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Associations of Polymorphisms in Four Immune-related Genes with Antibody Kinetics and Body Weight in Chickens

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ABSTRACT : Four biological candidate genes, natural resistance associated macrophage protein 1 (*SLC11A1* or *NRAMP*), prosaposin (*PSAP*), interferon Gamma (*IFNG*), and toll-like receptor 4 (*TLR4*), were examined to identify single nucleotide polymorphisms (SNP) and associations of the SNP with antibody response kinetics in hens. An F_2 population was produced by mating G_0 highly inbred (<99%) males of two MHC-congenic Fayoumi lines with highly inbred Leghorn hens. The F_2 hens (n = 158) were injected twice with SRBC and whole, fixed *Brucella abortus* (BA). Blood samples were obtained before each immunization, at 7 d after primary immunization, and at several time points after secondary immunization. Minimum titers (Ymin) and the time needed to reach them (Tmin), and maximum (Ymax) titers and the time needed to reach them (Tmax), were estimated from the seven post-secondary immunization titers using a non-linear regression model. The F_2 hens were genotyped for the four candidate genes by using PCR-RFLP for one SNP per gene, which identified the parental allele. General linear models were used to test associations of SNP genotypes with antibody response parameters and BW measured at 4 ages. The *IFNG* SNP was highly significantly ($p<0.0125$) associated with primary response to SRBC, Tmin to BA, Ymin to BA, and 12-week BW. The current study demonstrated that the novel *IFNG* promoter SNP was associated with antibody kinetics for BA and SRBC in laying hens, and also with BW, suggesting that this cytokine may play a pivotal role in the relationship between immune function and growth. (**Key Words :** Antibody Kinetics, Sheep Red Blood Cells, *Brucella abortus*, Interferon Gamma, Body Weight)

INTRODUCTION

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, 2003b). 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*), prosaposin (*PSAP*), interferon-γ (*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).

Natural resistance associated macrophage protein 1 (*SLC11A1* or *NRAMP1*) is a membrane phosphoglycoprotein of macrophages (Cellier et al., 1996). The SCL11A1 has a range of effects on macrophage activation including modulation of MHC expression, production of pro-inflammatory cytokines and chemokines and the production of antimicrobial effectors such as nitric oxide and oxidative burst (Blackwell et al., 2001). Association of a SNP polymorphism in a high conserved region of SCL11A1 with *Salmonella enteritidis* (SE) vaccine and pathogen challenge response in young chicken has been reported (Liu et al., 2003).

Prosaposin (*PSAP*) is a multifunctional protein encoded by a single-copy gene. It contains four domains occurring as

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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 *TLR* 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 F_1 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 F_2 population resulted from intercrossing between one male and eight females within each F_1 line. The F_2 population consisted of 71 females from the M5.1 grandsire and 87 from the M15.2 grandsire. The two separate lineages of the F_2 population genetically differed only in the MHC alleles contributed by the two MHC-congenic Fayoumi grandsires (Zhou et al., 2001). All tested F_2 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, F_2 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 $MgCl_2$, 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'- GCACGATGCCCACCCTTG- 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'- GTGCTGCTCAGTGGGTGTAA- 3'	676 bp	60°C
	Reverse	5'- GGAGGAAGGCAATCATCAAA-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 F_2 population

The DNA was isolated from F_2 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 I U *Bsl1*, 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, Tmin, Ymax, and Tmax.

Data were analyzed separately for F_2 progeny from the two 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 $p \geq 0.1$ were eliminated from each final model. The models were:

$$\text{M5.1 cross } Y_{ijklm} = \mu + SLC11A1_i + PSAP_j + IFNG_k + TLR4_l + SLC11A1_i \times TLR4_l + SLC11A1_i \times IFNG_k + e_{ijklm}$$

$$\text{M15.2 cross } Y_{ijklm} = \mu + SLC11A1_i + PSAP_j + IFNG_k + TLR4_l + TLR4_l \times IFNG_k + SLC11A1_i \times IFNG_k + e_{ijklm}$$

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 F_2 hens. The restriction enzyme *NlaIII*, which has a unique restriction site at base 309 in the Fayoumi lines, was used to genotype the F_2. 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 *BslI* 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 F_2 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

Table 2. Main effects of natural resistance associated macrophage protein 1 gene (SLC11A1), prosaposin gene (PSAP), interferon gamma gene (IFNG), and toll-like receptor 4 (TLR4) polymorphisms on chicken antibody parameters of primary and secondary phases in F_2 females of an inbred cross

Gene	SRBC					*Brucella abortus*				
	Y[1]	Tmin[2]	Ymin[3]	Tmax[4]	Ymax[5]	Y	Tmin	Ymin	Tmax	Ymax
M5.1 cross line										
SLC11A1	0.94	0.94	0.70	0.78	0.29	0.36	0.02	0.86	0.32	0.76
PSAP	0.62	0.76	0.21	0.72	0.18	0.10	0.16	0.22	0.46	0.44
IFNG	0.18	0.64	0.45	0.21	0.29	0.04	0.004	0.52	0.20	0.15
TLR4	0.75	0.70	0.62	0.23	0.47	0.25	0.50	0.55	0.03	0.48
SLC11A1*TLR4	0.17	0.64	0.96	0.03	0.21	0.04	0.60	0.45	0.17	0.57
SLC11A1*IFNG	0.76	0.80	0.96	0.79	0.13	0.13	0.02	0.29	0.11	0.17
M15.2 cross line										
SLC11A1	0.57	0.61	0.17	0.87	0.11	0.59	0.94	0.05	0.54	0.47
PSAP	0.95	0.24	0.14	0.15	0.30	0.54	0.10	0.06	0.37	0.30
IFNG	0.008	0.55	0.51	0.64	0.29	0.07	0.89	0.004	0.40	0.91
TLR4	0.42	0.14	0.47	0.06	0.07	0.63	0.47	0.76	0.75	0.77
SLC11A1*IFNG	0.70	0.32	0.85	0.88	0.41	0.09	0.97	0.05	0.72	0.29
TLR4*IFNG	0.22	0.56	0.61	0.80	0.20	0.33	0.75	0.01	0.65	0.79

[1] Primary antibody response. [2] Time required to reach minimum secondary antibody titers.
[3] Minimum secondary antibody titers. [4] Time required to achieve maximum secondary antibody titers.
[5] Maximum secondary antibody titers.

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 F_2 lineages are presented for the 3 traits with associations of significance at $p\leq0.0125$ (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 F_2 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 F_2 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 6 and 12 wk, F_2 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, F_2 hens of the M15.2 cross with the homozygous Leghorn (*IFNG*-LL) genotype had higher BW than the other two allelic combinations.

Table 3. Mean antibody parameters, by interferon gamma (IFNG) genotype, to SRBC and *Brucella abortus* in F_2 progeny of M5.1 and M15.2 grandsires

Gene	Trait	M5.1 cross line			M15.2 cross line		
		LL	LF	FF	LL	LF	FF
IFNG	Y SRBC[1] (titer log2)	5.50^{a}	5.60^{a}	4.44^{a}	5.26^{a}	4.80^{ab}	4.04^{b}
IFNG	Tmin BA[2] (day)	8.40^{a}	1.96^{b}	1.19^{b}	1.98^{a}	1.78^{a}	1.33^{a}
IFNG	Ymin BA[3] (titer log2)	7.19^{a}	4.04^{a}	6.53^{a}	6.55^{b}	6.87^{a}	6.73^{b}

[a, b] Means in a row within a grandsire MHC type with no common superscript differ significantly ($p\leq0.05$).
[1] Primary antibody titer to SRBC. [2] Time required to reach minimum secondary antibody titers to BA.
[3] Minimum secondary antibody titers to BA.

Table 4. Mean body weight (g), by interferon gamma (IFNG) genotype, of F_2 birds

Gene	Trait	M5.1 cross line			M15.2 cross line		
		LL[1]	LF[2]	FF[3]	LL	LF	FF
IFNG	BW 2 wk	109.4[a]	106.3[a]	105.3[a]	105.8[a]	103.2[b]	101.2[b]
IFNG	BW 6 wk	347.5[a]	333.1[a]	317.5[b]	340.6[a]	328.1[b]	316.8[b]
IFNG	BW 12 wk	814.2[a]	793.4[a]	755.5[b]	809.6[a]	823.0[a]	789.1[a]

[a,b] Means in a row within a grandsire MHC type with no common superscript differ significantly ($p \leq 0.05$).
[1] LL = Leghorn homozygote. [2] LF = heterozygote. [3] FF = Fayoumi homozygote.

DISCUSSION

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 F_2 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 F_2 population.

There were three SNP detected previously in the *IFNG* promoter region in the same F_2 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 F_2 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 F_2 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 F_2 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 F_2 lineages, which were founded by different *MHC*-congenic sires. Because the only difference between the genetics of the two F_2 lineages is expected to be in the MHC contribution of the grandsires, the different effects detected between the F_2 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

(Lochmiller and Deerenberg, 2000).

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.

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