Effect of Route of Inoculation on Humoral Immune Response of White Leghorn Chickens Selected for High or Low Antibody Response to Sheep Red Blood Cells

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ABSTRACT Effects of route of SRBC inoculation and antigen dosage on primary and secondary antibody response of White Leghorn lines selected for high (HA) or low (LA) 5-d antibody response to a single i.v. inoculation with 0.1 mL of a 0.25% suspension of SRBC were studied in two trials. In the first trial, chicks from parents of generation S_{24} of each line were randomly assigned to one of four treatments. At 35 d of age, they were inoculated into the brachial vein with 0.1 mL of 0.25% suspension of SRBC or into the breast muscle with 0.1 mL of 0.25, 2.50, or 25.00% SRBC. Plasma SRBC antibody was measured 3, 6, 10, and 20 d later. In the second trial, chicks from parents of generation S_{25} of each line were randomly assigned to treatment groups. At 28 d of age they were inoculated with 0.1 mL of 0.25% SRBC into the brachial vein, 0.1 mL of 25.00% SRBC into the thigh (T-L) or breast muscle (B-L), or 0.5 mL of 25.00% SRBC into the thigh (T-H) or breast muscle (B-H). Twenty-one days later, chicks (except five per group) were given a booster inoculation of 0.1 mL of 25.00% SRBC into the thigh muscle. Six and 10 d after each inoculation, plasma SRBC antibody, IgG, and IgM titers were measured.

The SRBC antibody titers after primary i.v. inoculation with SRBC were always higher for HA than LA chicks. When inoculations were i.m., differences between lines varied with dosage. Low dosages inoculated into the breast failed to induce line differences consistently, whereas at higher dosages, titers were greater for HA than LA chicks regardless of inoculation site. For Line LA, inoculation into the thigh elicited higher titers than inoculations into the breast. Antibody titers to the booster inoculation of SRBC were similar for the lines.

(*Key words*: sheep red blood cells, IgG, IgM, chicken, genetic line)

2001 Poultry Science 80:1073–1078

INTRODUCTION

The route by which an antigen enters the body influences the tissues where immune responses are mounted as well as the magnitude of response. After an i.v. inoculation with an antigen, the spleen is the major source of antibody production (White et al., 1975), whereas after an i.m. injection, the antigen may also be associated with lymphoid tissues (Donker et al., 1989; Kreukniet et al., 1992). Primary antibody response to i.v. injection was greater than responses to i.m. or i.p. injections of SRBC (Kreukniet et al., 1992). Differences among lines in response to an antigen may be altered by the route of administration. Inoculation s.c. failed to elicit line differences that were observed with natural and oral exposures to lymphomatosis (Heisdorf et al., 1947). In lines of chickens divergently selected for antibody response to an i.m. injection of SRBC, differences between lines could be detected in total primary titers within i.m. and i.v. groups but not in the i.p. group (Kreukniet et al., 1992). Such differences between lines, however, while absent for secondary responses to i.v., were evident in i.p. and i.m. groups (Kreukniet et al., 1992). Muir et al. (1995, 1998) reported that primary i.p. immunization followed by an oral secondary immunization with a nonreplicating antigen stimulated greater immune protection of chicks than repeat oral delivery of the same antigen. Martin et al. (1990) reported on lines of chickens selected for high or low antibody response to an i.v. injection of SRBC. The present study used these lines, which, as those used by Kreukneit et al. (1992), provide animal models for studying effects of route of inoculation on primary and secondary immune responses.

Received for publication July 3, 2000.
Accepted for publication April 9, 2001.
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Abbreviation Key: B = breast muscle; H = high antigen dose; HA = high antibody response line; L = low antigen dose; LA = low antibody response line; MER = 2-mercaptoethanol-resistant; MES = 2-mercaptoethanol-sensitive; PPI = post primary (initial) inoculation; PSI6 = 6-d total secondary antibody titer; T = thigh muscle.

MATERIALS AND METHODS

Trial 1

This trial was undertaken to study the antibody response of selected lines to different routes of antigen delivery. Chicks used in this trial were obtained from two White Leghorn lines divergently selected for 24 generations for high (HA) or low (LA) antibody titers 5 d after an i.v. inoculation with 0.1 mL of a 0.25% suspension of SRBC (Martin et al., 1990). Eggs from age-contemporary hens were incubated in the same machine. At hatching, chicks were wing-banded and vaccinated for Marek's disease. Rearing was on litter with a mash diet containing 22.4% CP and 3,108 kcal ME/kg provided ad libitum. Lighting was continuous through 14 d of age and from 0500 to 2100 h thereafter. At 35 d of age, 96 chicks were randomly selected from each line and randomly assigned to one of four SRBC treatments: 0.1 mL of 0.25% suspension of SRBC injected into the brachial vein and 0.1 mL of 0.25, 2.50, or 25.00% suspension of SRBC injected into the breast muscle. Twelve chicks from each of the four groups were bled via the brachial vein 3 and 10 d after injection with SRBC. The other 12 chicks were bled 6 and 20 d after injection with SRBC. Blood was collected into tubes with EDTA as the anticoagulant and refrigerated overnight; the plasma was tested for antibodies by the hemagglutination method of Wegmann and Smithies (1966). Titers were expressed as $\log_2$ of the reciprocal of the highest dilution giving visible agglutination.

Antibody titers 3, 6, 10, and 20 d after inoculation, designated as PPI3, PPI6, PPI10, and PPI20 respectively, were analyzed by ANOVA (SAS Institute, 1985). Within each PPI day, lines, SRBC treatments, and the interaction between them were the main sources of variation in a fixed effects model. Significance of effects was declared at $P \leq 0.05$ and $P \leq 0.01$. When SRBC treatments were significant, multiple means were separated by Duncan's multiple-range test. Lines were also compared within each SRBC treatment subclass using the chi-squared statistic for number of chicks responding to SRBC inoculation.

Trial 2

Chicks used for this trial were from parents of generation S_{25} of the same lines used in Trial 1. Eggs from age-contemporary hens were incubated in the same machine. At hatch, 150 chicks per line were wing-banded and vaccinated for Marek's disease. Details of rearing and feeding were as described for Trial 1. At 28 d of age, 13 to 22 chicks were randomly selected from each line and randomly assigned to one of five SRBC treatments: 0.1 mL of 0.25% SRBC injected into the brachial vein, 0.1 mL of 25.00% SRBC injected into the thigh (T-H) or breast (B-H) muscle, or 0.5 mL of 25.00% SRBC into the thigh or breast muscle. Six and 10 d after inoculation, chicks were bled, and SRBC antibody (Wegmann and Smithies, 1966) and 2-mercaptoethanol-resistant (MER) antibodies (Delhanty and Solomon, 1966) in the plasma were measured. The 2-mercaptoethanol-sensitive (MES) antibodies were estimated as the difference between the total and MER antibody titers. MER antibodies are presumed to be a measure of IgG response, whereas MES titers consist primarily of IgM (Delhanty and Solomon, 1966).

At 49 d of age (21 d after the initial inoculation), all chicks except five from each inoculation route-dosage subclass were given a booster inoculation of 0.1 mL of 25.00% SRBC into the thigh muscle. Six days later (6 d PSI), chicks given the booster as well as those not given the booster were bled to ascertain the effectiveness of the second inoculation. Chicks given the booster inoculation were again bled 4 d later (10 d PSI), and total secondary, MER, and MES antibodies were measured as for primary titers.

Total, MER, and MES antibody titers 6 and 10 d PPI were analyzed by ANOVA (SAS Institute, 1985) with line, route of SRBC delivery, sex, day bled, and interactions among them as the main sources of variation. The analysis was made for all chicks and also with nonresponders excluded. For the analysis of 6 d PSI, inoculation status (boosted or not boosted) was added to the statistical model used to analyze primary antibodies. The model used to compare 6- and 10-d antibodies PSI was the same as that used for primary antibody titers because the nonboosted chicks were not bled on Day 10 PSI. Out of a total of 44 nonresponders to the primary inoculation, only three were from Line HA. Therefore chi-squared analysis for route of antigen delivery on frequency of antibody responders was undertaken only for LA chicks. As in Trial 1, significance was considered at $P \leq 0.05$ and $P \leq 0.01$. When significant, multiple means were separated by Duncan's multiple-range test.

RESULTS

Trial 1

Because the interaction between lines and SRBC treatments was significant, lines were compared within SRBC treatment subclasses and treatments within lines. Within the HA line antibody titers were consistently higher for i.v. than i.m. injected chickens (Table 1). In the LA line this difference was present at 6 and 10 d, but not 3 and 20 d, after injection. HA chicks injected i.v. with 0.1 mL of 0.25% suspension of SRBC had higher antibody titers than LA chicks treated similarly at all times bled. For chicks injected i.m. with 0.25% SRBC, differences between lines (HA > LA) were present only at 10 d after inoculation. No line differences were detected in chicks inoculated i.m. with the SRBC at 2.50%. When inoculated i.m. with the 25.00% SRBC, however, 6 d after inoculation HA chicks had higher antibody titers than LA chicks. Frequency of response among chicks inoculated i.v. with SRBC was higher for Line HA than LA at 3 and 20 d after inoculation and was similar for the two lines at 6 and 10 d after inoculation (Table 2). Differences between lines in frequency of responders among chicks inoculated i.m.

TABLE 1. Means and SEM for antibody titers of chicks after inoculation with SRBC by treatment, line, and days after inoculation in Trial 1

Treatment			Days after inoculation			
Route[1]	Dosage[2]	Line[3]	3	6	10	20
i.v.	0.25	HA	1.9 ± 0.2 **	6.5 ± 0.2 **	4.9 ± 0.3 **	3.0 ± 0.2 **
		LA	1.2 ± 0.2	2.5 ± 0.3	2.8 ± 0.5	1.1 ± 0.1
i.m.	0.25	HA	1.3 ± 0.2	1.0 ± 0.0	2.0 ± 0.4 *	1.2 ± 0.1
		LA	1.0 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
	2.50	HA	1.0 ± 0.0	1.3 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
		LA	1.0 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.0
	25.00	HA	1.1 ± 0.1	2.5 ± 0.4 **	2.5 ± 0.6	1.4 ± 0.2
		LA	1.0 ± 0.0	1.2 ± 0.1	1.5 ± 0.2	1.3 ± 0.2

[1]i.v. and i.m. are intravenous and intramuscular routes of inoculation respectively, of 0.1 mL of the SRBC dosages.

[2]% SRBC.

[3]Lines have undergone 24 generations of selection, respectively, for high (HA) or low (LA) antibody response to an i.v. inoculation of 0.1 mL of 0.25% SRBC.

*$P \leq 0.05$; **$P \leq 0.01$ are for a specific comparison of lines HA and LA within a route of inoculation, dosage, and day after inoculation subclass.

with the different dosages of SRBC were significant (HA > LA) only in chicks given 25.00% SRBC and bled 6 d afterwards.

Trial 2

Primary Antibody Titers. Out of 44 nonresponder chicks, only 3 were from Line HA, and these had been inoculated into the breast muscle with 0.1 mL of 25.00% SRBC (B-L). There was, however, a highly significant association between route of SRBC delivery and antibody response for LA chicks with percentage of responders being 86, 82, 30, 59, and 40, respectively, for i.v., T-H, B-H, T-L, and B-L route-dosage combinations.

None of the higher order interactions was important for total primary antibody titers. Of the two-factor interactions, line × day bled and line × route of antigen delivery were significant. Line interacted with day because, whereas antibody titers for Day 6 PPI exceeded those for Day 10 PPI for HA chicks, there was no such difference in LA chicks (Table 3). Although titers were always higher for HA than LA chicks, route of delivery ranked differently for the lines. In Line HA, antibody titers for i.v., T-H, and B-H did not differ. Also, titers for T-H, T-L, and B-H were similar. However, chicks inoculated i.v. had higher titers than those inoculated T-L and B-L, these latter two routes being similar. In Line LA, i.v., T-H, and T-L routes elicited similar levels of antibody, which were higher than those from inoculations into breast muscle, regardless of dosage (B-H, B-L).

Primary IgG and IgM titers were higher for HA than LA chicks (Table 4). Day of bleeding after primary inoculation

TABLE 2. Percentage of chicks showing SRBC antibody titers by treatment, line, and days after inoculation in Trial 1

Treatment			Days after inoculation			
Route[1]	Dosage[2]	Line[3]	3	6	10	20
i.v.	0.25	HA	80 **	100	100	100 **
		LA	8	80	77	10
i.m.	0.25	HA	17	0	42	17
		LA	0	7	8	25
	2.50	HA	0	17	27	15
		LA	0	33	27	0
	25.00	HA	8	73 **	67	27
		LA	0	17	42	17

[1]i.v. and i.m. are intravenous and intramuscular routes of inoculation, respectively, of 0.1 mL of the SRBC dosages.

[2]% SRBC.

[3]Lines have undergone 24 generations of selection, respectively, for high (HA) or low (LA) antibody response to an i.v. inoculation of 0.1 mL of 0.25% SRBC.

**$P \leq 0.01$ is for a specific comparison of lines HA and LA within a route of inoculation, dosage, and day after inoculation subclass.

TABLE 3. Means and SEM for antibody titers of chicks after inoculation with SRBC by line, days after inoculation, and route and dosage of inoculation in Trial 2

	Line[1]		
	HA		LA
Days after inoculation			
6	6.8 ± 0.4	**	2.8 ± 0.1
	**		
10	5.3 ± 0.3	**	2.8 ± 0.2
Route and dosage of inoculation[2]			
i.v.	7.5 ± 0.6[a]	**	3.1 ± 0.2[a]
i.m. T-H	6.4 ± 0.3[ab]	**	3.1 ± 0.2[a]
i.m. T-L	5.1 ± 0.6[bc]	**	2.8 ± 0.2[a]
i.m. B-H	6.4 ± 0.6[ab]	**	2.1 ± 0.2[b]
i.m. B-L	3.4 ± 0.5[c]	**	2.1 ± 0.1[b]

[a–c]Means within a column for route and dosage with different superscripts differ at $P \leq 0.05$.

[1]Lines have undergone 25 generations of selection, respectively, for high (HA) or low (LA) antibody response to an i.v. inoculation of 0.1 mL of 0.25% SRBC.

[2]i.v. is an intravenous inoculation of 0.1 mL of 0.25% SRBC. i.m. is an intramuscular inoculation of 25.00% SRBC into the thigh (T) or breast (B) at 0.5 mL (H) or 0.1 mL (L) volume of SRBC.

**$P \leq 0.01$ is for a specific comparison between lines HA and LA or between Days 6 and 10 after inoculation.

had no effect on IgG titers, whereas levels of IgM declined from 6 to 10 d after inoculation. Route of SRBC delivery did not influence the magnitude of IgM produced. For IgG titers, inoculation via the i.v., T-H, and B-H were similar and higher than those from the B-L inoculation. Regardless of dosage, inoculations into the thigh resulted in similar IgG levels, whereas inoculations into the breast resulted in differences among doses (B-H > B-L).

Secondary Antibody Titers. Of all factors considered in analyzing 6-d total secondary antibody titers (PSI6), only inoculation status (boosted = 7.2 ± 0.3; nonboosted = 2.1 ± 0.1) was significant. The line difference observed for primary response was not apparent for the secondary titers (HA = 6.0 ± 0.5; LA = 5.6 ± 0.1). For IgG at PSI6, however, there was a difference between lines for the chicks that were boosted (HA = 5.8 ± 0.5; LA = 3.4 ± 0.2) but not for those that did not receive the booster (HA = 1.6 ± 0.2; LA = 1.2 ± 0.1). There was also a significant interaction for IgM 6 d PSI because of the line difference for boosted chicks (HA = 2.2 ± 0.3; LA = 3.4 ± 0.2) but not for those not boosted (HA = 0.8 ± 0.3; LA = 0.7 ± 0.1).

Line HA chicks had higher IgG and lower IgM titers than LA chicks PSI (Table 4). This inverse relationship canceled a line effect for total antibody titers because IgM titers were calculated as the difference between IgG and total titers. Route, day bled, sex, and the sex × dosage-route interaction were significant for IgG, IgM, and total SRBC antibody PSI. All titers declined between 6 and 10 d after the second inoculation. Results presented in Table 5 show the significant sex × dosage-route interactions. Although dose-route of delivery did not influence IgM of males, both IgG and total SRBC for the B-L route were lower than those for the other routes (Table 5). In females however, i.v. inoculation elicited the higher titers. Between sexes, IgM and SRBC levels were higher for females than males for i.v. and B-L but not for the other routes.

DISCUSSION

The effectiveness in eliciting an immune response in different host genotypes is influenced, among other fac-

TABLE 4. Means and SEM for IgG and IgM titers after the first (primary) and second (secondary) inoculation of SRBC and total antibody titers of chicks after the second inoculation with SRBC by line, days after inoculation, and route and dosage of inoculation in Trial 2

	Primary titers		Secondary titers		
	IgG	IgM	IgG	IgM	Total
Line[1]					
HA	3.8 ± 0.3	2.2 ± 0.2	4.8 ± 0.2	1.9 ± 0.2	6.7 ± 0.5
	**	**	**	**	
LA	1.2 ± 0.1	1.5 ± 0.1	2.8 ± 0.1	3.2 ± 0.2	6.1 ± 0.3
Days after inoculation					
6	2.7 ± 0.2	2.1 ± 0.1	4.3 ± 0.2	3.0 ± 0.2	7.2 ± 0.3
		**	**	*	**
10	2.3 ± 0.2	1.7 ± 0.1	2.8 ± 0.2	2.5 ± 0.2	5.4 ± 0.2
Route and dosage of inoculation[2,3]					
i.v.	3.1 ± 0.4[a]	1.8 ± 0.2[a]			
i.m. T-H	2.5 ± 0.2[ab]	2.0 ± 0.2[a]			
i.m. T-L	2.1 ± 0.3[bc]	1.9 ± 0.2[a]			
i.m. B-H	3.0 ± 0.4[a]	2.1 ± 0.3[a]			
i.m. B-L	1.6 ± 0.2[c]	1.5 ± 0.2[a]			

[a–c]Means within a column for route and dosage with different superscripts differ at $P \leq 0.05$.

[1]Lines have undergone 25 generations of selection, respectively, for high (HA) or low (LA) antibody response to an i.v. inoculation of 0.1 mL of 0.25% SRBC.

[2]i.v. is an intravenous inoculation of 0.1 mL of 0.25% SRBC. i.m. is an intramuscular inoculation of 25.00% SRBC into the thigh (T) or breast (B) at 0.5 mL (H) or 0.1 mL (L) volume of SRBC.

[3]There was a significant interaction between route of inoculation and dosage with sex for secondary titers. See Table 5 for results.

*$P \leq 0.05$; **$P \leq 0.01$ are for a specific comparison between lines HA and LA and between Days 6 and 10 after inoculation.

TABLE 5. Means and SEM for IgG, IgM, and total antibody titers of chicks after the second inoculation with SRBC by route and dosage of inoculation[1] and sex[2] in Trial 2

Route and dosage of inoculation	IgG		IgM			Total		
	Female	Male	Female		Male	Female		Male
i.v.	4.8 ± 0.5[a]	3.6 ± 0.5[a]	5.2 ± 0.5[a]	**	2.2 ± 0.5[a]	10.1 ± 0.5[a]	**	5.8 ± 0.7[a]
i.m. T-H	3.7 ± 0.4[ab]	4.1 ± 0.4[a]	2.1 ± 0.3[c]		2.2 ± 0.4[a]	5.8 ± 0.4[b]		6.3 ± 0.5[a]
i.m. T-L	3.6 ± 0.4[ab]	3.7 ± 0.4[a]	2.8 ± 0.5[bc]		2.3 ± 0.4[a]	6.4 ± 0.6[b]		6.0 ± 0.6[a]
i.m. B-H	2.9 ± 0.5[b]	3.5 ± 0.4[a]	2.3 ± 0.3[bc]		2.8 ± 0.2[a]	5.2 ± 0.6[b]		6.2 ± 0.4[a]
i.m. B-L	3.2 ± 0.5[b]	2.3 ± 0.3[b]	3.5 ± 0.5[b]	**	1.7 ± 0.4[a]	6.5 ± 0.8[b]	**	4.0 ± 0.4[b]

[a–c]Means within a column for route and dosage with different superscripts differ at $P \leq 0.05$.

[1]i.v. is an intravenous inoculation of 0.1 mL of 0.25% SRBC. i.m. is an intramuscular inoculation of 25.00% SRBC into the thigh (T) or breast (B) at 0.5 mL (H) or 0.1 mL (L) volume of SRBC.

**$P \leq 0.01$ is for a specific comparison between males and females.

tors, by the route of entry (Heisdorf et al., 1947; Kreukniet et al., 1992; Muir et al., 1998). The present experiment studied the effect that selecting chickens divergently for antibody response to a single i.v. inoculation of SRBC antigens had on i.m. routes of antigen delivery as well as to a booster inoculation of SRBC. These two lines are known to have diverged in antibody response to the selection protocol (Martin et al., 1990; Boa-Amponsem et al., 1997). Results of the present work regarding primary antibody responses to an i.v. inoculation at the dosage used under selection (0.1 mL of 0.25% SRBC) was consistent with the characteristic responses of the lines in that the high antibody line surpassed the low line at all times after inoculation.

Line differences in antibody responses to i.m. inoculation varied with dosage. When low dosages of SRBC were inoculated into the breast (Trial 1), line differences virtually disappeared, suggesting that the threshold for i.m. inoculation may be higher than for i.v. inoculation for the lines. It is noteworthy that differences between these lines tended to be larger for the i.v. route at lower dosages than that used in this investigation (Ubosi et al., 1985; Gross, 1986). At higher SRBC dosages, the specific i.m. site (breast or thigh) rather than dosage (T-H, T-L > B-H, B-L) appeared to have a greater influence on both frequency and magnitude of the response of Line LA. For Line HA however, both site of delivery and dosage of SRBC (T-H, T-L, B-H > B-L) were important in antibody response. This observation for i.m. inoculation is not consistent with reports that Line HA is less influenced by dosage when SRBC is delivered by the i.v. route (Boa-Amponsem et al., 1997, 1999), suggesting that body tissues might have been influenced to different extents by the selection. This inference is supported by the finding that HA chicks have larger spleens than LA chicks (Boa-Amponsem et al., 1998) and is corroborated by Kreukniet et al. (1992) who observed that differences between their lines divergently selected for SRBC antibody were affected by route of immunization.

That secondary titers were similar for both lines regardless of primary route of immunization and that Line LA exhibited memory response is consistent with previous reports on these (Martin et al., 1989; Boa-Amponsem et al., 1999) and other similarly selected lines (Kreukniet et al., 1992; Pinard et al., 1992; Mashaly et al., 2000). It has been suggested that a high primary antibody response characteristic of Line HA precludes resources available for anamnestic responses (Boa-Amponsem et al., 1999). Line differences in IgG for primary and secondary titers support earlier findings that Line HA probably achieves high antibody response through increases in IgG rather than IgM (Martin et al., 1989; Boa-Amponsem et al., 1999). In Line HA the ranking of route of inoculation for total primary antibody titers was similar to that for IgG titers. Muir et al. (1998) also observed that route of antigen delivery influenced IgG response and may provide additional opportunity for establishing passive protection through transfer of maternal IgG to chicks.

Overall, our results show a higher sensitivity of Line HA than LA to SRBC antigen injected i.m., which prompts the inference that although different in degree, the two lines have diverged in the ability to respond to SRBC antigens, regardless of route of entry into the body.

ACKNOWLEDGMENTS

This research was supported in part by a grant from The Virginia Agricultural Council. Thanks is expressed to S. I. Jackson for manuscript preparation.

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