Diet and Immunological Memory of Lines of White Leghorn Chickens Divergently Selected for Antibody Response to Sheep Red Blood Cells

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ABSTRACT Antibody responses to a first, second, and third injection with SRBC, and growth were studied in lines of White Leghorn chickens selected for high (HA) or low (LA) 5-d antibody titers to an i.v. inoculation with 0.1 mL of a 0.25% suspension of SRBC. The experiment involved parallel studies on two groups of chicks hatched from the same matings of parental lines HA and LA at a 14-d interval. Chicks of each age-line subclass were fed either a high or low nutrient density diet from hatch onwards. When chicks of Hatches 1 and 2 were 28 and 14 d of age (doa) respectively, they were injected with 0.1 mL of 0.25% suspension of SRBC, and antibody titers measured 3 and 6 d later. A second and a third injection of the same concentration of SRBC was given to chicks of each age-line-diet subclass at 10-d intervals and antibody titers measured 3 and 6 d after each injection in different chicks randomly sampled from each age-line-diet subclass.

After the first injection, antibody (primary) responses of HA chicks were higher than those of LA chicks regardless of age and diet. This difference (HA > LA) observed for the primary response was seldom evident in the responses to the second (secondary) and third (tertiary) injections. Antibody responses of LA chicks after the second and third injections were anamnestic. For HA chicks given the first injection at 28 doa, neither the secondary nor tertiary responses suggested anamnestic capacities, whereas there was apparent memory exhibited by the secondary and tertiary responses of HA chicks initially injected at 14 doa.

The LA chicks were significantly heavier than HA chicks at all ages. Even though the higher nutrient density diet increased BW of chicks of both lines, its effect on memory responses was sporadic. The results of this experiment show that, even though divergent selection has been successful in the primary responses, correlated responses in immunological memory were not always observed, suggesting that the two types of responses might be under different genetic control.

(*Key words*: sheep red blood cells, diet, body weight, antibody responses, immunological memory)

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INTRODUCTION

Vaccination, an important tool in disease control, depends upon immunological memory to be effective. Recall antibody responses differ from primary antibody responses in several respects. They occur more rapidly, persist longer, attain higher titers, and are of higher affinity, and isotype class switching takes place (Roitt, 1994; Ahmed and Gray, 1996). Immunological memory would seem to be influenced by genetic selection. Parmentier *et al.* (1996) reported that a line of chickens selected for high humoral response to SRBC antigen responded better to vaccination with viral antigens than a line selected in the opposite direction.

Ubosi *et al.* (1985) studied the kinetics of primary and secondary immunizations of a pair of chicken lines divergently selected for antibody response to SRBC. They reported that antibody titers peaked about 3 d after secondary immunization rather than the 5-d peak following primary inoculation. An anamnestic response to the secondary inoculation was, however, observed only in the low antibody response line (Ubosi *et al.*, 1985). Responses of these lines to secondary inoculations with SRBC were also studied by Martin *et al.* (1989), who found memory response only in the low response line. According to Pinard *et al.* (1992) the lower secondary response in their high antibody line might adversely affect the effectiveness of vaccination. None of the above studies measured responses to further inoculations or the effect of nutrition, which may influence immune responses in chickens (Cook, 1991); however, in com-

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Abbreviation Key: doa = days of age; HA = line selected for high antibody response to SRBC; LA = line selected for low antibody response to SRBC; PPI = post primary injection; PSI = post secondary injection; PTI = post tertiary injection.

mercial poultry production, diets vary and it is common to give sequential vaccinations of particular antigens (Bains, 1979). Also, the effect of a factor such as age on immunological memory in chickens needs elucidation. This paper reports on an investigation into the effects of nutrient density on memory responses of lines of chickens divergently selected for antibody response to SRBC.

MATERIALS AND METHODS

Stocks, Diets, and Husbandry

The chicks used in this experiment were progeny from White Leghorn lines that had undergone 23 generations of selection for high (HA) or low (LA) antibody response to 0.1 mL of a 0.25% suspension of SRBC given intravenously between 41 and 51 d of age (doa; Martin *et al.*, 1990). Two diets that differed in nutrient density were fed in mash form throughout the experiment. The lower density diet, which was also the diet under which the lines had been selected, contained 20% CP and 2,685 kcal ME/kg. The higher nutrient density diet contained 24% CP and 3,146 kcal ME/kg.

Two groups of chicks (192 total) were hatched at a 14-d interval from the same matings of Lines HA and LA. At hatch, 96 chicks per line were wing-banded, vaccinated for Marek's disease, randomized into groups of 48, and placed in floor pens covered with litter. For each hatch, half of the chicks from each line was randomly assigned to each diet. Feed and water were consumed *ad libitum* and lighting was continuous. These husbandry practices were consistent with those under which the lines were selected. Chicks were individually weighed at 14, 28, and 38 doa and sex determined on Day 38.

When chicks from Hatches 1 and 2 were 28 and 14 doa, respectively, they were injected with 0.1 mL of 0.25% suspension of SRBC. Three and 6 d later, eight of the injected chicks from each line-diet-age subclass were randomly chosen and bled (0.5 mL of blood). Blood was refrigerated for 24 h and the plasma tested for total SRBC antibody (Wegmann and Smithies, 1966). Antibody titers were expressed as the $\log_2$ of the reciprocal of the last dilution in which agglutination was macroscopically observed. Ten days after the first (primary) injection, chicks not bled for post primary antibody response (PPI) were injected again with 0.1 mL of 0.25% SRBC suspension. Three and 6 d later, blood was obtained from 16 different, but randomly chosen, chicks from each line-diet-age subclass of those given the second as well as those given only the first inoculation. Plasma was tested for SRBC antibody as before (PSI).

Ten days after the second injection, the 16 remaining chicks from each line-diet-age subclass that had not been bled, were given a third injection of SRBC. Eight of these chicks, together with eight each of those that received first and second injections respectively, were randomly selected and bled 3 and 6 d later (PTI). Throughout the experiment, no chick was bled on consecutive bleeding days.

Statistical Analysis

Individual BW were transformed to common logs and subjected to ANOVA (SAS Institute, 1985) with line, diet, sex, age at initial injection, type of response, and interactions among them as the main sources of variation. A preliminary analysis of antibody titers using the model for BW showed a lack of sex effects and interactions involving sex. Because of significant interactions involving age at initial injection, subsequent analyses were carried out within age at first injection (14 or 28 d) with line, diet, type of response, and interactions among them as the main sources of variation. When chicks were bled after repeated injections, multiple types of responses resulted (i.e., primary, secondary, and tertiary). Comparisons between lines were biased by the extreme differences between them for primary antibody responses to SRBC. Therefore a particular type of response at a given bleeding time was analyzed by ANOVA within age with line, diet, and the interaction between them as the main sources of variation. Tests of significance were at $P < 0.05$.

RESULTS

Body Weight at Various Ages

The number of injections of SRBC a chick received, and the group in which it was hatched, had no influence on the BW at 14, 28, or 38 doa. Thus, hatches were pooled for data summarized in Table 1. Only 5 of 78 possible interactions were significant and they were considered unimportant for further analysis. At all ages Line LA males and females were heavier than those of Line HA, chicks fed the high density diet were heavier than those fed the low density diet, and males were heavier than females.

Antibody Responses to Primary Inoculation

Antibody titers measured 3 d PPI were the lowest throughout the study (Table 2). For both age groups HA chicks produced higher titers than LA chicks, with no difference between diets. Six days PPI, antibody titers increased several fold over those for 3 d PPI. The increase was noted especially in HA chicks of both ages. Line HA chicks maintained higher titers 6 d PPI than those of Line LA regardless of diet or age of initial injection. Moreover, the older HA chicks had a greater antibody response 6 d PPI than the younger HA chicks (12.6 ± 0.4 vs 7.9 ± 0.6), whereas in LA chicks, 6 d PPI titers were not influenced by age at initial injection. Also, the 6-d titers for HA chicks were the highest primary response observed in the experiment. The highest primary responses for LA chicks, however, occurred after 6 d PPI (Table 2).

TABLE 1. Means and SEM for body weights of chicks by sex, line, diet, and age

			Age		
Sex	Line[1]	Diet	14 d	28 d	38 d
			(g)		
Male	HA		96 ± 2	197 ± 6	306 ± 9
			**	**	**
	LA		106 ± 2	222 ± 5	334 ± 6
		Low density	98 ± 2	199 ± 5	300 ± 6
			**	**	**
		High density	104 ± 2	220 ± 6	340 ± 9
Female	HA		88 ± 3	165 ± 5	245 ± 7
			**	**	**
	LA		101 ± 2	189 ± 5	281 ± 6
		Low density	91 ± 2	166 ± 4	248 ± 6
			**	**	**
		High density	98 ± 3	188 ± 6	278 ± 7

[1]Lines HA and LA were selected for high and low response to SRBC, respectively.

**Within an age, differences between lines and between diets are significant ($P \leq 0.01$).

Antibody Responses to Secondary Inoculation

In all comparisons, 3 d PSI responses were similar to or higher than 3 d PPI and 6 d PPI responses (Table 2). The increase in the case of LA chicks was three- to fourfold for the chicks given the initial injection at 14 doa. For those LA chicks given the initial injection at 28 doa, however, the increase was less.

Primary responses of chicks not given the second injection (13 d PPI) were declining in HA but not LA chicks. For chicks injected initially at 14 doa, the line by type of response interaction was significant because in HA chicks, 3 d PSI titers were similar to the declining primary titers (13 d PPI), whereas in the LA chicks, titer 3 d PSI far exceeded those 13 d PPI. For chicks injected initially at 28 doa, differences due to line (HA > LA) were significant. Types of response differed when chicks were fed the high density diet (3 d PSI > 13 d PPI) but were similar when fed the low density diet.

When types of responses were compared in chicks initially injected at 14 doa, there was a line difference (HA > LA) for 13 d PPI but not for 3 d PSI. However, for chicks injected initially at 28 doa, line differences (HA > LA) were observed for both 13 d PPI (9.8 ± 0.8 vs 2.9 ± 0.4) and 3 d PSI (11.9 ± 0.6 vs 5.5 ± 1.2). For the 13 d PPI titers of these chicks, those fed the lower density diet (the one in which the lines had been selected) generally sustained higher antibody titers (7.1 ± 0.7 vs 5.6 ± 0.6) than those fed the higher density diet.

Six days after the second injection, antibody responses (16 d PPI, 6 d PSI) of chicks initially injected at 14 doa reflected line by diet and line by type of response interactions (Table 3). The line by diet interaction occurred because, although there was no line difference for chicks fed the low density diet, titers were higher for HA than LA chicks when fed the high density diet. Conversely, titers were higher for HA chicks fed the high density diet than for those fed the low density diet, whereas no diet differences were observed for LA chicks. The line by type of response interaction was due to a line difference (HA > LA) in titers of chicks given only the first injection (16 d PPI), but not for those given the second injection.

For chicks injected initially at 28 doa, the magnitude of the residual primary inoculation 16 d PPI compared with 6 d PSI depended upon diet. The interactions occurred because chicks fed the low density diet had higher primary titers than those fed the high density diet (6.6 ± 0.7 vs 4.6 ± 0.6), whereas no dietary differences (low = 10.9 ± 0.5 vs high = 11.5 ± 0.4) were found for the secondary responses.

Antibody Responses to the Third Injection

The pattern of response after the third injection showed consistent increases in titers from 3 to 6 d PTI (Table 2). Comparisons among 3 d PTI, 13 d PSI, and 23 d PPI for chicks bled on the same day, revealed a line by diet by type of response interaction for chicks initially injected at 14 doa. Subsequent analyses were undertaken within line-diet-type of response subclasses. A consistent pattern emerged for all cases in that 3 d PTI titers were higher than 13 d PSI, which in turn exceeded titers of chicks injected only once 23 d PPI. In contrast, for the older chicks (initially injected at 28 doa), rankings of the type of response depended upon the line-diet subclass (Table 2). Except for LA chicks fed the low density diet, whose titers 13 d PSI were lower than those 3 d PTI, the titers at 13 d PSI and 3 d PTI were similar and both higher than at 23 d PPI. A more detailed analysis of individual types of response revealed that for 23 d PPI titers of both age groups, the higher density diet was associated with higher titers than the lower density diet. Line effects (HA > LA) influenced

TABLE 2. Means and SEM for SRBC antibody titers of chicks by age at first injection, line,[1] diet, and type of response[2]

Age at first injection	Line	Diet	Type of response[2]	Days after SRBC injection: 3 d PPI / . . . / . . .	6 d PPI / . . . / . . .	13 d PPI / 3 d PSI / . . .	16 d PPI / 6 d PSI / . . .	23 d PPI / 13 d PSI / 3 d PTI	26 d PPI / 16 d PSI / 6 d PTI
(d)									
14	HA	Low density	PPI	1.5 ± 0.3^{a}	7.5 ± 0.2^{a}	7.0 ± 0.8	4.5 ± 0.5^{x}	2.9 ± 0.2^{x}	2.8 ± 0.3^{x}
			PSI	. . .	. . .	8.5 ± 1.1	10.0 ± 0.7^{y}	6.1 ± 0.7^{y}	5.9 ± 0.4^{y}
			PTI	. . .	. . .	. . .	. . .	8.1 ± 0.9^{z}	11.0 ± 0.5^{z}
		High density	PPI	1.6 ± 0.2^{a}	8.3 ± 0.9^{a}	6.8 ± 0.9	6.5 ± 0.3^{x}	5.3 ± 0.6^{x}	2.9 ± 0.3^{x}
			PSI	. . .	. . .	9.0 ± 1.1	12.3 ± 0.5^{y}	7.1 ± 0.5^{y}	6.1 ± 0.6^{y}
			PTI	. . .	. . .	. . .	. . .	9.6 ± 0.7^{z}	11.4 ± 0.5^{z}
	LA	Low density	PPI	1.0 ± 0.6^{b}	2.8 ± 0.4^{b}	3.1 ± 0.4^{x}	2.8 ± 0.5^{x}	2.1 ± 0.3^{x}	2.1 ± 0.2^{x}
			PSI	. . .	. . .	11.1 ± 0.6^{y}	10.5 ± 0.5^{y}	5.8 ± 0.6^{y}	4.0 ± 0.5^{y}
			PTI	. . .	. . .	. . .	. . .	7.8 ± 0.8^{z}	9.4 ± 0.9^{z}
		High density	PPI	1.0 ± 0.6^{b}	2.3 ± 0.3^{b}	2.6 ± 0.6^{x}	2.3 ± 0.6^{x}	1.3 ± 0.2^{x}	2.1 ± 0.2^{x}
			PSI	. . .	. . .	8.8 ± 1.2^{y}	9.6 ± 1.2^{y}	5.7 ± 0.6^{y}	5.4 ± 1.0^{y}
			PTI	. . .	. . .	. . .	. . .	10.3 ± 0.6^{Z}	11.9 ± 1.0^{z}
28	HA	Low density	PPI	2.4 ± 0.3^{a}	13.0 ± 0.0^{a}	10.8 ± 1.0	9.0 ± 0.7^{x}	3.0 ± 0.4^{x}	4.4 ± 0.6^{x}
			PSI	. . .	. . .	12.0 ± 0.4	11.9 ± 0.6^{y}	8.0 ± 0.5^{y}	6.5 ± 0.7^{y}
			PTI	. . .	. . .	. . .	. . .	8.5 ± 0.8^{y}	11.8 ± 0.5^{z}
		High density	PPI	3.0 ± 0.5^{a}	12.1 ± 0.7^{a}	8.8 ± 0.8^{x}	6.0 ± 0.6^{x}	4.8 ± 0.6^{x}	4.0 ± 0.5^{x}
			PSI	. . .	. . .	11.6 ± 0.8^{y}	12.5 ± 0.3^{y}	7.1 ± 0.8^{y}	3.6 ± 0.3^{x}
			PTI	. . .	. . .	. . .	. . .	8.8 ± 0.8^{y}	11.8 ± 0.5^{y}
	LA	Low density	PPI	1.5 ± 0.3^{b}	2.4 ± 0.3^{b}	3.4 ± 0.4	4.3 ± 0.4^{x}	1.4 ± 0.3^{x}	2.1 ± 0.2^{x}
			PSI	. . .	. . .	5.9 ± 1.8	10.1 ± 0.7^{y}	4.1 ± 0.6^{y}	3.9 ± 0.5^{x}
			PTI	. . .	. . .	. . .	. . .	7.6 ± 0.8^{z}	9.1 ± 1.2^{y}
		High density	PPI	1.1 ± 0.3^{b}	2.1 ± 0.4^{b}	2.4 ± 0.3^{x}	3.3 ± 0.7^{x}	2.1 ± 0.4^{x}	2.0 ± 0.3^{x}
			PSI	. . .	. . .	5.0 ± 0.6^{y}	10.5 ± 0.6^{y}	5.7 ± 0.9^{y}	4.9 ± 0.6^{y}
			PTI	. . .	. . .	. . .	. . .	7.1 ± 0.8^{y}	11.1 ± 0.7^{z}

[a,b]Means in a column within an age group with no common superscript differ significantly ($P \leq 0.05$).

[x–z]Means in a column within an age-line-diet subclass with no common letter among type of response differ significantly ($P \leq 0.05$).

[1]Lines HA and LA were selected for high and low response to SRBC, respectively.

[2]Responses PPI, PSI, and PTI represent days after chicks given one, two, or three injections of SRBC. Subsequent injections were given 10 and 20 d after the first injection.

23 d PPI and 13 d PSI but not 3 d PTI titers in the 28 doa injected group, and only 23 d PPI titers in chicks initially injected at 14 doa.

In the younger chicks injected initially at 14 doa, rankings of the type of response were: 6 d PTI >16 d PSI > 26 d PPI. When lines and diets were compared for each of the three antibody responses, it was evident that HA chicks had higher residual primary responses (26 d PPI) than LA chicks (2.9 ± 0.3 vs 2.1 ± 0.2), whereas line differences were lacking for 16 d PSI (6.0 ± 0.5 vs 4.7 ± 0.8) and 6 d PTI (11.2 ± 0.5 vs 10.7 ± 1.0).

For chicks injected initially at 28 doa and bled 6 d PTI, antibody titers were higher than the 16 d PSI and 26 d PPI measured that day. Chicks of Line HA fed the high density diet and LA chicks fed the low density diet had similar 16 d PSI and 26 d PPI responses. In contrast, for HA chicks fed the low density diet and LA chicks fed the high density diet, the 16 d PSI titers surpassed the 26 d PPI titers (Table

TABLE 3. Means and SEM for line by diet and line by type of response of SRBC titers

Line[1]	Diet: Low density		Diet: High density	Type of response[2]: 16 PPI		Type of response[2]: 6 PSI
HA	7.3 ± 0.8	*	9.4 ± 0.8	5.5 ± 0.4	**	11.1 ± 0.5
	NS		**	**		NS
LA	6.6 ± 1.1	NS	5.9 ± 1.1	2.5 ± 0.4	**	10.1 ± 0.6

[1]Lines HA and LA were selected for high and low response to SRBC, respectively.

[2]Chicks were 14 d of age when given the first inoculation and 24 d of age when given the second inoculation of SRBC. PPI and PSI represent SRBC antibody titers measured 16 and 6 days after receiving the first and second inoculation of SRBC.

*$P \leq 0.05$.

**$P \leq 0.01$.

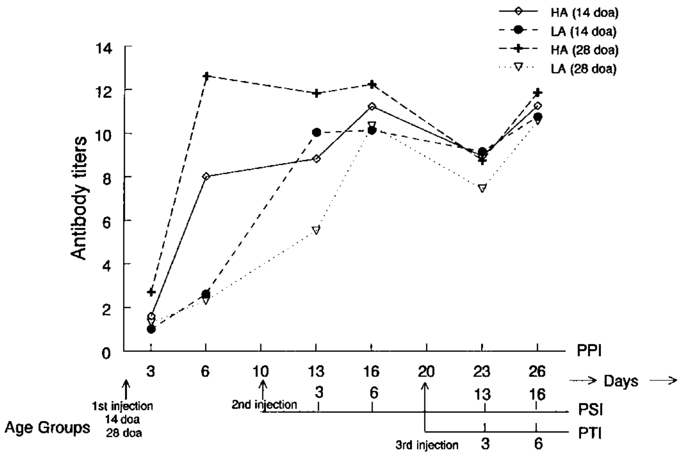

FIGURE 1. Antibody responses of HA and LA chicks injected initially at 14 or 28 d of age (doa) and subsequently injected 10 and 20 d later with SRBC. Responses were measured at 3 and 6 d post primary (PPI), post secondary (PSI), and post tertiary (PTI) injections. HA = line selected for high antibody response to SRBC; LA = line selected for low antibody response to SRBC.

2). When analyzed separately, the differences between lines (HA > LA) influenced 26 d PPI titers of both age groups, as well as the 6 d PTI titers of the group injected at 28 doa. There was no difference between the secondary residual (16 d PSI) and the tertiary (6 d PTI) titers of chicks initially injected at 14 doa.

DISCUSSION

The lines involved in this experiment have been developed through selection for high (HA) or low (LA) primary antibody responses 5 d after a single injection with SRBC administered i.v. between the ages of 41 and 51 d (Siegel and Gross, 1980). The LA chicks were heavier than the HA chicks, a result consistent with previous data for these lines (Martin *et al.*, 1990; Boa-Amponsem *et al.*, 1998) and for similar lines developed in the Netherlands (Kreukniet *et al.*, 1994; Parmentier *et al.*, 1996, 1998). Also males were heavier than females and chicks fed higher nutrient density diets had higher BW than those fed lower nutrient diets, well documented phenomena for chickens. Several reports on these lines indicate the HA line reaches maximum titers at between 5 and 7 d PPI with SRBC (Siegel and Gross, 1980; Ubosi *et al.*, 1985; Martin *et al.*, 1989). The present report is consistent with these findings regarding peak antibody response of the HA line and further indicates that this peak period was not influenced by diet or age of chicks.

The benefits of repeated inoculations with the same antigen in maintaining high levels of antigen-specific antibody appeared consistent throughout the experiment when the booster responses were compared with titers of chicks that did not receive boosters, as have been previously reported for these lines (Ubosi *et al.*, 1985; Martin *et al.*, 1989). The question arises, however, as to whether the response to repeated inoculations resulted from immunological memory mechanisms or was simply an enhanced primary response to each antigen injected due to further maturation of the immune system with age. Accelerated responses associated with re-exposure to antigen are due principally to increases in the frequency of antigen-specific immune cells that are of higher affinity than those involved in primary responses (Roitt, 1994; Ahmed and Gray, 1996).

The results of the present work suggest that both 3 and 6 d PSI responses in LA chicks exhibited clear, definite immunological memory regardless of age of initial injection with SRBC antigens (Figure 1). The responses were rapid and achieved higher titers than the primary titers. In the case of the HA chicks initially injected at 28 doa, neither the secondary nor the tertiary responses suggested an anamnestic response. The secondary responses of chicks of the HA line injected initially with SRBC at 14 doa, however, appeared to be anamnestic. It is noteworthy that these chicks were given the second injection at 24 doa. The similarity of the secondary titers of these chicks with the primary titers of chicks of the same line injected initially at 28 doa suggests that antibody responses in this line are probably due to primary mechanisms influenced by age.

The memory responses of these lines as discussed above so far agree with those of Ubosi *et al.* (1985) and Martin *et al.* (1989), as well as lines reported by Pinard *et al.* (1992). They also suggest that a high initial response may preclude resources available for anamnestic responses from immunological memory. Similar to the pattern observed between primary and secondary antibody levels, is a further observation that tertiary responses observed in our experiment did not exceed secondary responses in most cases, which is contrary to expectations (Roitt, 1994; Coligan *et al.*, 1994). Such response patterns may have been due to the presence of high levels of residual antigen-specific antibody at the time of repeated antigen administration, which thus neutralized a proportion of SRBC antigen.

Although the higher nutrient density diet improved BW of both lines, its effect on memory responses appears sporadic. The results of this experiment show that even though divergent selection has been successful in the primary responses, no correlated responses in immunological memory were observed. A possibility exists that the hemagglutination assay used in our study could not detect nonagglutinating antibodies such as IgG/IgA, which are produced following isotype switching in secondary and tertiary responses. Martin *et al.* (1989) measured the kinetics of IgG and IgM in primary and secondary responses of Lines HA and LA. Total and IgG levels were higher for HA than LA chicks, with immunological memory exhibited in LA chicks. Thus, it is also likely that the two types of responses are under different genetic control.

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