Antibody Responses and Body Weights of Chicken Lines Selected for High and Low Humoral Responsiveness to Sheep Red Blood Cells. 2. Effects of Separate Application of Freund's Complete and Incomplete Adjuvant and Antigen

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ABSTRACT Antibody (Ab) responses to SRBC, BSA, *Mycobacterium butyricum*, and *Escherichia coli* lipopolysaccharide (LPS) were measured in two chicken lines divergently selected for high and low Ab responses to SRBC, and in a randombred control line.

Levels of Ab binding SRBC, BSA, and *Mycobacterium* protein, but not LPS were higher in the high Ab producing (H) line than in the control (C) and low Ab producing (L) lines ($P < 0.05$), and at almost every time, the L line showed significantly lower titers than the H and C lines. In the H and C lines, Ab responses to SRBC were enhanced when Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) were simultaneously administered on a separate location than SRBC. In the L line, Ab titers to SRBC and BSA were enhanced when antigen was administered emulsified in CFA.

At all times until 28 d after sensitization the C and L line birds were significantly heavier than birds of the H line. Body weight, body growth, and percentage body growth were impaired in birds that received antigen emulsified in CFA, which suggested a negative relationship between BW gain and immune responses to *Mycobacteria* protein.

Prolonged divergent selection for Ab responses to SRBC resulted into two lines that not only differ in Ab responses to T cell-dependent antigens but also in BW. In contrast to previous findings with the current lines, line differences with respect to Ab responses were not abolished by CFA treatment.

(*Key words*: sheep red blood cells, bovine serum albumin, *Mycobacterium*, antibody, body weight)

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INTRODUCTION

Two layer lines have been established that, at 37 d of age, exhibit either high (H line) or low (L line) antibody (Ab) responses to i.m. injected SRBC (Van der Zijpp and Nieuwland, 1986). A randombred control (C line) resembling the genetic pool of the original parental stock (Pinard *et al.*, 1993) was also maintained. Line differences in Ab responses to several unrelated T cell-dependent antigens and vaccines (Kreukniet *et al.*, 1996; Parmentier *et al.*, 1996), but not T cell-independent antigens (Kreukniet *et al.*, 1992) were found. Birds of the fourth generation of the current H and L lines responded equally well to SRBC administered in Complete Freund's Adjuvant (CFA), but line differences were still found when antigens were administered in Incomplete Freund's Adjuvant (IFA) (Kreukniet *et al.*, 1992).

A higher proportion of CD8+ cells and cells expressing γ/δ (TCR-1+) T cell receptors were found in the present L line than in the H line (Parmentier *et al.*, 1995). Recognition of Mycobacterial antigens and heat-shock proteins (HSP) by murine and human γ/δ T cells suggest a role of these cells in immune surveillance and immune regulation (Janeway *et al.*, 1988).

Adjuvants stimulate specific immunity to an antigen incorporated in that adjuvant, either by improved macrophage activation, lymphocyte trapping, improved antigen presentation, and/or depot formation, respectively. Adjuvants, including derivatives of microorganisms, but also nonimmunogenic water-in-oil emulsions, have been found to stimulate or suppress humoral and cellular responses of mice when administered separately

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Abbreviation Key: Ab = antibody; C = control line; CFA = Complete Freund's Adjuvant; H = line with high antibody responses to SRBC; HSP = heat shock proteins; IFA = Incomplete Freund's Adjuvant; Ig = immunoglobulin; IL = interleukin; L = line with low antibody responses to SRBC; LPS = lipopolysaccharide; PPD = purified protein derivative.

from antigen in time and place (Schwab, 1975; Hilgers *et al.*, 1984; Van der Heijden *et al.*, 1986).

The purpose of the present study was threefold. First, effects of type of adjuvant (with or without *Mycobacterium* components in water-in-oil emulsion) on the Ab response to the particulate antigen SRBC and the soluble antigen BSA were measured in birds of the three lines. Second, effects of *Mycobacterium* antigen, such as antigenic promotion (Wu and Cinader, 1971) on Ab responses of the three lines was studied by administration of antigen and CFA at the same, but also at separate location. Third, as line differences with respect to BW and live weight gain were found (Parmentier *et al.*, 1996) we measured the effects of all sensitization procedures on BW. Finally, effects of all treatments on Ab titers to *Escherichia coli* lipopolysaccharide (LPS) due to continuous sensitization by ubiquitous microorganisms were determined.

TABLE 1. Sensitization treatments

	Treatment[2]	
Treatment group[1]	Left breast	Right breast
1[3]	CFA[4]	SRBC / BSA
2[3]	CFA / SRBC / BSA	. . .
3	IFA[4]	SRBC / BSA
4	IFA / SRBC / BSA	. . .
5	PBS / SRBC / BSA	. . .
6	PBS	. . .

[1]Treatment groups consisted of 10 hens of each line.

[2]CFA = Complete Freund's Adjuvant; IFA = Incomplete Freund's Adjuvant.

[3]Contrasts 1 vs 2, and 3 vs 4 (i.e., different or similar location of adjuvant and antigen) reveal either general systemic activation of the immune system, or local activation, for instance by antigen presentation / depot formation.

[4]Contrasts 1 and 2, vs 3 and 4, and vs 5 indicate the effects of either the *Mycobacterium* components on the immune response, or the effect of oil / depot.

MATERIALS AND METHODS

Chickens

The experiment was conducted with 180 female chicks originating from an ISA Warren cross (medium heavy layers), which had been selected in the past for H and L primary antibody responses at Day 5 after primary i.m. immunization with SRBC at 37 d of age, and the randombred C line (Van der Zijpp and Nieuwland, 1986). From the 15th generation, 60 chicks of each line, 5 wk of age, from one hatch were used. Chicks were housed in battery cages with a maximum bird density of 12 chicks per cage and free access to feed (152 g / kg CP, 2,817 kcal / kg ME) and water. The birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at 0, 2, and 15 d of age, respectively.

General Reagents

Bovine serum albumin[2] (Factor V), CFA,[3] IFA,[3] *E. coli*-derived LPS (LPS, 055:B5),[2] and lyophilized *Mycobacterium butyricum* (84383 JC)[3] were used.

Experimental Design

Effects of type of adjuvant and location of adjuvant administration on Ab responses to SRBC and BSA were studied using a 3×6 factorial arrangement of treatments as shown in Table 1. The treatment-groups consisted of three different lines (H, C, and L lines), three different adjuvants (CFA, IFA, or PBS), and two locations of administration of antigen and adjuvant (antigen in the right breast muscle and simultaneously adjuvant in the left breast muscle, or antigen with adjuvant together in the left breast muscle). The negative control groups consisted of birds of all three lines that received only PBS i.m. in the left breast muscle. These groups were identical to the negative control groups described in the accompanying paper (Parmentier *et al.*, 1998).

At 5 wk of age, 10 birds per line (H, C, and L) were sensitized i.m. in the left breast muscle either with 0.5 mL adjuvant CFA, IFA, or PBS. Simultaneously, they were sensitized in the right breast muscle with 1 mL PBS containing a mixture of 1 mg BSA and 25% packed SRBC. Another 10 birds of each line received similar amounts of BSA and SRBC mixed with either CFA, IFA, or PBS, in the left breast muscle.

Humoral Immune Response SRBC, BSA, Mycobacterium butyricum, and LPS

Total Ab titers to SRBC were determined by agglutination according to Van der Zijpp and Leenstra (1980) in serum from all 180 birds 1 d before, and at 2, 5, 7, 9, 14, 21, and 28 d after sensitization. Antibody titers measured against SRBC were expressed as the $\log_2$ of the reciprocal of the highest serum dilution giving complete agglutination. Titrations were assessed the same day in 96-well microtiter plates, using the same SRBC that were used for the immunizations.

Total Ab titers to i.m. administered BSA, *M. butyricum*, and *E. coli* LPS, were determined by ELISA in serum from all 180 birds 1 d before, and at 2, 5, 7, 9, 14, 21, and 28 d after sensitization. Briefly, 96-well plates were coated with either 4 μg / mL BSA, 4 μg / mL *M. butyricum*, or 4 μg / mL LPS, respectively. After subsequent washing with PBS and, 0.05% Tween, the plates were incubated with serial dilutions of serum. Binding of Ab to BSA, *M. butyricum*, and LPS antigen was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase.[4] After washing, tetramethylbenzidine and 0.05% H_2O_2 were

[2]Sigma Chemical Co., St. Louis, MO 63178-9916.
[3]Difco Laboratories, Detroit, MI 48232-7058.
[4]Nordic, Tilburg, The Netherlands.

TABLE 2. Serum antibody titers from High (H), Control (C), and Low (L) line chickens after sensitization to SRBC, BSA within or simultaneously with Freund's Complete (CFA)/Incomplete (IFA) Adjuvant[1]

Group[2]		Antigen			
Line (Li)[3]	Treatment (Tr)	SRBC	BSA	*Mycobacterium butyricum*	Lipopolysaccharide
H	1	5.73[a]	4.11[b]	9.14[a]	7.47
	2	3.61[b]	4.97[b]	8.89[a]	6.52
	3	5.26[a]	4.47[b]	2.25[b]	6.10
	4	3.06[b]	4.65[b]	2.12[b]	6.40
	5	4.16[b]	4.37[b]	ND	6.47
	6	0.65[c]	0.30[c]	2.77[b]	6.18
C	1	4.01[a]	3.65[b]	7.43[a]	6.32
	2	2.59[b]	4.25[ab]	8.19[a]	6.50
	3	2.89[b]	3.32[b]	2.61[b]	6.19
	4	2.28[b]	4.38[a]	2.22[b]	6.78
	5	2.49[b]	3.66[b]	ND	6.31
	6	0.60[c]	0.37[c]	3.80[c]	7.11
L	1	0.68[bc]	1.73[b]	6.17[a]	6.96
	2	1.64[a]	2.61[a]	6.40[a]	5.97
	3	1.40[ab]	2.06[ab]	1.14[b]	6.37
	4	1.08[ab]	1.73[b]	1.25[b]	6.88
	5	0.54[bc]	1.81[b]	ND	6.42
	6	0.16[c]	0.10[c]	2.04[c]	7.63
	SEM	0.37	0.23	0.27	0.44
	Main effects				
	Li	***	***	***	NS
		H > C > L	H > C > L	H, C > L	H, C ≤ L
	Tr	***	***	***	NS
		1, 3 > 2, 4, 5 > 6	2 > 1, 3, 4, 5 > 6	1, 2 > 6 > 3, 4	
	Li × Tr	***	***	***	NS
	Ti	***	***	***	***
	Li × Ti	***	***	***	***
	Tr × Ti	***	***	***	***
	Li × Tr × Ti	***	***	*	*

[a,b,c]Means per antigen within line with no common superscript differ significantly ($P < 0.05$).

[1]Least squares means of the entire observation period.

[2]Ten birds per group (Line, Treatment).

[3]H = High line; C = Control line; L = Low line.

[4]Treatment 1 = SRBC/BSA simultaneously with CFA; Treatment 2 = SRBC/BSA within CFA/ Treatment 3 = SRBC/BSA simultaneously with IFA; Treatment 4 = SRBC/BSA within IFA; Treatment 5 = SRBC/BSA in PBS; Treatment 6 = PBS.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

added and incubated for 10 min at room temperature. The reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan[5] at a wavelength of 450 nm. Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of a standard positive serum present on every microtiter plate.

Body weights were measured 1 d before the day of sensitization, 24 h after sensitization, and at 9 wk of age, respectively.

Statistical Analysis

Total serum antibody titers to SRBC, BSA, *M. butyricum* protein, and LPS were analyzed by a three-way ANOVA for the effect of line, type of sensitization (with or without adjuvant; separate or similar location of adjuvant, and antigen administration), time, and their interactions using the repeated measurement procedure (SAS Institute, 1985). Body weights at 5 and 9 wk of age were analyzed by a two-way analysis of variance for the effect of line, type of sensitization, and their interactions (SAS Institute, 1985). Differences between the eight types of immunization and lines were tested with the Bonferroni's test.

RESULTS

Humoral Immune Response to SRBC, BSA, M. butyricum, *and* E. coli *LPS*

Least squares means of mean serum Ab titers of all lines to SRBC, BSA, *Mycobacterium* protein, and *E. coli* LPS during 4 wk after sensitization at 5 wk of age are shown in Table 2.

[5]Labsystems, Helsinki, Finland.

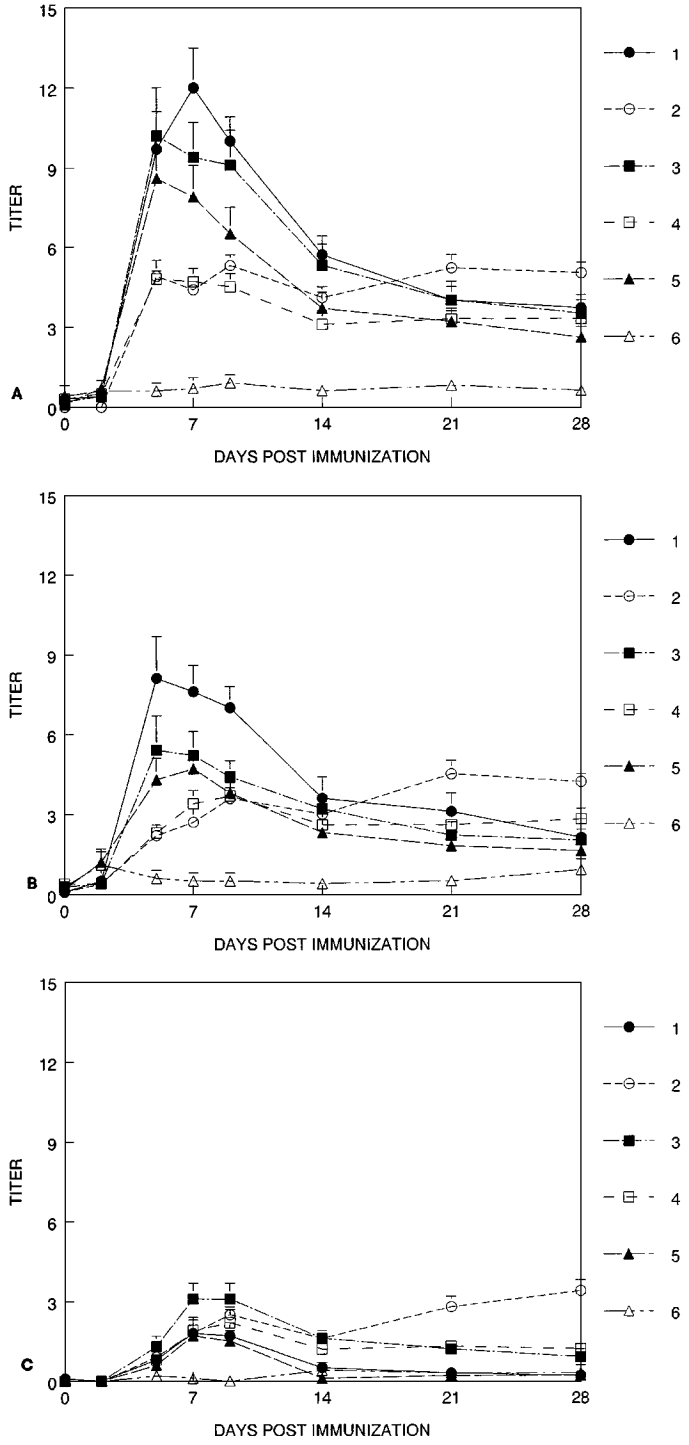

FIGURE 1. Antibody titers in serum from High line (A), Control line (B), and Low line (C) chickens to SRBC after i.m. sensitization at 5 wk of age with SRBC and BSA simultaneously with Complete Freund's Adjuvant (CFA) at different locations (Treatment 1, ●), or emulsified in CFA (Treatment 2, o), or simultaneously with Incomplete Freund's Adjuvant (IFA) at different location (Treatment 3, ■), or emulsified in IFA (Treatment 4, □), or emulsified in PBS (Treatment 5, ▲), or sensitized solely with PBS (Treatment 6, △). Data represent the mean + SEM of 10 hens per group.

SRBC. Anti-SRBC serum antibody titers in H, C, and L lines during 4 weeks after sensitization are shown in Figure 1. All three lines mounted Ab responses to SRBC; however, the Ab response to SRBC was significantly affected by a line by treatment by time interaction. During the complete experimental period, levels of Ab binding SRBC were higher in the H line than in the C and L lines ($P < 0.05$), and at almost every moment after sensitization, the L line showed significantly lower titers than the H and C lines. Treatment 1 (SRBC administered simultaneously with, but at different location than CFA) and Treatment 3 (SRBC administered simultaneously with, but at separate location than IFA) significantly enhanced titers as compared to Treatment 5 (SRBC in PBS) in the H line (Figure 1A), whereas Treatments 2 and 4 (SRBC administered within CFA, or IFA) did not enhance the Ab response to SRBC in the H line (Figure 1A) and C line (Figure 1B). The enhancing effect of separately administered CFA on the Ab response to SRBC was only significant in the H and C lines ($P < 0.05$), especially during the first 14 d after sensitization. In the L line, administration of SRBC within CFA was the only treatment that significantly enhanced the Ab response to SRBC as compared to Treatment 5, whereas administration of SRBC within IFA (Treatment 4) or simultaneously with IFA (Treatment 3) also enhanced Ab responses ($P < 0.05$) as compared to the negative control (Treatment 6) (Figure 1C).

In all lines, administration of SRBC within CFA (Treatment 2) significantly enhanced (prolonged) the Ab response as compared to administration of SRBC alone. Higher antibody responses were still present 4 wk after sensitization in Treatment 2 groups of all lines (Figure 1).

BSA. Anti-BSA serum antibody titers in H, C, and L lines during 4 weeks after sensitization are shown in Figure 2. All lines mounted Ab responses to BSA when sensitized to BSA, but, as was true for SRBC, the humoral response to BSA was significantly affected by a line by treatment by time interaction. Significantly higher titers ($P < 0.05$) were found in the H line than in the C and L lines, and the C line also differed significantly ($P < 0.05$) from the L line. Treatment 2 (BSA administered within CFA) significantly enhanced titers compared to the positive control (Treatment 5, BSA in PBS), whereas Treatment 1 (BSA administered simultaneously with, but at different location than CFA) did not enhance the Ab response to BSA. Also Treatments 3 and 4 (BSA simultaneously with or within IFA) did not enhance the Ab response to BSA. In the H line, none of the treatments significantly enhanced the response to BSA compared to Treatment 5 (Figure 2A). In the C line, Ab titers were higher in birds of Treatments 2, and 4, respectively; but only BSA within IFA enhanced the Ab response significantly (Figure 2B). Treatment 2 ($P < 0.05$) significantly enhanced the Ab response to BSA in the L line (Figure 2C). This response was especially true during the first 14 d after sensitization. None of the treatments resulted in disappearance of significant differences between the L line on the one hand and the H and C lines on the other hand.

Administration of BSA within CFA (Treatment 2) significantly enhanced (prolonged) the Ab response compared to administration of BSA in PBS alone in all lines (Treatment 5).

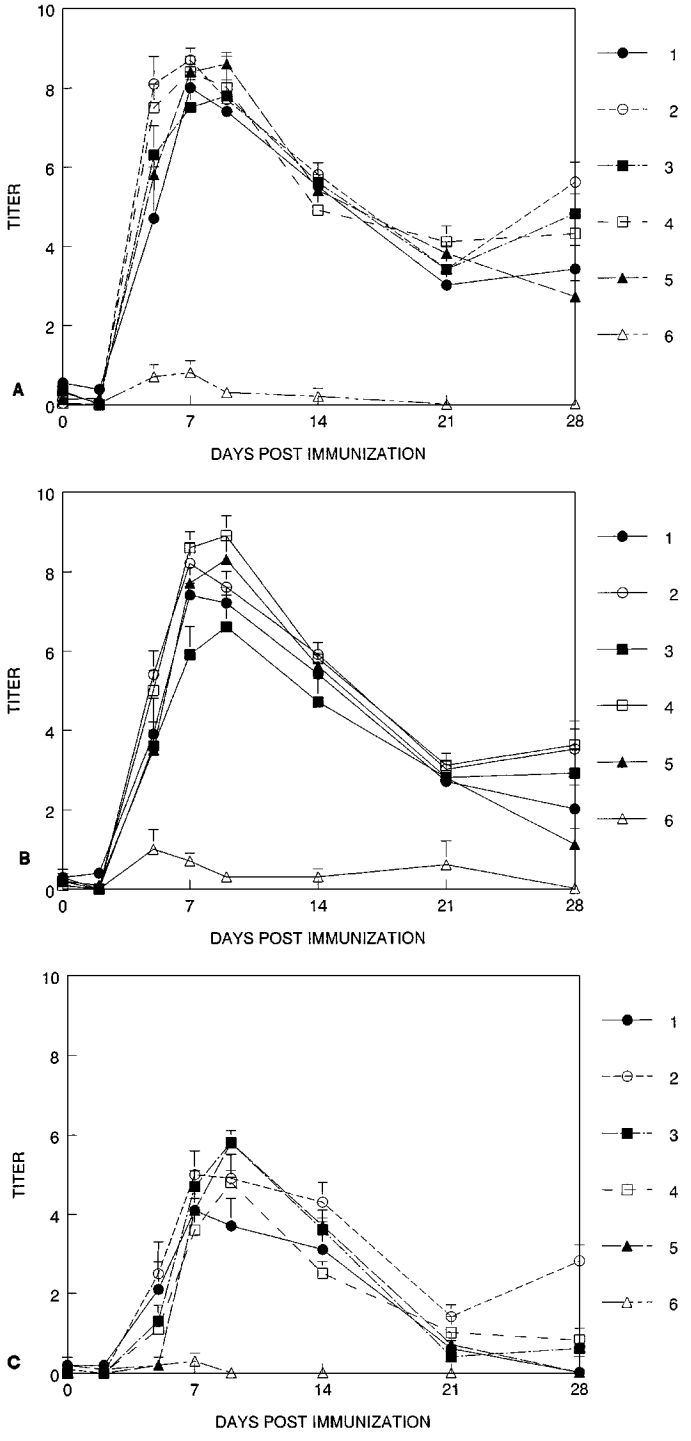

FIGURE 2. Antibody titers in serum from High line (A), Control line (B), and Low line (C) chickens to BSA after i.m. sensitization at 5 wk of age with SRBC and BSA simultaneously with Complete Freund's Adjuvant (CFA) at different location (Treatment 1, ●), or emulsified in CFA (Treatment 2, o), or simultaneously with Incomplete Freund's Adjuvant (IFA) at different location (Treatment 3, ■), or emulsified in IFA (Treatment 4, □), or emulsified in PBS (Treatment 5, ▲), or sensitized solely with PBS (Treatment 6, △). Data represent the mean + SEM of 10 hens per group.

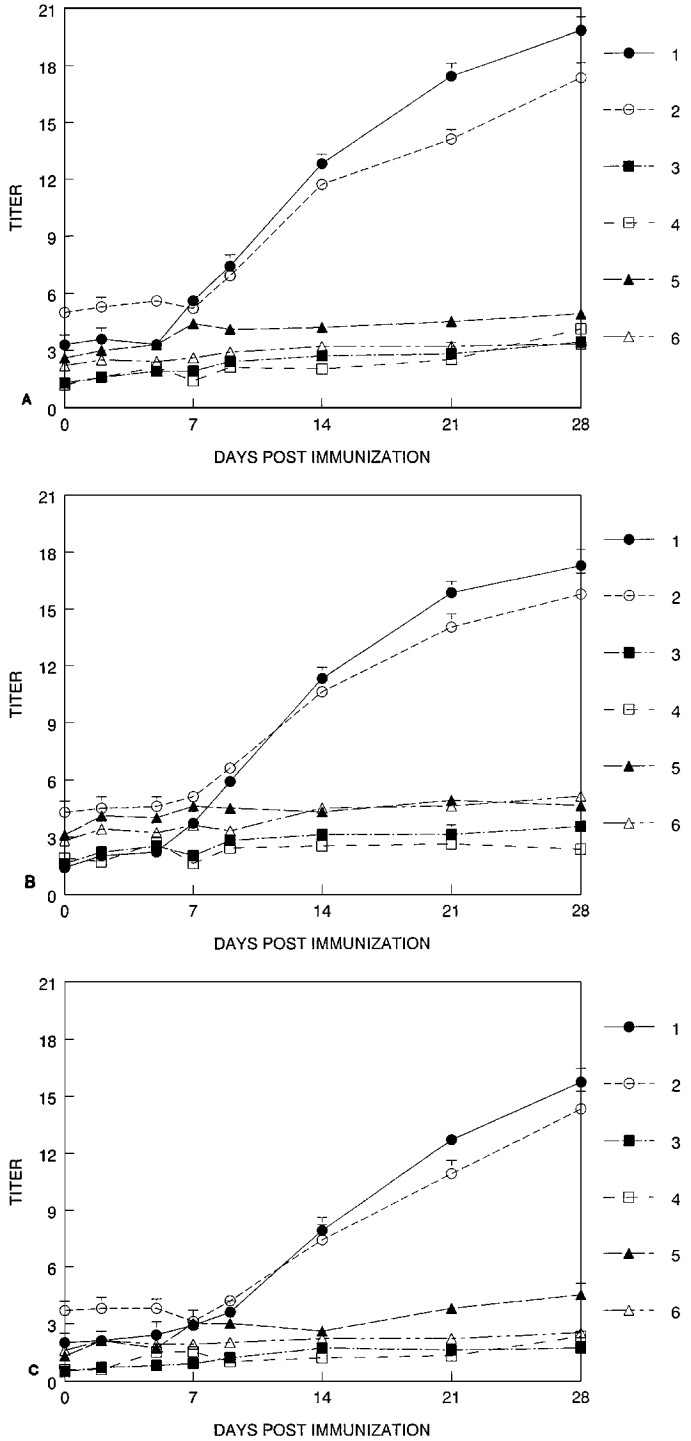

FIGURE 3. Antibody titers in serum from High line (A), Control line (B), and Low line (C) chickens to *Mycobacterium* protein after i.m. sensitization at 5 wk of age with SRBC and BSA simultaneously with Complete Freund's Adjuvant (CFA) at different location (Treatment 1, ●), or emulsified in CFA (Treatment 2, o), or simultaneously with Incomplete Freund's Adjuvant (IFA) at different location (Treatment 3, ■), or emulsified in IFA (Treatment 4, □), or emulsified in PBS (Treatment 5, ▲), or sensitized solely with PBS (Treatment 6, △). Data represent the mean + SEM of 10 hens per group.

M. butyricum. Anti-*Mycobacterium* protein serum antibody titers in H, C, and L lines during the 4 wk after sensitization are shown in Figure 3. With respect to the entire experimental period, significantly enhanced ($P < 0.05$) Ab responses to *Mycobacterium* protein were found in birds of all lines that had received CFA (Treatments 1 and

2). A line by treatment by time interaction was found, higher responses were found in the H line (Figure 3A) and C line (Figure 3B), which both differed significantly from the L line (Figure 3C) during the entire observation period ($P < 0.05$) (Table 2). At all days, significantly enhanced Ab responses ($P < 0.05$) were found in birds that had been sensitized with CFA (Treatments 1 and 2).

E. coli *LPS.* Anti-LPS serum antibody titers in H, C, and L lines during 4 wk after sensitization are shown in Figure 4. In sera from all three lines, slowly but steadily increasing Ab titers to *E. coli* LPS were observed. No main effects (line, treatment) or interactions between line and treatment were found, but a line by treatment by time interaction was present (Table 2). The L line (Figure 4C) tended to have higher Ab titers to LPS than the H line (Figure 4A). No treatment effects were found during the entire period, but in the L line significantly lower Ab titers to LPS were found in birds that had received CFA (Treatments 1 and 2) at Days 2, 5, and 9 after sensitization. At D 28 (9 wk of age) postsensitization, however, enhanced Ab responses to LPS were found in H and L line birds that had received CFA and SRBC / BSA (Treatments 1 and 2).

Body Weights

At 5 wk of age, BW of chicks of the H line were significantly lower than BW of chicks of the C and L lines (Table 3). Sensitization (treatment groups) did not affect BW at 24 h after sensitization nor 48-h body growth (live weight gain). Only in the C line birds did Treatment 3 tend to induce a higher percentage 48-h body growth. There was a tendency for retarded growth, both absolute and as percentage, in birds that were sensitized with CFA (Treatment 1).

At 9 wk of age, i.e., at D 28 after various antigen and adjuvant sensitizations, live weights of birds of the H line were still significantly lower ($P < 0.05$) than BW of the C and L lines. During the period from 5 to 9 wk of age relatively higher growth was found in the H line (Table 4). Birds of Treatment 2 (antigen mixed with CFA) appeared to grow significantly slower ($P < 0.05$) than birds injected with PBS. This result was true for all lines, but significantly retarded absolute and percentage growth was found in birds of the C and L lines.

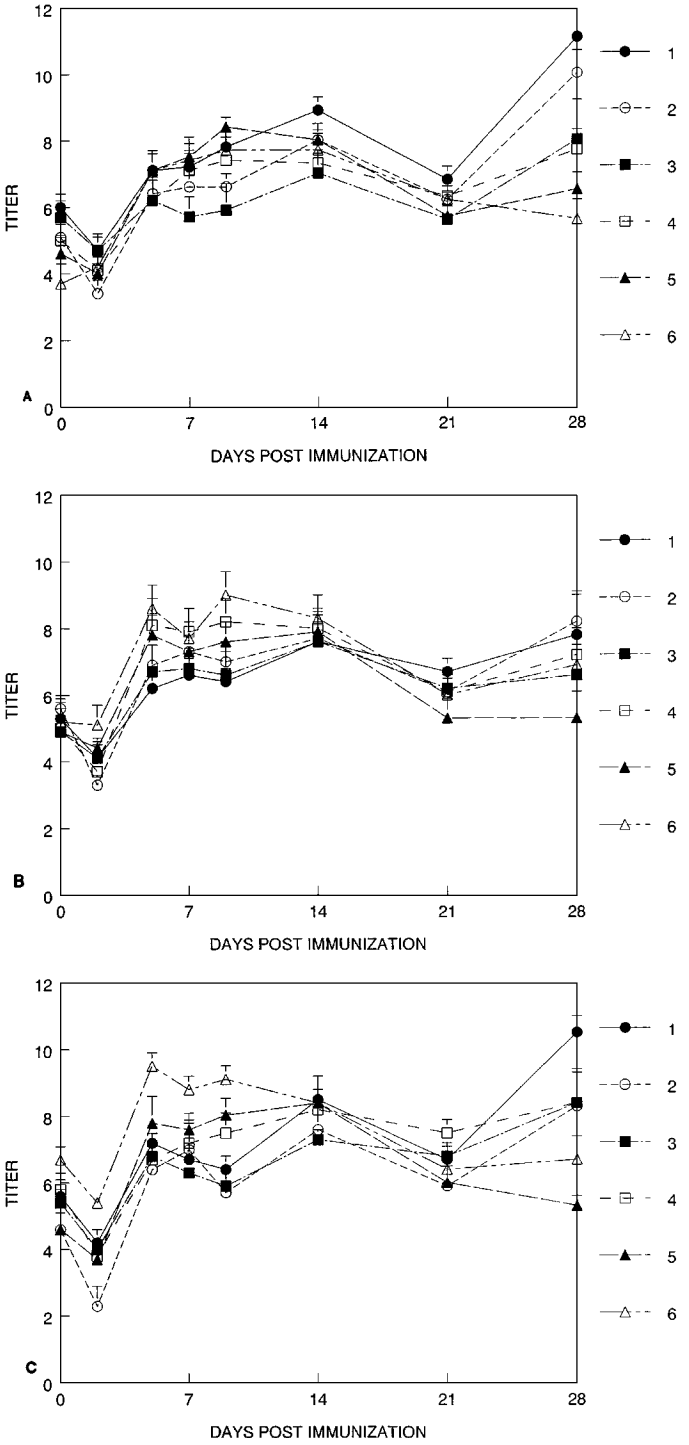

FIGURE 4. Antibody titers in serum from High line (A), Control line (B), and Low line (C) chickens to *Escherichia coli* lipopolysaccharide (LPS) after i.m. sensitization at 5 wk of age with SRBC and BSA simultaneously with Complete Freund's Adjuvant (CFA) at different location (Treatment 1, ●), or emulsified in CFA (Treatment 2, ○), or simultaneously with Incomplete Freund's Adjuvant (IFA) at different location (Treatment 3, ■), or emulsified in IFA (Treatment 4, □), or emulsified in PBS (Treatment 5, ▲), or sensitized solely with PBS (Treatment 6, △). Data represent the mean + SEM of 10 hens per group.

DISCUSSION

Two chicken lines were established that clearly differed in their capacity to produce Ab responses to SRBC and other T cell-dependent antigens such as BSA (Kreukniet *et al.*, 1992; Parmentier *et al.*, 1994). In the present study, these lines also mounted different Ab titers to *M. butyricum*. Antibody titers of the three lines to environmental LPS did not differ. These results indicated that 1) the absence of line differences to T cell-independent antigens, as found in the fourth generation (Kreukniet *et al.*, 1992) was not affected by continued selection, and 2) line differences probably do not comprise the capacity to produce Ab, as well rest on differences in functions of T cells or antigen presenting cells.

TABLE 3. Acute effects of sensitization on BW and 48-h growth at 5 wk of age of High (H), Control (C), and Low (L) line hens of the 15th generation

Line (Li)	Treatment[1] (Tr)	BW[2] Day–1	BW[2] Day+1	Growth[3]	Percentage growth[4]
		(g)			(%)
H	1	557	581	23	3.9
	2	506	549	43	8.6
	3	530	584	54	10.2
	4	546	588	42	7.8
	5	548	585	37	6.7
	6	519	563	44	8.6
C	1	600	658	58	9.7[ab]
	2	617	669	52	8.4[ab]
	3	597	660	63	10.7[a]
	4	589	646	57	9.7[ab]
	5	639	690	51	7.9[ab]
	6	612	670	58	9.4[ab]
L	1	583	629	46	8.0
	2	595	638	43	7.3
	3	639	700	61	9.7
	4	599	653	53	8.8
	5	589	641	53	9.0
	6	587	639	53	9.0
	SEM	19.8	21.5	6.3	1.1
	Main effects				
	Li	***	***	***	NS
		H < C, L	H < C, L	H < C, L	
	Tr	NS	NS	**	**
				3 ≥ 6	3 ≥ 6
				6 ≥ 1	6 ≥ 1
	Li × Tr	NS	NS	NS	NS

[a,b]Means per antigen within line with no common superscript differ significantly ($P \leq 0.05$).

[1]Treatment 1 = SRBC/BSA simultaneously with CFA; Treatment 2 = SRBC/BSA within CFA; Treatment 3 = SRBC/BSA simultaneously with IFA; Treatment 4 = SRBC/BSA within IFA; Treatment 5 = SRBC/BSA in PBS; Treatment 6 = PBS.

[2]Mean estimates of BW.

[3]Forty-eight hour increase in BW.

[4]Percentage increase in BW after 48 h.

**$P < 0.01$.

***$P < 0.001$.

In the fourth generation, no line differences in Ab titers were found when SRBC were administered in a CFA emulsion. When IFA was used as an adjuvant, line differences in Ab titers to SRBC were still found (Kreukniet *et al.*, 1992). The *Mycobacterium* component of CFA may enhance the immune system of the L line differently than the H line. In the present study, no treatment resulted in abolishment of line differences. This effect was true for Ab responses to SRBC and BSA, but also for Ab titers to *Mycobacterium* protein present in CFA. Emulsions of SRBC, BSA, and CFA enhanced Ab responses to SRBC and BSA significantly in the L line, but in contrast to the fourth generation, line differences were found. Continuous selection may have resulted in decreased genetic variability of the H and L lines, which may be required to modify immune responses and to diminish line differences.

In mice, polyclonal activation of humoral immune responses was described after application of CFA (Rosenberg, 1981), LPS (Björklund and Coutinho, 1983), or after immunization with SRBC (Rosenberg and Chiller, 1979). Simultaneous administration of (nonimmunogenic) water-in-oil emulsions at different locations or times enhanced Ab responses of the immunoglobulin M (IgM) isotype in mice (Van der Heijden *et al.*, 1986). In the present study, Ab levels to *E. coli* LPS were enhanced at the end of the observation period, but only in birds that had received antigen in CFA. Adjuvant enhanced Ab responses to SRBC, which are probably of the IgM type (Parmentier *et al.*, 1997), in the H line (CFA and IFA), and C line (CFA) when administered separately; emulsions of adjuvant and antigen did not enhance responses to SRBC and BSA in these lines. Neither separate administration, nor emulsions of adjuvant and antigen affected Ab titers to BSA in the H line, probably being of the IgM and IgG isotype (Parmentier *et al.*, 1994), only the BSA in IFA emulsion enhanced titers to BSA in the C line. Adjuvants containing antigen (such as CFA) may stimulate B cells by antigenic promotion (Wu and Cinader, 1971); however, IFA cannot give rise to antigenic promotion. The enhancing effect of separately administered CFA and IFA on Ab

TABLE 4. Body weights and growth of High (H), Control (C), and Low (L) line hens of the 15th generation at 9 wk of age

Line (Li)	Treatment (Tr)[1]	BW[2]	Growth[3]	Percentage growth[4]
		(g)		(%)
H	1	1,078	520	93.7
	2	1,002	495	98.0
	3	1,033	497	93.2
	4	1,068	521	95.2
	5	1,081	533	97.2
	6	1,039	521	102.8
C	1	1,089	489	81.5[b]
	2	1,121	504	81.6[b]
	3	1,109	512	86.0[ab]
	4	1,169	580	99.4[a]
	5	1,182	543	85.4[ab]
	6	1,171	559	91.2[ab]
L	1	1,093[ab]	511[ab]	88.5
	2	1,036[b]	441[b]	74.2
	3	1,215[a]	576[a]	90.8
	4	1,134[ab]	535[ab]	89.8
	5	1,103[ab]	515[ab]	88.3
	6	1,124[ab]	538[ab]	91.9
	SEM	35.7	21.9	3.8
	Main effects			
	Li	***	NS	***
		H < C, L		H > C, L
	Tr	*	**	**
		6 ≥ 2	4, 6 > 2	4, 6 > 2
	Li × Tr	NS	NS	NS

[a,b]Means per antigen within line with no common superscript differ significantly ($P < 0.05$).

[1]Treatment 1 = SRBC/BSA simultaneously with CFA; Treatment 2 = SRBC/BSA within CFA; Treatment 3 = SRBC/BSA simultaneously with IFA; Treatment 4 = SRBC/BSA within IFA; Treatment 5 = SRBC/BSA in PBS; Treatment 6 = PBS.

[2]Mean estimates of BW.

[3]Net increase in BW from 5 to 9 wk of age (grams).

[4]Percentage increase in BW from 5 to 9 wk of age.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

responses to SRBC in the H and C lines, but not in the L line, may rest on activated macrophages as antigen-presenting cells, or enhanced lymphocyte trapping, as proposed before for separately administered water-in-oil adjuvants such as IFA (Van der Heijden *et al.*, 1986).

Antibody responses to SRBC and BSA in the L line were enhanced after administration of antigen within CFA. Different responses of the L line as compared to the H or C lines to *Mycobacterium*, or antigens administered together with *Mycobacterium* were expected. Higher proportions of splenic CD8+ cells and TCR-1+ (γ/δ+) cells were found in L line birds, in contrast to more CD4+ T cells in H line birds (Parmentier *et al.*, 1995). The function of γ/δ T cells is unknown, but they may act as suppressor cells in chicken spleen and intestines (Bucy *et al.*, 1988; Quere *et al.*, 1990). Murine γ/δ T cells/hybridomas can be activated by *Mycobacterium tuberculosis* derived components such as purified protein derivative (PPD) and HSP (Janis *et al.*, 1989; O'Brien *et al.*, 1989; Haas *et al.*, 1990; Kaufmann and Kabelitz, 1991). In chickens, a transient increase of peripheral γ/δ T cells and activation of splenic γ/δ T cells by mycobacterial antigens, PPD, and the 65-kDa HSP65 was found after CFA immunization (Petter Arstila *et al.*, 1995). Whether the enhanced response in the L line to SRBC and BSA depended on polyclonal activation by CFA, enhanced antigen presentation, antigenic promotion, or is related with the higher proportions of γ/δ T-cells, that may release interleukin (IL)-2 like reagents upon activation with *Mycobacterium*, remains to be determined.

Administration of adjuvants a few days before antigen may lead to suppression due to antigenic competition, temporary unresponsiveness of antigen-reactive B cells, or an increased activity of scavenger cells, resulting in a diminished efficiency as antigen-presenting cells (Hilgers *et al.*, 1984; Schwab, 1975). In mammals, *Mycobacterium* components initiate γ-interferon and IL-12, which initiate T-helper-1-dependent inflammatory responses, but suppress T-helper-2-dependent antibody responses (Mosmann and Sad, 1996). Regardless of location and type of adjuvant

administration, sensitization of the current birds did not result in lower Ab responses to the particulate antigen SRBC, which is a T-helper-2 antigen in mammals, nor to the soluble antigen BSA, as compared to birds solely immunized with SRBC/BSA. Also, SRBC and BSA administered simultaneously at similar, or separate location apparently did not affect the responses to *M. butyricum* in all lines.

Chicks of the current H line showed significantly lower BW than chicks of the C and L lines. Allocation or prioritization of resources may have occurred in the L line similarly as in chickens artificially selected for body growth (Dunnington and Siegel, 1996). Alternatively, reduced growth and feed intake of immune-stimulated chickens may rest on cachectin activities of IL-1 and Tumor necrosis factor-α (Klasing *et al.*, 1987). In the present study immunization treatments did not affect body growth 24 h after sensitization. However, a significant decrease in BW and BW gain was found, especially in L line birds that mounted enhanced Ab to SRBC and BSA after sensitization with antigen within CFA, but not when immunized with these antigens in IFA or PBS. This result suggested that enhanced immune responsiveness in the L line may affect body weight via enhanced or prolonged IL-1 release by *Mycobacterium*-activated macrophages. A transient decrease in live BW gain of the current lines was found after i.p. administration of LPS (Parmentier *et al.*, 1998). The increasing anti-LPS titers in CFA-treated birds suggested an indirect relationship between immune responses to antigens as Mycobacteria on the one hand and BW on the other hand. This possibility will be the subject of further studies.

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