Zhao, N. Li, N. Yang, W. Dong, S. Hu, C. Zeng, W. Zheng, B. Hao, L. W. Hillier, S. P. Yang, W. C. Warren, R. K. Wilson, M. Brandstrom, H. Ellegren, R. P. Crooijmans, J. J. van der Poel, H. Bovenhuis, M. A. Groenen, I. Ovcharenko, L. Gordon, L. Stubbs, S. Lucas, T. Glavina, A. Aerts, P. Kaiser, L. Rothwell, J. R. Young, S. Rogers, B. A. Walker, A. van Hateren, J. Kaufman, N. Bumstead, S. J. Lamont, H. Zhou, P. M. Hocking, D. Morrice, D. J. de Koning, A. Law, N. Bartley, D. W. Burt, H. Hunt, H. H. Cheng, U. Gunnarsson, P. Wahlberg, L. Andersson, K. Institutet, E. Kindlund, M. T. Tammi, B. Andersson, C. Webber, C. P. Ponting, I. M. Overton, P. E. Boardman, H. Tang, S. J. Hubbard, S. A. Wilson, J. Yu and H. Yang. 2004. A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature 432:717-722.

Ye, X., S. Avendano, J. C. M. Dekkers and S. J. Lamont. 2006. Association of twelve immune-telated genes with performance of three broiler lines in two different hygiene environments. Poult. Sci. 85:1555-1568.

Zhou, H., A. J. Buitenhuis, S. Weigend and S. J. Lamont. 2001. Candidate gene promoter polymorphisms and antibody response kinetic in chicken: interferon-gamma, interleukin-2, and immunoglobulin light chain. Poult. Sci. 80:1679-1689.

Zhou, H. and S. J. Lamont. 2003a. Chicken MHC class I and II gene effects on antibody response kinetic in adult chicken. Immunogenetics 55:133-140.

Zhou, H. and S. J. Lamont. 2003b. Association of six candidate genes with antibody response kinetic in hens. Poult. Sci. 82:1118-1126.

Zhou, H., H. S. Lillehoj and S. J. Lamont. 2002. Associations of interferon-γ genotype and protein level with antibody response kinetics in chickens. Avian Dis. 46:869-876.