IMMUNOLOGY, HEALTH, AND DISEASE

Natural Humoral Immune Competence and Survival in Layers

L. Star,*[1] K. Frankena,† B. Kemp,* M. G. B. Nieuwland,* and H. K. Parmentier*

*Adaptation Physiology Group, †Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences, Wageningen University, PO Box 338, 6700 AH Wageningen, the Netherlands

ABSTRACT The relation between survival and levels of humoral components of innate (and specific) immune competence of laying hens was investigated in a population of 1,063 laying hens from 12 purebred layer lines. Natural immune competence of the chickens was studied by measuring levels of natural antibodies (NAb) binding to keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS), respectively, and hemolytic (classical and alternative) complement activity at 20, 40, and 65 wk of age. In addition, levels of antibodies binding a Newcastle disease vaccine strain as a measure of specific immunity were investigated at 20 wk of age. A distinction could be made between lines showing high or low immune competence with respect to NAb, complement activity, and specific antibodies. Within lines, significant correlations were found for each of the innate parameters among the 3 ages. The innate and specific parameters were, however, not correlated with each other. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. However, regardless of line, low levels of NAb binding to KLH or high levels of NAb binding to LPS were detected in chickens that did not survive the laying period. The major difference between the responses of NAb binding to KLH or LPS was that the chickens probably did not encounter KLH, which suggests a reflection of the capacity to respond, whereas the chickens most probably did encounter LPS, which suggests a reflection of the active status of the innate humoral immune system. In conclusion, we propose that levels (KLH) and activation (LPS) of components of natural antibodies are indicative for the probability that chickens survive a laying period.

Key words: natural immunity, natural antibody, hemolytic complement activity, specific antibody, survival rate

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INTRODUCTION

Innate (natural) immunity is the most universal, rapid acting, and probably, the most important part of immunity. Many organisms survive through innate (-like) immune systems alone; in vertebrates, however, an additional acquired immunity evolved (Beutler, 2004). In the absence of an innate immune system, however, acquired immunity would offer weak or delayed protection because skewing and maintenance of the acquired immune response and even the most pronounced characteristic of specific immunity, memory, rests to an important degree on innate mechanisms (Bernasconi et al., 2003). Finally, the innate immune system usually acts effectively without previous exposure to a pathogen and confers broad protection against a variety of pathogens (Kimbrell and Beutler, 2001).

Among the first line of innate immune defense 2 (interrelated) humoral components can be distinguished: natural antibodies (**NAb**) and the complement system. The NAb are defined as antigen-binding antibodies present in nonimmunized individuals, which have a broad specificity repertoire and usually a low binding affinity, and which can be directed to exogenous as well endogenous antigens (Ochsenbein et al., 1999; Boes, 2000). The NAb are potentially important biological agents, prevalent in the healthy immune repertoire (Bayry et al., 2005) and are proposed to participate in the maintenance of immune homeostasis by exposure to environmental stimulations (Coutinho et al., 1995). It has been shown that NAb enhance specificity on the humoral and cellular levels in chicken (Lammers et al., 2004). In addition, NAb might be prerequisite to modulate the T-helper 2 route of specific immunity by maturation of dendritic cells (Bayry et al., 2004, 2005). The complement system is a complex enzymatic system consisting of more than 30 proteins. These proteins participate in a cascade to defend the host against invading pathogens. There are 3 pathways for activating the complement system: the classical (**CPW**), alternative (**APW**), and mannan-binding lectin pathway, all resulting in formation of a membrane attack complex (Walport, 2001a; Parmentier et al., 2002). Formation of an antibody-antigen complex is the principal way of activating the classical complement pathway (Walport, 2001b), whereas the alternative path-

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[1]Corresponding author: laura.star@wur.nl

way is activated directly by foreign microbial particles (Parmentier et al., 2002), and the mannan-binding lectin pathway is activated by foreign microbial particles following their binding by recognition molecules such as mannan-binding lectin (Carroll and Prodeus, 1998; Laursen and Nielsen, 2000). The NAb (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Stäger et al., 2003) and complement (Thorbecke et al., 1994) have been shown to perform important functions in the subsequent activation of specific humoral and cellular immune responses in mammals. The presence and activities of NAb (Lammers et al., 2004; Parmentier et al., 2004b) and complement (Laursen and Nielsen, 2000; Parmentier et al., 2004a) in poultry were reported earlier.

Innate immunity might play an important role in enhancing survival of the host by providing early resistance against infection (Ochsenbein and Zinkernagel, 2000). Low levels of innate immunity, cellular as well as humoral, may be related with disease susceptibility and high levels with disease resistance (Parmentier et al., 2004a); however, a relation between innate immunity and survival has not been shown before.

An experiment was conducted to investigate a relation between survival and humoral components of innate (natural) as well as specific immune competence of laying hens during 1 laying period. Natural immune competence of the chickens was studied by measuring levels of NAb and hemolytic complement activity (CPW and APW). Antigens were chosen to estimate levels of NAb binding to an exo-antigen (keyhole limpet hemocyanin, **KLH**), which the chickens have probably not encountered before nor will encounter during life, or an environmental-antigen (lipopolysaccharide, **LPS**) derived from the intestinal gram-negative micro biota. Specific immune reactivity was studied by measuring antibody levels binding to Newcastle disease (**NCD**) virus vaccine strain to which chickens have been routinely vaccinated at an earlier age. In the present study we established differences between layer lines in levels of natural and specific humoral immune competence, but most importantly, our data suggests that, regardless of line, levels of NAb binding to KLH or LPS were related to the probability of surviving the laying period.

MATERIALS AND METHODS

Chickens, Housing, and Feed

A population of 1,063 laying hens was used to establish natural and specific antibody levels and hemolytic complement activity. Within this population 12 purebred layer lines (Hendrix Genetics, Boxmeer, the Netherlands) could be distinguished: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE).

Chickens arrived at the laying facility at 17 wk of age and were housed in battery cages with 4 chickens of the same line in each cage (44 cm height × 46 cm depth × 39 cm width; equals 448.5 cm^2 per chicken). All chickens were housed in the same facility to minimize variation in environmental influences.

At the start of the laying period (at 19 wk of age) until 42 wk of age, chickens were fed a standard commercial phase 1 diet (159 g/kg of CP, 43 g/kg of crude fiber, and 11.17 MJ of ME/kg). From 42 wk until the end of the laying period (at 70 wk of age), chickens were fed a standard commercial phase 2 diet (152 g/kg of CP, 47.0 g/kg crude fiber and 11.01 MJ of ME/kg). Chickens had free access to feed and water.

At 17 wk of age chickens were kept at a 9L:15D light scheme. After 1 wk, the light period was increased with half an hour. Hereafter, the light period was increased with approximately 10 min/d. From 30 wk onwards chickens were kept at a 16L:8D light scheme.

Chickens were not debeaked. Chickens received routine vaccinations to Marek's disease (d 1), NCD (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15).

Experimental Design

The observational period consisted of 1 laying period, from 20 until 70 wk of age. During this period a population of 2,504 chickens [range 180 to 244 chickens per line (respectively 45 to 61 cages per line) at the start of lay] was monitored, and from chickens that died during the laying period the day of mortality was registered (cause of death was not determined).

From this population of 2,504 chickens, 1,063 chickens were used to establish natural and specific antibody levels and hemolytic complement activity. Blood samples from approximately 80 chickens per line [range 74 to 86 per line (respectively, 18.5 to 21.5 cages per line) at the start of the experiment] were taken from a wing vein at 20, 40, and 65 wk of age for measurement of immune parameters. At each sample moment the same chickens were used. Serum was collected and stored at –20°C for further processing.

The NAb binding to KLH or LPS, and complement activity of the classical and alternative pathways, were determined at 20, 40, and 65 wk of age. Specific antibodies binding to NCD vaccine were determined at 20 wk of age.

Immune Parameters

Natural Antibodies. The NAb binding to KLH or LPS were determined in individual samples by an indirect ELISA procedure as published earlier (Parmentier et al., 2004b). Flat-bottomed 96-well medium binding ELISA-plates were coated with 1 μg/mL of KLH (MP Biomedicals Inc., Aurora, OH), or 8 μg/mL of *Escherichia coli* LPS (serotype 055:B5, Sigma Chemical Co., St. Louis, MO). After subsequent washing the plates were filled with 100 μL of PBS + Tween (0.05%) + newborn calf serum (2%) per well. Serum samples were stepwise diluted (1:30, 1:90, 1:270, and 1:810), and the plates were

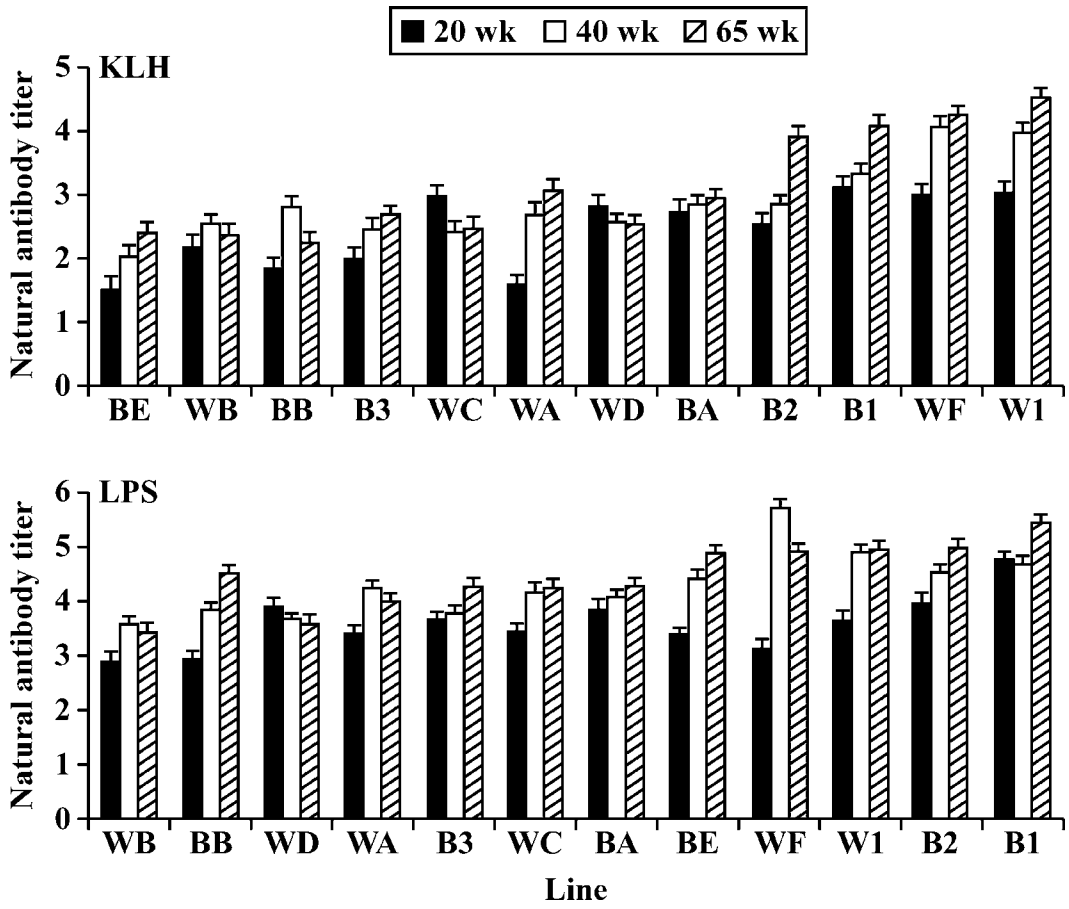

Figure 1. Levels of natural antibodies binding to keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS) in 12 purebred layer lines: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means (log_2 value + SE) of natural antibody titers determined in serum samples collected from approximately 80 chickens per line at 20, 40, and 65 wk of age (at each sample moment the same chickens were used). Lines are distributed according to total rank number per tested antigen (sum of rank numbers determined at 20, 40, and 65 wk of age).

incubated for 1 h at room temperature. Binding of the antibodies to KLH or LPS antigen was visualized using a 1:20,000 diluted rabbit antichicken IgG_{H+L} labeled with peroxidase (RACh/IgG_{H+L}/PO; Nordic, Tilburg, the Netherlands). After washing, substrate (tetramethylbenzidine and 0.05% H_2O_2) was added, and 10 min later, the reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Levels (titers; i.e., equivalent to double amounts of antibodies in serum) were calculated based on log_2 values of the dilutions that gave extinction closest to 50% of EMAX, where EMAX represents the highest mean extinction of a standard positive serum present on each plate.

Complement Assay. Sera were diluted in appropriate buffers in flat-bottomed 96-well micro titer plates and incubated with sensitized (Haemolysin, Biomerieux, ref. no. 72202) SRBC to measure CPW, or bovine red blood cells to measure APW as published earlier (Demey et al., 1993; Parmentier et al., 2002). During 1.5 h of incubation the plates were shaken every 30 min. After that the plates were read with a Multiskan at a wavelength of 690 nm. Readings were transformed by log-log equation (Von Krogh, 1916), and the hemolytic titer was expressed as the titer that lyses 50% of the red blood cells (CH50 U/mL).

Specific Antibody Response to Newcastle Disease Vaccine. Sera collected at 20 wk of age (i.e., 5 wk after the last NCD vaccination) were tested for the levels of specific antibodies to the vaccine strain (NCD clone 30, Intervet International BV, Boxmeer, the Netherlands) using an indirect ELISA as described above. Plates were coated with 100 μL/well of a solution of 1,000 doses of NCD dissolved in 200 mL of carbonate buffer. Sera were diluted 1:40, 1:160, 1:640, and 1:2,560, respectively.

Survival

The population of 2,504 chickens (range 180 to 244 chickens per line at the start of lay) was monitored, including the chickens that were used for blood collection. From chickens that died during the laying period the day of mortality was registered (cause of death was not determined). In addition, for the same 12 lines mortality data had been registered in the laying period of 1 yr earlier. Mortality data during the earlier laying period were based on a different population of chickens (between 144 and 488 chickens per line at the start of lay).

Table 1. Pearson correlation coefficients (r) (and *P*-values) between 20 and 40, 20 and 65, and 40 and 65 wk of age for keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS) in 12 purebred layer lines: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)

	KLH						LPS					
	20 × 40		20 × 65		40 × 65		20 × 40		20 × 65		40 × 65	
Line	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
B1	0.54	<0.0001	0.47	<0.0001	0.65	<0.0001	0.23	0.0415	0.19	0.1037	0.59	<0.0001
B2	0.46	<0.0001	0.44	0.0002	0.49	<0.0001	0.50	<0.0001	0.52	<0.0001	0.72	<0.0001
B3	0.60	<0.0001	0.49	<0.0001	0.68	<0.0001	0.47	<0.0001	0.48	<0.0001	0.79	<0.0001
BA	0.49	<0.0001	0.46	<0.0001	0.82	<0.0001	0.41	0.0003	0.57	<0.0001	0.46	<0.0001
BB	0.33	0.0044	0.25	0.0448	0.68	<0.0001	0.39	0.0005	0.27	0.0300	0.35	0.0049
BE	0.27	0.0246	0.11	0.3808	0.66	<0.0001	0.29	0.0151	0.35	0.0026	0.58	<0.0001
W1	0.33	0.0047	0.20	0.1231	0.51	<0.0001	0.41	0.0004	0.19	0.1462	0.56	<0.0001
WA	0.43	0.0001	0.46	<0.0001	0.67	<0.0001	0.50	<0.0001	0.46	<0.0001	0.61	<0.0001
WB	0.30	0.0130	0.32	0.0114	0.53	<0.0001	0.59	<0.0001	0.40	0.0012	0.65	<0.0001
WC	0.49	<0.0001	0.53	<0.0001	0.84	<0.0001	0.21	0.0896	0.22	0.0929	0.62	<0.0001
WD	0.33	0.0035	0.28	0.0168	0.74	<0.0001	0.36	0.0013	0.50	<0.0001	0.62	<0.0001
WF	0.35	0.0030	0.28	0.0252	0.66	<0.0001	0.42	0.0003	0.35	0.0033	0.50	<0.0001
Overall	0.44	<0.0001	0.40	<0.0001	0.69	<0.0001	0.35	<0.0001	0.38	<0.0001	0.62	<0.0001

The main difference between the earlier laying period and the laying period in the present study was that the chickens in the earlier laying period were debeaked, and in the laying period of the present study chickens were not debeaked.

Statistical Analysis

A 1-way ANOVA was performed to investigate differences in levels of NAb binding to KLH or LPS, and in activity of the classical and alternative complement pathway in the 12 purebred layer lines at each sample time (20, 40, and 65 wk of age). Differences among the 12 lines for specific antibodies binding to NCD were only investigated at 20 wk of age. Mean differences among lines were tested with Bonferroni's test.

Differences in rank number of the lines for the various natural immune parameters were tested with the Wilcoxon signed-rank test for each parameter at 20, 40, and 65 wk of age. At 20 wk of age, differences in rank number were tested between the natural immune parameters and specific antibodies binding to NCD. At 40 and 65 wk of age, differences in rank number were tested between the natural immune parameters.

Correlation between levels of NAb binding to KLH or LPS and activity of the classical and alternative complement pathway at 20, 40, and 65 wk of age were analyzed for each of the 12 layer lines by Pearson product-moment correlation. Correlation between the natural immune parameters and specific antibodies binding to NCD were analyzed for each of the 12 layer lines at 20 wk of age.

To study the relation between survival [binary variable taking the values 0 (survived) or 1 (died)], and natural (levels of NAb and complement activity) or specific immune competence (level of antigens directed to NCD), univariable logistic regression analysis was applied first. Variables with $P < 0.25$ were included in a multivariable logistic regression model:

$$\text{logit}(\pi) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon$$

where $\text{logit}(\pi) = \ln\left(\frac{\pi}{1-\pi}\right)$ and π is the probability to die given a set of explanatory variables: P(Y=1|X), x_1 = effect of line, x_2 = effect of natural immune parameter 1 (at 20 or 40 wk of age), and x_3 = effect of natural immune parameter 2 (at 20 or 40 wk of age). Outcomes will be presented as odds ratios, which indicate the relative change in risk to die dependent on the levels of natural immune parameters.

For the univariable as the multivariable logistic regression analysis, the laying period was divided in 3 parts (20 to 39 wk of age, 40 to 64 wk of age, and 65 to 70 wk of age) depending on the time of blood sampling. Chickens that died between 20 and 39 wk of age were tested against surviving chickens for natural immune parameters measured in serum collected at 20 wk of age, and chickens that died between 40 and 64 wk of age were tested against surviving chickens for natural immune parameters measured in serum collected at 40 wk of age. Chickens that died after 65 wk of age were not tested because of the low mortality rate in the last weeks of lay. A logistic regression analysis to test the predictive value of natural immunity at 20 wk of age at survival during the whole laying period was also performed.

Analysis of line differences, differences in rank number, and correlations were carried out using SAS (SAS Institute, 2004). Logistic regression analysis was carried out using Stata 8 (StataCorp, 2003). Effects were considered significant at $P < 0.05$.

RESULTS

Immune Competence

Natural Antibodies. Average levels of NAb binding to KLH or LPS in chickens at 20, 40, and 65 wk of age differed between lines (Figure 1). In 10 of the 12 and 11 of the 12 lines, respectively, an increase in levels of NAb binding to KLH and LPS was found with increasing age (from 20 to 65 wk of age). Ranking of the lines for NAb

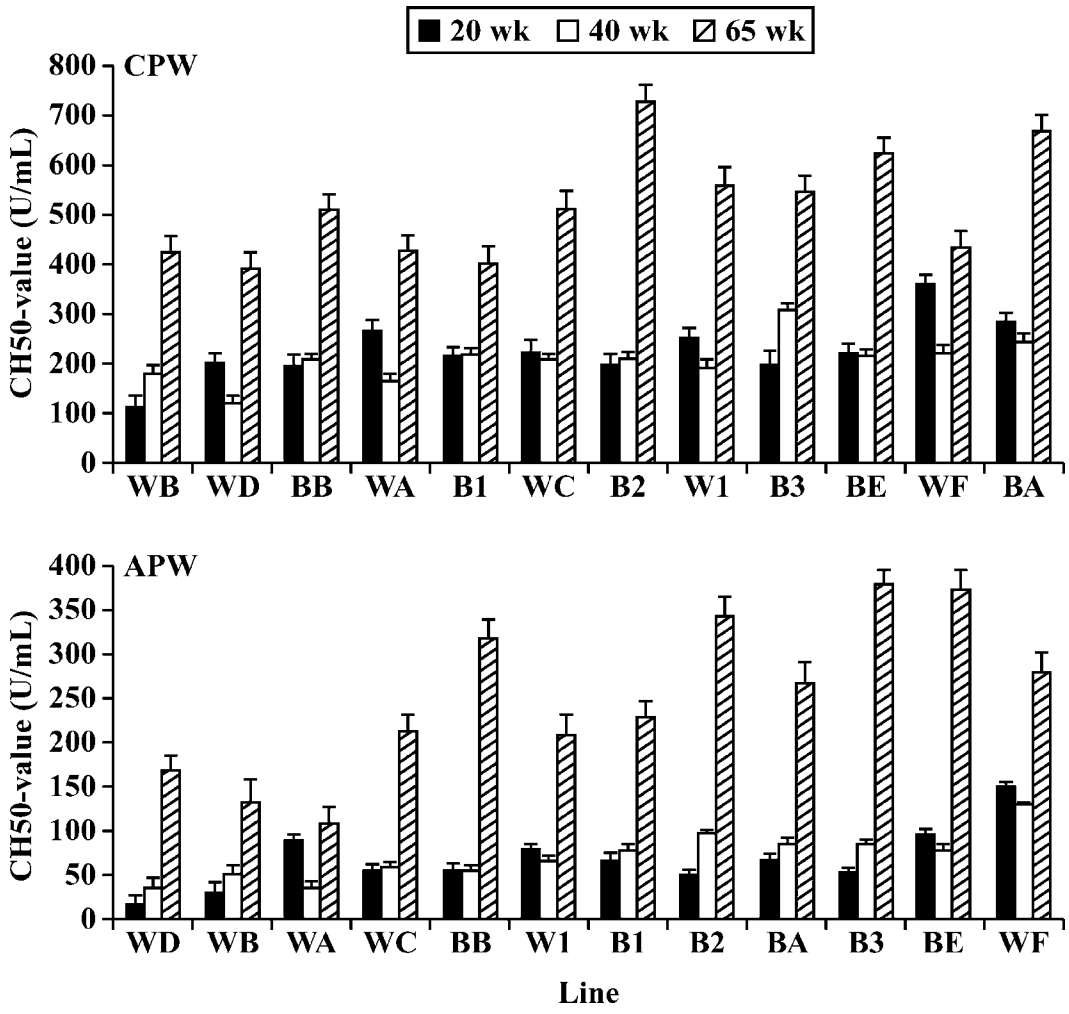

Figure 2. Hemolytic complement activity of the classical and alternative complement pathway (CPW and APW) in 12 purebred layer lines: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means [CH50-value (U/mL) + SE] of hemolytic complement activity determined in serum samples collected from approximately 80 chickens per line at 20, 40, and 65 wk of age (at each sample moment the same chickens were used). Lines are distributed according to total rank number per tested complement pathway (sum of rank numbers determined at 20, 40, and 65 wk of age).

binding to KLH or LPS were similar ($P > 0.05$) irrespective of aging. Chickens of lines B1, W1, and WF showed the highest levels of NAb for each antigen at each age (except line WF for NAb binding to LPS at 20 wk of age). In contrast to these high lines were lines BB and WB; chickens of these lines showed the lowest levels of NAb for each antigen at each age.

Within each line, significant correlations were found for levels of NAb binding to KLH or LPS between 20 and 40 wk of age and 40 and 65 wk of age. Correlations between NAb binding to KLH or LPS at 20 and 65 wk of age were significant in 10 of the 12 lines and 9 of the 12 lines, respectively. The age-related correlations for NAb binding to KLH or LPS are given in Table 1. There were no correlations of interest between NAb binding to KLH and LPS (data not shown).

Hemolytic Complement Activity. Average hemolytic complement activity of the classical and alternative complement pathways in chickens at 20, 40, and 65 wk of age differed between lines (Figure 2). In each line an increase in CPW and APW activity was found with increasing age (from 20 to 65 wk of age). This was mainly due to the strong increase of CPW and APW activity at 65 wk of age compared with CPW and APW activity at 20 and 40 wk of age. Ranking of the lines over the 3 ages seems less constant, although the ranking of lines for complement activity was not significantly different among the 3 ages and between complement activity and NAb levels. Again, line WF was among the highest ranked lines for CPW (except at 65 wk of age) and APW. Lines WB and WD were ranked among the lowest lines.

Correlations for CPW or APW between 20 and 40 wk of age were only significant in 3 of the 12 and 6 of the 12 lines, respectively. Between 40 and 65 wk of age, significant correlations for CPW or APW were found in 8 of the 12 and 9 of the 12 lines, respectively. For CPW no significant correlations were between 20 and 65 wk of age, whereas for APW between 20 and 65 wk of age a significant correlation was found in 4 of the 12 lines. The age-related correlations for CPW and APW are given in Table 2. Besides correlations for CPW or APW, correlations were also significant between CPW and APW at

Table 2. Pearson correlation coefficients (r) (and *P*-values) between 20 and 40, 20 and 65, and 40 and 65 wk of age for the classical (CPW) and alternative (APW) complement pathway in 12 purebred layer lines: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)

	CPW						APW					
	20 × 40		20 × 65		40 × 65		20 × 40		20 × 65		40 × 65	
Line	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
B1	0.24	0.0658	0.24	0.1266	0.53	<0.0001	0.18	0.3574	0.48	0.0160	0.50	0.0001
B2	0.19	0.2848	–0.07	0.7317	0.16	0.2449	0.32	0.0953	0.21	0.3639	0.31	0.0488
B3	–0.03	0.8930	0.07	0.7220	0.39	0.0006	0.45	0.0035	0.45	0.0290	0.42	0.0029
BA	0.26	0.0441	0.27	0.0672	0.47	0.0001	0.35	0.0215	0.14	0.5161	0.53	0.0008
BB	0.58	<0.0001	0.14	0.4280	0.34	0.0114	0.51	0.0057	0.27	0.2330	0.36	0.0410
BE	0.01	0.9654	0.13	0.3492	0.13	0.3492	0.31	0.0318	0.25	0.0793	0.15	0.2608
W1	0.21	0.1598	0.22	0.1634	0.53	<0.0001	0.27	0.1614	0.56	0.0083	0.38	0.0260
WA	0.20	0.2513	0.12	0.5214	0.33	0.0149	0.58	0.0178	0.28	0.1585	0.47	0.0276
WB	0.24	0.1681	–0.11	0.5623	0.11	0.4050	—	—	–0.18	0.7751	–0.23	0.4974
WC	0.38	0.0353	0.13	0.5339	0.06	0.6487	0.27	0.3027	0.49	0.0292	0.69	<0.0001
WD	0.03	0.8389	0.04	0.8112	0.38	0.0031	—	—	0.35	0.3282	0.46	0.2072
WF	0.26	0.0575	0.26	0.0653	0.33	0.0079	0.43	0.0010	0.29	0.0500	0.44	0.0012
Overall	0.23	<0.0001	0.13	0.0044	0.31	<0.0001	0.41	<0.0001	0.16	0.0042	0.09	0.0472

40 and 65 wk of age in 11 of the 12 and 9 of the 12 lines, respectively (data not shown). There were no correlations of interest between CPW and APW at 20 wk of age. Furthermore, there were no correlations between complement activity (CPW or APW) and levels of NAb (binding to KLH or LPS; data not shown).

Specific Antibody Response to Newcastle Disease Vaccine. Levels of specific antibodies binding to NCD vaccine were only analyzed at 20 wk of age because the chickens were vaccinated for the last time with NCD 5 wk earlier. The highest and lowest antibody level was found in, respectively, lines B1 (average level of 7.46) and WB (average level of 4.61; Figure 3). Although correlations were not significant between specific antibodies binding to NCD and each of the natural immune parameters at 20 wk of age, ranking of the lines for average antibody levels to NCD was similar to ranking of the lines for NAb.

Survival

Survival rate of the 12 layer lines in this experiment was between 81.5% (line WC) and 95.6% (line B3; Table 3) with an overall survival rate of 88.7%. In the earlier (trimmed beaks) and current (intact beaks) laying periods, the lines had a similar rank number with respect to survival, except line B3 that showed a large increase in survival rate, 88.9 and 95.6% for trimmed and intact beaks, respectively, resulting in a large difference in rank number for survival rate. Lines WA and WF had a high survival rate in both laying periods, and therefore overall a high rank number, whereas lines WC and WB had a low survival rate in both periods and therefore a low rank number.

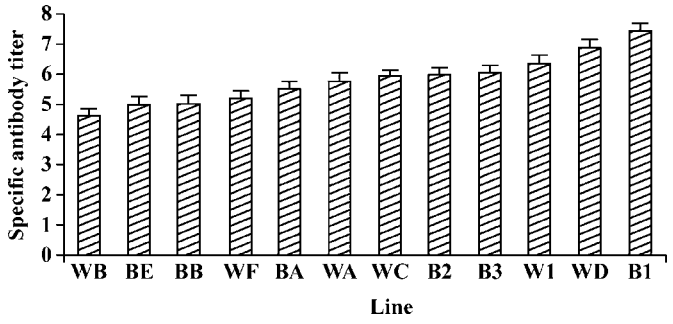

Figure 3. Specific antibodies binding to a vaccine for Newcastle disease (NCD) in 12 purebred layer lines: 6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means ($\log_2$ value + SE) of specific antibody titers determined in serum samples collected from approximately 80 chickens per line at 20 wk of age. Lines are distributed according to rank number for specific antibody titer to NCD.

Natural Immune Competence Indicative for the Probability to Survive

To investigate the relation between survival and immune competence a logistic regression analysis was performed. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. Therefore, the logistic regression analysis was performed over lines, where the statistical model was corrected for line. For the logistic regression analysis the laying period was divided in 3 parts based on the time of blood sampling. Survival rates for the period from 20 to 39 wk of age and 40 to 64 wk of age were 96.2 and 94.1%, respectively. Chickens that died after 65 wk of age were not tested because of the low mortality rate in the last weeks of lay.

The level of NAb binding to KLH or LPS at 20 wk of age was, regardless of line, significantly related to the probability to survive the first period of lay (between 20 and 39 wk of age). Chickens with a lower level of NAb binding to KLH or a higher level of LPS had a lower probability to survive this period (Figure 4). For NAb binding to KLH an odds ratio of 0.80 was found (Table 4), which means that if NAb binding to KLH increases with 1 unit (titer; i.e., equivalent to double the amount of serum NAb) the relative change in risk to die during the first period of lay increases 0.80-fold (i.e., decreases

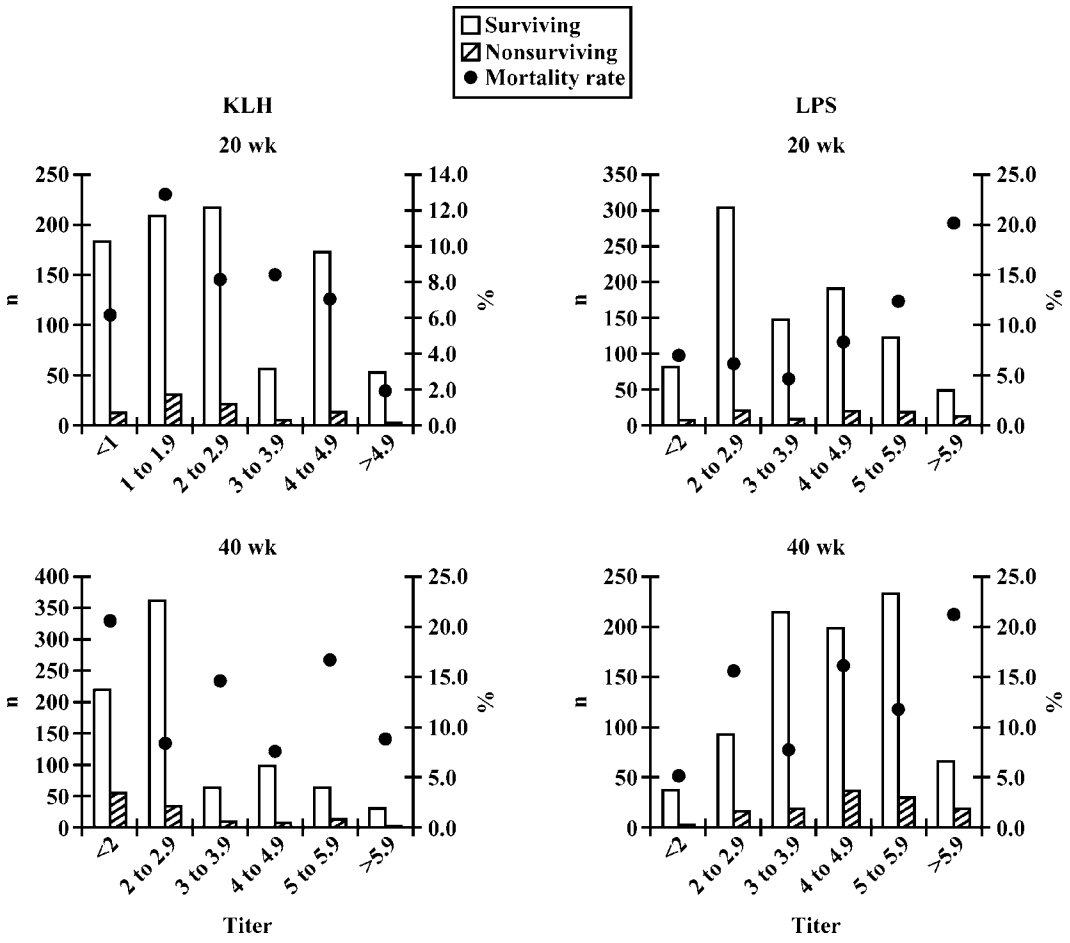

Figure 4. Mortality rate (%) and number (n) of surviving and nonsurviving chickens during the first period (20 to 39 wk of age) and the second period of lay (40 to 64 wk of age) classified by levels of natural antibodies (log_2 value) binding to keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS).

with 20%). For NAb binding to LPS an odds ratio of 1.42 was found, which means that if NAb binding to LPS increases with 1 unit (titer) the relative change in risk to die during the first period of lay increases 1.42-fold (i.e., increases with 42%).

The level of NAb binding to KLH or LPS at 40 wk of age was, regardless of line, significantly related to the probability to survive the second period of lay (between 40 and 64 wk of age), but this was not a linear effect (Figure 4). Therefore NAb binding to KLH and LPS was categorized into 6 classes (titer < 2, titer 2 to 3, titer 3 to 4, titer 4 to 5, titer 5 to 6, and titer > 6; Table 4). For NAb binding to KLH all classes had an odds ratio lower than 1 compared with the reference category (titer < 2), which means that hens with low levels of NAb binding to KLH have a higher chance to die between 40 and 64 wk of age. For NAb binding to LPS all classes had an odds ratio higher than 1 compared with the reference category (titer < 2), which means that hens with high levels of NAb binding to LPS have a higher chance to die between 40 and 64 wk of age.

Levels of NAb binding to KLH or LPS at 20 wk of age were not significantly related to the probability to survive the whole laying period. Furthermore, neither activity of the classical and alternative complement pathway at 20 and 40 wk of age nor levels of specific antibodies binding to NCD vaccine at 20 wk of age were significantly related to the probability to survive (data not shown).

DISCUSSION

To study a relation between levels of innate (natural) humoral immune competence and survival in laying hens, levels of NAb and complement activity were investigated at 3 ages during 1 laying period. Antigens were chosen to estimate levels of NAb binding to an exoantigen (KLH), which the chickens most probably have not encountered before nor will encounter during life, or an environmental-antigen (LPS) derived from the intestinal micro biota. It is likely that the chickens encountered LPS, but we have chosen to use the word natural for LPS-binding antibodies according to the definition that there is no intentional nor controllable challenge with the antigen leading to the formation of antibodies. Furthermore, because all chickens were housed in the

Table 3. Number of animals at the start of the laying period (n), survival rate (%) (rank number), and survival days (±SD) of 12 purebred layer lines [6 White Leghorn lines (W1, WA, WB, WC, WD, and WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)] as found in an earlier laying period done by Hendrix Genetics in beak-trimmed chickens and within the laying period of the present study (experiment) in chickens with intact beaks

	Hendrix Genetics			Experiment		
Line	n	Survival, %	Survival days	n	Survival, %	Survival days
B1	235	89.8 (6)	477	200	86.5 (8)	345 ± 58
B2	340	93.8 (2)	486	200	92.0 (4)	351 ± 57
B3	144	88.9 (8)	486	180	95.6 (1)	360 ± 34
BA	488	90.4 (5)	484	200	91.0 (6)	349 ± 58
BB	266	87.6 (10)	478	244	87.3 (7)	348 ± 57
BE	385	88.6 (9)	483	230	82.2 (11)	338 ± 75
W1	249	89.6 (7)	484	197	86.3 (9)	347 ± 57
WA	250	97.6 (1)	492	210	94.8 (2)	357 ± 43
WB	340	87.1 (11)	479	204	85.8 (10)	343 ± 68
WC	378	87.0 (12)	474	233	81.5 (12)	339 ± 72
WD	279	92.1 (4)	487	206	92.3 (5)	352 ± 49
WF	212	92.4 (3)	483	200	93.0 (3)	351 ± 58

same facility it was assumed that they were exposed to highly similar levels of environmental airborne or manure-derived LPS and that differences in levels of NAb binding to LPS could be attributed to line differences.

Most chickens, regardless of line, showed an increase in levels of NAb binding to KLH or LPS with aging. Higher levels of NAb with increasing age were reported before (Parmentier et al., 2004b), which correspond with the idea that exogenous stimuli enhance the formation of NAb (Prokešová et al., 1997) or maintain nonantigen-specific memory toward a variety of antigens that the individual has not encountered before and will be recognized by nonspecific innate pathogen recognition receptors such as Toll-like receptors present on B-cells (Bernasconi et al., 2003). In this respect, levels of NAb binding to LPS may reflect environmental stimulation by ubiquitous bacteria present in micro biota or the air.

Complement activity (CPW and APW) was studied at 3 ages during the laying period. Demey et al. (1993) demonstrated that complement activity of chicken sera may decline due to storage time and storage temperature. In this experiment all serum samples were stored at –20°C, and storage time was comparable for all samples, enabling us to measure line differences. Line differences were significant for CPW and APW at 20, 40, and 65 wk of age. Furthermore, the lower complement activity as observed in younger birds suggested an effect of age. Little is known about the effect of age on complement activity, but an increase of total complement activity was found for human (Nagaki et al., 1980) and ovine (Oswald et al., 1990).

The NAb binding to KLH or LPS and complement activity (CPW and APW) revealed a similar ranking depending on lines. A distinction could be made between lines showing high or low levels of NAb and complement activity. However, no significant correlations were found between the 2 types of NAb (directed to KLH or LPS), nor between NAb on the one hand and complement activity on the other hand. A relation between NAb and complement was expected because formation of an antibody-antigen complex is the principal way of activating the classical complement pathway (Walport, 2001b). Within lines, however, there were positive correlations for these parameters between the different ages. These results suggest that 1) NAb binding to KLH and LPS represents different functional B cell activities, 2) high levels of NAb can be found in chicken lines with high complement activity and low levels of NAb can be found in chickens with low complement activity, which fit with earlier observations of Parmentier et al. (2002, 2004b), suggesting different mechanisms underlying the formation of NAb or complement, and 3) most importantly, chickens regardless of age act immunologically consistent over time (i.e., our findings are not due to an artifact caused by age), and therefore they may represent an immune phenotype and possibly genotype.

Table 4. Outcome of the logistic regression model expressed as odds ratio and *P*-value of natural antibodies binding to keyhole limpet hemocyanin (KLH) or lipopolysaccharide (LPS) measured at 20 and 40 wk of age in surviving and nonsurviving chickens of all 12 purebred layer lines during, respectively, the first period of lay (20 to 39 wk of age; natural humoral immune parameters included as continuous) and the second period of lay (40 to 64 wk of age; natural humoral immune parameters included as categorical)

	20 wk			40 wk	
Antigen	Odds ratio	*P*-value	Category	Odds ratio	*P*-value
KLH	0.80	0.008	Titer < 2[1]		
			Titer 2 to 3	0.33	<0.0001
			Titer 3 to 4	0.66	0.256
			Titer 4 to 5	0.25	0.001
			Titer 5 to 6	0.61	0.159
			Titer > 6	0.26	0.035
LPS	1.42	<0.0001	Titer < 2[1]		
			Titer 2 to 3	4.35	0.060
			Titer 3 to 4	2.03	0.359
			Titer 4 to 5	4.73	0.039
			Titer 5 to 6	3.60	0.092
			Titer > 6	7.86	0.009

[1]Reference category.

Innate and specific immunity are linked by complement and NAb (Carroll and Prodeus, 1998; Ochsenbein and Zinkernagel, 2000). The NAb and complement were shown to perform important functions in the subsequent activation of specific humoral and cellular immune responses after vaccination (Stäger et al., 2003; Lammers et al., 2004). In the present study, chickens were not challenged with antigen, but specific antibody responses to NCD vaccination could be measured. Lines that showed high levels of NAb binding to KLH and LPS were also high responders for specific antibodies to NCD. Although there were no significant correlations between the natural immune parameters (NAb binding to KLH or LPS, and CPW and APW) and levels of specific antibodies to NCD, these data suggest (based on ranking) a genetic or functional linkage between natural immune competence and specific immune competence. It is worth mentioning that NAb may be prerequisite to modulate the T-helper 2 route of specific immunity by maturation of dendritic cells (Bayry et al., 2004, 2005).

Although the cause of death is unknown in this experiment, it is certain that most of the chickens died because of nonspecific causes (no disease-related mortality or cannibalism). For nonspecific mortality, therefore, nonspecific (innate) immunity might play an important role in enhancing survival of the host by providing early resistance against infection (Ochsenbein and Zinkernagel, 2000). Low levels of innate immunity, cellular as well as humoral, may be related with disease susceptibility and high levels with disease resistance (Parmentier et al., 2004a); however, a relation between levels of or activation of natural immunity and survival has not been shown before. In knock-out mice (BALB/c), however, absence of NAb caused a delayed specific (T-dependent) response resulting in increased mortality (Baumgarth et al., 1999), which indicates the important role of the natural immune system for survival.

The major difference between the responses of NAb binding to the exo-antigens, KLH or LPS, is that chickens did not, and probably will not encounter KLH, thus reflecting a capacity to respond, whereas the chickens probably did encounter LPS, thus the latter reflects an active status of the innate immune system. The present data suggest, regardless of line, a relation between levels (KLH) and activation (LPS) of humoral components of innate immunity and survival. Low levels of NAb binding to KLH were detected in chickens that did not survive, which suggests the (lack of) capacity to maintain NAb to KLH. Because KLH is a classical antigen for NAb and because it seems that (too) low levels of NAb binding to KLH are not in favor of survival, it is supposed that NAb binding to KLH reflects the capacity to mount an appropriate level of natural immune defense. Conversely, NAb binding to LPS reflect the immune reactivity of the chicken. The LPS is a gram-negative bacterial membrane molecule, but when released by damaged or dead bacteria, LPS could act as a danger signal crucial for stimulation of the innate immune response (Reid et al., 1997; Matzinger, 2002). Therefore it is supposed that chickens that did not survive had physiological or immunological problems at the time of blood sampling, and this is reflected by the levels of NAb binding to LPS.

In conclusion, a distinction could be made between lines showing high or low immune competence, with respect to NAb, complement activity, and specific antibodies. Within lines significant correlations were found for each of the innate and specific parameters among the 3 ages. The innate and specific parameters were, however, not correlated with one another. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. However, we found a relation, regardless of line, between natural immune competence (in the form of NAb binding to KLH) and activation of natural immunity (in the form of NAb binding to LPS) and the probability to survive. The NAb reflecting immune competence or immune activation could thus be indicative factors for the chickens' abilities to survive a laying period within a genetic setting. To our knowledge this is the first study indicating a relation between innate immunity and survival. In further research, relations between NAb levels, disease susceptibility, and environmental influences (such as exposure to LPS) should be further unraveled.

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