Effect of Duration of Cold Stress on Plasma Adrenal and Thyroid Hormone Levels and Immune Responses in Chicken Lines Divergently Selected for Antibody Responses

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ABSTRACT There is increasing evidence that stress affects various immune processes. Some of these changes are due to hormonal changes involving corticosterone (CORT), triiodothyronine (T_3), and thyroxine (T_4). Effects of stress depend on the nature of specific stressors (e.g., thermal extremes, diet, pollutants), and stress-modifiers (e.g., genetic make-up, duration and severity of the stressors). We studied the effects of a specific stress (cold stress) with stress-modifiers (duration of stress and genotype of the bird) on immune responses and plasma adrenal and thyroid hormone levels in 3 layer-type chicken lines. Two lines were divergently selected for high (H line) or low (L line) antibody responses to SRBC, and the third line was a randombred control (C) line. Growing chicks (3- to 4-wk-old) of the 3 lines were feed-restricted at 80% of ad libitum consumption, and subjected to cold stress (CS) at 10°C continuously for 7, 5, 3, 1, or 0 d before immunization with keyhole limpet hemocyanin (KLH). Specific antibody titers to KLH, and in vitro lymphocyte proliferation (LP) upon mitogen stimulation were measured. In addition, adrenal and thyroid hormone levels were measured in the plasma samples collected at the end of CS.

No significant effect of duration of CS on specific antibody titers was found in the 3 lines. A significant enhancing effect of CS was found on LP. A significant dose-dependent suppressive effect of CS was found on plasma CORT levels. One day of CS had a significant enhancing effect on T_3 levels. There was no significant effect of duration of CS on T_4 levels. We conclude that CS does not affect specific antibody responses, but may have a modulating effect on cellular immunity and plasma CORT levels, depending on the duration of the stress. The present study suggests an inverse relationship between LP and CORT. This is the first study that reveals an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines.

(*Key words*: cold stress, antibody response, hormone level, layer chicken)

2004 Poultry Science 83:1644–1649

INTRODUCTION

Stressors can alter immune function by several potential mechanisms. One of these mechanisms involves alterations of the endocrine system that in turn modulate immune function (Blalock, 1994; Savino and Dardenne, 1995). Various studies have reported activation of the hypothalamic-pituitary-adrenal axis, the sympathetic-adrenal-medullary axis (Maier et al., 1994; Mills et al., 1997; Azpiroz et al., 1999), and the hypothalamic-pituitary-thyroid axis (Silberman et al., 2002) by stressors resulting in hormonal stress responses.

There are reports about interactions between the thyroid axis and adrenocortical function (Sanchez-Franco et al., 1989; Kühn et al., 1998; Lo et al., 1998). Moreover, the modulation of immune function by glucocorticoids (Wilckens and De Rijk, 1997), and thyroid hormones (Madden and Felten, 1995) is well known.

It has been hypothesized that the genotype or phenotype of an animal influences its hormonal reaction to stress stimulations, which in turn alters the animal's behavioral adaptability and well being (Siegel, 1995; Mench and Duncan, 1998). Understanding the genetic basis for different individual responses to stress is critical in preventing harmful management practices and enhancing

Received for publication January 21, 2004.
Accepted for publication July 11, 2004.
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Abbreviation key: C = randombred layer chicken line; CORT = corticosterone; CS = cold stress; H = layer chicken line selected for high antibody response to SRBC; KLH = keyhole limpet hemocyanin; L = layer chicken line selected for low antibody response to SRBC; LP = lymphocyte proliferation; T_3 = triiodothyronine; T_4 = thyroxine.

productivity in the poultry industry (Mench, 1992; Craig and Swanson, 1994).

The impact of stress on the development of an immune response depends on the nature of specific stress (e.g., thermal extremes, diet, pollutants), and stress-modifiers (e.g., genetic make-up, duration or severity of the stressor) (Dohms and Metz, 1991). However, this hypothesis has not been tested experimentally. Therefore, the present study was undertaken to analyze the impact of the duration of cold stress (CS) on immune responses and the interrelationships with plasma corticosterone (CORT), thyroxine (T_4), and triiodothyronine (T_3) levels of genetically selected chicken lines for immune responsiveness. In addition, we examined whether these differences reflect genetic variation in the plasma CORT, T_4, and T_3 levels in response to CS.

To achieve these objectives, we studied effects of a specific stressor (cold stress) with stress-modifiers (duration of stress and genotype of the bird) on plasma CORT, T_4, and T_3 hormone levels and specific antibody responses and lymphocyte proliferative responses in 3 layer-type chicken lines. Two lines were divergently selected for high (H) or low (L) antibody responses to SRBC, and the third line was a random bred control (C) line. The phenotypic differences of 2 lines have been reported previously (Hangalapura et al., 2003).

MATERIALS AND METHODS

Chickens, Housing, and Experimental Design

The birds, their housing, and treatments used in the present experiment were previously described (Hangalapura et al., 2003). Briefly, 180 23-d-old ISA Brown (Warren) medium heavy layer hens from 3 lines were used. The first 2 lines were divergently selected for 21 generations for high or low primary antibody responses at 5 d after i.m. immunization with SRBC at 35 d of age. The third line was randombred, originating from the same parental line, and served as control (C) line (van der Zijpp and Nieuwland, 1986). The C line resembles the genetic pool of the original parental stock of layers (Pinard et al., 1993). These birds were fed once a day at 80% ad libitum. Birds had free access to water throughout the experiment. The photoperiod was 14L:10D, with light provided from 0400 to 1800 h.

At 23 d of age (experimental d 9), 5 groups of 12 hens of each (H, C, and L) line were randomly assigned to 1 of 5 treatment groups that each received a different duration of CS. Birds were allowed to acclimate to the climate chambers for 2 d. The 5 treatment groups were subjected to 7 (7CS), 5 (5CS), 3 (3CS), and 1 (1CS) d of CS of 10.4 ± 0.5(C (RH = 76.1 ± 1.1%), or no CS (0CS). Before and after the CS episode, all birds were kept at 22.5 ± 2.2(C (RH = 70 ± 0.2%) until the last experimental day.

At experimental d 0 (33 d of age), all birds were injected s.c. with 1 mg of keyhole limpet hemocyanin (KLH[2]) in 1 mL of PBS (pH 7.2) per bird. Blood samples collected from all birds at experimental d 0 (before immunization), and on d 1, 8, 11, and 32 postimmunization were used to measure various immune parameters (Hangalapura et al., 2003). Blood samples collected on d 1 postimmunization were also used to measure CORT, T_3, and T_4 levels. The experimental protocol was approved by The Institutional Animal Care and Use Committee of Wageningen University.

ELISA

Antibodies binding to KLH were determined in individual plasma samples obtained from all birds using an indirect 2-step ELISA procedure. Plates were coated with 1 μg/mL KLH, and after subsequent washing, incubated with serial 2-fold dilutions of plasma. Binding of antibodies to the antigens was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase.[3] After washing, tetramethylbenzidine and 0.05% H_2O_2 were added, and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan[4] at a wavelength of 450 nm. The titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of 2-fold diluted standard positive plasma present on every microtiter plate.

Lymphocyte Stimulation Test

An in vitro lymphocyte stimulation test was performed to determine effects of duration of cold stress on in vitro mitogen-stimulated T cell proliferation. Aliquots of 200 μL of whole blood were diluted 1:30 in RPMI tissue culture medium and cultured for 72 h at 41°C and 5% CO_2 in a humidified atmosphere. Medium was supplemented with 2 m*M* L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin, in 96-well flat bottom plates, with 10 μg/mL Concanavalin A.[5] The last 12 h before harvesting, cultures set up in triplicates were pulsed with 0.5 μCi of methyl-[^{3}H]-thymidine.[6] Tritiated thymidine uptake by cultures was determined with a Beckman beta-scintillation counter.

Results were expressed as mean cpm in mitogen- or antigen-stimulated cultures minus cpm in unstimulated cultures (ΔCPM) and as mean stimulation indices. The stimulation index = cpm in mitogen- or antigen-stimulated cultures/cpm in unstimulated cultures.

Plasma Hormone Determinations

Plasma corticosterone was determined using a radioimmunoassay kit.[7] Plasma 3,5,3′-triiodothyronine (T_3) and

[2]Cal Biochem, Novabiochem Co., San Diego, CA.
[3]Nordic, Tilburg, The Netherlands.
[4]LabSystems, Helsinki, Finland.
[5]Sigma Chemical Co., St. Louis, MO.
[6]ICN Biochemicals, Inc., Aurora, OH.
[7]IDS, Inc., Bolton, UK.

thyroxine (T_4) concentrations were measured by radioimmunoassay as described by (Darras et al., 1992). Intraassay coefficients of variation were 4.5 and 5.4% for T_3 and T_4, respectively. Standards for antisera, T_3, and T_4 were purchased from Byk-Belga.[8]

Statistical Analysis

Differences in titers of plasma antibodies binding KLH were analyzed by 3-way ANOVA for the effect of cold treatment, line, time, and their interactions using the repeated measurement procedure using a 'bird nested within treatment and line' option. A 2-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphoproliferation, CORT, T_3, and T_4 levels. Simple correlation between different immune parameters, CORT, T_3, and T_4 were calculated using Pearson's correlation coefficients. All analyses were according to SAS procedures (SAS Institute, 1990). Mean differences of treatment and line were tested with Bonferroni's test.

RESULTS

Specific Antibody Responses to KLH

Results of specific antibody responses to KLH have been published previously (Hangalapura et al., 2003). Briefly, no significant effect of duration of CS on antibody responses to KLH was found (Table 1). There was a significant line effect; the H line had the highest antibody titers followed by the C and the L lines.

In Vitro Lymphocyte Proliferation

Results of in vitro lymphocyte proliferation (LP) have been published previously (Hangalapura et al., 2003). Briefly, stimulation indices of in vitro LP to Concanavalin A were significantly affected by the duration of CS before immunization ($P < 0.05$, Table 1). Seven days of CS significantly ($P < 0.05$) enhanced in vitro LP to Concanavalin A compared with no CS. In addition, higher, but not significantly different LP was found in birds subjected to 5, 3, and 1 d of CS.

Effect of CS Duration on Adrenal Hormone Levels

Cold stress significantly depressed plasma CORT levels, and the depressive effect was more pronounced with increasing duration of CS (Table 2). All 3 lines responded similarly to the CS, as there was no line effect or line by CS treatment effect (Table 2).

TABLE 1. Average total plasma antibody titers[1] binding keyhole limpet hemocyanin (KLH) and stimulation indices of in vitro mitogen-stimulated lymphocyte proliferation of whole blood from 3 layer-type chicken lines subjected to 1 of 5 durations of cold stress treatment before immunization with KLH (adopted from Hangalapura et al., 2003)

Line[2]	Treatment[3]	Antibody titer	Stimulation index[4]
High (H)	7 CS	5.55	109.2[a]
	5 CS	5.50	81.0[ab]
	3 CS	5.22	54.2[b]
	1 CS	5.68	65.9[ab]
	0 CS	5.65	48.6[b]
Control (C)	7 CS	4.72	128.9[a]
	5 CS	5.46	54.5[bc]
	3 CS	5.24	98.4[ab]
	1 CS	5.46	73.1[bd]
	0 CS	4.74	43.4[cd]
Low (L)	7 CS	3.57	85.7
	5 CS	4.69	90.7
	3 CS	4.25	43.8
	1 CS	3.86	80.1
	0 CS	4.28	55.8
SEM		0.30	19.4
Main effects			
Treatment (T)		NS	*
			0 ≤ 3 ≤ 1 ≤ 5 ≤ 7
Line (Li)		***	NS
		H > C > L	
T × Li		NS	NS
Time (Ti)		***	—
Ti × T		*	—
Ti × Li		***	—
Ti × Li × T		***	—

[a–c]Means within a parameter and line group with no common superscript differ significantly ($P < 0.05$).

[1]Titers were expressed as the 2log values of the highest dilution giving a positive reaction. Values are least square means of the complete experimental period.

[2]For line and treatment, n = 12 hens per group.

[3]7CS, 5CS, 3CS, 1CS = birds subjected to cold for 7, 5, 3, 1 d before immunization, respectively; 0CS = birds kept in climate chamber maintained at 22.5 ± 2.2°C continuously (control).

[4]Values are least square means of stimulation indices.

*$P < 0.05$; ***$P < 0.001$.

Effect of CS Duration on Thyroid Hormone Levels

One day of CS before immunization resulted in a significant increase in circulating T_3 concentrations in plasma compared with levels of the 0CS group, whereas longer durations of CS did not significantly affect T_3 concentrations. No line effect or interaction term was found (Table 2). Thyroxine concentrations were not affected by duration of CS, line, or interaction between CS treatment and line (Table 2).

Effect of Genetic Selection on Adrenal and Thyroid Hormone Levels

Genetic selection for high or low primary antibody response did not significantly affect the plasma CORT, T_3, and T_4 levels in the described selection lines (Table 3).

[8]Byk-Belga, Brussels, Belgium.

TABLE 2. Corticosterone (CORT), 3,5,3′-triiodothyronine (T_3), and thyroxine (T_4) hormone levels[1] in plasma samples from high (H), control (C), and low (L) line hens subjected to 1 of 5 durations of cold stress prior to immunization with keyhole limpet hemocyanin

Line[2]	Treatment[3]	CORT	T_3	T_4	T_3/T_4
H	7 CS	8.16	0.35^{ab}	9.38^{cb}	0.042
	5 CS	16.10	0.23^{a}	22.39^{a}	0.012
	3 CS	15.45	0.96^{ab}	15.02^{ac}	0.080
	1 CS	13.59	1.10^{b}	5.78^{b}	0.190
	0 CS	14.39	0.83^{ab}	8.65^{cb}	0.104
C	7 CS	11.05^{a}	0.25^{a}	14.78	0.027
	5 CS	13.91^{ab}	0.16^{a}	10.25	0.018
	3 CS	10.36^{a}	0.51^{a}	9.86	0.062
	1 CS	15.90^{ab}	2.02^{b}	7.17	0.294
	0 CS	25.84^{b}	0.53^{a}	8.79	0.064
L	7 CS	9.38^{a}	0.17^{a}	8.57	0.020
	5 CS	11.68^{a}	0.65^{ab}	6.96	0.093
	3 CS	20.47^{ab}	0.47^{ab}	6.19	0.073
	1 CS	22.62^{ab}	1.36^{b}	7.77	0.184
	0 CS	32.62^{b}	0.58^{ab}	9.07	0.062
Pooled SEM		5.55	0.36	3.28	0.041
Main effects					
Treatment		*	***	NS	***
		$0 \geq 1 \geq 3 \geq 5 \geq 7$	$1 > 0 \geq 3 \geq 5 \geq 7$		
Line		NS	NS	NS	NS
Treatment × line		NS	NS	NS	NS

[a–c]Means within a parameter and a line group with no common superscript differ significantly ($P \leq 0.05$).

[1]Values are least square means of hormone levels (ng/mL).

[2] For treatment and line, n = 5 hens per group.

[3]7CS, 5CS, 3CS, 1CS = birds subjected to cold for 7, 5, 3, 1 d before immunization, respectively; 0CS = birds kept in climate chamber maintained at 22.5 ± 2.2°C continuously (control).

*$P < 0.05$; ***$P < 0.001$.

DISCUSSION

In the present study, the effect of different durations of CS on specific antibody and in vitro LP responses and their relationship with plasma CORT, T_4, and T_3 levels in genetically selected chicken lines were studied. There was no significant effect of duration of CS on specific antibody responses to KLH. Phenotypic differences in specific antibody responses in the present lines of chicken were not altered by the duration of CS. On the other hand, the duration of CS significantly enhanced mitogen-induced LP. This finding is in agreement with our previous findings with the same selection lines, which mounted higher in vitro mitogen responses when kept in the cold under free-range conditions (van Loon et al., 2004), and is also in agreement with the findings of Kubera et al. (1998). However, our finding contradicts the reported suppressed humoral immunity and lymphocyte proliferation reported in rats subjected to CS (Rybakina et al., 1997) and suppressed cell mediated immunity reported in chickens subjected to CS (Regnier and Kelley, 1981).

TABLE 3. Corticosterone (CORT), 3,5,3′-triiodothyronine (T_3), and thyroxine (T_4) hormone levels[1] at 23 d of age in plasma samples from High (H), Control (C), and Low (L) line hens divergently selected for antibody responses

Line[2]	CORT	T_3	T_4
H	37.2	1.2	5.0
C	37.9	1.1	6.0
L	42.7	1.6	6.3
SEM	6.0	0.2	0.6
Line effect	NS	NS	NS

[1]Values are least square means of hormone levels (ng/mL).

[2]n = 20 hens per line.

It is well established that stress is related to neurochemical and hormonal changes including alterations in adrenal and thyroid hormone levels (Lasser and Baldessarini, 1997; Servatius et al., 2000). The interaction between the adrenal and thyroid axis with the immune system, on the basis of existence of adrenal and thyroid receptors on lymphocytes as well as the frequent immune alteration associated with physiological and pathological fluctuation of adrenal and thyroid hormones, was suggested. Over the past years, strong evidence of participation of the neuroendocrine system in the modulation of humoral immunity (Haddad and Mashaly, 1991; Mashaly et al., 1998) and lymphocyte activity (Haddad and Mashaly, 1991; Blalock, 1994; Savino and Dardenne, 1995; Trout and Mashaly, 1995) has accumulated.

Glucocorticoids are considered immunosuppressive (Chrousos, 1995). Several studies have revealed immune enhancing (Jefferies, 1991) and immunomodulating effects of glucocorticoids (Wilckens, 1995). Moreover, it was demonstrated that lymphoid cells possess glucocorticoid receptors and therefore are sensitive for glucocorticoid action (Plaut, 1987). In the present study, exposure of birds to different durations of CS suppressed the CORT levels in a dose-dependent manner. The results of CORT and lymphoproliferation taken together suggest an in-

verse relationship. Considering the results of corticosterone and lymphoproliferation suggests that the intensity of CS used in the present experiment is mild. Silberman et al. (2002) reported an early increment of CORT levels that returned to normal values after 3 wk in mice exposed to a chronic mild stress. Ayensu et al. (1995) reported higher CORT levels in rats after 4 wk of chronic mild stress. However, others have not found elevated levels of this hormone in humans after prolonged stress situations (Mills et al., 1997). Moreover, it was demonstrated that chronic stress induces a hypersuppressive state for induced CORT secretion in response to acute stress, which is caused by partial habituation, coping, and adaptation to the stressors (Mizoguchi et al., 2001).

There is a close interaction between the thyroid axis and the hypothalamic-pituitary-adrenal axis. It has been reported that T_3 and T_4 influence plasma and adrenal corticosterone levels (Lo et al., 1998), and have direct immunomodulating activities (Madden and Felten, 1995). Moreover, chronic stress has been generally associated with a suppression of thyroid axis function. During stress, a suppressed secretion of thyroid-stimulating hormone and decreased conversion of the relatively inactive T_4 to the more biologically active T_3 has been described (Stratakis and Chrousos, 1995). Silberman et al. (2002) reported that exposure to chronic stress was able to induce a reduction of thyroid hormone levels in mice. Therefore, we determined T_3 and T_4 levels in the same samples in which CORT was determined. Levels of T_3 were significantly enhanced in birds exposed to the shortest duration of CS before immunization, whereas T_3 levels were unaffected in birds subjected to the longer duration of CS. The increased circulating T_3 levels in chickens exposed to a mild cold period (Kühn and Nouwen, 1978) could be due to an enhanced conversion of T_4 to T_3, a process that is known to be sensitive to ambient temperature (Kohrle, 1999). Apart from that, the significant increase in plasma T_3 levels after 1 d of CS suggests an increase in metabolic heat production. However, prolonged CS results in acclimation and restoration of heat balance as can be inferred from the normalized plasma T_3 levels as well as progressively decreased plasma CORT levels.

In the present study, there was no significant effect of CS on T_4 levels. A clear decrease in lymphoid proliferative response to mitogens and a depression of primary humoral immune response in hypothyroid animals were reported (Fabris et al., 1995). However, the effect of hyperthyroidism provoked by T_3 or T_4 administration on humoral and cellular immunity is still controversial (Silberman et al., 2002). The lack of correlation between T_3 or T_4 levels and proliferative responses in the present study suggests that the effect of CS on proliferative responses is unrelated to changes in T_3 or T_4 levels.

In addition, we studied the effect of genetic selection of chicken lines for antibody responses on levels of plasma CORT, T_3, and T_4. This study revealed an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines. Similar results were reported in broilers selected for high or low fat content (Buyse et al., 1987). However, differences may exist in daily rhythms in concentration of hormones in plasma or even with life stage of the selection lines.

In conclusion, CS does not affect specific antibody responses. However, CS does have a modulating effect on cellular immunity and plasma CORT levels, depending on the duration of the stress. There were no direct correlations between any immune parameters and the adrenal or thyroid hormone levels. However, results of CORT and lymphoproliferation taken together suggest an inverse relationship. In addition, this is the first study that reveals an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines. Further studies are needed, however, to confirm the effect of the CS on hormone levels, immune performance, and their interactions.

ACKNOWLEDGMENTS

The authors thank Ger Vries Reilingh and G. Nackarerts for their skilled technical assistance and the animal caretakers of De Haar for assistance provided during the study.

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