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Selection for Increased Nitric Oxide Production Does Not Increase Resistance to Marek's Disease in a Primary Broiler Breeder Line

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SUMMARY. Two primary broiler breeder lines, A and B, were examined for their potential to produce nitric oxide (NO) after stimulating splenocytes from 20-day-old embryos with lipopolysaccharide and interferon-γ. Significant differences were found between lines A and B. Overall, line A had a higher response than line B, but line A also had a large degree of variation between individual sire families. Selection for high and low responders within line A resulted in the segregation of high- and low-responder sire families. Offspring from sire families selected for high and low NO responses and from a nonselected control group from line A were challenged with RB-1B Marek's disease (MD) virus to determine whether these differences could be used to select for improved resistance to MD. Virus isolation rates at 6 and 10 days postinfection were not significantly different, but unexpectedly, the MD incidence in the high-responder group was significantly higher than in the other two groups.

RESUMEN. La selección para la producción incrementada de óxido nítrico no incrementa la resistencia para la enfermedad de Marek en una línea primaria de reproductores pesados.

Dos líneas primarias de reproductores pesados A y B fueron examinadas en su potencial para producir oxido nítrico después de estimular esplenocitos procedentes de embriones de 20 días de edad con lipopolisacáridos e interferón-gama. Se encontraron diferencias significativas entre las líneas A y B. Especialmente, la línea A tuvo una respuesta más alta que la línea B, pero la línea A también tuvo una mayor cantidad de variaciones entre las familias individuales de machos. La selección para respuesta alta y baja dentro de los integrantes de la línea A resultó en una segregación de familias de machos con respuesta alta y baja. La progenie de familias de machos seleccionados para respuesta alta y baja de óxido nítrico y de un grupo control no seleccionado de la línea A fueron desafiados con el virus de la enfermedad de Marek cepa RB-1B, para determinar si estas diferencias podían ser aplicadas para mejorar la resistencia a la enfermedad de Marek. Los porcentajes de aislamiento viral a los 6 y 10 días después la infección no fueron significativamente diferentes, pero inesperadamente la incidencia de la enfermedad de Marek en el grupo de alta respuesta fue significativamente mayor que en los otros dos grupos.

Key words: genetic selection, herpesvirus, inducible nitric oxide synthase, interferon-γ, lipopolysaccharide, Marek's disease, nitric oxide

Abbreviations: CEF = chicken embryo fibroblasts; CKC = chick kidney cells; FFU = focus forming units; H = high level of NO production; IFN-γ = interferon-γ; iNOS = inducible nitric oxide synthase; L = low level of NO production; LPS = lipopolysaccharide; MD = Marek's disease; MDV = Marek's disease herpesvirus; MHC = major histocompatibility complex; NO = nitric oxide; PBS = phosphate-buffered saline; PI = postinfection; QTL = quantitative trait loci; vv+ = very virulent plus strain of MDV

Marek's disease (MD), caused by MD herpesvirus (MDV), remains an economically important disease in chickens, although it is currently well controlled by vaccination *in ovo* or at 1 day of age (1,32). Since the introduction of vaccination, the pathogenicity of MDV strains has continuously increased. Currently, four pathotypes are recognized, ranging from mild to very virulent plus (vv+) strains (31). The increase in pathogenicity not only led to the introduction of new vaccines but also reemphasized the need to continue selection for enhanced genetic resistance. Witter (32) suggested that enhanced genetic resistance and vaccine-induced immunity appear to be additive or complementary.

Genetic resistance to MD is complex and partly based on the major histocompatibility complex (MHC) of the chickens with specific MHC haplotypes linked to the degree of resistance (2). The MHC-based resistance is likely linked to acquired cell-mediated immune responses (21,25) and perhaps natural killer cells (2,8). Selection for increased MD resistance on the basis of MHC haplotyping could lead to an increase in susceptibility to other diseases. For example, Macklin *et al.* (20) reported that chickens with the $B^{13}B^{13}$ haplotype had a significantly lower incidence of cellulitis than chickens with the $B^{21}B^{21}$ haplotype; these haplotypes are associated with susceptibility and resistance to MD, respectively (28). On the other hand, Groot and Albers (10) suggested that resistance to MD is mostly linked to genes other than MHC genes. Several quantitative trait loci (QTL) have been identified that are linked to MD susceptibility (18,34). Although the use of QTL might become useful for selection for increased resistance, the lack of specific information on the actual genes involved in the QTL limits the application at this time.

Genetic resistance to MD has also been linked to the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). Xing and Schat (33) showed that chicken embryo fibroblast (CEF) cultures from genetically resistant N2a chickens ($B^{21}B^{21}$) produced significantly higher levels of NO after stimulation with lipopolysaccharide (LPS) and interferon (IFN)-γ than CEF cultures from genetically susceptible P2a ($B^{19}B^{19}$) and S13 ($B^{13}B^{13}$) chickens. *In vivo* inhibition of iNOS resulted in increased viremia levels after challenge with the JM-16 strain of MDV. In addition, Djeraba *et al.* (6) and Jarosinski *et al.* (16) also noted that genetically resistant lines

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of the $B^{21}B^{21}$ MHC haplotype had, in general, a higher NO response early after infection with MDV than susceptible lines of the $B^{13}B^{13}$ or $B^{19}B^{19}$ haplotypes, suggesting that selection for high levels of NO production might increase protection to MDV replication.

In addition to CEF, chicken macrophages can be stimulated to produce NO by induction of iNOS by LPS (12). Genetic differences in NO production were shown between chicken lines with the use of abdominal exudate cells from 3-to-5-week-old chickens as a source of macrophages (13). Djeraba *et al.* (5) found that bone marrow–derived macrophages from 2-wk-old chickens and CEF are both good producers of NO.

In this paper, we tested the hypothesis whether selection for high levels of NO in primary broiler lines could be used to increase resistance for MD. First we wanted to determine if there were differences between and within two primary broiler breeder lines in inducible NO production. If differences were detected within a line, we wanted to determine if these differences could be exploited to increase genetic resistance to MDV. Although we had used CEF cultures to analyze chicken lines for genetic differences in NO production (33), this approach was not feasible to develop a high-throughput screening assay that can be used by breeders in a selection program. Because macrophage precursors and most likely macrophages can be obtained from bone marrow and spleens from 16-to-20-day-old embryos (23,24), we decided to use splenocytes from 20-day-old embryos as a source of cells for NO production.

Table 1. NO production by spleen cells from 20-day-old embryos obtained from specific sires and dams from line A. Spleen cells were stimulated for 48 hr with lipopolysaccharide and recombinant chicken interferon-γ (Expt. 2).

Parents[A]		NO (μM)[B]		
Sire	Dam	First test	Second test	Classification[C]
126	89	9	7.8	L
127	103	3	15	L
	104	4	8	L
128	120	40	4	?
129	131	4	21	?
	132	8	8	L
133	191	18	9	I
135	225	9	15	I
136	238	52	11	?
137	247	27	34	H
	252	9	24	?
	255	6	20	?
138	266	25	31	H
	267	23	30	H
	268	68	26	H
139	280	19	13	I

[A]The sires and dams were selected on the basis of Expt. 1.
[B]Three embryos per hen per test were used for each sire/dam combination in each test.
[C]L = low, I = intermediate, H = high levels of NO production; ? = unable to classify because of a large variation between the two tests.

MATERIALS AND METHODS

Embryos and experimental chickens. Twenty-day-old pedigreed embryos were received from Cobb-Vantress (Siloam Springs, AR), and individual spleens were harvested from all embryos. For the challenge experiments, 1-day-old pedigreed birds were wing-banded and shipped by Cobb-Vantress to Cornell University (Ithaca, NY). The parent flocks were vaccinated with HVT/SB-1 and probably also infected with serotype 1 MDV. Therefore, the chicks were assumed to be positive for maternal antibodies against all three serotypes. Chickens were fed a commercial diet *ad libitum*. The animal experiments were approved by the Cornell University Institutional Animal Care and Use Committee.

Cell cultures. Chick kidney cell (CKC) cultures were prepared from 2-week-old specific-pathogen-free chicks from the Departmental flocks and cultured as described (30). Embryonal spleen cell suspensions were prepared from individual pedigreed embryos by gently forcing the spleens through an autoclavable-sterile 60 μm nylon bolting screen (Sefar America, Depew, NY). Cells were washed with phosphate-buffered saline (PBS), counted according to the Trypan blue exclusion method, and resuspended in LM10 medium (30) without phenol red at 10^6 cells/ml/well and 2–3 wells/embryo. After 48 h in culture, the spleen cells were stimulated with 50 U/ml recombinant chicken (rCh)IFN-γ and 25 ng/ml LPS. NO production was measured at 72 h posttreatment.

Virus and virus isolation. RB-1B passage 17 (27) was propagated in CKC cultures. For virus isolation, CKC cultures were inoculated with spleen lymphocytes, and foci were enumerated as described (29).

Reagents. LPS (*Escherichia coli* serotype O55:B5) was obtained from Sigma Chemical Co (St. Louis, MO). *Escherichia coli*–derived rChIFN-γ (4) with a specific activity of 10^6 U/ml was kindly provided by John Lowenthal (Australian Animal Health Laboratory, CSIRO, Geelong, Victoria, Australia).

Nitrite determination. Nitrite, which is produced from NO in the presence of H_2O and O_2, accumulates in the culture medium and reflects the amount of NO production. NO is a free radical with a very short half-life; therefore, the end product nitrite was used as an indicator for NO production (9). Briefly, the concentration of nitrite was determined by mixing 100 μl of culture medium or 50 μl of heparinized blood plasma with 100 μl of Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, 0.1% naphthylethylene diamine) in 96-well microtiter plates (19). After 5 min, the color development was measured by absorbance at 550 nm (A_{550}) with a spectrometer (Bio-Tek Instruments, Winooski, VT). The concentration of nitrite in the medium was calculated with the use of a standard curve generated by mixing 0–250 μM sodium nitrite solutions with Griess reagent. Standard curves are typically linear between 0 and 200 μM nitrite. All measurements were done in triplicate.

Experimental design. Experiment 1 was designed to determine whether two primary broiler breeder lines, identified as line A and line B, from Cobb-Vantress differed in their potential to produce NO with the use of splenocytes from 20-day-old individual embryos stimulated with LPS and rChIFN-γ. Between 4 and 16 embryos were tested from 20 sire families of line A, and between 2 and 12 embryos from 15 sire families of line B were tested. Each sire was mated to two to five dams, and eggs were collected from trap nests.

In Expt. 2, the genetic contributions to produce NO was determined for individual dams of line A. Three embryos were obtained on two occasions from specific sires and specific dams from this line. The specific dam/sire combinations are listed in Table 1.

In Expt. 3, embryos from the next generation of line A were analyzed to select specific sire-dam combinations to generate sublines with high (H) and low (L) levels of NO production to produce offspring for the animal experiments. To select potential H and L NO combinations, one embryo per dam was tested from 20 sires, with 240 dams producing between 6 and 15 eggs/sire family for a total of 240 embryos. On the basis of these results, 12 hens and six sires were selected for the H subline, and 12 hens and 8 sires for the L subline. In Expt. 4, one to seven additional embryos were tested from 12 H and 12 L NO hens for the final selection of sires to generate the birds for Expts. 5 and 6. Nonselected sire-dam combinations were used to provide the control line (C subline).

Experiment 5 was designed to determine whether the embryonal splenocyte NO response correlated with NO plasma levels and virus isolation rates after MDV challenge. One-day-old, wing-banded, nonvaccinated chicks ($n = 122$) hatched from H, L, and C sire families were shipped by Cobb-Vantress to Cornell University. The chicks were

Table 2. Average concentration of NO production (Avg. NO conc.) by spleen cells from 20-day-old embryos obtained from sire families representing line A and line B. Spleen cells were stimulated for 48 hr with lipopolysaccharide and recombinant chicken interferon-γ (Expt. 1).

Line A				Line B			
Sire	No. of hens/sire	No. embryos	Avg. NO conc. (μM)	Sire	No. of hens/sire	No. of embryos	Avg. NO conc. (μM)
121	2	3	25.9	241	4	8	4.6
122	3	6	13.5	242	2	3	5.6
123	2	4	21.6	243	2	4	10.0
124	3	5	15.2	244	2	4	7.2
125	2	4	19.2	245	3	10	5.9
126	4	8	8.2	247	2	4	12.9
127	2	4	3.7	248	2	4	6.8
128	2	4	41.0	249	1	2	7.7
129	2	4	5.9	250	3	9	4.4
130	2	8	14.4	252	2	7	6.4
131	2	8	18.6	253	2	4	5.2
132	4	7	23.1	254	2	4	6.2
133	3	8	21.2	255	4	11	8.0
134	3	8	16.4	256	2	4	6.5
135	5	9	14.3	257	2	12	7.6
136	3	12	28.8				
137	5	12	19.5				
138	5	16	37.9				
139	3	8	15.0				
140	2	8	15.0				
Avg. ± SE			18.9 ± 2.1*				7.0 ± 0.6*

*$P < 0.05$ according to a two-sample Student's *t*-test assuming unequal variance.

placed into two multitier brooders, each placed in an isolation room with filtered air. At 2 days of age, all birds were inoculated intra-abdominally with 1000 focus forming units (FFU) of RB-1B. At 6 and 10 days postinfection (PI), plasma samples were collected to measure NO levels, birds were euthanized by CO_2 inhalation, and spleens were aseptically collected for virus isolation.

Experiment 6 was conducted to determine whether the selection for H and L NO production by embryonal spleen cells influenced the development of MD. One-day-old, wing-banded chickens ($n = 161$) hatched from H, L, and C sire families were shipped to Cornell University. The chicks were not vaccinated against MD or other diseases at the hatchery. The Cornell investigators were blinded to the NO status of the sire families, and I. Pevzner decoded the groups on completion of the experiment. The chicks were housed in multitier brooders placed in three isolation rooms, and 156 birds were inoculated at 7 days of age with 1000 FFU of RB-1B. Five birds were excluded from the study because of early mortality. The birds were observed daily, and chickens showing clinical symptoms for MD were immediately euthanized. The experiment was terminated at 50 days PI when survivors were euthanized by CO_2 inhalation. All birds were examined for gross lesions, which were classified as 1) bursal and or thymus atrophy (BA/TA) without other lesions, 1) neural lesions with or without BA/TA, 3) clinical paralysis but without gross neural lesions on postmortem examination, and 4) tumors.

Statistical analysis. Differences in NO production were analyzed by two sample Student's *t*-tests assuming unequal variance. Differences in viremia levels and MD incidence were analyzed by the Wilcoxon ranking test and chi-square test, respectively. Differences were considered significant at $P < 0.05$.

RESULTS

NO production by embryonal spleen cells (Expts. 1–4). Twenty sire families of line A and 15 sire families of line B were tested in Expt. 1. Results for individual sire families, number of hens per sire and number of embryos tested per sire are summarized in Table 2. The average NO production was significantly different between the two lines (mean ± SE: 18.9 ± 2.1 compared with 7.0 ± 0.6 μM for lines A and B, respectively, $P < 0.05$ Student's *t*-test assuming unequal variance). Interestingly, the range for line A was very large, with mean values of 3.7–37.9 μM NO/sire, which was in contrast with line B (4.4–12.9 μM NO/sire), suggesting a segregation for NO production in line A.

Table 3. Selection for high and low NO–producing sire families within line A. Embryonal spleen cells were stimulated for 48 hr with lipopolysaccharide and recombinant chicken interferon-γ (Expts. 3 and 4).

	NO (μM) ± SE[AB]	
Subline	Expt. 3	Expt. 4
Low	1.7 ± 0.4[a]	12.2 ± 1.9[a]
High	28.3 ± 2.9[b]	18.6 ± 3.3[b]

[A]In Expt. 3, 240 embryos representing 20 sires and 240 dams were used, and in Expt. 4, between one and seven embryos were used for the Low (eight sires and 12 dams) and High (6 sires and 12 dams) sublines.
[B]Values in columns with a different superscript are statistically different (*t*-test: $P < 0.05$).

A second set of embryos was examined from line A to determine more specifically the contribution of individual hens to NO production (Expt. 2). The results are summarized in Table 1. Results were classified arbitrarily as low when both tests gave values ≤10 μM NO, resembling the majority of the values for line B. Values between 10 and 20 μM NO were classified as intermediate, which would place only one or two sire/dam combinations of line B (Expt. 1) in this category, and >20 μM NO as high. In several cases, we were unable to classify specific sire/dam combinations because the values of the first and second test varied greatly.

In Expts. 3 and 4, embryos from the next generation of line A were analyzed in more detail to determine whether it was possible to confirm the segregation into high and low NO–producing sire families. The results of Expts. 3 and 4 are summarized in Table 3. Although the difference between the high and low NO–producing sire families is less pronounced in Expt. 4 than in Expt. 3, the differences are still significant. This probably reflects the fact that more embryos per dam were used in Expt. 4 than in Expt. 3. However, the combined data from these two experiments confirm that sire families can be selected for significant differences in NO production in the next generation of line A.

NO production and viremia in MDV-infected chickens (Expt. 5). The results of Expt. 5 are summarized in Table 4. NO production by splenocytes stimulated with LPS and rChIFN-γ was similar for the H, L, and nonselected sublines at 6 and 10 days PI. Virus isolation rates did not differ between the three sublines at 6 and 10 days PI. However, all three lines increased at 10 days PI compared with 6 days PI, but because of the large variation within sublines, the increase was not statistically significant.

MD incidence in the sublines (Expt. 6). The total incidence of MD was significantly higher in subline H than in the L and nonselected sublines (Table 5). Similarly, the percentage of birds that died or needed to be euthanized before 50 days PI was significantly higher for subline H than the other two sublines. Tumor incidence was significantly higher in subline H than in the nonselected subline, but the differences in other lesions were not statistically significant.

Table 4. NO production and Marek's disease (MD) virus viremia levels in three sublines of broiler line A at 6 and 10 days postinfection (PI) with the RB-1B strain of MD virus.

Subline[A]	Days PI[B]	No. of birds	NO production[C]		Viremia (No. foci/10^6 cells)[C]	
			Average	Range	Average	Range
L	6	24	4.9	3.0–8.3	6.3	0–31
C		21	4.6	3.1–12.5	9.9	0–45
H		21	4.0	0.0–8.3	5.8	1–13
L	10	19	5.3	4.6–6.0	21.6	0–57
C		19	5.7	5.0–6.6	33.1	2–88
H		18	5.4	4.3–6.5	34.1	14–82

[A]The sublines were classified as low (L) and high (H) nitric oxide (NO) producers on the basis of NO production by embryonal spleen cells. Offspring of nonselected offspring served as the control (C) line.
[B]All chicks were inoculated with 1000 focus-forming units of RB-1B at 2 days of age.
[C]The differences between H, C, and L chickens were not statistically different for NO production and viremia.

DISCUSSION

Several lines of evidence have suggested that the expression level of iNOS is under genetic control in chickens. Hussain and Qureshi (12,13) reported that macrophages from the K strain produced higher levels of iNOS than the two congenic G-B1 and G-B2 lines. Interestingly, the K strain is highly resistant to MD (14), whereas G-B1 and G-B2 are moderately resistant to challenge with the Md5 strain of MDV (17). Xing and Schat (33) and subsequently Jarosinski *et al.* (16) and Djeraba *et al.* (6) reported that the MD-resistant $B^{21}B^{21}$ lines produce higher levels of NO than MDV-susceptible, MHC-defined lines after challenge with MDV. On the basis of these publications, we first determined whether it is feasible to detect differences in NO production between primary broiler breeder lines with spleen cells from 20-day-old embryos as a source of NO-producing cells. Line B showed little variation in NO production between embryos from different sire families. In contrast, embryos from different sire families from line A showed significant differences in NO production in repeated experiments and with the use of different generations. The data suggest that sires and dams both contribute to the genetic diversity in NO production. Clearly, 20-day-old embryos from broiler breeder lines can be used for the characterization of the genetic potential to produce NO for selection programs.

The second objective was to determine whether selection for NO production can be used to improve genetic resistance to MD. On the basis of the available data in the literature, we expected to find increased resistance in the H subline. Unexpectedly, the results clearly demonstrated that the H subline had a significantly higher incidence of tumors and MD-associated mortality than the L and C sublines, whereas the NO levels in plasma and spleen cell–associated viremia were not significantly different among the three lines.

Several potential explanations exist for these unexpected *in vivo* results. *In vitro* studies by Djeraba *et al.* (5) had shown that NO was not the only pathway through which IFN-γ inhibited replication of MDV. It is therefore feasible that selection for high NO production interfered with other antiviral pathways or, conversely, that these other pathways might be more functional in line A birds selected for low production. Alternatively, Djeraba *et al.* (7) also suggested that LPS and IFN-γ are strong inducers of iNOS and arginase, with both competing for L-arginine as the substrate. The balance between iNOS and arginase depends on the type of macrophage that is activated. Moreover arginase is upregulated by Th2 cytokines in murine macrophages (22). Interestingly, Heidari *et al.* (11) recently reported that MDV induces a Th2 response during the cytolytic phase of infection. A Th2 response is linked to a shift toward antibody production rather than cell-mediated immune responses, which are driven by a Th1 response. The latter is more important for protective immunity in MD than the former, which probably plays a minor role in reducing the cytolytic infection phase (reviewed in Schat and Markowski-Grimsrud, 29). Increased virus replication during the cytolytic infection phase has been linked to an increased risk for tumor development (reviewed in Calnek, 3). It is therefore feasible that the selection procedure we applied with embryonal spleen cells to select the H subline selected in unexpected ways for increased arginase production by macrophages in chickens, causing an increase in susceptibility to MD tumor development.

It is also possible that NO production in the H subline became too high after the initial cytolytic infection, leading to a strong proinflammatory response, resulting in deregulation of the immune response and increased risk of tumor development. Indeed, the evidence is that high levels of NO are associated with MDV-related pathology. Jarosinski *et al.* (15) found a greater increase in NO in plasma between 4 and 15 days PI after infection with vv+ strains than after infection with less virulent strains, with only a minimal increase after infection with the latter strains. They also reported that high levels of NO in the brain were associated with neurological problems, especially in the resistant N2a line. These high levels were also associated with a very strong proinflammatory response, which might be responsible for the neural lesions. Although we did not find differences in NO levels between the three sublines at 6 and 10 days PI (Expt. 5), it is not known whether high levels of NO developed in chickens of the H subline after 10 days PI, which could in turn have contributed to lesion development through an overreaction of proinflammatory cytokines.

We were able to select for differences in NO production in line A by using embryonal spleen cells stimulated with LPS and IFN-γ.

Table 5. Marek's disease (MD) in nonvaccinated chickens of three sublines of broiler line A inoculated with the RB-1B strain of MD virus.

Subline[A]	No. of birds	No. dead or euthanatized (%)[B]	No. of birds with Marek's disease[CD]				Total No. with MD (%)[B]
			Clinical	TA/BA	Neural	Tumors[D]	
L	46	10 (21.7)[a]	0	2	4	29[ab]	35 (76.1)[a]
C	53	11 (20.8)[a]	1	3	6	30[a]	40 (75.5)[a]
H	57	20 (35.1)[b]	2	3	2	45[b]	52 (91.2)[b]

[A]The sublines were classified as low (L) and high (H) nitric oxide (NO) producers on the basis of NO production by embryonal spleen cells. Offspring of nonselected offspring served as the control (C) line.
[B]Values with different superscripts are statistically significant at $P < 0.05$ (chi-square test).
[C]Marek's disease lesions were classified as clinical paralysis without gross neural lesions (Clinical); thymus atrophy, bursa atrophy, or both, without neural lesions or tumors (TA/BA); gross neural lesions with or without TA/BA (Neural); and tumors (Tumors).
[D]All chickens were inoculated intra-abdominally at 6 days of age with 1000 focus-forming units of RB-1B.

However, the selection for higher levels of NO did not result in an increase in genetic resistance to MD in line A. Contrary to the expected results we found an inverse relationship between MD resistance and NO production. Offspring from birds producing relatively low levels of NO were more resistant than birds producing relatively high levels of NO.

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