

Cortisol circadian rhythms and stress responses in infants at risk of allergic disease

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Background: The cortisol circadian rhythm and response to stressful stimuli are altered in children and adults with allergic disease, including asthma. It is not known whether these alterations precede or follow the onset of allergic disease.

Objective: We sought to evaluate the cortisol circadian rhythm and stress response among infants at risk for the development of allergic disease.

Methods: Infants with and without risk factors for allergic disease were evaluated at age 6 months. Saliva was obtained at 8 AM, 2 PM, and 8 PM at home ($n = 68$) by parents when their infants were comfortable and in the clinic ($n = 88$) before and after their physical examination and vaccinations. Information regarding parental allergy and exposure to other children at home or in child care were obtained by questionnaire.

Results: In multivariate analysis the circadian rhythm of cortisol was flattened because of the lack of the expected morning surge of cortisol, resulting in decreased diurnal variation of cortisol in infants of mothers with allergy ($P = .035$) or asthma ($P = .002$) or an asthmatic father ($P = .022$). The cortisol stress response was greater in infants of mothers with allergy ($P = .045$) or asthma ($P = .039$), those with fewer siblings ($P = .066$), and those not entering day care early in life ($P = .017$).

Conclusions: These alterations in both basal and stress levels of endogenous cortisol among infants predisposed to allergic disease might affect the development of allergic immune responses early in life through interactions with inflammatory mediators. (J Allergy Clin Immunol 2006;117:306-11.)

Key words: Endogenous cortisol, atopy, sibling, childcare, birth order, day care, hypothalamic-pituitary-adrenal axis

Firstborn children and children with allergic parents are more likely to have allergic diseases, such as asthma.^{1,2} Although the mechanism underlying the sibling effect is unknown, the hygiene hypothesis suggests that immune systems of firstborn children mature in a more hygienic environment, experiencing less microbial exposure, which in turn predisposes to an allergic phenotype.¹ The inheritance of an allergic predisposition from one's parents is undoubtedly primarily caused by genetic susceptibility.

Abbreviation used

HPA: Hypothalamic-pituitary-adrenal

However, because maternal allergy generally infers greater risk of asthma in children than paternal allergy, the intrauterine environment has been speculated to play an additional role in the risk of asthma development.^{2,3}

Altered hypothalamic-pituitary-adrenal (HPA) function is a possible link between these risk factors and the development of allergic disease. Both intrauterine and neonatal events affect the development of HPA function, which in turn is related to allergic disease. Maternal asthma can suppress fetal HPA function during pregnancy by altering fetal estriol production, a derivative of fetal adrenal dehydroepiandrosterone sulphate,⁴ and is a risk factor for the development of asthma in offspring.² Endotoxin exposure, a marker of microbial exposure, is associated with alterations of both basal circadian rhythm and stress responsiveness of cortisol in rats⁵ and with a decreased risk of allergy in children.⁶

Although the recent focus of HPA function in asthmatic subjects has primarily been on the potential side effects of corticosteroid therapy, studies have recently found that asthmatic subjects not treated with glucocorticoids are more likely to have alterations in the circadian rhythms and stress response of cortisol compared with nonasthmatic subjects.^{7,8} It is not known whether these alterations in HPA function precede or follow the onset of asthma. Historically these changes have been interpreted as being due to the chronic stress of disease, as was hypothesized by Selye⁹ more than 50 years ago. However, it is also plausible that altered HPA function precedes the development of allergic disease, such as asthma. Cortisol responses to the stress of a blood draw in newborns at risk for the subsequent development of allergic disease by virtue of a parental history of allergy and increased cord IgE levels differed from responses of newborns with low cord IgE levels and nonallergic parents.¹⁰ Because of the critical role of cortisol in organ maturation, its distribution throughout body tissues, and the known effect on large numbers of genes, cytokines, and other inflammatory mediators,¹¹ altered HPA function during early development could potentially have a significant effect on the maturing immune system.

We are unaware of previous investigations evaluating risk factors for asthma in regard to the development of both the cortisol circadian rhythm and stress response in

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infants before the onset of allergic disease. This study investigates the cortisol circadian rhythm and stress response of cortisol among a group of infants who vary in their familial predisposition to allergic disease and exposure to other children at home or in day care. The study hypothesis was that infants with allergic parents or little exposure to other children would have a lower basal circadian rhythm of cortisol and a blunted cortisol response to the stress of vaccination.

METHODS

Infants were recruited between 2 and 4 months of age during routine care at the pediatric clinic of the University of Arizona Health Sciences Center from April 2003 through August 2004. Children born at less than 36 weeks' gestation, with admission to the newborn intensive care unit for more than 6 hours, with any disease known to affect HPA function, or who had received medications, such as corticosteroids, that affect HPA function were excluded from study participation. The study protocol was approved by the University of Arizona Institutional Review Board, and all parents provided signed informed consent for their child's participation.

Parental allergy was determined by parent report of a previous diagnosis of asthma, hay fever, eczema, or other allergic disorder. Maternal age, other children less than 18 years of age living in the household, parental ethnicity, and gestational age were also reported on entry into the study. Day care was defined as any child care with one or more children occurring outside of the home during the first 6 months of life. Because both the circadian rhythm and stress response of cortisol mature after 2 months of age,^{12,13} *a priori* day-care entry was categorized as occurring in the first 2 months of life, 3 to 6 months of life, or never during the first 6 months of life.

Study protocol

At study entry, parents completed questionnaires regarding their medical histories, their study child's medical history, and their infant's child-care history and were instructed on the proper collection and storage of saliva. At the routine 6-month clinic visit, both the child's medical history and child-care history were updated. One infant had a febrile illness at the time of his 6-month visit, and therefore the examination, vaccinations, and data collection occurred the following week, when the child was well. One week before this visit, the family was sent a home saliva collection kit along with written instructions on proper collection and storage of saliva samples. These specimens were transported from home on ice when brought to the 6-month clinic visit. On arrival to the clinic, the study coordinator collected the previsit saliva specimen. The infant was then weighed and measured, examined by his or her physician, and given the age-appropriate vaccinations, which did not change throughout the study. Ten minutes after the receipt of the vaccinations, the study coordinator collected a second postvaccine saliva specimen.

Saliva collection

All saliva collections were performed by suctioning saliva from the infant's mouth with a soft plastic transfer pipette, attempting to collect 1 mL of saliva. All saliva specimens at home and in the clinic were performed when the child's mouth was free of food or milk. Parents were also instructed to collect the saliva at 8 AM, 2 PM, and 8 PM on a day with normal activities when the child was comfortable and without illness and on the weekend before the clinic visit so that employed parents could collect specimens in a similar manner as those children with a parent staying at home. Parents were also instructed to make an appointment between 1:30 and 2:30 pm for the

6-month clinic visit. All specimens were immediately refrigerated or frozen; those refrigerated were transferred to a freezer within 20 minutes.

Cortisol measurement

All specimens were transferred to a -80°C freezer for long-term storage. For measurement of cortisol, all specimens were thawed and spun at 3000 rpm for 15 minutes. Cortisol was measured by means of ELISA specifically designed for saliva specimens (Salimetrics LLC, State College, Pa), according to the manufacturer's instructions.¹⁴ All samples were assayed in duplicate, with 25 μL for each assay. All paired specimens (eg, each pair of clinic specimens and each group of 3 home specimens) were assayed together on the same ELISA plate. The manufacturer's reported sensitivity is 0.007 $\mu\text{g/dL}$, with a 0.31% cross-reactivity with salivary cortisone. The intra-assay and interassay precision was found to have approximately a 6% coefficient of variation.

Statistical analysis

Saliva cortisol concentrations were not normally distributed and therefore were natural log transformed for analysis. Geometric means and 95% CIs are reported. The Student *t* test was used to compare means of continuous variables. Pearson correlation was used to determine correlations between normally distributed continuous variables, and Spearman correlation was used for nonnormally distributed data.

Linear regression models were performed for the prediction of the diurnal variation of cortisol from the morning to the evening and the cortisol stress response to the clinic visit and vaccination. The primary predictive factors of interest were parental allergy and exposure to children at home, in child care, or both. Other potentially confounding variables considered for inclusion in the linear regression models were maternal age, infant's sex, parental ethnicity, and gestational age. In addition, the time of collection of previsit saliva (done on arrival to the clinic), the time from the previsit to postvaccine saliva collections, the minutes after the vaccine of the second clinic saliva collection, and the infant's actual age at the 6-month clinic visit were considered confounding variables in predicting the cortisol stress response. The time of the home morning saliva collection, time from the morning to the evening saliva collections, and the infant's actual age at the time of the collection of the home specimens were also considered as confounding variables in the linear regression model predicting the diurnal variation of cortisol. Categorical variables of maternal allergy (none, allergy without asthma, and asthma), paternal allergy (none, allergy without asthma, and asthma), and time of day-care entry were entered into the model as dummy variables. All potential confounding variables related to either outcome with a *P* value of less than .20 were entered into the appropriate multivariate model.

Using previously published data on a group of 3- to 4-year-old children,¹⁵ we estimated that a sample size of 72 children would yield a power of 80% to detect a difference between children with high versus low exposure to other children with a 2-sided α value of .05. Because we anticipated dropout and missing samples, we sought to enroll more than 90 infants.

An α value of less than .05 was considered significant in all analyses. All statistical tests reported are 2-sided.

RESULTS

There were 152 children referred by their physician to the study coordinator. Of these, 50 declined participation; the primary reasons being that the parents were "too

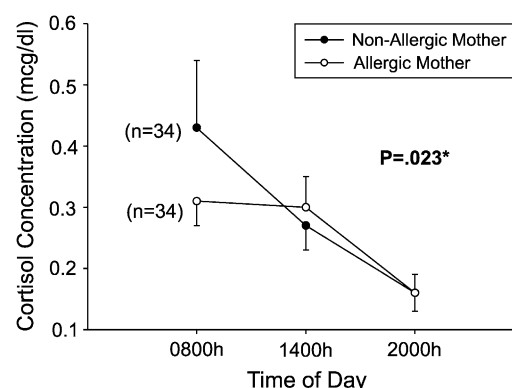
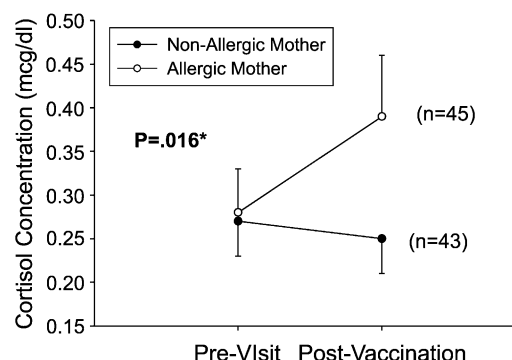
TABLE I. Characteristics of the study population

Characteristic	% (n/n)
Female sex	51.1 (45/88)
Ethnicity	
Both parents Anglo	30.7 (27/88)
One parent Anglo	18.2 (16/88)
Neither parent Anglo	47.7 (42/88)
Father's ethnicity missing	3.4 (3/88)
Mother's allergy history	
Asthma	14.8 (13/88)
Allergic disease without asthma	34.1 (30/88)
No allergic disease	51.1 (45/88)
Allergic father	
Asthma	9.1 (8/88)
Allergic disease without asthma	17.0 (15/88)
No allergic disease	67.0 (59/88)
Father's allergy history missing	6.8 (6/88)
No. of household children	
1	42.0 (37/88)
2	35.2 (31/88)
3	14.8 (13/88)
≥4	8.0 (7/88)
Month of entry into day care	
First 2 mo of life	10.2 (9/88)
3-6 mo of life	11.4 (10/88)
Not during first 6 mo of life	78.4 (69/88)
	Mean (SD)
Gestational age (wk)	39.0 (1.6)
Actual age (mo) at the 6-mo visit	6.5 (0.7)
Actual age (mo) of collection of home specimens	6.5 (0.7)
Maternal age (y)	25.4 (6.1)

TABLE II. Times of collection and cortisol concentrations of salivary specimens

Factor	N	Time, mean (SD)	Cortisol ($\mu\text{g/dL}$), geometric mean (95% CI)	P value
Home collection				
Morning	68	8:21 (0:48)	0.36 (0.11-1.20)	
Afternoon	68	14:19 (0:39)	0.28 (0.10-0.81)	
Evening	68	20:04 (0:48)	0.16 (0.05-0.47)	.001
Clinic collection				
Time of arrival	88	12:34 (2:22)	0.28 (0.11-0.74)	
Time of post-vaccine sample	88	13:38 (2:19)	0.31 (0.11-0.91)	.298
Minutes after vaccination	88	11.4 (3.3)		

busy” or “not interested.” Seven did not provide saliva specimens at 6 months and 3 were born at less than 36 weeks’ gestational age and were therefore excluded from this study. Of the remaining 92 infants, 88 provided paired clinic saliva samples, and 68 provided 3 home samples and were therefore included in this study. There were no differences in parental ethnicity, parental allergy, sex, day-care attendance, or number of household children among those children included and excluded from this analysis (data not shown).

**FIG 1.** Diurnal variation of cortisol by maternal allergy. *P value of difference between diurnal variation (morning cortisol – evening cortisol) of infants of allergic mothers compared with infants of nonallergic mothers.**FIG 2.** Change in cortisol with examination and vaccination by maternal allergy. *P value of difference between change in cortisol during examination and vaccination of infants of allergic mothers compared with infants of nonallergic mothers.

A majority of the infants had 1 or 2 non-Anglo parents, primarily Hispanic, and almost one half of the mothers reported having a history of allergic disease (Table I). The home collections of saliva were collected close to the requested times, and the clinic visits occurred primarily in the afternoon, as planned per the study protocol (Table II). As expected for a group of 6-month-old infants, the mean cortisol concentrations decreased significantly throughout the day ($P = .001$), and the cortisol concentrations showed a small increase after receipt of the vaccinations, although this change was not statistically significant ($P = .298$).

When the infants were stratified by the presence of allergic disease in their mother, there was a marked difference in both their cortisol circadian rhythm (Fig 1) and stress response to the clinic visit (Fig 2). Compared with children of nonallergic mothers, children with an allergic mother had a lower diurnal variation of their cortisol circadian rhythm from the morning to evening ($P = .023$) and a greater cortisol response to vaccination ($P = .006$). Both of these effects were more pronounced for infants of mothers with asthma compared with infants of mothers

TABLE III. Cortisol circadian rhythm and stress response by potential risk factors

Factor	N	Diurnal variation* (μg/dL), mean (SD)	P value	N	Stress change† (μg/dL), mean (SD)	P value
Sex						
Male	37	0.52 (0.88)		43	0.10 (0.41)	
Female	31	0.20 (0.40)	.052	45	0.04 (0.35)	.520
Ethnicity						
Mother Anglo	33	0.43 (0.90)		40	0.20 (0.39)	
Mother non-Anglo	35	0.31 (0.50)	.477	48	−0.04 (0.40)	.005
Father Anglo	26	0.35 (0.64)		31	0.26 (0.41)	
Father non-Anglo	40	0.39 (0.79)	.821	54	−0.04 (0.38)	.001
Maternal allergic disease						
Asthma	12	0.09 (0.14)		13	0.30 (0.48)	
Allergy, not asthma	22	0.21 (0.35)		30	0.14 (0.42)	
No allergy	34	0.57 (0.94)	.025	45	−0.05 (0.35)	.003
Paternal allergy						
Asthma	7	−0.17 (0.64)		8	0.02 (0.42)	
Allergy, not asthma	13	0.27 (0.31)		15	0.19 (0.45)	
No allergy	44	0.42 (0.61)	.017	59	0.04 (0.42)	.643
Household children						
1	31	0.44 (0.87)		37	0.17 (0.32)	
2	24	0.20 (0.53)		31	0.07 (0.40)	
3	8	0.38 (0.48)		13	−0.12 (0.63)	
≥4	5	0.75 (0.79)	.743	7	−0.16 (0.28)	.009
Day care entry						
First 2 mo	9	0.40 (0.58)		9	−0.12 (0.21)	
3–6 mo	8	0.18 (0.08)		10	0.06 (0.17)	
None	51	0.39 (0.79)	.825	69	0.09 (0.45)	.188
		Pearson <i>r</i>			Pearson <i>r</i>	
Maternal age	67	0.215	.081	87	−0.085	.433
Age at 6-mo visit		—	—	88	−0.153	.157
Age of 6-mo home collection	67	−0.215	.080		—	—
Gestational age	68	0.074	.550	88	−0.109	.314
Time of previsit saliva collection		—	—	88	−0.031	.776
Time from previsit collection to postvaccine saliva collection		—	—	88	−0.172	.110
Minutes after vaccine of postvaccine saliva		—	—	88	0.329	.002
Time of morning collection	68	−0.042	.735		—	—
Time from morning to evening collections	68	−0.045	.716		—	—

*Diurnal variation was calculated by subtracting the evening from the morning cortisol concentration.

†Stress response was calculated by subtracting the previsit cortisol concentration from the postvaccine cortisol concentration.

with other allergic disease or no allergic disease (Table III). Among infants of asthmatic mothers, there was no relation between maternal use of glucocorticoids during pregnancy and either the diurnal variation or stress response of cortisol (data not shown). These 2 characteristics of HPA function, diurnal variation and stress response, were not significantly correlated with each other in the total population (Pearson $r = -0.170$, $P = .172$). However, when stratified by maternal allergy, the 2 outcomes were inversely correlated among infants of allergic mothers (Pearson $r = -0.343$, $P = .050$) but not among infants of nonallergic mothers (Pearson $r = -0.025$, $P = .890$).

The diurnal variation was also significantly decreased in female infants, infants of allergic fathers, infants of younger mothers, and infants who were older at the time of

their 6-month visit (Table III). The cortisol response to the stress of examination and vaccination was increased in infants of Anglo parents, infants of families with fewer household children, and those who donated saliva later after vaccination.

Predictors of a decreased diurnal variation that remained significant in multivariate linear regression were having a mother with allergies or asthma, having a father with asthma, and being older at the time of the 6-month visit (Table IV). Significant predictors of an increased cortisol response to examination and vaccination in multivariate linear regression were having a mother with allergies or asthma, lack of early entry into day care, having a non-Anglo father, and having the postvaccine saliva collected later after vaccination (Table V). Being exposed to fewer household children was associated with an

TABLE IV. Predictive factors of the diurnal variation of cortisol in multivariate linear regression model*

Factor	Coefficient (95% CI)	P value
Mother's allergy history		
Not allergic	Referent	
Allergic without asthma	-0.335 (-0.645 to -0.025)	.035
Asthmatic	-0.692 (-1.110 to -0.273)	.002
Allergic father		
Not allergic	Referent	
Allergic without asthma	0.030 (-0.322 to 0.382)	.864
Asthmatic	-0.533 (-0.985 to -0.081)	.022
Male sex	0.231 (-0.043 to 0.505)	.097
Age (d) at collection of specimens	-0.009 (-0.017 to 0.000)	.041

*Model also adjusted for the mother's age at birth of study infant.

increased cortisol response, but this was of borderline significance ($P = .066$). After adjustment for maternal allergy, maternal ethnicity was no longer a significant predictor of cortisol stress change. There was no effect modification of these relationships by the infant's sex (data not shown).

DISCUSSION

The primary findings of this study were that infants of allergic mothers were more likely to have a greater cortisol response to the stress of examination and vaccination and a flattened cortisol circadian rhythm caused by a lack of the expected morning cortisol surge, resulting in a lower diurnal variation of cortisol than seen in infants with nonallergic mothers. In addition, paternal asthma was related to lower diurnal variation of cortisol, whereas early day-care entry and increased number of household children were related to a decreased cortisol response to the stress of examination and vaccination.

Endogenous cortisol is pervasive throughout the human body, with glucocorticoid receptors in virtually all cells. Through binding with these intracellular receptors, cortisol affects the transcription and translation of many inflammatory mediators. Recently, by using DNA microarray technology, it was shown that glucocorticoids affected 20% of the expressed human leukocyte genome.¹⁶ Varying cortisol levels caused by altered HPA function during infancy and early childhood might play a critical role through altered modulation of glucocorticoid-responsive genes in the development of immune responses at a time when the allergic phenotype is developing.¹⁷

Recently, both the cortisol circadian rhythm and stress response were shown to differ between nonasthmatic subjects and asthmatic subjects who were not receiving glucocorticoid therapy.^{7,8} Altered stress response of cortisol has also been found among subjects with allergic rhinitis and atopic dermatitis but not among atopic subjects without clinical allergies.^{18,19} This suggests that endogenous cortisol might be related to the expression of allergic disease but not atopy per se. In all cases, the

TABLE V. Predictive factors of cortisol stress response to clinic visit with vaccinations in multivariate linear regression model*

Factor	Coefficient (95% CI)	P value
Allergic mother		
Not allergic	Referent	
Allergic without asthma	0.191 (0.004-0.378)	.045
Asthmatic	0.264 (0.013-0.515)	.039
Each household child	-0.086 (-0.178 to 0.006)	.066
Month of day care entry		
Not during first 6 mo	Referent	
During 3rd to 6th mo	-0.093 (-0.364 to 0.178)	.496
During first 2 mo	-0.324 (-0.588 to -0.060)	.017
Minutes after vaccine to saliva collection	0.026 (-0.001 to 0.053)	.049
Father's ethnicity		
Anglo	Referent	
Non-Anglo	-0.228 (-0.446 to -0.011)	.040

*Model also adjusted for actual age at 6-month visit, time from previsit saliva collection to postvaccine saliva collection, infant's sex, and mother's ethnicity.

cortisol stress response was blunted among those with allergic disease compared with nonallergic control subjects. However, it is not known whether this blunted stress response was present before the onset of disease or was a result of the presumed chronic stress of allergic disease.

Previous studies in rats have suggested that neonatal treatment with endotoxin increased the circulating level of endogenous corticosterone and increased the corticosterone response to stress.⁴ Our original hypothesis was based on these findings in rats and the abovementioned findings of a blunted cortisol stress response among adults and children with allergic diseases. Given that children of allergic parents are at risk of allergic disease, the circadian rhythm findings are consistent with our original hypothesis. The increased stress response among infants at risk of allergic disease because of their parents' allergic status was opposite to our expectations. However, it is consistent with a recent study that found increased cortisol response to the stress of the heel stick associated with a blood draw in newborns with allergic parents and increased cord IgE levels.¹⁰ It is not clear whether these differences in cortisol response between healthy infants predisposed to allergic disease and older children and adults with allergic disease are due to age- or disease-related factors. The speculation that chronic hyperactivity of the HPA axis might ultimately lead to hypocortisolism through the downregulation of corticotrophin-releasing hormone receptors in the pituitary might explain these findings.²⁰

Infants do not exhibit the usual diurnal decrease in cortisol levels at birth; it usually develops between the first 2 and 6 months of life.^{21,22} The cortisol response to examination and vaccination gradually decreases from 2 to 6 months of age.¹³ Because both the circadian rhythm and stress response of cortisol are controlled at the level of the hypothalamus and were correlated among infants of allergic mothers, we speculate that both of our findings

are secondary to altered neuroendocrine maturation of the hypothalamus, hippocampus, or both in infants at risk for allergic disease. It is not clear whether these findings represent delayed maturation of the HPA axis or a fixed alteration of HPA function among infants 6 months of age predisposed to allergy. The same pattern of flattened circadian rhythm of cortisol was found in asthmatic children and adults not treated with exogenous glucocorticoids.^{7,23,24} Longer follow-up is necessary to differentiate between these 2 possible outcomes.

This study has several potential limitations. The reproducibility of measuring the circadian rhythm of cortisol during infancy over 1 day has recently been questioned.¹² Although this continues to be a methodologic challenge in this field of research, we did find consistent results with both the circadian rhythm and stress responsiveness, and it is unlikely an actual bias between risk groups existed in the measures. If random error occurred in our cortisol measures, this would have biased the results toward the null hypothesis. There were inevitable variations in the study protocol regarding the time of arrival, length of the clinic visit, and time of collection of the home salivary specimens. However, our results were not altered when adjusted for these variables. Consistent with previous epidemiologic studies, we used parental report to ascertain medical histories in both parents.²⁵

Regardless of the underlying mechanism, variations in endogenous cortisol could interact with genes critical to the development of the allergies, possibly through pervasive transcriptional or posttranscriptional effects on the expression of cytokines and other inflammatory mediators. It remains to be determined whether these findings are due to dysregulation within the integrated system of immune signaling and HPA function that begins *in utero* and continues to develop early in life.

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