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Comparison of Immune Responses of Inducible Nitric Oxide Synthase (iNOS) Hyper-and Hypo-responsive Genotypes of Chickens

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Abstract: Cornell K-strain ($B^{15}B^{15}$) is hyper and GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) are hypo responder chicken genotypes for iNOS gene expression and activity. Two trials were conducted to compare these lines for immune parameters. For mononuclear phagocytic function quantification, 5 chickens per group were injected i.v. with Black India Ink at a rate of 1 mL/kg body weight (BW) at 4 wk. Blood samples were drawn and optical density measured at 640 nm on plate reader. K-strain was significantly better in clearing carbon (P = 0.04) than the GB1 and GB2 chickens at 10 min post i.v. injection. Lymphoproliferation was measured *in vitro* by incubating 0.2 x 10^6 peripheral blood leukocytes with 25 µg Concanavalin A. K-strain chicks had the highest lymphoproliferation index than the GB1 and GB2. On the contrary, K-strain chicks had a weaker PHAP-mediated toe-web swelling response as compared with the GB1 and GB2 chicks. No differences were observed between genotypes for the total or IgG anti-SRBC antibodies in primary or secondary immunization response (one trial). However, K-strain exhibited greater persistence (P < 0.05) of IgM levels than GB2 chicks during the decline phase of the booster response. While K chicks had heavier BW (P < 0.01), they had smallest bursal, thymic and splenic weights (relative to BW) vs. GB1 and GB2 in both trials. These findings show that the iNOS hyper responder K-strain chicken also perform better for monocyte/macrophage, T-lymphocyte and perhaps even B-lymphocyte functions even though their lymphoid organ growth lagged behind the iNOS hyporesponder (GB1 and GB2) genotypes.

Key words: Chickens, iNOS, immune functions, genotypes

Introduction

Selection for good immune response is an important factor in poultry breeding programs. Bird's immune response depends upon the combination of three factors; antibody response (humoral immunity), T cell-mediated immunity and phagocytosis. Genetic control of these facets of the immune system may be independent of each other (Cheng and Lamont, 1988; Sarker *et al.*, 2000; Li *et al.*, 2001). Several chicken lines selected for high antibody response to sheep red blood cells (SRBC) have been shown to be more resistant to *Eimeria spp.*, but, in contrast, appeared more susceptible to *Escherichia coli* and *Staphylococcus aureus* infections (Gross *et al.*, 1980; Dunnington *et al.*, 1986). Also, Parmentier *et al.* (2001) confirmed that selection for enhanced humoral immunity to SRBC did not result in enhanced resistance to *E. acervuline* in terms of fecal oocyst output. Dil and Qureshi (2002a,b) stated that macrophages from Cornell K-strain ($B^{15}B^{15}$) were hyper-responders to *Escherichia coli* lipopolysaccharide (LPS) whereas macrophages from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens were hypo-responders for inducible nitric oxide synthase (iNOS) expression and activity.

Significant effects of hatch, line sex and B-group haplotype on response to SRBCs have also been reported (Gross *et al.*, 1980; Van der Zijpp and Leenstra, 1980; Pinard *et al.*, 1993; Kundu *et al.*, 1999). Blood monocytes derived from two inbred chicken lines carrying the same MHC (B^2) haplotype exhibited different inflammatory cell profile and possessed different phagocytic characteristics, suggesting that these macrophage function parameters are likely to be influenced primarily by non-MHC genes (Powell *et al.*, 1983). Previous work with chicken macrophages has demonstrated that various chicken lines may be classified as hypo- or hyper-responders based on LPS-mediated induction of inducible form of iNOS. For example, macrophages from the MQ-NCSU macrophage cell line and Cornell K-strain ($B^{15}B^{15}$) chickens produced almost three-fold higher nitrite levels in their culture supernatants after LPS stimulation compared to macrophages isolated from GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) chickens (Hussain and Qureshi, 1998). The purpose of the present study was to examine any possible difference in immunogenetic traits among three different genetic groups [K-strain ($B^{15}B^{15}$), GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$) strains] of chickens. This question was asked based on the fact that all of these three chicken genotypes differ in the induction and expression of the iNOS gene leading to the differential

enzyme (i.e. nitric oxide synthase) and activity (i.e., nitrite production) (Hussain and Qureshi, 1997, 1998; Dil and Qureshi, 2002 a, b, 2003).

Materials and Methods

Birds and Management: Three different strains of chicken, namely Cornell K-strain ($B^{15}B^{15}$), GB1 ($B^{13}B^{13}$) and GB2 ($B^{6}B^{6}$), were used in this study. All birds were maintained at the Department of Poultry Science, North Carolina State University under the Institutional Animal Care and Use Committee's approval. Upon hatch, the chicks were placed in electrically heated Petersime batteries with raised wire floors under continuous light. Room temperature was maintained at 21-24 °C; battery temperature was maintained at 32-35 °C. Feed (North Carolina State University chick starter diet) and water were available *ad libitum* in stainless-steel troughs. Two hatches of chicks were evaluated in this study.

Sheep Red Blood Cell Challenge (Antibody production assay): For assessing humoral immunity response, 10 chicks per strain were randomly assigned. The sheep red blood cells (SRBCs), collected in an anti-coagulant (potassium oxalate solution), were washed 3 times in phosphate-buffer saline (PBS, pH 7.4). After that, the packed cells were brought to a 7% vol/vol solution in the PBS. At one week of age, chicks were given the first injection of 1 mL suspension of 7% SRBC intravenously. The second (booster) injection was given at 3 weeks of age. Blood samples were drawn at 7, 14 days post first and second injection. Serum was recovered from clotted blood by centrifugation and was stored at -20 °C until tested. The antibody levels against SRBC were measured by hemagglutination test using 2% SRBCs suspension. The serum was heat-inactivated at 56 °C for 30 min and analyzed for total, mercaptoethanol (ME)-sensitive (presumably IgM) and ME-resistant (IgG) anti-SRBC antibodies as previously mentioned (Delhanty and Solomon, 1966). The antibody titer was expressed as the $\log_2$ of the reciprocal of the highest dilution giving a positive reaction.

Phytohemagglutinin Injection (*In vivo* cell-mediated immunity assay): To examine the cell-mediated immune response, 10 chicks from each strain at 3 weeks of age were used. Each chick was intradermally injected in the toe-web (between the second and the third digit) of the left foot with 100 µg Phytohemagglutinin-P (PHAP) (Sigma Chemical Co., St. Louis, MO 63178) in 0.1 mL sterile saline. The control toe web of the right foot received 0.1 mL of sterile saline in an identical manner. The thickness of toe-webs was measured with a constant tension caliper before injection and at 24, 48 and 72 h after PHAP injection. The toe web swelling was calculated as the difference between the thickness of the toe web before and after injection.

Relative Weight of Lymphoid Organs: After completion of phytohemagglutinin assay, the same chicks were weighed and euthanatized by CO_2. The bursa of Fabricius, spleen and thymus (all lobes from left side of the neck) were removed and weighed to the nearest milligram. The relative weights of lymphoid organs were compared among the strains.

Carbon Clearance (Mononuclear phagocytic system function assay): The phagocytic ability of chicks was determined by the carbon clearance assay (CCA) based on the method of Cheng and Lamont (1988) with some modification. Briefly, the supernatant fraction of Black India Ink (Design/Higgins, 4415, Sanford, Bellwood. Illinois 60104) was obtained through centrifugation (3000-x g for 30 min). At 4 weeks of age, 5 chicks from each strain were injected with ink at the rate of 1 mL/kg body weight into the jugular vein. Blood samples (100µL/chick) were collected from brachial vein before injection (0 min) and from other side of jugular vein at 3 and 10 min after ink injection. The blood samples were immediately transferred into 2 mL of 1% sodium citrate. The samples were then centrifuged at 50-x g for 4 min. The relative amount of carbon particles remaining in the supernatant was measured spectrophotometrically at a wavelength of 640 nm using the samples at 0 min as the zero value.

***In Vitro* Concanavalin Exposure (Peripheral Blood Lymphocytes blastogenesis assay):** At 4 weeks of age, five birds per strain were randomly assigned for lymphoblastogenesis assay *in vitro*. Blood samples (1 mL/chick) were withdrawn with a heparinized syringe and diluted with equal volume of PBS. The peripheral blood lymphocytes (PBLs) were separated from the whole blood by Ficoll (Atlanta Biologicals, Norcross, GA) density gradient by centrifuging at 2000 rpm for 20 min. The cells were washed twice and cell count was adjusted to $2x10^6$/mL in RPMI-1640 growth medium (Fisher, Norcross, GA) supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic. One hundred µL PBLs, containing $0.2x10^6$ cells from each sample were added to each of the four wells of a 96-well plate. Concanavalin A (ConA) (type IV; Sigma) was added at a concentration of 25 µg/100µL/well. The plates were incubated at 41 °C in a humidified incubator in the presence of 5% CO_2. After 22 h of incubation, 50µL of a 1-mg/mL stock of MTT (3-[5,5-dimethylthiazol-2-yl]-2,5-[diphenyltetrazolium]) (Sigma) was added to each well and the plates were reincubated for additional 4 h. After incubation, all liquid from the wells was carefully removed, then 150 µL of MTT acid (200 mL 2-propanol [isopropyl alcohol] + 1.32 mL conc. HCl + 100 mL PBS) was added to each well and blue formazan crystals were dissolved by repeated pipetting. The optical density (OD) was read on a plate reader at 540 nm wavelength. The

Table 1: LS Means for IgG, IgM and total antibody titers (log_2) of chicks from iNOS hypo-and hyper-responder genotypes

	IgG				IgM				Total			
	Strain											
Days post-injection	K	GB1	GB2	Pooled SEM	K	GB1	GB2	Pooled SEM	K	GB1	GB2	Pooled SEM
7	0.0	0.0	0.0	0.0	1.5	0.4	1.0	0.28	1.5	0.4	1.0	0.28
14	0.0	0.0	0.0	0.0	0.4	0.3	0.6	0.18	0.4	0.3	0.6	0.18
21	2.0	2.2	2.3	0.17	2.9	2.6	2.2	0.39	4.9	4.8	4.5	0.35
28	0.6	0.9	1.1	0.19	2.0[a]	1.5[ab]	1.2[b]	0.26	2.6	2.4	2.3	0.25

[a,b] The means for a given type of antibody within a row with no common superscript differ significantly ($p<0.05$)

Table 2: LS Means for body weight and relative weight of lymphoid organs from iNOS hypo-and hyper-responder chicken genotypes

	BW		Bursa		Thymus		Spleen	
Strain	(g)		%BW					
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
K	169.0[a]	172.8[a]	0.30[c]	0.37	0.14[c]	0.15[c]	0.11[b]	0.10[c]
GB1	110.9[c]	133.3[c]	0.36[b]	0.40	0.20[b]	0.22[b]	0.20[a]	0.20[b]
GB2	146.7[b]	149.2[b]	0.44[a]	0.42	0.25[a]	0.27[a]	0.23[a]	0.25[a]
Pooled SEM	±5.26	±4.70	±0.02	±0.02	±0.02	±0.01	±0.01	±0.01

[a,b,c] The means within columns with no common superscript differ significantly ($p<0.01$) n= 10 birds per strain

mean OD values from every strain were used to calculate stimulation indices by the following formula: Con A-stimulated - unstimulated/unstimulated.

Statistical Analysis: All data were subjected to a one-way ANOVA with the main effect of strain using the general linear model procedure (Proc GLM), SAS software (SAS Institute, 1995). Least squares means procedure was used to test the significance between means. Pearson correlation coefficients (Proc Corr) were calculated to analyze the relationship between relative weight of lymphoid organs and the response to phytohemagglutinin injection. Since no significant difference among strains for saline injection control swelling was found, the data of PHAP was therefore expressed as the PHAP-mediated in millimeter.

Results and Discussion

Humoral immune response: Least squares means for Cornell K-strain, GB1 and GB2 genotypes are presented in Table 1. The results revealed that K-strain had the highest antibody titers when compared with GB1 and GB2 strains, but these were not statistically significant until 21 days post challenge with SRBCs. At 28 days post-injection, the K-strain chicks had higher IgM titers than GB1 or GB2 strain chicks. Moreover, the difference between K-strain and GB2 was statistically significant ($P<0.05$). Generally, the antibody response of all different strains reached to its maximum level at 21 days and then declined by 28 days. Genetic differences in antibody response between different strains of chickens have been reported previously. For example, Boa-Amponsem *et al.* (2001) found a higher sensitivity of line HA than LA to SRBC antigen injected intramuscularly. Since SRBCs are considered as a T-dependent antigen, the process of antibody response against SRBC would therefore involve not only the B-lymphocytes responsible for antibody synthesis but also the macrophage-type cells which would engulf, process and present the antigen as well as the T-lymphocytes which would provide the cytokine-mediated help for expansion of antibody synthesis process. It is interesting to speculate that all of these mechanistic pathways are functionally better in K-strain chicks than in GB1 and GB2 chicks

Relative weight of lymphoid organs: Body weight at 3 weeks of age and relative weight of the lymphoid organs for each strain are shown in Table 2. In the two trials K-strain had a significantly higher body weight as compared to the other two strains. With respect to relative weight of lymphoid organs significant differences among strains were observed. These differences were more pronounced for thymus and spleen in the two trials. However, GB1 strain chicks had the highest percentage weights as compared to K-strain whereas GB1 chicks were intermediate. The bursa, thymus and spleen are the important lymphoid organs involved in the development and differentiation of B or T lymphocytes (Eerola *et al.*, 1987; Toivanen *et al.*, 1987). In chickens, Muir and Jaap (1967) reported that bursa of Fabricius

Table 3: LS Means for PHAP-mediated swelling response in the Toe-webs of iNOS hypo-and hyper-responder chicken genotypes

Strain	24 h		48h		72h	
	mm					
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
K	0.14[b]	0.10[b]	0.12	0.08	0.09	0.04
GB1	0.29[a]	0.21[ab]	0.16	0.17	0.11	0.12
GB2	0.19[b]	0.26[a]	0.13	0.10	0.10	0.08
Pooled SEM	±0.23	±0.04	±0.02	±0.03	±0.01	±0.02

[a,b] The means within columns with no common superscript differ significantly (P<0.01). n= 10 birds per strain

Table 4: Phenotypic correlation coefficients between relative lymphoid organs and toe web response from iNOS hypo- and hyper-responder chicken genotypes

Variable	T	B	S	T_{24}	T_{48}	T_{72}	Strain
Thymus % (T)	1.00	-0.21	0.48	-0.14	-0.25	-0.28	GB1
	1.00	-0.12	-0.38	0.67*	0.48	0.60	GB2
	1.00	0.16	0.04	0.04	-0.01	0.03	K
Bursa % (B)		1.00	0.30	-0.33	-0.41	-0.45	GB1
		1.00	0.28	-0.20	0.28	-0.37	GB2
		1.00	-0.38	0.22	-0.07	-0.09	K
Spleen % (S)			1.00	-0.48	-0.65*	-0.71*	GB1
			1.00	-0.65*	-0.37	-0.56	GB2
			1.00	-0.68*	-0.56	-0.69*	K
Toe web increase				1.00	0.87***	0.86***	GB1
After 24 h (T_{24})				1.00	0.12	0.75**	GB2
				1.00	0.62	0.80**	K
Toe web increase					1.00	0.94***	GB1
After 48 h (T_{48})					1.00	0.31	GB2
					1.00	0.85**	K
Toe web increase						1.00	GB1
After 72 h (T_{72})						1.00	GB2
						1.00	K

* p<0.05, ** p<0.01, *** p<0.001

Table 5: Lymphoblastogenic response of leukocytes against Concanavalin-A from iNOS hypo-and hyper-responder chicken genotypes

Lymphoblastogenesis Stimulation Index			
Trial 1		Trial 2	
Strain	Mean	Strain	Mean
K	0.44	K	0.77[a]
GB1	0.31	GB1	0.53[a]
GB2	0.44	GB2	0.26[b]
Pooled SEM	±0.06		±0.11
Probability	0.28		0.01

Peripheral blood leucocytes were separated out of whole blood by ficoll gradient (5 birds/strain). The concentration of cells were adjusted to $2x10^6$ /mL and incubated with 25 µg/100 µL of Con-A mitogen. After 22 hrs of incubation, lymphoblastogenesis was quantitated using MTT assay. Lymphoproliferative index was calculated as: (stimulated–unstimulated)/unstimulated. [a,b] Indicate significant difference among strains.

weight at hatching was negatively associated with post-hatching body weight. Similar relationship was observed for turkey (Li *et al.*, 2001). In the present study, K-strain had the higher antibody titer although its lymphoid organs were relatively smaller. It seems that the size of lymphoid organs may not necessarily be associated with antibody titers. Yamamoto and Glick (1982) found that a chicken line selected for small bursa size had higher total and 2-mercaptoethanol-resistant antibody titers in the primary response to SRBC and also had higher total antibody titers in the secondary response compared to the counterparts in the line selected for larger bursa size. Ubosi *et al.* (1985) observed that a chicken line selected for high response to SRBC had a larger bursa size than the line selected for low response. In turkeys, Li *et al.* (2001) reported that the F line had a higher antibody response to SRBC and higher serum IgM concentration than the RBC2 line. While for relative weight of spleen the opposite was true. It is difficult to speculate what effect, if any, or correlation, the lower lymphoid organ weights in K-strain might have

Table 6: Carbon clearance by chicks from iNOS hypo-and hyper-responder genotypes after intravenous injection

	OD increase %			
Strain	Trial 1		Trial 2	
	T3[b]	T10	T3	T10
K	58.17[a]	13.0[b]	50.31[b]	14.89[b]
GB1	23.79[b]	40.14[a]	45.53[b]	32.46[a]
GB2	54.69[a]	26.73[ab]	82.60[a]	30.08[a]
Pooled SEM	±8.37	±5.66	±9.08	±4.65
Prob.	0.05	0.04	0.03	0.04[a,b]

[a,b]Indicate significant difference among strains. OD increase % = [(OD reading at a considered time - OD reading at 0 min)/OD reading at 0 min] x 100

with the hyper-responsiveness for iNOS induction in K-strain as compared to the GB1 and GB2 chicks.

Cell-mediated immune response: The data on the response of chickens to PHAP injection in two trials is provided in Table 3. The chicks from K-strain had lower PHAP-mediated swelling in toe web as compared to GB1 and GB2 counterparts at all tested times post-injection. This finding is rather surprising since the K-strain chicks are high-responders for iNOS production whereas for the PHAP challenge the K-strain birds were consistently low responders. On the contrary, the GB1 and GB2, the iNOS hypo-responders, had a better PHAP response at all time points. Kean *et al.* (1994) had observed that the chicken lines selected for high immunoresponsiveness had a significantly higher wing web response to PHAP injection after 5 generations of selection compared to the other chicken lines selected for low immunoresponsiveness. Our data therefore suggest that the iNOS responsiveness and PHAP-mediated *in vivo* lymphoproliferative responses may not be positively correlated in chickens. It must be pointed out that the immune cell types in this comparison would be the macrophages (for iNOS) and presumably T-lymphocytes (in PHAP-mediated assay).

As expected, there were significantly positive correlation coefficients between the swelling in toe web measured after 72 hrs post-injection and the swelling after 24 and 48 hrs for almost all strains (Table 4). Negative relationship between the percent bursal weight and the swelling of toe web was observed at all times, especially for GB1 strain. This suggests that the size of bursa did not affect the cell-mediated immune response. The same trend was noticed for the relationship between the percent thymic weight and the swelling of toe web after injection for GB1 only. Inversely, GB2 strain had a positive correlation, whereas K-strain had almost no relationship between thymus size and toe web swelling. A pronounced negative relationship between spleen percent weight and swelling of toe web for all different strains was detected.

Lmphoblastogenesis response: The lymphoblastogenic response of peripheral blood lymphoctes (PBL) from various genetic groups against Con-A is presented in Table 5. In Trial 1, there was no significant difference among groups. However, Cornell K-strain and GB2 chicks recorded the highest indices. As seen in Trial 2, the responsiveness of PBLs was significantly lower in GB2 chickens as compared to the other two strains. Again, the K-strain recorded the highest index than that of GB1 and GB2 strains. Taken together, the PHAP and Con-A experiments data clearly show that these two responses are opposite to each other in the iNOS hyper-responder chickens (i.e., K-strain). One explanation for this observation may be the fact that the *in vitro* and *in vivo* micro environments may impart a differential responsiveness on the effector cells. Secondly, it is also possible that the target cells for both mitogens may be different, thereby inducing a variation between the *in vivo* and *in vitro* response to the T-cell mitogens.

Phagocytic ability: This function was measured by injecting a particulate material, India Ink, into the birds from all three strains and comparing their ability to clear the injected carbon from circulation over a period of time. This was accomplished by obtaining optical density of the plasma samples collected at zero and after 3 and 10 min of ink injection. The data are presented in Table 6. An increase in the percentage of OD value would be indicative of more carbon present in the sample at the time of quantification. There were significant differences among strains for different times post-injection. For example, after 3 min post-injection, all strains had a high level of circulating carbon in both trials. However, at 10 min post-injection when the injected carbon is supposed to be trapped by the cells of the mononuclear phagocytic system, i.e., blood monocytes and fixed tissue macrophages (Qureshi *et al.*, 2000), the K-strain chicks had significantly lower levels of carbon in their circulation as compared with the GB1 and GB2 chicks. These data clearly show that the Cornell K-strain mononuclear phagocytic system is functionally much more efficient than the one in GB1 and GB2. This would then be consistent with the fact that the iNOS expression

and activity in macrophages (members of the mononuclear phagocytic system) from K-strain chicks is significantly higher than in the GB1 and GB2 chickens (Hussain and Qureshi, 1997, 1998; Dil and Qureshi, 2002a,b; 2003).

In conclusion, it is clear from these data and the previously published studies that the Cornell K-strain White Leghorn chickens have a superior mononuclear phagocytic function as measured by the *in vivo* carbon clearance as well as the expression and activity of iNOS in purified macrophages. Furthermore, Dil and Qureshi (2002a,b; 2003) have shown that K-strain macrophages express the LPS-binding (CD14) and signaling molecules (TLR4) much more efficiently leading to improved LPS-mediated signal transduction for iNOS expression and activity. Both GB1 and GB2 chickens were clearly low responder in such signaling functions. While macrophage functions are clearly more efficient in K-strain chicks, the lymphoid organs such as bursa, thymus and spleen were much smaller indicating slower development than in the GB1 and GB2 birds. Interestingly, K-strain birds had a slightly better humoral immune response and a higher lymphoproliferative response against a T-cell mitogen, Con-A. This suggests that K-strain chickens have the potential to mount a well-balanced immune response involving all three cell types, i.e., macrophages, B - and T-lymphocytes.

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