Diisocyanate asthma and gene-environment interactions with *IL4RA*, *CD-14*, and *IL-13* genes

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Background: Diisocyanate asthma (DA) affects 2% to 10% of exposed workers, yet the pathogenetic mechanisms underlying this disorder remain ill defined.

Objective: To determine if specific single nucleotide polymorphisms (SNPs) of interleukin 4 receptor α (*IL4RA), IL-13,* and *CD14* promoter genes are associated with DA.

Methods: Sixty-two workers with DA confirmed by specific inhalation challenge (SIC) and 75 diisocyanate-exposed, SIC-negative workers were analyzed for SNPs associated with *IL4RA*, *IL-13,* and *CD14* promoter genes.

Results: No associations were found with individual SNPs and DA. When stratified according to specific diisocyanate exposure, a significant association was found between *IL4RA* (I50V) II and DA among individuals exposed to hexamethylene diisocyanate (HDI) (odds ratio [OR], 3.29; 95% confidence interval [CI], 1.33–8.14; $P = .01$) only. Similarly, the *IL4RA* (I50V) II and *IL-13* (R110Q) RR combination was significantly associated with DA in HDI-exposed workers (OR, 4.13; 95% CI, 1.35–12.68; $P = .01$), as was the *IL4RA* (I50V) II and *CD14* (C159T) CT genotype combination (OR, 5.2; 95% CI, 1.82–14.88; $P = .002$) and the triple genotype combination *IL4RA* (I50V) II, *IL-13* (R110Q) RR, and *CD14* (C159T) CT (OR, 6.4; 95% CI, 1.57–26.12; $P = .01$).

Conclusions: Gene-environmental interactions may contribute to the pathogenesis of DA, and gene-gene interactions may modulate this relationship.

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INTRODUCTION

Occupational asthma (OA) is the most common occupational lung disorder,[1] and diisocyanate chemicals are among the most frequent causes.[2] Several clinical observations suggest that immune mechanisms contribute to diisocyanate asthma (DA), including occurrence in a small proportion of exposed workers, a latency period of diisocyanate exposure preceding respiratory sensitization, and elicitation of asthmatic responses by subirritant levels of chemical. Respiratory sensitivity to diisocyanates persists years after exposure cessation, suggesting immunologic memory.[3–6] However, evidence of specific humoral or cellular responses directed against diisocyanate antigens is absent in many affected workers. Serum specific IgE antibodies for diisocyanate human serum albumin conjugates have been detected in no more than 40% of confirmed cases of OA.[7–9] We have previously reported that in vitro production of monocyte chemoattractant protein by peripheral mononuclear cells stimulated with diisocyanate human serum albumin can be demonstrated in 79% of workers with specific inhalation challenge (SIC)–confirmed DA[8] compared with 8.3% of unexposed asthmatic patients and 8.6% of diisocyanate-exposed workers with negative SIC test results.

Partly because of the limitations of immunologic tests, investigators have performed genetic association studies in attempts to define genetic markers that identify those diisocyanate-exposed populations at risk for OA. The HLA class II alleles, *HLA-DQA1*0104* and *HLA-DQB1*0503*, are significantly increased in workers with DA, whereas the *HLA-DQA1*0101* and *HLA-DQB1*0501* alleles were increased among asymptomatic workers.[10,11] Investigators in Finland reported that diisocyanate workers who possessed the null

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genotype (M1) of the antioxidant enzyme, glutathione-s-transferase, are 1.89-fold more likely to develop DA,[12,13] although this finding has not yet been replicated in other populations. Nevertheless, these studies suggest that susceptibility for DA may be genetically determined.

The binding of interleukin 4 (IL-4) to its IL-4 receptor α chain (*IL4RA*) is essential for the development of airway inflammation in murine models of allergen-induced airway inflammation.[14,15] Candidate genes located on chromosome 5q, which encode the T_H2 cytokines IL-4 and IL-13, have been identified in patients with nonoccupational or atopic asthma.[16–19] In multiple studies, 2 of the *IL4RA* allelic variants, (I50V) V and (Q551R) R alleles, have been associated with asthma and atopy.[20] Although studies of *IL-4* receptor and *IL-13* polymorphisms have been conducted in atopic populations, these genes have not been actively investigated in workers with OA. Murine models of isocyanate-induced airway inflammation demonstrate the presence of $CD4^+$ cells that produce T_H2 cytokines (eg, IL-4, IL-13). Full expression of the latter phenotype was shown to be heavily influenced by the genetic mouse strain used.[21–24] Despite these findings in animals, IL-4 is either transiently or rarely expressed in bronchial tissue of workers with toluene diisocyanate (TDI) asthma.[25,26] Nevertheless, these findings suggest that human investigations of T_H2 cytokine gene polymorphisms conducted in workers with DA might be informative. In the present study, we hypothesized that DA could be associated with unique alleles and genotypes on the *IL-4* receptor and *IL-13* genes. Because of evidence that DA is more often IgE independent, genetic markers for DA would likely differ from those reported in allergic asthma. Following the same rationale, we also investigated interaction of a *CD14* (C159T) gene polymorphism (shown to modify the atopic phenotype) with *IL4RA* genotypes and the DA phenotype.

METHODS

Study Participants

Workers with exposure to diisocyanates who were referred for evaluation of reported lower respiratory tract symptoms were prospectively recruited from the Respiratory Clinics at Hôpital du Sacré-Coeur de Montréal, Université de Montréal, Laval Hospital in Sainte-Foy, and at the Gage Occupational and Environmental Health Unit in Toronto, Ontario. All were sent for SIC to work-relevant diisocyanate chemicals, the diagnostic gold standard for establishing OA.[27,28] Data regarding age, sex, race, smoking status, time of exposure, and other factors were collected by questionnaire, and atopy was evaluated by skin prick testing to common aeroallergens.

Patients were evaluated and divided into DA-positive and DA-negative groups based on positive and negative responses to SIC, respectively. Evaluation included methacholine challenge testing performed before SIC.[29] A decrease in forced expiratory volume in 1 second of at least 20% from baseline was defined as a positive SIC test result.[30] Ethics committees at each participating institution approved the study protocol, and informed consent was obtained from all participants.

Genotyping of IL4RA, CD14*, and* IL-13 *Single Nucleotide Polymorphisms*

Genomic DNA was isolated from EDTA anticoagulated whole blood, and the *IL4RA* variants were genotyped as previously described.[31] The *CD14*−159°C 224 T single nucleotide polymorphism (SNP) was genotyped for the presence of −159°C or −159T alleles by polymerase chain reaction using the following primers: sense 5′-GTGCCAA-CAGATGAGGTTCAC-3′ and antisense 5′-GCCTCTGA-CAGTTTATGTAATC-3′. A 497-bp segment of the *CD14* promoter from −517 to –19 was amplified, and the C and T alleles were distinguished by digestion with *Ava*II. The *IL-13* (R110Q) SNP was genotyped as previously described.[18]

Statistical Analyses

Hardy-Weinberg equilibrium was tested for each examined SNP in DA-positive and DA-negative groups, respectively, by using χ^2 statistics for contingency tables. The program PHASE v.2.1 was used to reconstruct the haplotypes of the SNPs in *IL4RA*.[32] The Fisher exact test was used to test for differences in SNP genotype, allele, and haplotype frequencies between the DA-positive and DA-negative groups. Logistic regressions with the outcome of diisocyanate asthma were performed to examine the main effects of the genes and hexamethylene diisocyanate (HDI) exposure, respectively, and the gene-HDI exposure interaction. A significant *P* value (<.05) for the interaction term determined by the logistic regression indicates that there is gene-environment interaction underlying diisocyanate asthma. The SE for the proportion of participants who carried the particular genotype (or the genotype combination) was obtained by $\sqrt{p(1-p)/n}$, where *p* represents the observed proportion, and *n* represents the sample size. All analyses were conducted using SAS version 9.1 statistical software (SAS Institute Inc, Cary, NC).

RESULTS

Characteristics of the Diisocyanate-Exposed Population

Table 1 summarizes characteristics of 137 individuals, 62 with positive SIC results (DA positive) and 75 diisocyanate-exposed workers with negative SIC results (DA negative) (37% early responses, 43% late responses, and 20% dual asthmatic responses). All study participants were white and primarily of French Canadian descent. The frequency of atopy in DA-positive and DA-negative groups was equivalent (39%). The geometric mean methacholine provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20% (PC_{20}) was 1.2 mg/mL in DA-positive vs 4.7 mg/mL in DA-negative workers. Positive methacholine test results (PC_{20}, <8 mg/mL) were detected in 51 (85%) of 60 DA-positive workers and 35 (47%) of 74 DA-negative workers. The duration of work exposure that preceded the occupational evaluation was greater in the DA-negative worker group (169.5 vs 151.7 months; *P* = .45). There was a

Table 1. Characteristics of DA-Positive (SIC-Positive) and DA-Negative (SIC-Negative) Workers

SIC results	N	Sex, M/F	Atopy, %*	Specific diisocyanate exposure, %	Geometric mean PC_{20}, mg/mL	Mean time of exposure before evaluation, mo
Positive	62	54/8	39	55 HDI; 18 MDI; 27 TDI	1.2	151.7‡
Negative	75	69/6	39	83 HDI; 12 MDI; 5 TDI	4.7	169.5

Abbreviations: DA, diisocyanate asthma; HDI, hexamethylene diisocyanate; MDI, methylene-diphenyl diisocyanate; PC_{20}, provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20%; SIC, specific inhalation challenge; TDI, toluene diisocyanate.
* Atopy is defined as skin prick test positive to 2 or more aeroallergens.
‡ $P = .50$ (Mann-Whitney *U* test).

higher frequency of smokers in the DA-negative group (31% vs 10% in the DA-positive group; $P = .01$). Mean age was also higher in the DA-positive group (47.7 vs 38.8 years; $P < .001$).

Frequencies of Alleles and Genotypes in Workers Evaluated for DA

We examined alleles and genotype frequencies of *IL4RA, IL-13,* and *CD14* SNPs in DA-positive (n = 62) and DA-negative workers (n = 75). Four known *IL4RA* variants were evaluated, including I50V, E375A, C406R, and Q551R. In addition, an atopy-associated SNP in *IL-13* (R110Q) and an atopy-associated SNP in the *CD14* promoter region (C159T) were evaluated. Except for the *IL-13* (R110Q) SNP in DA-negative workers ($P = .04$), other SNPs were in Hardy-Weinberg equilibrium in each group. Genotype and allele frequency differences for each SNP between the 2 groups are given in Table 2. No association was observed with individual alleles and DA.

Table 2. Number of Workers Who Expressed Individual Genotypes and Alleles and Their Frequencies Found Among DA-Positive and DA-Negative Groups

SNP	Genotype/allele	DA positive, No. (%)	DA negative, No. (%)	*P* value*
I50V	II	24 (39)	19 (25)	.25
	IV	29 (47)	43 (57)	
	VV	9 (15)	13 (17)	
	I	77 (62)	81 (54)	.22
	V	47 (38)	69 (46)	
E375A	EE	49 (79)	60 (80)	.35
	EA	11 (18)	15 (20)	
	AA	2 (3)	0 (0)	
	E	109 (88)	135 (90)	.70
	A	15 (12)	15 (10)	
C406R	CC	53 (85)	63 (85)	.62
	CR	8 (13)	11 (15)	
	RR	1 (2)	0 (0)	
	C	114 (92)	137 (93)	>.99
	R	10 (8)	11 (7)	
Q551R	QQ	40 (66)	43 (57)	.22
	QR	17 (28)	30 (40)	
	RR	4 (7)	2 (3)	
	Q	97 (80)	116 (77)	.77
	R	25 (20)	34 (23)	
IL13 (R110Q)	RR	32 (52)	48 (64)	.18
	RQ	26 (42)	20 (27)	
	QQ	4 (6)	7 (9)	
	R	90 (73)	116 (77)	.40
	Q	34 (27)	34 (23)	
CD14 (C159T)	CC	17 (27)	15 (20)	.60
	CT	33 (53)	45 (60)	
	TT	12 (19)	15 (20)	
	C	67 (54)	75 (50)	.55
	T	57 (46)	75 (50)	

Abbreviations: DA, diisocyanate asthma; SNP, single nucleotide polymorphism.
* *P* value was determined by the Fisher exact test.

Specific genotypes and genotype combinations of all SNPs were evaluated in the entire study population of workers exposed to any diisocyanate chemical (TDI, methylene-diphenyl diisocyanate [MDI], or HDI) by univariate analysis. No significant associations were detected between any genotype or combination of genotypes and DA among all isocyanate-exposed workers (data not shown).

Most DA-positive workers were exposed to HDI. Therefore, gene-environment interaction between genotypes or genotype combinations and DA status were examined by logistic regression in HDI workers. When the main effect of HDI exposure was considered, a significant association was found between genotype *IL4RA* (I50V) II only and the DA outcome (Table 3). The interaction variate of HDI exposure and genotype was modeled by logistic regression. Genotype *IL4RA* (I50V) II and genotype combinations *IL4RA* (I50V) II and *IL-13* (R110Q) RR, *IL4RA* (I50V) II and *CD14* (C159T) CT, and *IL4RA* (I50V) II and *IL-13* (R110Q) RR-*CD14* (C159T) CT were significantly associated with DA.

As shown in Figure 1 (panel A), when genotypes were evaluated in HDI-exposed workers, DA was associated with genotype *IL4RA* (I50V) II in 48% of the workers vs 22% in the DA-negative group (odds ratio [OR], 3.29; 95% confidence interval [CI], 1.33–8.14; P = .01). In HDI-exposed workers, the genotype combination (I50V) II and *IL-13* (R110Q) RR was present in 30% of the DA-positive workers vs 10% of DA-negative workers (OR, 4.1; 95% CI, 1.35–12.68; P = .01) (Fig 1, panel B). For the genotype combination of (I50V) II and *CD14* (C159T) CT, the frequency among DA-positive workers exposed to HDI was 39% vs 11% among the DA-negative workers (OR, 5.2; 95% CI, 1.82–14.88; P = .002) (Fig 1, panel C). The OR for the triple genotype combination (I50V) II, *IL-13* (R110Q) RR, and *CD14* (C159T) CT in the HDI-exposed cohort was 6.4 (95% CI, 1.57–26.12; P = .01) (Fig 1, panel D), and the frequency of this combination among the DA-positive workers was 24% vs 5% among the DA-negative group. A complete haplotype analysis of *IL4RA* did not yield additional DA-associated haplotypes (data not shown).

There were no significant associations among atopy, methacholine PC_{20}, *IL4RA, IL13,* and *CD14* SNP alleles alone or in combination. Serum samples were not available in all workers, precluding evaluation of diisocyanate-specific antibodies.

DISCUSSION

OA accounts for 9% to 15% of asthma in adults,[33] and isocyanates are among the most frequently identified causes. Although genetic susceptibility markers, including HLA class II and glutathione-s-transferase alleles, have previously been associated with DA,[10,13] it is likely that DA represents a complex disease phenotype determined by multiple genes. In this study, the (I50V) II genotype alone and in combination with *IL-13* and *CD14* promoter genotypes were significantly associated with DA in HDI-exposed workers.

The worker population in this study was uniquely homogeneous with respect to ethnicity in that nearly all were of French Canadian descent and resided in the Province of Quebec. Thus, unknown effects of different background allele frequencies from other populations were less likely to confound our data.[34] In addition, a clear strength of this study was rigorous definition of both DA-positive and DA-negative phenotypes accomplished by using SIC testing, an approach that is superior to subjective tools such as occupational questionnaires.[30,35,36] It is likely that this approach reduced misclassification of disease phenotypes, which is a concern in genetic case-control association studies.[34] We recognize that a clear limitation of this study is the relatively small sample sizes of our affected and comparator groups. However, the scarcity of workers with well-defined DA precluded our ability to recruit larger study cohorts from the current population, much less from a second background population. On the other hand, these findings are strengthened by our ability to recruit optimally phenotyped individuals with a rare condition that come from a relatively homogeneous French Canadian background population.

The T_H2 cytokines, IL-4 and IL-13, play key roles in B-cell IgE isotype class switching and are believed to at least partially determine expression of airway inflammation and allergic diseases.[37] Therefore, many investigators have evaluated associations between *IL-13* and the *IL4RA* gene polymorphisms (ie, SNPs) and atopy and asthma phenotypes.[18–20,38–42] We have reported that the *IL4RA* Q551R variant is significantly associated with atopy[40] and severe asthma.[43] However, few if any studies have examined relationships of the latter genes with expression of other asthma phenotypes, including DA. In HDI-exposed workers, we found that DA was associated with the *IL4RA* (I50V) II

Table 3. *P* Values for 2-Way Gene-Environment Interaction and the DA-Positive Outcome in a Logistic Regression Model

Genotype or genotype combination	Main effect for genotype or genotype combination	Main effect for HDI exposure	Genotype or genotype combination and HDI exposure interaction
IL4RA (I50V) II	.51	.004	.03
IL4RA (I50V) II and *IL13* (R110Q) RR	.51	.10	.04
IL4RA (I50V) II and *CD14* (C159T) CT	.86	.23	.001
IL4RA (I50V) II, *IL13* (R110Q) RR, and *CD14* (C159T) CT	.77	.63	.006

Abbreviations: DA, diisocyanate asthma; HDI, hexamethylene diisocyanate.

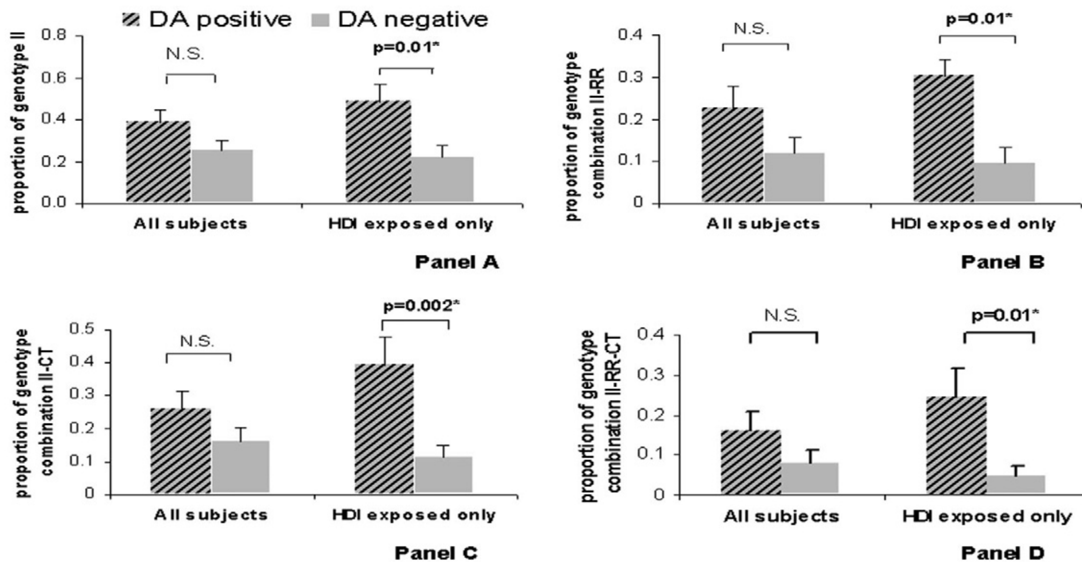

Figure 1. Proportion (±SE) of genotype-genotype combination in all study participants and in hexamethylene diisocyanate (HDI)–exposed worker group stratified by diisocyanate asthma (DA) status. Significant differences in genotype frequencies between DA-positive and DA-negative groups were observed only in the HDI-exposed group for *IL4RA* (I50V) II (A; odds ratio [OR], 3.29; 95% confidence interval [CI], 1.33–8.14; $P = .01$); *IL4RA* (I50V) II and *IL13* (R130Q) RR (B; OR, 4.13; 95% CI, 1.35–12.68, $P = .01$); *IL4RA* (I50V) II and *CD14* (C159T) CT (C; OR, 5.2; 95% CI, 1.82–14.88; $P = .002$); and *IL4RA* (I50V) II, *IL13* (R130Q) RR, and *CD14* (C159T) CT (OR, 6.4; 95% CI, 1.57–26.12; $P = .01$). N.S. indicates nonsignificant.

genotype and that gene-gene interactions associated with DA included *IL4RA* (I50V) II and *IL-13* (R110Q) RR genotypes; the *IL4RA* (I50V) II and *IL-13* (R110Q) RR combination; the *IL4RA* (I50V) II and *CD14* (C159T) CT combination; and the triple combination of *IL4RA* (I50V) II, *IL-13* (R110Q) RR, and *CD14* (C159T) CT. Because these associations were significant among HDI-exposed workers, our data suggest gene-environment interactions linked to the HDI-asthma phenotype. It must be emphasized that similar analyses of genotype-disease associations with TDI and MDI exposure were not possible because of inadequate cohort sizes of workers exposed to the latter agents.

It is uncertain why these gene associations were found exclusively among HDI-exposed workers (as opposed to workers exposed to any diisocyanate chemical). In support of this hypothesis, Herrick et al[21] demonstrated that induction of airways inflammation after epicutaneous sensitization of mice with HDI was genetically determined, because only 1 (BALB/c) of 6 mouse strains exhibited optimal specific antibody and airway eosinophilic responses. Wisnewski et al and other investigators[44–46] have reported that HDI-conjugated antigens induce proliferation of T cells isolated from HDI-exposed workers and uniquely elicit expression of oligoclonal γ/δ vs common α/β T-cell receptors observed in response to allergens. The HDI-stimulated γ/δ T cells produced interferon-γ but not IL-5 or IL-13. It is unknown whether similar specific immune responses would occur in response to MDI or TDI antigens. However, it is possible, although unproven, that unique genotypes could determine in vivo specific immune responses in HDI workers, whereas other genes could influence immune responses in TDI- or MDI-exposed workers.

In chronic DA, the role of IL-4 may be less important than for other asthma phenotypes. $CD8^+$ T-cell clones isolated from bronchial biopsy tissue of DA-positive patients after TDI inhalation produced abundant IL-5 but little IL-4.[47] Although IL-4 and IL-5 are expressed in bronchial tissue at 48 hours after isocyanate inhalation challenge, only IL-5 persists in patients with chronic TDI asthma after removal from the workplace.[25] Cells that express IL-4 were not detected in another bronchial biopsy study conducted in 5 workers with DA at 24 hours after SIC.[26] Our data show that HDI asthma is associated with the I (I50V) allele of *IL4RA*; this wild-type allele is functionally associated with less IL-4 cell signaling capacity than the more active V (I50V) variant, which enhances cellular expression of CD23.[31] Similarly, DA was also associated with the *IL-13* (R110Q) RR and *CD14* (C159) CT genotypes when combined with the *IL4RA* (I50V) II genotype in the subset of workers exposed to HDI. These gene-exposure associations found by univariate analysis were confirmed by multivariate analysis (Table 4). Studies that have examined the functional relevance of the *IL-13* R110Q polymorphism have demonstrated that products of the Q variant allele are more active than the wild-type allele in stimulating *IL4RA*-dependent STAT6 phosphorylation and up-regulation of CD23 molecules.[48,49] Another study suggested that the V variant but not the I allele of the I50V *IL4RA* SNP contributes to IL-4–dependent CD23 expression.[31] Considering the putative functional roles of these variant alleles, our genetic data, which identified associations with wild-type genotypic

markers, suggest that the pivotal pathogenetic roles attributed to IL-4 and IL-13 in allergic asthma may be different and less robust than in DA.

The CT genotype (C159T) combined with *IL4RA* (I50V) II and *IL-13* (R110Q) RR genotypes was associated with HDI asthma. Koppelman et al[50] reported that the CC genotype (but not CT or TT) was associated with elevated total IgE and atopy in an adult population. The significance of the finding in our study of an association of DA with the combinations of CT and the *IL4R* and *IL-13* genotypes is uncertain.

In conclusion, our data clearly demonstrate that combinations of *IL4RA, IL-13,* and *CD14* polymorphisms may represent genetic susceptibility markers of HDI-induced asthma. The present study was conducted in a relatively small population compared with the size often used in genetic association studies. However, it is a unique group in that extensive clinical data enabled precise definition of the DA phenotype. Almost all other reported association studies of asthma (eg, allergic asthma) have inferred phenotypes based on subjective or limited clinical information (eg, questionnaires or lung function) and, in fact, may represent heterogeneous disorders that could result in type I errors.[51] Expanded studies in different background populations are necessary to address the clinical utility of these and other genetic markers that have been associated with diisocyanate asthma.

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