Animal Science Publications   Animal Science

2002

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Abstract

Although previous studies have demonstrated an association between interferon-gamma (IFN-gamma) promoter genotype and antibody response kinetics in chickens, the protein levels that may mediate such a gene-trait association have not been determined. The objective of this study, therefore, was to determine the correlation of circulating IFN-gamma levels with both the IFN-gammaIFN-gamma promoter polymorphisms and antibody response in order to evaluate the potential role of IFN-gamma protein in mediating genetic control of antibody response in chickens. Antibody response after Salmonella enteritidis (SE) vaccination at day 10, antibody response to sheep red blood cells (SRBCs) and killed Brucella abortus after immunizations at 19 wk and 22 wk, and serum IFN-gamma protein level were measured in an F2 population derived from inbred lines. A single nucleotide polymorphism in the IFN-gamma promoter region was associated with IFN-gamma protein expression as measured by an enzyme-linked immunosorbent assay after both primary and secondary immunizations. Higher IFN-gamma protein level was correlated with higher antibody level to SE and with increased maximum level and decreased time to reach the maximum secondary antibody response to SRBCs. These results suggest that one of the mechanisms by which promoter polymorphism of IFN-gamma affects antibody production in chickens may involve the circulating level of IFN-gamma protein.

Keywords

interferon gamma, antibody response, protein level, promoter polymorphism, inbred lines

Disciplines

Agriculture | Animal Sciences | Genetics and Genomics | Poultry or Avian Science

Comments

This article is from *Avian Diseases* 46 (2002): 869.

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Associations of Interferon-γ Genotype and Protein Level with Antibody Response Kinetics in Chickens

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SUMMARY. Although previous studies have demonstrated an association between interferon-gamma (IFN-γ) promoter genotype and antibody response kinetics in chickens, the protein levels that may mediate such a gene–trait association have not been determined. The objective of this study, therefore, was to determine the correlation of circulating IFN-γ levels with both the IFN-γ promoter polymorphisms and antibody response in order to evaluate the potential role of IFN-γ protein in mediating genetic control of antibody response in chickens. Antibody response after *Salmonella enteritidis* (SE) vaccination at day 10, antibody response to sheep red blood cells (SRBCs) and killed *Brucella abortus* after immunizations at 19 wk and 22 wk, and serum IFN-γ protein level were measured in an F_2 population derived from inbred lines. A single nucleotide polymorphism in the IFN-γ promoter region was associated with IFN-γ protein expression as measured by an enzyme-linked immunosorbent assay after both primary and secondary immunizations. Higher IFN-γ protein level was correlated with higher antibody level to SE and with increased maximum level and decreased time to reach the maximum secondary antibody response to SRBCs. These results suggest that one of the mechanisms by which promoter polymorphism of IFN-γ affects antibody production in chickens may involve the circulating level of IFN-γ protein.

RESUMEN. Asociación del genotipo del interferón-γ y los niveles de proteína con la cinética de la respuesta de anticuerpos en pollos.

Aunque estudios anteriores han demostrado que existe una relación entre el genotipo del promotor del gen del interferón-γ (IFN-γ) y la cinética de la respuesta de los anticuerpos en pollos, los niveles de proteína que pueden mediar dicha asociación por tendencia genética no han sido determinados. Por tanto, el objetivo de este estudio fue la determinación de la correlación entre los niveles circulantes de IFN-γ con el polimorfismo del promotor del gen y la respuesta de los anticuerpos, para así estudiar el papel potencial de la proteína IFN-γ en la mediación del control genético de la respuesta de los anticuerpos en pollos. Se midió la respuesta de los anticuerpos en las aves después de la aplicación de vacunas contra la *Salmonella enteriditis* a los 10 días de edad, y después de la aplicación de eritrocitos de ovejas y la vacunación mediante el uso de una cepa inactivada de *Brucella abortus* a las 19 y 22 semanas, y también se midieron los niveles séricos de IFN-γ en una población F_2 derivada de líneas de aves de alta consanguinidad. El polimorfismo de un solo nucleótido en el promotor del gen que codifica por el IFN-γ fue asociado a la expresión de dicha proteína, lo cual fue medido mediante la técnica de inmunoensayo ligado a enzimas (ELISA) después de inmunizaciones primarias y secundarias. Se pudieron correlacionar niveles más altos de IFN-γ con niveles más altos de anticuerpos contra la *S. enteriditis,* así como con niveles máximos de anticuerpos más altos y menor tiempo en alcanzar niveles máximos de los mismos en la respuesta secundaria contra eritrocitos de oveja. Estos resultados sugieren que uno de los mecanismos por los cuales el polimorfismo del promotor del gen del IFN-γ afecta los niveles de producción de anticuerpos en pollos puede deberse a los niveles circulantes de la proteína IFN-γ.

Interferon-gamma (IFN-γ), a virus inhibitory molecule, was first reported to be induced from human leukocytes by phytohemagglutinin in 1965 (35). IFN-γ, also known as type II interferon, is an important immunoregulatory protein that exerts multiple effects on the development, maturation, and function of the immune system. IFN-γ acts as a mild inhibitor of proliferation for most cell types, whereas it stimulates the proliferation of mitogen-activated primary T cells as well as a variety of T-cell lines (36). The properties of IFN-γ include regulation of several aspects of the immune response, stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, orchestration of leukocyte–endothelium interactions, effects on cell proliferation, and apoptosis (4,5,7,13,20,30). IFN-γ also mediates immunoenhancement against tumors, bacteria, viruses, and parasites (8,10,18,25,26,27,31,40).

An antiviral factor is produced by chicken thymocytes and spleen cells after stimulation with mitogens (34). This factor has been defined as chicken IFN-γ in 1995 (9). Chicken IFN-γ is biochemically and functionally homologous to mammalian IFN-γ; however, the chicken IFN-γ gene has been cloned and characterized, and amino acid sequence identity with humans is only 35.2% (9,19). Chicken IFN-γ plays a primary role in many economically important poultry disease and antibody response (17).

A single nucleotide polymorphism (SNP) in the promoter of the IFN-γ gene was found between two highly inbred chicken lines. This sequence polymorphism was previously associated with primary and secondary antibody response to both sheep red blood cell (SRBC) and *Brucella abortus* (BA) antigen in an F_2 population derived from these two inbred lines (38). Resistance to a wide range of diseases may be provided by selection for correlated antibody response to foreign proteins (6). Understanding relationships between general humoral immune response and disease resistance will be very beneficial for commercial breeding populations, which cannot be exposed to infectious agents.

Salmonella enteritidis (SE) is one of a group of emerging foodborne zoonotic poultry pathogens, which is a public health problem to humans in the world. The special biology of the SE infection in the avian host makes standard control measures to SE very difficult. The transmission of SE is not only vertical from parent to chick but also horizontal through environment (28).

An *in vitro* virus neutralization assay to measure chicken IFN-γ production during cell-mediated immunity requires the use of live virus (33). To avoid use of live virus, a simple, specific, and sensitive *in vitro* chicken IFN-γ assay utilizing mouse monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) was developed to measure IFN-γ levels in serum (37). The association of the IFN-γ promoter-region polymorphism with circulating IFN-γ protein level and with SE antibody as well as associations between circulating IFN-γ protein levels and antibody response to SRBCs and BA are unknown. In the present study, a monoclonal antibody–based ELISA was used to investigate associations among IFN-γ promoter mutation, antibody response to SE, circulating IFN-γ protein levels, and antibody response kinetics to SRBCs and BA in chickens.

MATERIALS AND METHODS

Experimental animals. Genetically distinct, highly inbred (>99%) chicken lines, the leghorn G-B1 and MHC-congenic Fayoumi M15.2 and M5.1 lines, were used as parental lines (39). One sire from each Fayoumi line was mated to nine dams each of the G-B1 line to produce an F_1 generation. From F_1 offspring of each Fayoumi sire, four sires and eight dams were used to produce 158 females of an F_2 generation. The two separate branches, therefore, of the F_2 population reflect the two MHC congenic Fayoumi sires, M5.1 and M15.2 (38).

Antigen administration and sample collection. At 10 days of age, birds of the F_2 population were vaccinated with SE, and blood samples were collected 11 days after vaccination. At 19 and 23 wk of age, the same chickens were injected intramuscularly with SRBCs and BA (38). Blood samples were obtained from the peripheral vein of the wing of each bird preceding each immunization, at 7 days after primary immunization, and at 4, 7, 10, 18, 32, and 63 days after secondary immunization. Sera were collected after centrifuging the blood samples and stored at −20 C until all assays were run simultaneously.

SE antibody and agglutination assays. The SE antibodies were measured by a commercial ELISA with modification as described by Kaiser *et al.* (16). This ELISA is a competitive assay, thus antibody response to SE was adjusted as follows: Antibody level = $1 - (Sample_{OD630}/Neg_{OD630})$, where $Sample_{OD630}$ is the sample optical density (OD) measurement at 630 nm and Neg_{OD630} is the mean of the OD measurements at 630 nm of the three negative controls. The transformed data represented the relative SE antibody level in serum. The SRBC and BA antibodies were assayed by agglutination (29).

IFN-γ ELISA. Chicken sera collected at 7 days after primary and secondary immunizations were used to measure circulating IFN-γ protein levels by the direct binding ELISA because significantly elevated levels of serum IFN-γ were detected around day 7 postinfection with *Eimeria maxima* (37). Flat-bottom, 96-well microtiter plates were coated with 60 μl of sera in 40 μl of 0.1 M sodium carbonate buffer, pH 9.6, for 18 hr at 4 C, blocked with 200 μl of phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumin for 1 hr at room temperature, and washed three times with PBS containing 0.05% Tween-20 (PBS-T), pH 7.2. One hundred microliters of anti-chicken IFN-γ monoclonal antibody (Mab) (YC1) was added, incubated 18 hr at 4 C, and washed three times with PBS-T, and bound antibody was detected at 450 nm with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin (Ig)G (H+L) (Sigma, St. Louis, MO) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride by an automated microtiter plate reader (37). For each

plate, two reference chicken sera and five chicken IFN-γ MAb plasmids were measured simultaneously as controls. Plasmid assay measurements were used to validate the efficacy of the assay system. Circulating IFN-γ protein levels in serum were adjusted by reference samples as follows because of variations between different measurements: Protein level = $Sample_{OD450}/Ref_{OD450}$, where $Sample_{OD450}$ is the sample OD measurement at 450 nm and Ref_{OD450} is the mean of the two reference chicken serum control OD measurements at 450 nm.

Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) for the chicken IFN-γ promoter region. There were three base pair mutations in the chicken IFN-γ promoter region between the leghorn and both Fayoumi lines. A SNP at site -318 was evaluated, by PCR-RFLP assay with restriction enzyme digestion in the F_2 population (38). The three levels of IFN-γ genotype were defined as homozygote from leghorn allele (LL), heterozygote of both leghorn and Fayoumi alleles (LF), and homozygote from Fayoumi allele (FF).

Statistical analysis. The analyses of antibody response were conducted separately by antigen (SE, SRBC, and BA) and by phase (primary, secondary, and equilibrium) for SRBC and BA. The single time-point measurement taken at 7 days post primary immunization was used for the primary phase parameters (Y) for SRBC and BA. The secondary phase parameters of maximum titers (Ymax), time (Tmax) needed to achieve maximum titers, minimum titers (Ymin), and time (Tmin) needed to achieve minimum titers were estimated from seven individual time-point, post secondary titer values by a nonlinear regression model (38). The equilibrium phase parameter (Equil.) was calculated as the mean of the titers of the last three sample times.

The JMP® program (SAS Institute, Inc., Cary, NC) was used to conduct the general linear model test for associations of IFN-γ genotype with IFN-γ serum protein level and SE antibody level; the multivariate pairwise correlation test was used between IFN-γ protein level and SE antibody level or antibody parameters to SRBC and BA antigens. Because grandsire by IFN-γ protein level and grandsire by SE antibody interactions ($P < 0.05$, data not shown) were found, data were thereafter analyzed separately for F_2 individuals derived from the two MHC-congenic grandsires.

RESULTS

Main effect of IFN-γ promoter genotype on antibody response to SE. The IFN-γ promoter polymorphism was associated with SE antibody level ($P = 0.04$) of F_2 offspring of the M15.2 grandsire. The allelic effect of IFN-γ genotype on SE antibody level is presented in Table 1. For F_2 offspring of the

Table 1. IFN-γ promoter allelic effect (mean ± SEM) on IFN-γ protein level and antibody response to *Salmonella enteritidis* in F_2 offspring of M5.1 and M15.2 grandsires.[A]

Trait	M5.1 Grandsire			M15.2 Grandsire		
	LL[B]	LF[C]	FF[D]	LL	LF	FF
PL1[E]	0.84 ± 0.07[a]	0.74 ± 0.05[a]	0.75 ± 0.05[a]	0.81 ± 0.04[a]	0.60 ± 0.04[b]	0.61 ± 0.05[b]
PL2[F]	1.09 ± 0.05[a]	0.93 ± 0.04[b]	0.92 ± 0.04[b]	0.95 ± 0.04[a]	0.93 ± 0.04[a]	0.92 ± 0.04[a]
SE[G]	0.42 ± 0.03[a]	0.37 ± 0.02[a]	0.38 ± 0.02[a]	0.30 ± 0.03[a]	0.25 ± 0.03[a]	0.35 ± 0.03[b]

[A]Means in a row within a grandsire MHC type with no common lowercase superscript differ significantly ($P < 0.05$).
[B]Leghorn homozygote.
[C]Heterozygote of leghorn and Fayoumi genotype.
[D]Fayoumi homozygote.
[E]IFN-γ protein level at 7 days after first injection.
[F]IFN-γ protein level at 7 days after second injection.
[G]Antibody level to *Salmonella enteritidis* vaccination.

M15.2 grandsire, the SE antibody level of Fayoumi homozygous IFN-γ promoter SNP hens (0.35) was higher than the other two allelic combinations (0.30 and 0.25; $P = 0.05$). There were 17% and 40% increases of the SE antibody level for Fayoumi homozygous individuals compared with leghorn homozygous and heterozygous birds, respectively.

Main effect of IFN-γ promoter genotype on circulating IFN-γ protein level. The ranges, means, and standard errors of the mean of IFN-γ protein level with sample sera at 7 days after primary and secondary immunization and reference chicken serum are presented in Table 2.

The IFN-γ promoter polymorphism was associated with the IFN-γ protein level at 7 days after primary immunization ($P = 0.002$) of F_2 offspring of the M15.2 grandsire and at 7 days after secondary immunization ($P = 0.002$) of F_2 offspring of the M5.1 grandsire. The allelic effect of IFN-γ genotype on IFN-γ protein level is presented in Table 1. For F_2 offspring of the M15.2 grandsire, the IFN-γ protein level of leghorn homozyous IFN-γ promoter SNP (LL) hens (0.81) at 7 days after primary immunization was higher (33%) than the other two allelic combinations (0.60 and 0.61; $P = 0.002$). For F_2 offspring of the M5.1 grandsire, in contrast, the leghorn homozygous IFN-γ promoter type (LL) (1.09) had a higher (18%) IFN-γ protein level at 7 days after secondary immunization than hens from the other two allelic combinations (0.93 and 0.92; $P = 0.05$).

Phenotypic correlation of chicken IFN-γ protein level with antibody production. IFN-γ protein level after primary immunization was positively correlated with IFN-γ protein level after post secondary immunization (0.31; $P = 0.002$). Phenotypic correlations between chicken IFN-γ protein level and SE antibody level or antibody parameters of primary, secondary, and equilibrium phases to SRBC and BA of all F_2 females are shown in Table 3. The IFN-γ protein level at 7 days post secondary immunization was positively correlated with SE antibody level ($P = 0.01$), maximum titer to SRBC ($P = 0.02$), and equilibrium antibody response to SRBC ($P = 0.08$) and negatively correlated with time required to achieve maximum titer to SRBC ($P = 0.07$) and primary antibody response to BA ($P = 0.04$). The level of IFN-γ protein at 7 days post primary immunization was positively correlated with time to reach the minimum titer to SRBC ($P = 0.08$) and BA ($P = 0.09$) and negatively correlated with time to achieve the maximum titer to SRBC ($P = 0.03$).

DISCUSSION

The important role of IFN-γ has been documented in many diseases in poultry

Table 2. Serum IFN-γ protein level (optical density at 450 nm) in F_2 females of an inbred cross.

	IFN-γ protein level 7 days postimmunization			
	Sample chicken serum		Reference chicken serum	
	Primary	Secondary	Primary	Secondary
Range	0.12–0.66	0.06–0.27	0.32–0.49	0.13–0.16
Mean ± SEM	0.29 ± 0.01	0.14 ± 0.003	0.40 ± 0.04	0.15 ± 0.006

Table 3. Phenotypic correlation between chicken IFN-γ protein level and antibody response to *Salmonella enteritidis,* sheep red blood cells, and *Brucella abortus* in F_2 females of an inbred cross.

		SE[A]	Sheep red blood cells						*Brucella abortus*					
			Y[B]	Tmin[C]	Ymin[D]	Tmax[E]	Ymax[F]	Equil.[G]	Y	Tmin	Ymin	Tmax	Ymax	Equil.
PL1[H]	Correlation	0.09	0.04	−0.12	0.15	−0.20	0.10	0.12	0.06	0.16	−0.05	0.06	0.03	−0.11
	P-value	NS[I]	NS	0.17	0.08	0.03	NS	0.16	NS	0.09	NS	NS	NS	0.17
PL2[J]	Correlation	0.20	−0.10	−0.08	0.04	−0.16	0.20	0.14	−0.17	−0.02	−0.07	−0.08	−0.04	−0.02
	P-value	0.01	0.20	NS	NS	0.07	0.02	0.08	0.04	NS	NS	NS	NS	NS

[A]Antibody response to *Salmonella enteritidis.*
[B]Primary antibody response.
[C]Time required to achieve minimum secondary antibody titers.
[D]Minimum secondary antibody titers.
[E]Time required to achieve maximum secondary antibody titers.
[F]Maximum secondary antibody titers.
[G]Equilibrium phase antibody titers.
[H]IFN-γ protein level at 7 days after primary injection.
[I]NS = not significant (*P*-value > 0.20).
[J]IFN-γ protein level at 7 days after secondary injection.

(8,17,18,25,31). Sequence polymorphisms in the promoter of mammalian IFN-γ genes have also been shown to play a role in resistance to disease (1,32). Disease resistance is often associated with higher antibody levels against specific pathogens, such as *Escherichia coli* and *Pasteurella multocida* (15,24). Also, after divergent selection for response to SRBC, the high antibody line displayed higher antibody to Newcastle disease virus and greater resistance to Marek's disease virus, *Eimeria tenella, Mycoplasma gallisepticum,* and *Eimeria necatrix* than the low antibody selected line (11,12,14). In chickens, IFN-γ promoter polymorphisms have previously been demonstrated to be associated with chicken primary and secondary antibody response to both SRBC and BA antigens (38). Thus, the potential role of promoter polymorphisms of the chicken IFN-γ gene in chicken disease resistance led us to investigate the role of the IFN-γ polymorphism in modulating antibody level.

Salmonella bacteria are a major foodborne infectious pathogen in humans. IFN-γ plays a critical role in host defense and intestinal immunity against *Salmonella typhimurium* in the mouse (2,3). There was positive correlation between antibody response to SE and circulating IFN-γ protein level at both post primary and post secondary times in the current study. Serum antibody level to SE vaccine has a high negative genetic correlation with SE burden in cecal content (16). Therefore, high IFN-γ protein level in serum may be associated with reduced gastrointestinal SE burden in chickens.

Both IFN-γ mRNA and protein are expressed predominantly by T cells and large granular lymphocytes (36). IFN-γ affects antigen-presenting cells, T cells, and B cells, each of which participates in the complex chain of events after antigen exposure to the immune system. In mice, blockage of endogenous IFN-γ reduces the level of primary IgM antibody responses to sheep erythrocytes *in vitro.* IFN-γ is a necessary component of T-cell-derived helper factors for antibody induction in *in vitro* immunization systems, and IFN-γ stimulates polyclonal Ig production by resting or activated human B cells (36). Therefore, expression of IFN-γ may affect antibody response via modulation of antigen presentation. Lymphocytes need to be activated to produce IFN-γ, which involves cooperation of accessory cells, mostly

mononuclear phagocytes (4). Many diverse, convergent signal transduction pathways have been implicated in the transcriptional control of IFN-γ (36). Therefore, many different genetic and cellular factors control the production of IFN-γ. Because the promoter region of a gene can regulate gene expression, we hypothesized that mutation in the promoter region would affect IFN-γ gene expression. The allelic effects of promoter polymorphism on IFN-γ protein level were very consistent, in that birds with IFN-γ LL genotype had higher IFN-γ protein levels than both other genotypes after both primary and secondary immunization and in F_2 offspring of both M5.1 and M15.2 grandsires.

IFN-γ production, as a quantitative trait, is affected by many genetic and nongenetic factors. On the basis of the general linear model analysis of IFN-γ genotype with protein level in serum, the IFN-γ promoter polymorphism determined 15.8% of the phenotypic variation in IFN-γ protein level at 7 days after primary immunization in F_2 offspring of the M15.2 grandsire, and 8.5% for IFN-γ production at 7 days after secondary immunization in F_2 offspring of the M5.1 grandsire. Accounting for this large percentage of variation strongly suggests that DNA sequence polymorphism in or near the IFN-γ promoter region might influence IFN-γ protein level after both primary and secondary immunizations.

There were consistently negative correlations of IFN-γ protein level after secondary immunization with primary antibody response to SRBC and BA. However, there was a positive correlation of IFN-γ protein level after both primary and secondary immunizations with maximum secondary antibody response to SRBC and negative correlations ($P = 0.03$, 0.07) with the time required to achieve maximum secondary antibody response to SRBC. These results suggest that augmented IFN-γ protein levels can increase maximum secondary antibody response to SRBC and decrease the time needed to reach maximum secondary antibody response to SRBC. Thus, exogenous administration of IFN-γ might be an effective method to increase antibody response to booster (second or later) vaccinations.

The different correlation directions of IFN-γ protein level between the two antibody response phases with SRBC antigen might arise

from the different cell types involved in primary and secondary antibody production. When a naive T cell recognizes an antigen-MHC complex on an appropriate antigen-presenting cell or target, an activated T cell will initiate a primary immune response. The memory T cell and B cell, however, are involved in the secondary immune response (21). The factors related to IFN-γ expression and antibody production might thus have different effects between primary and secondary phases of immune response.

The chicken MHC plays an important role in antibody production (22,23). Interferon-stimulated response elements mediate the induction of MHC class I expression by type I and type II IFN. In particular, IFN-γ regulates the expression of multiple components in the MHC class I–restricted antigen presentation circuit in the human cell line (13). The direction of allelic effects of the IFN-γ promoter genotype on chicken antibody response to SE was different between the F_2 branches produced from the two MHC-congenic grandsires. This result suggests a genetic interaction between the chicken IFN-γ promoter polymorphism and MHC haplotype on antibody response to SE vaccine. Therefore, effects of IFN-γ polymorphism must be defined in the context of the population's MHC alleles.

Adequate functioning of the IFN-γ and macrophage system is essential for innate, as well as acquired, resistance to infection (4). The results of the current study, which clearly demonstrated the relationship of a promoter polymorphism with circulating IFN-γ levels, and also of IFN-γ protein levels with antibody response parameters, sets the stage for enhancement of humoral immunity via genetic selection of optimal IFN-γ genotypes.

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