Antibody responses to keyhole limpet hemocyanin, lipopolysaccharide and Newcastle disease virus vaccine in F2 and backcrosses of White Leghorn lines selected for two different immune response traits

Giulietta Minozzi, H.K. Parmentier, M.G.B. Nieuwland, Bertrand B. Bed'Hom, Francis F. Minvielle, David Gourichon, Marie-Hélène Pinard-van Der Laan

Antibody Responses to Keyhole Limpet Hemocyanin, Lipopolysaccharide, and Newcastle Disease Virus Vaccine in F_2 and Backcrosses of White Leghorn Lines Selected for Two Different Immune Response Traits

G. Minozzi,*[1] H. K. Parmentier,† M. G. B. Nieuwland,† B. Bed'hom,* F. Minvielle,* D. Gourichon,‡ and M. H. Pinard-van der Laan*

*UMR1236 Génétique et Diversité Animales, Institut National de la Recherche Agronomique/AgroParisTech, F-78352 Jouy en Josas, France; †Adaptation Physiology Group, Department of Animal Sciences, Wageningen Institute of Animal Sciences, Wageningen University, 6700 AH, the Netherlands; and ‡Unité Expérimentale de Génétique Avicole, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

ABSTRACT Planned crosses were designed to produce an F_2 and 2 backcross populations from 2 lines of White Leghorn chickens previously selected over 10 generations for 2 different in vivo immune responses. The selection criteria applied on the 2 grandparental lines were as follows: high antibody response to Newcastle disease virus vaccine 3 wk after vaccination (ND3) and high cell-mediated immune response [response to phytohemagglutinin]. Furthermore a control line was kept by random breeding. The objective of the study was to estimate if the 2 selection criteria applied on the pure lines had changed the level of and type of immune (humoral) response to a new antigen, keyhole limpet hemocyanin (KLH), in the various second-generation progeny groups. In addition, correlations between parameters of acquired and innate immunity were tested. Primary total (IgT) and isotype-specific (IgG and IgM) antibody response to KLH 1 wk after immunization and levels of natural antibodies (NAB) binding to *Salmonella enteriditis*-derived lipopolysaccharide (LPS) were measured. Although no differences were present between IgM and IgG antibodies to KLH and the phytohemagglutinin skin-swelling response, significant differences were present between all the progeny groups for IgT to KLH and ND3 and NAB binding to LPS. The mean values for IgT to ND3 and KLH were significantly different between the crosses using the selected lines compared with the control line, indicating a contribution of the previous selection. In addition, a sex effect was found for IgM to KLH and NAB to LPS, for which females had a higher response than males in both cases. No interaction between progeny type and sex was found. Furthermore, significant positive correlations were found between NAB to LPS and specific antibody titers to KLH. Finally, the results of the present study demonstrated an interaction between innate and acquired immunity under this strategy of selection and crossbreeding and confirmed the effect of selection on general immune response to a new antigen in second-generation crosses.

Key words: primary antibody response, chicken, keyhole limpet hemocyanin, *Salmonella enteriditis*-derived lipopolysaccharide, selection

2007 Poultry Science 86:1316–1322

INTRODUCTION

In recent years, genetic selection of farm animals has led to impressive improvements in animal production and especially in traits of economic importance in poultry (Flock and Heil, 2002). The selection objectives now tend toward a better sustainability of livestock production, and selection for disease resistance is a major challenge for animal geneticists studying microsatellite markers (McElroy et al., 2005) and candidate genes associated with resistance to diseases (Kramer et al., 2003).

There are several possible strategies for developing chicken lines selected for disease resistance in poultry (Lamont et al., 2003). Direct strategies range from divergent selection for a single immune response trait to selection based on an index of various different immune responses. These approaches can also be combined by crossing lines, each developed by selection on a different set of traits or on a different, single immune trait, as it was done in the present work after a number of within-line generations of selection.

The hemocyanin of the marine gastropod *Megathura crenulata*, keyhole limpet hemocyanin (**KLH**), is a Cu-containing respiratory protein that has been used in vari-

Received January 17, 2007.
Accepted March 17, 2007.
[1]Corresponding author: Giulietta.Minozzi@jouy.inra.fr

Table 1. Means ± SD and overall ANOVA of total (IgT) and isotype-specific (IgM and IgG) plasma antibody titers[1] to keyhole limpet hemocyanin (KLH), levels of natural antibodies (NAB) binding *Salmonella enteriditis*-derived lipopolysaccharide (LPS) 1 wk after immunization with KLH, total antibody titers against Newcastle disease virus vaccine (ND3), wing web response to phytohemagglutinin (PHA) at 9 wk of age, and 8-wk BW

Overall ANOVA			Trait						
			KLH IgT	KLH IgM	KLH IgG	ND3[2]	PHA (mm)	LPS NAB	BW (g)
Significance of main effects									
Sex			NS	***	NS	NS	***	NS	***
Group			***	NS	NS	***	NS	***	NS
Sex × group			NS	NS	NS	NS	NS	NS	NS
Sire family			NS	**	***	**	NS	NS	**
R^2			0.46	0.38	0.40	0.61	0.57	0.38	0.74
Progeny type	n	Group[3] (sire parental lines; dam parental lines)	Group means						
F_2	34	1 (ND3-L × PHA-L; ND3-L × PHA-L)	12.07 ± 1.75^{abc}	7.43 ± 1.83	8.23 ± 2.11	6.88 ± 1.75^{a}	1.41 ± 0.52	5.08 ± 2.09^{abcd}	907.0 ± 123.5
F_2	20	2 (ND3-L × PHA-L; PHA-L × ND3-L)	12.47 ± 1.33^{a}	7.69 ± 1.63	8.58 ± 1.99	6.25 ± 1.33^{ab}	1.42 ± 0.67	6.21 ± 1.78^{ab}	914.4 ± 146.4
F_2	20	3 (PHA-L × ND3-L; ND3-L × PHA-L)	11.57 ± 0.97^{abcd}	6.76 ± 1.08	7.91 ± 1.05	6.75 ± 1.61^{a}	1.37 ± 0.71	4.37 ± 1.79^{de}	925.9 ± 131.0
F_2	28	4 (PHA-L × ND3-L; PHA-L × ND3-L)	11.34 ± 1.35^{bcde}	6.92 ± 1.69	7.21 ± 1.55	6.14 ± 1.75^{ab}	1.59 ± 0.63	6.32 ± 2.10^{a}	944.8 ± 123.7
BC1	30	5 (ND3-L × PHA-L; ND3-L)	11.41 ± 1.32^{bcde}	7.32 ± 1.05	8.18 ± 1.65	6.97 ± 1.65^{a}	1.27 ± 0.61	5.13 ± 1.79^{abcd}	896.5 ± 119.1
BC1	20	6 (PHA-L × ND3-L; ND3-L)	11.16 ± 1.12^{cde}	7.17 ± 1.21	8.95 ± 1.41	6.90 ± 1.37^{a}	1.56 ± 0.85	4.86 ± 1.92^{cd}	884.4 ± 103.9
BC1	22	7 (ND3-L; ND3-L × PHA-L)	11.69 ± 1.25^{abcd}	7.55 ± 1.16	8.24 ± 1.90	6.25 ± 1.57^{ab}	1.46 ± 0.78	5.79 ± 2.26^{abc}	842.4 ± 94.9
BC1	32	8 (ND3-L; PHA-L × ND3-L)	11.21 ± 1.56^{bcde}	7.21 ± 1.13	7.61 ± 1.42	6.50 ± 1.50^{ab}	1.51 ± 0.47	5.02 ± 2.01^{bcd}	927.4 ± 130.4
BC2	29	9 (ND3-L × PHA-L; PHA-L)	10.96 ± 2.00^{de}	6.43 ± 1.57	7.74 ± 2.23	5.65 ± 1.56^{b}	1.72 ± 0.81	4.48 ± 2.16^{de}	934.2 ± 117.1
BC2	24	10 (PHA-L × ND3-L; PHA-L)	12.17 ± 1.39^{ab}	7.96 ± 1.27	8.61 ± 1.42	4.67 ± 2.07^{c}	1.56 ± 0.62	5.48 ± 1.73^{abcd}	895.9 ± 113.3
BC2	27	11 (PHA-L; ND3-L × PHA-L)	11.57 ± 1.46^{abcd}	7.13 ± 1.08	7.83 ± 1.71	4.28 ± 1.40^{c}	1.71 ± 0.60	4.43 ± 2.04^{d}	946.8 ± 137.7
BC2	22	12 (PHA-L; PHA-L × ND3-L)	10.53 ± 1.63^{e}	7.13 ± 1.70	7.22 ± 2.49	4.35 ± 1.24^{c}	1.37 ± 0.56	4.61 ± 2.07^{cd}	905.4 ± 145.3
Control	42	13 (control-L; control-L)	9.41 ± 2.06^{f}	6.68 ± 2.09	7.98 ± 2.82	3.19 ± 1.25^{d}	1.38 ± 0.70	3.27 ± 1.89^{e}	932.1 ± 126.2

[a–e]Means in the same column with no common superscripts differ significantly ($P \leq 0.05$) based on Duncan's multiple range test.

[1]Titers for IgT, IgM, and IgG to KLH and LPS NAB are the base-2 logarithm of the reciprocal of the antibody dilution.

[2]Antibody titers in plasma measured by hemagglutination inhibition test; the titer is expressed as the highest dilution giving total inhibition of hemagglutination.

[3]ND3-L = line selected for high antibody response to ND3 three weeks after vaccination; PHA-L = line selected for high cell-mediated immune response to PHA at 9 wk of age; control-L = line selected at random.

**$P \leq 0.01$; ***$P \leq 0.001$.

ous scientific and biomedical studies (Harris and Markl, 1999), including a study aimed at identifying QTL for both natural as well as specific immune responses in chicken (Siwek et al., 2003). The KLH is a T-cell-dependent antigen that neither mammals nor birds usually have ever been exposed to. It is a potent immunostimulator used as a hapten carrier to assess the magnitude of immune responses (Macia et al., 2006) and to study cancer (Ragupathi et al., 2006). Recently, higher levels of natural antibodies (**NAB**) and specific antibodies to KLH were found in chickens selected for high antibody responses to SRBC than in the corresponding low-antibody-producing and control chickens (Parmentier et al., 2004a; Siwek et al., 2006), suggesting that NAB might be a useful tool to estimate (humoral) immune competence.

Lipopolysaccharide (**LPS**) is a homotope or similarly called pathogen-associated molecular pattern. It is a component of the cell wall shared by all gram-negative bacteria and a stimulator of the innate immune system (Tang et al., 2006). Lipopolysaccharide is commonly present in the chicken intestinal microflora as well as in the normal husbandry environment (Parmentier et al., 2006). Lipopolysaccharide acts by binding to Toll-like receptor 4 on antigen, presenting cells that may induce release of Th-1-like cytokines facilitating cell-mediated immune responses. Lipopolysaccharide negatively affects antibody production in poultry to KLH (Parmentier et al., 2004b).

A selection experiment based on 3 different immune response traits was conducted for 12 discrete generations of selection at the Institut National de la Recherche Agronomique in Jouy en Josas. Two of the selection criteria were as follows: high antibody response to Newcastle disease virus (HB1 vaccine; **ND3**) and high cell-mediated immune response [skin-swelling response to phytohe-

magglutinin (**PHA**)]. In addition, since the beginning of the selection experiment, a control line was kept by random breeding. Each line was selected on 1 immune trait, but all traits were measured in all lines. Heritabilities estimated for the selection criteria ND3 and PHA were 0.35 and 0.13, respectively, and correlation between the traits was not significant (Pinard-van der Laan, 2002).

In the present work, reciprocal crosses with birds from the high antibody response and the high cell-mediated response lines were made to produce an F_2 and 2 backcrosses. In addition, a contemporary replicate of the control line was produced. The objectives of the present study were to estimate the effect of selection for general immune response on the specific antibody production to a new antigen in second-generation crosses between the 2 selected lines and to gain insight on the correlated effects of selection on the immune system, using total and isotype-specific primary antibodies to KLH. Also, a humoral component of innate immunity was assessed measuring levels of *Salmonella enteriditis*-derived LPS-binding NAB. The interaction between innate and acquired immune responses was evaluated through the correlation between the level of LPS-binding NAB and the specific antibody titers to KLH and ND3.

MATERIALS AND METHODS

Lines

The F_2 and backcross populations used in the current study were produced from 2 selected lines of White Leghorn chickens derived from the same base population and measured for 3 different immune response criteria for 12 discrete generations of selection. The immune response traits selected in the 2 purebred lines were as follows:

1) High antibody response to ND3 (HB1 vaccine) 3 wk after vaccination in line 1 (**ND3-L**), measured at 6 wk of age by hemagglutination inhibition test, in which the plasma antibody titer was the highest dilution giving total inhibition of hemagglutination.

2) High cell-mediated immune response, measured using the wing web 24-h delayed-type hypersensitivity response to the mitogen PHA in line 2 (**PHA-L**). The difference in the thickness of the wing webs was measured at 9 wk of age by a micrometer at 0 and 24 h after injection of 1 mg of PHA (Sigma-Aldrich GmbH, Schnelldorf, Germany) in 0.1 mL of PBS in 1 wing and 0.1 mL of PBS in the other wing, which was used as a control. The PHA skin-swelling response was calculated as the difference between the PHA-injected and PBS-injected responses.

In addition, a control line was kept by random selection. The selection procedure was described by Pinard-van der Laan (2002). Briefly, each year, 200 chicks per line were hatched in a single batch, and chicks from all lines were assigned to every group cage. Selection for each trait was done by within-family mass selection based on individual phenotype, and mating was performed at random, but half-sib and full-sib matings were avoided to limit inbreeding.

In 2005, animals selected from generation 9 (15 males and 30 females from each line) were chosen to produce the purebreds of the following generation (generation 10) and the F_1 crossbreds. All possible reciprocal crosses among the 3 pure lines were made to generate the F_1 generation, and a contemporary replicate of the control line was produced. The F_1 population was composed of 800 animals: 200 per cross, with 100 for each reciprocal cross and 200 birds from the control line. Each sire was mated to 2 dams, and matings between half-sibs or full-sibs were avoided.

For the present study, the F_1 progeny from the crosses of lines 1 (ND3-L) and 2 (PHA-L) and the purebred breeders of lines 1 and 2 at generation 10 were crossed to produce 200 reciprocal F_2 and 400 backcrosses. In addition, a control line composed of 200 contemporary unselected birds was produced. To obtain the F_2, 15 males of the F_1 between lines 1 and 2 were mated to 2 F_1 females each. Breeders were chosen randomly, but mating of individuals with a common grandparent was avoided, and the breeding plan was designed to generate all 4 different possible groups (Table 1). The backcross to line 1 (**BC1**; ND3-L) was produced by mating 8 males (4 per reciprocal cross) of the F_1 group and 7 pure line males to 2 females each of the opposite population to obtain 4 progeny groups (Table 1). The backcross to line 2 (**BC2**; PHA-L) was obtained in a similar way. A contemporary replicate of the control line, composed of 200 animals, was produced. The experiment was conducted on 350 fourteen-week-old animals, 175 females, and 175 males. Both sexes and as many families as possible were included in the experimental population tested for antibody response to KLH and for level of NAB binding to LPS.

Husbandry

After hatching, chicks were housed and genetic groups intermingled in a 3-tier battery of group cages, in which they remained until the end of the experiment. Each cage housed about 13 animals randomly selected from each progeny group. Both sexes were kept in separate cages but housed in the same room. Artificial light was 16 h/d. The birds were fed a layer diet (2,685 kcal of ME/kg and 175 g/kg of CP) ad libitum with free access to water. Birds were vaccinated against Marek's disease at hatch by i.m. injection; spray-vaccinated for infectious bronchitis at d 1 and 63; vaccinated against Gumboro disease via drinking water at d 19, 37, and 84; vaccinated against Newcastle disease by intraocular method (eye drop) at d 26 and in drinking water at d 62; vaccinated against avian encephalomyelitis via drinking water at d 98; and spray-vaccinated for avian infectious rhinotracheitis or swollen head syndrome at d 77. All birds were weighed at 8 wk of age.

Table 2. Comparisons between mating types for total (IgT) and isotype-specific (IgM and IgG) plasma antibody titers to keyhole limpet hemocyanin (KLH), levels of natural antibodies binding *Salmonella enteriditis*-derived lipopolysaccharide (LPS) 1 wk after immunization with KLH, total antibody titers against Newcastle disease virus vaccine (ND3) vaccination, wing web response to phytohemagglutinin (PHA), and 8-wk BW

	Trait						
Linear combination of progeny types[1]	(KLH) IgM	(KLH) IgT	(KLH) IgG	LPS	ND3	PHA	BW
F_2 – backcross groups	–0.30	1.71*	–0.72	1.96	2.75***	–0.21	35.26
F_2 – control-L	0.60*	2.41***	0.20	2.22***	3.21***	0.02	–3.71
Backcross to ND3-L – control-L	0.73**	1.99***	0.54	1.95***	3.53***	–0.02	–30.32*
Backcrosses to PHA-L – control-L	0.63*	1.98***	0.22	1.57***	1.52***	0.17	5.26
Backcrosses to PHA-L – backcrosses to ND3-L	–0.41	–0.02	–1.29	–1.49	–8.03***	0.78*	142.3**

[1]ND3-L = line selected for high antibody response to Newcastle disease virus (HB1 vaccine) 3 wk after vaccination; PHA-L = line selected for high cell-mediated immune response, using the wing web response to PHA at 9 wk of age; control-L = line selected at random.

*$P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

Humoral Immune Response to KLH and LPS

Total IgG and IgM antibody titers to KLH (Sigma-Aldrich GmbH) and levels of NAB binding to LPS (Sigma Chemical Co., St Louis, MO) in plasma of 350 birds previously immunized at 14 wk of age with 1 mg/mL of KLH were measured by an indirect 2-step ELISA procedure at d 7 postimmunization. Briefly, 96-well plates were coated with 1 μg of KLH/mL and 4 μg of LPS/mL. After washing with tap water and 0.05% Tween, plates were incubated with serial dilutions of plasma. Binding of total antibodies to KLH and of NAB to LPS was detected using 1:20,000 diluted rabbit antichicken (IgG_{H+L}; Nordic, Tilburg, the Netherlands) coupled to peroxidase (**PO**). Binding of IgG and IgM antibodies to KLH was detected by using, respectively, goat antichicken (IgG-Fc; Bethyl Inc., Montgomery, TX) coupled to PO and goat antichicken (IgM; Bethyl Inc.) labeled with PO. Tetramethylbenzidine and 0.05% of H_20_2 were added and incubated for 10 min at room temperature. The reaction was stopped with 1.25 *M* H_2SO_4, and extinctions were measured with a Multiscan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as the base-2 logarithm values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of a positive control serum present on every microtiter plate.

Statistical Analysis

Plasma primary antibody titers to KLH, ND3, skin swelling to PHA, BW at 8 wk of age, and levels of NAB binding to LPS were analyzed by a mixed 3-way ANOVA with the sex and the progeny group as fixed effects, the sire as a random effect nested within the progeny group, and with all possible interactions. All analyses were done with the GLM procedure of SAS (SAS Institute, 2001). The sire MS error was used to test the overall significance of the effect of the progeny group. The MS error of the interaction between sex and sire within progeny group was used to test the significance of effect of sex and of the interaction between the sex and the progeny group. When a main effect was found to be significant, the corresponding group means were compared by using Duncan's multiple range test.

RESULTS

Means and SD of IgT, IgM, and IgG antibody titers to KLH, IgT to LPS 1 wk after KLH immunization, IgT to ND3 3 wk after vaccination, PHA, and 8-wk BW for all 13 groups are shown in Table 1. Females had higher levels ($P < 0.001$) of specific IgM antibodies to KLH and higher PHA response than males. The effect of the progeny group on IgT against KLH, LPS, and ND3 was significant ($P < 0.001$), but there were no group differences for IgM and IgG antibodies to KLH, BW, and for the PHA response. No interactions were significant.

Progeny Types

The comparisons among the 4 progeny types (F_2, BC1, BC2, and control) obtained by linear combinations of the group least squares means are shown in Table 2 for each immune trait and for BW. Differences between the 2 backcross populations (BC1 – BC2) were absent for IgT and isotype-specific antibody titers to KLH and levels of NAB binding to LPS but marked for IgT to ND3 (–8.03, $P < 0.001$) for BW (142.3, $P < 0.01$) and PHA skin swelling (0.78, $P < 0.05$). The control line differed from all the other 3 progeny groups for NAB binding to LPS, IgT, and IgM titers to KLH and only from the backcross population to line ND3 for BW. Differences between the mean of the F_2 group and the mean of the 2 backcross populations were found for IgT antibody titers to KLH (1.71, $P < 0.05$), and antibody titers to ND3 (2.75, $P < 0.001$). The latter difference was mainly contributed by the BC1 progeny type.

A Posteriori Comparisons of Means

The mean values for ND3 and for IgT to KLH were lower ($P < 0.05$) in the control population than in all other progeny groups, and means for IgM and IgG to KLH and PHA were similar in all groups (Table 1). Only few significant differences were found for IgT to KLH. Means were higher in group 2 (ND3-L × PHA-L; PHA-L × ND3-

Table 3. Correlation coefficients of natural antibody (NAB) titers[1] to *Salmonella enteriditis*-derived lipopolysaccharide (LPS) with total (IgT) and isotype-specific (IgM and IgG) antibody titers to keyhole limpet hemocyanin (KLH) 1 wk after immunization with KLH, IgT against Newcastle disease virus vaccine (ND3), and 8-wk BW, in the 13 groups

Progeny type	n	Group[2] (sire parental lines; dam parental lines)	Correlation coefficient (KLH) IgT	(KLH) IgM	(KLH) IgG	ND3[3]	BW
F_2	34	1 (ND3-L × PHA-L; ND3-L × PHA-L)	0.67***	0.66***	0.61***	0.35*	1.41
F_2	20	2 (ND3-L × PHA-L; PHA-L × ND3-L)	0.63**	0.55*	0.58**	–0.19	0.02
F_2	20	3 (PHA-L × ND3-L; ND3-L × PHA-L)	0.36	0.49*	0.30	0.11	–0.191
F_2	28	4 (PHA-L × ND3-L; PHA-L × ND3-L)	0.59***	0.75***	0.47*	–0.09	0.01
BC1	30	5 (ND3-L × PHA-L; ND3-L)	0.21	0.37*	0.15	0.12	0.25
BC1	20	6 (PHA-L × ND3-L; ND3-L)	0.53*	0.54**	–0.48*	0.09	–0.32
BC1	22	7 (ND3-L; ND3-L × PHA-L)	0.52**	0.58**	0.32	0.17	–0.09
BC1	32	8 (ND3-L; PHA-L × ND3-L)	0.55***	0.42*	0.57***	0.17	–0.18
BC2	29	9 (ND3-L × PHA-L; PHA-L)	0.72***	0.57***	0.69***	0.16	–0.20
BC2	24	10 (PHA-L × ND3-L; PHA-L)	0.57**	0.28	0.64***	0.38	–0.01
BC2	27	11 (PHA-L; ND3-L × PHA-L)	0.62***	0.39*	0.64***	0.31	–0.17
BC2	22	12 (PHA-L; PHA-L × ND3-L)	0.74***	0.76***	0.27	–0.52**	–0.14
Control	42	13 (control-L; control-L)	0.66***	0.47***	0.24	0.13	0.11

[1]Titers for IgT, IgM, and IgG to KLH and LPS NAB are the base-2 logarithm of the reciprocal of the antibody dilution.

[2]ND3-L = line selected for high antibody response to Newcastle disease virus (HB1 vaccine) 3 wk after vaccination; PHA-L = line selected for high cell-mediated immune response, using the wing web response to phytohemagglutinin at 9 wk of age; control-L = line selected at random.

[3]Antibody titers in plasma measured by hemagglutination inhibition test; the titer is expressed as the highest dilution giving total inhibition of hemagglutination.

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

L) than in groups 4, 5, 6, 8, 9, and 12 and higher in groups 1, 2, and 10 than in groups 9 (ND3-L × PHA-L; PHA-L) and 12 (PHA-L; PHA-L × ND3-L). Higher values in NAB for LPS were found in the 2 different F_2 reciprocal crosses (groups 4 and 2), because their means were larger $P < 0.05$) than that of groups 3, 6, 9, 11, and 12. On the other hand, means of groups 1, 2, 4, 5, 7, and 10 were similar. Dams of both groups 2 and 4 were PHA-L × ND3-L crossbreds. Higher titers of IgT to ND3 ($P < 0.05$) were obtained for groups 1 to 8 (having a larger genetic contribution from ND3-L) than for groups 10 to 12, and the means of groups 2, 4, 7, 8, and 9 were similar.

Correlations

NAB Titers to LPS. Significant correlations were found between NAB to LPS and the titers of IgT and isotype-specific antibodies against KLH in most groups (Table 3). Positive values ranged from 0.37 to 0.76, and the only negative value was –0.48. On the other hand, most correlations of NAB binding to LPS and antibody titers with ND3 and BW were not significant.

PHA. Few correlations with PHA and the other immune traits were significant (Table 4). A negative correlation between PHA and IgT titers to KLH was found in the control line only. Correlation between PHA and IgG antibodies to KLH was positive in the control group (0.31, $P < 0.05$) and negative in the backcross population to the PHA-L line (–0.49, $P < 0.05$). On the other hand, negative correlations ranging from –0.45 and –0.63 were found with BW in 7 out of the 13 groups.

DISCUSSION

The objectives of the present study were 3-fold. First, using total and isotype-specific primary antibodies to KLH, the effect of selection for general immune response on the specific antibody production to a new antigen in second-generation crosses between the 2 immune-selected lines was studied to gain insight on the correlated effects of selection on the immune system. Second, correlated responses between antibody titers to ND vaccine (humoral immunity) and skin-swelling responses to PHA (nonspecific cellular immunity) on the one hand and between the level of specific antibody production to KLH and the levels of NAB to LPS on the other hand was tested in the crossbred and F_2 populations.

Third, correlations between parameters of innate (NAB) and acquired (specific antibodies) immunity of animals under this strategy of selection and crossbreeding were estimated. Previous studies (Parmentier et al., 2004a) have suggested a significant relationship between the levels of NAB and acquired humoral immune responses. Results obtained from the present study may provide new information on genetic components of these traits in crossbred animals as well as new insights on genetic selection for general immune responses.

With respect to the antibody titers to the ND3 measure, the level of responses in the different progeny types was clearly influenced by the different contribution of the selected line in the different crosses. The main difference between the progeny types was seen in the 2 backcross populations, in which the line backcrossed to the ND3-selected line responded better than the backcross to the cell-mediated selected pure line, which indicates a significant contribution of the previous selection.

Within the different backcross populations, the 4 reciprocal crosses to the ND3 line (selected on antibody titers to ND3) responded in an equal fashion, indicating that in the future, any 1 of the 4 reciprocal crosses could be used. For the other 4 backcross groups to line PHA-L or line 2 (cellular immunity to PHA), however, the highest

Table 4. Correlation coefficients of wing web response to phytohemagglutinin (PHA) with total (IgT) and isotype-specific (IgM and IgG) antibody titers[1] to keyhole limpet hemocyanin (KLH), levels of natural antibodies (NAB) binding to *Salmonella enteriditis*-derived lipopolysaccharide (LPS) 1 wk after immunization with KLH, antibody titers (ND3) to Newcastle disease virus vaccine, and 8-wk BW

Progeny type	n	Group[2] (sire parental lines; dam parental lines)	Correlation coefficient					
			(KLH) IgT	(KLH) IgM	LPS	(KLH) IgG	ND3[1]	BW
F_2	34	1 (ND3-L × PHA-L; ND3-L × PHA-L)	–0.24	–0.02	–0.12	–0.29	0.07	–0.23
F_2	20	2 (ND3-L × PHA-L; PHA-L × ND3-L)	–0.16	0.03	–0.04	0.12	0.11	–0.38
F_2	20	3 (PHA-L × ND3-L; ND3-L × PHA-L)	0.16	0.40	0.04	0.24	–0.53*	–0.30
F_2	28	4 (PHA-L × ND3-L; PHA-L × ND3-L)	0.13	0.24	–0.03	0.16	0.29	–0.48**
BC1	30	5 (ND3-L × PHA-L; ND3-L)	–0.24	0.09	0.00	–0.09	–0.08	–0.58***
BC1	20	6 (PHA-L × ND3-L; ND3-L)	–0.07	0.06	–0.01	–0.16	0.11	–0.42
BC1	22	7 (ND3-L; ND3-L × PHA-L)	–0.29	–0.30	–0.02	–0.22	0.05	–0.37
BC1	32	8 (ND3-L; PHA-L × ND3-L)	–0.03	0.12	0.16	–0.03	0.19	–0.63***
BC2	29	9 (ND3-L × PHA-L; PHA-L)	0.05	0.14	–0.03	0.03	–0.21	–0.52**
BC2	24	10 (PHA-L × ND3-L; PHA-L)	0.20	0.34	–0.05	0.28	0.28	–0.54**
BC2	27	11 (PHA-L; ND3-L × PHA-L)	0.32	0.12	0.27	0.30	–0.06	–0.09
BC2	22	12 (PHA-L; PHA-L × ND3-L)	–0.12	0.26	0.05	–0.49*	0.21	–0.45**
Control	42	13 (control-L; control-L)	–0.37**	–0.14	–0.26	0.31*	–0.29	–0.45**

[1]Titers for IgT, IgM, and IgG to KLH and LPS NAB are the base-2 logarithm of the reciprocal of the antibody dilution.

[2]ND3-L = line selected for high antibody response to Newcastle disease virus (HB1 vaccine) 3 wk after vaccination; PHA-L = line selected for high cell-mediated immune response to PHA at 9 wk of age; control-L = line selected at random.

[3]Antibody titers in plasma measured by hemagglutination inhibition test, the titer is expressed as the highest dilution giving total inhibition of hemagglutination.

**$P \leq 0.01$; ***$P \leq 0.001$.

value was obtained by crossing an F_1 male (ND3-L; PHA-L) to a PHA-L female. All the mating types differed from the control line, underlining the contribution of the current selection procedure. In addition, the present study confirmed that after a crossbreeding design based on the selection for humoral response to ND3 and cellular response to PHA in the pure lines (Pinard-van der Laan, 2002), independence of the traits in the F_2 and crossbred progeny was still observed. This gives further possibilities for using single traits independently in each grand parental line in 4-way crosses.

Although the trend for the primary antibody production to ND3 vaccine showed marked differences, the PHA value in the different mating types was similar overall. The different rearrangement of the genotypes and the relative different pressure of selection for PHA responsiveness did not enhance the particular cellular immune response compared with the unselected control line. A sex effect was present that was not found in the corresponding pure line (Pinard-van der Laan, 2002). No phenotypic correlation was found between the PHA skin-swelling value and all the other (humoral) immune traits, suggesting the possibility of introducing these criteria in a multiselection program. Whether the absence of any correlation between PHA and the other traits is based on the specific character of the humoral immune responses on the one hand and the nonspecific mitogenic character of the PHA response on the other hand remains to be elucidated.

The KLH was used as a model of T-cell-dependent antigen to test correlated effects of selection on general immune responses of the grand parental animals [i.e., whether selection for high antibody production to a specific antigen (ND3) could be associated with general humoral immune responsiveness]. In addition, we wanted to test the correlation of this trait with the other criteria as a possible candidate trait for additional selection criteria in the future. An effect of the crosses was found for IgT against KLH only. The IgG and IgM responses did not significantly vary among the different crosses, although they were positively correlated with the level of IgT. Characterization of a secondary immune response to KLH, consisting mainly of IgG, or alternatively measuring kinetics of the antibody responses of the pure lines, however, might reveal differences between the crosses that went unnoticed until now. The present data suggest that the different genotypic backgrounds do not cause differential isotype-specific responses measured at d 7. Females performed significantly better than males in IgM to KLH, confirming prior results on antibody production to SRBC (Pinard-van der Laan et al., 1993).

The different levels of NAB binding to LPS in the different crosses showed clearly an effect of the genotype on this trait. This demonstrates that selection on the pure lines gave rise to these variations in the F_2 and crossbreds. Levels in all crossbreds are higher than those found in the control unselected line. This is in agreement with previous studies in which higher levels of NAB to a variety of antigens were found in chicken lines selected for high specific antibody response to SRBC compared with the low responders (Parmentier et al., 2004a). Levels of LPS to which the birds were exposed in this experiment were not expected to be different, because all birds were kept in the same environment, given the same food, and all immunized with the same dose of KLH. Thus, the different immune selection procedures may have determined a different magnitude of antibody production to LPS present in the environment or intestinal microflora. Earlier, levels of antibodies to LPS were shown to be positively influenced by KLH pretreatment (Parmentier et al., 2004b). In addition, a positive phenotypic correlation was found between the level of specific antibodies

against KLH and NAB to LPS. In lines selected for SRBC, correlations between primary antibody response to SRBC and NAB to LPS were very low but significant (Siwek et al., 2006). The positive association found between humoral innate (NAB binding to LPS) and acquired immunity (IgT to KLH) may be due, in this case, to a particular rearrangement in the F_2 and backcross populations. From the present results, it is tempting to speculate on a possible coselection of innate and acquired immunity, but further investigations are needed to check if this correlation is due to one or more of the selected traits or if it results form the combined effects of the selection and crossbreeding schemes.

No correlation between immune response traits and BW was found in the F_2 and backcross populations, except for PHA skin-swelling responses. With respect to the latter, a negative correlation was found in 7 out of the 13 crosses, whereas positive phenotypic (0.16) and genetic (0.09) correlations were found previously in the pure lines (Pinard-van der Laan et al., 1998). The present findings do not contradict the concept of an energy-demanding cellular or innate immune response, as opposed to a non-energy-demanding humoral immune response, in poultry (Klasing, 1998; Parmentier et al., 2002).

In conclusion, the current study further demonstrated the possibility of genetically enhancing the variability of immune responses in poultry, using direct selection or various crossbreeding designs. Using lines selected for high antibody production to a specific antigen and for high cell-mediated response to a T-cell mitogen, respectively, gave rise to an F2 and backcross populations that allowed the estimation of positive correlated effects on the specific antibody response to KLH antigens and the levels of NAP binding LPS.

ACKNOWLEDGMENTS

The research project was supported by a Marie Curie Early Stage Research Training Fellowship of the European Community's Sixth Framework Program under contract number MEST-CT-2004-504854 and by EADGENE STS funding opportunities. The assistance of all the staff from the GFA Experimental Unit at the Institut National de la Recherche Agronomique in Nouzilly is gratefully acknowledged.

REFERENCES

Flock, D. K., and G. Heil. 2002. A long-term analysis of time trends in the performance profile of white-egg and brown-egg hybrid laying strains based on results of official German random sample tests from 1974/75 to 1997/99. Arch. Geflugelkd. 66:1–20.

Harris, J. R., and J. Markl. 1999. Keyhole limpet hemocyanin (KLH): A biomedical review. Micron 30:597–623.

Klasing, K. C. 1998. Nutritional modulation of resistance to infectious diseases. Poult. Sci. 77:1119–1125.

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

Lamont, S. J., M.-H. Pinard-van der Laan, A. Cahaner, J. J. van der Poel, and H. K. Parmentier. 2003. Selection for disease resistance: Direct selection on the immune response. Pages 399–418 in Poultry Genetics, Breeding and Biotechnology. W. M. Muir and S. E. Aggrey, ed. CABI Publ., Cambridge, MA.

Macia, L., M. Delacre, and G. Abboud. 2006. Impairment of dendritic cell functionality and steady-state number in obese mice. J. Immunol. 177:5997–6006.

McElroy, J. P., J. C. Dekkers, J. E. Fulton, N. P. O'Sullivan, M. Soller, E. Lipkin, W. Zhang, K. J. Koehler, S. J. Lamont, and H. H. Cheng. 2005. Microsatellite markers associated with resistance to Marek's disease in commercial layer chickens. Poult. Sci. 84:1678–1688.

Parmentier, H. K., S. Bronkhorst, M. G. B. Nieuwland, G. De Vries Reylingh, J. M. van der Linden, M. J. W. Heetkamp, B. Kemp, J. W. Schrama, M. W. A. Verstegen, and H. van den Brand. 2002. Increased fat deposition after repeated immunization in growing chickens. Poult. Sci. 81:1308–1316.

Parmentier, H. K., A. Lammers, J. J. Hoekman, G. De Vries Reilingh, I. T. Zaanen, and H. F. J. Savelkoul. 2004a. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. Dev. Comp. Immunol. 28:39–49.

Parmentier, H. K., L. Star, S. C. Sodoyer, M. G. Nieuwland, G. De Vries Reilingh, A. Lammers, and B. Kemp. 2006. Age- and breed-dependent adapted immune responsiveness of poultry to intratracheal-administered, pathogen-associated molecular patterns. Poult. Sci. 85:2156–2168.

Parmentier, H. K., W. J. A. Van Den Kieboom, M. G. B. Nieuwland, G. De Vries Reilingh, B. N. Hangalapura, and H. F. J. Savelkoul. 2004b. Differential effects of lipopolysaccharide and lipoteichoic acid on the primary antibody response to keyhole limpet hemocyanin of chickens selected for high and low antibody responses to sheep red blood cells. Poult. Sci. 83:1133–1139.

Pinard-van der Laan, M. H. 2002. Immune modulation: The genetic approach. Vet. Immunol. Immunopathol. 87:199–205.

Pinard-van der Laan, M. H., P. B. Siegel, and S. J. Lamont. 1998. Lessons from selection experiments on immune response in the chicken. Poult. Avian. Biol. Rev. 9:125–141.

Pinard-van der Laan, M. H., J. A. M. van Arendonk, M. G. B. Nieuwland, and A. J. Van der Zijpp. 1993. Divergent selection for humoral immune responsiveness in chickens: Distribution and effects of major histocompatibility complex. Genet. Sel. Evol. 25:191–203.

Ragupathi, G., F. Koide, and P. O. Livingston. 2006. Preparation and evaluation of unimolecular pentavalent and hexavalent antigenic constructs targeting prostate and breast cancer: A synthetic route to anticancer vaccine candidates. J. Am. Chem. Soc. 128:2715–2725.

SAS Institute. 2001. The SAS System for Windows. Release 8.02. SAS Inst. Inc., Cary, NC.

Siwek, M., A. J. Buitenhuis, S. J. Cornelissen, M. G. Nieuwland, H. Bovenhuis, R. P. Crooijmans, M. A. Groenen, G. de Vries-Reilingh, H. K. Parmentier, and J. Van der Poel. 2003. Detection of different quantitative trait loci for antibody responses to keyhole lympet hemocyanin and *Mycobacterium butyricum* in two unrelated populations of laying hens. Poult. Sci. 82:1845–1852.

Siwek, M., B. Buitenhuis, S. J. Cornelissen, M. G. B. Nieuwland, E. F. Knol, R. P. M. A. Crooijmans, M. A. M. Groenen, H. K. Parmentier, and J. Van der Poel. 2006. Detection of QTL for innate: Non-specific antibody levels binding LPS and LTA in two independent populations of laying hens. Dev. Comp. Immunol. 30:659–666.

Tang, X., D. Metzger, S. Leeman, and S. Amar. 2006. LPS-induced TNF-α factor (LITAF)-deficient mice express reduced LPS-induced cytokine: Evidence for LITAF-dependent LPS signalling pathways. Proc. Natl. Acad. Sci. USA. 12:13777–13782.