Polymorphisms in the novel gene acyloxyacyl hydroxylase (AOAH) are associated with asthma and associated phenotypes

Kathleen C. Barnes, PhD, Audrey Grant, MS, Peisong Gao, MD, PhD, Daniela Baltadjieva, MD, PhD, Tiina Berg, BS, Peter Chi, MHS, Shu Zhang, MA, April Zambelli-Weiner, PhD, Eva Ehrlich, MS, Omeed Zardkoohi, MD, Mary E. Brummet, MS, Maria Stockton, MA, Tonya Watkins, BS, Li Gao, MD, PhD, Marquita Gittens, PhD, Marsha Wills-Karp, PhD, Christopher Chadee, PhD, Lisa A. Beck, MD, Terri H. Beatty, PhD, Kevin G. Becker, PhD, Joe G. N. Garcia, MD, and Rasika A. Mathias, ScD

Background: The gene encoding acyloxyacyl hydroxylase (AOAH), an enzyme that hydrolyzes secondary fatty acyl chains of LPS, is localized on chromosome 7p14-p12, where evidence for linkage to total IgE (tIgE) concentrations and asthma has been previously reported.

Objective: We hypothesized that variants in AOAH are associated with asthma and related phenotypes. Because both AOAH and soluble CD14 respond to LPS, we tested for gene-gene interaction.

Methods: We investigated the association between 28 single nucleotide polymorphisms throughout the AOAH gene and asthma, concentrations of tIgE, the ratio of IL-13/IFN-γ, and soluble CD14 levels among 125 African Caribbean, multiplex asthmatic pedigrees (n = 834). Real-time PCR was used to assess whether AOAH cDNA expression differed with AOAH genotype.

Results: Significant effects were observed for all 4 phenotypes and AOAH markers in 3 distinct regions (promoter, introns 1-6, and the intron 12/exon 13 boundary/intron 13 region) by means of single-marker and haplotype analyses, with the strongest evidence for a 2-single-nucleotide-polymorphism haplotype and log[tIgE] (P = .006). There was no difference in AOAH expression levels by AOAH genotype for any of the markers.

Conclusion: Our results indicate that polymorphisms in markers within the AOAH gene are associated with risk of asthma and associated quantitative traits (IgE and cytokine levels) among asthmatic subjects and their families in Barbados, and there is an interactive effect on tIgE and asthma concentrations between an AOAH marker and the functional CD14 (−260)C>T polymorphism.

Clinical implications: AOAH is a novel innate immunity candidate gene associated with asthma and related phenotypes in an African ancestry population. (J Allergy Clin Immunol 2006;118:70-7.)

Key words: CD14, acyloxyacyl hydroxylase, association, asthma, total IgE, soluble CD14, family-based association test

The interface between innate (natural) immunity and the more recently evolved acquired (adaptive) immune response might be critical in the development and manifestation of several allergic diseases. Chronic exposure to domestic endotoxin (soluble fragments of bacterial LPS) appears to influence the risk for asthma. Several innate immunity receptors that bind endotoxin have been proposed as candidate genes for allergic airway disease. For example, CD14 is a cell-surface antigen preferentially expressed on mature monocyte cells, which initiate antimicrobial host defense responses characterized by release of TLR1-type inflammatory cytokines (eg, TNF-α, IL-1, IL-6, and INF-γ) and subsequently send inhibitory signals to T<sub>H</sub>2 lymphocytes (eg, IL-4 and IL-13). Baldini et al demonstrated that the −159 (subsequently designated −260 [rs2569190])C>T allele of the CD14 gene was significantly associated with lower serum total IgE (tIgE) levels, a finding replicated by several groups. Significant associations have also been observed between this...
AOAH is an effective LPS antagonist.20,21 Recently, it was demonstrated that AOAH also appears to modulate B-cell proliferation for asthma and its associated traits.

Another interesting candidate, but one for which relatively little is known, is acyloxyacyl hydroxylase (AOAH), a highly conserved,15 eukaryotic lipase that releases secondary acyl chains from the LPS found on cell walls of gram-negative bacteria.16-18 A unique leukocyte enzyme, AOAH is potentially an important host defense molecule because deacylated LPS is at least 100-fold less potent than intact LPS.19 In vitro assays show deacylated LPS is an effective LPS antagonist.20,21 Recently, it was demonstrated that AOAH also appears to modulate B-cell proliferation and polyclonal antibody production in response to gram-negative bacterial infection.22

AOAH is located on chromosome 7p14-p12,23 a region where evidence for linkage to asthma, bronchial hyperreactivity, and tIgE concentrations has been reported.24-26 Collectively, these observations suggest that AOAH is a candidate gene in the LPS signaling pathway for tIgE levels in particular and possibly for other allergic diseases, including asthma.

METHODS

Study subjects

Nuclear and extended asthmatic families were recruited in Barbados as a part of an ongoing asthma genetics study (described elsewhere72,73). Patients with a positive family history of asthma (≥1 asthmatic siblings) were referred by physicians cooperating with coinvestigators at the University of the West Indies. Blood samples were collected from all subjects for serum extraction of DNA for genotyping, and total RNA was collected from a subset (n = 30) for expression experiments.

Asthma and tIgE concentrations were measured, as described previously.27,28 IgE values were log transformed to minimize skewness and then adjusted for age and sex. Soluble CD14 levels were measured with a commercially available ELISA kit supplied by Biosource (Europe S.A.), and concentration was estimated to be 1 ng/mL and defined as the sCD14 concentration corresponding to the average OD of 20 replicates of the zero standard +2 SD. Serum IL-13 and IFN-γ levels were measured by means of ELISA with specific anti-IL-13 and anti-IFN-γ mAbs obtained from BD Biosciences (San Diego, Calif), according to the manufacturer’s instructions. Serum IL-13 and IFN-γ levels were log transformed to approximate a normal distribution. Family-based association tests (FBATs) on the qualitative phenotype asthma and the quantitative traits were performed on 834 persons from 125 pedigrees (both nuclear and extended).

All subjects provided verbal and written consent, as approved by the Johns Hopkins Bayview Hospital Institutional Review Board and the Ministry of Health in Barbados.

Genotyping

A total of 29 single nucleotide polymorphisms (SNPs) from the gene encoding AOAH (accession no. NT_007819.13) were genotyped in these data. Of these, 26 were validated SNPs selected from Applied Biosystems (Foster City, Calif) by using SNPBrowser software 3.0 (www.allsnps.com/snpbrowser), a freely available Applied Biosystems tool. Of these, one nonsynonymous variant (Gly/Ala) was not polymorphic in this African Caribbean population. Two additional SNPs that were part of an initial pilot study on AOAH were also included. The final panel comprised 28 SNPs, all of which were intronic, with the exception of four 5′ UTR SNPs; this, however, reflects the lack of reported SNPs in exons in this gene (~1% exonic).

DNA was extracted by using standard protocols. When necessary, whole-genome amplification of DNA was prepared by OmniPlex Technology (Rubicon Genomics Inc, Ann Arbor, Mich). Genetic screening for the CD14(−260)C>T polymorphism is one of the most reproducible associations for asthma and its associated traits.


genes in treated samples

average fold change

average fold change

5′ nucleic acid assay (TaqMan probes). Probes and primers were designed and synthesized by Applied Biosystems. Relative gene expressions were calculated by using the 2−ΔΔCt method, in which ΔΔCt indicates cycle threshold, the fractional cycle number at which the fluorescence signal reaches a detection threshold.30 The normalized ΔCt value of each sample was calculated by using a total of 3 endogenous human control genes (GAPDH, ACTB, and PGK1). Fold change values are presented as average fold change = 2−ΔΔCt for genes in treated samples relative to control samples. Error bars represent the SEM for multiple biologic replicates (10 for each group).

Statistical analyses

Clinical characteristics of the study population, including mean age, proportion by sex, proportion of asthmatic subjects, and mean values with SDs for serum measures and quantitative traits considered (log(tIgE), sIL-13, sIFN-γ, the IL-13/IFN-γ ratio, and sCD14), were calculated for all subjects with available data who were genotyped for AOAH, for founder members of the population, and for asthmatic and nonasthmatic subjects by using STATA 8.2 (StataCorp, College Station, Tex). Equality of means was tested between asthmatic and nonasthmatic subjects for serum measures and quantitative traits by

Mechanisms of asthma and allergic inflammation
using linear regression, and proportions by $CD14(-260)$ genotype were also calculated for these subsets of the study population.

Gene-gene interaction was investigated by using logistic regression models for asthma with interaction terms according to genetic transmission model, applying the robust "sandwich" estimator of variance in STATA 8.2. Both regression coefficients and within-family correlation were estimated by using generalized estimating equations in regression models for quantitative traits.

Mendelian inconsistencies were identified with Sib-Pair (version 99.9). Departures from Hardy-Weinberg equilibrium proportions at each marker were tested among founders. Pairwise linkage disequilibrium (LD) was measured by HaploView (http://www-genome.wi.mit.edu/personal/jcbarret/haplo). Association between markers in $CD14$ and AOAH and asthma, log[lgE], sCD14, log[IL-13]/[IFN-γ], and haplotype associations were tested by using the FBAT (version 1.5.5). For haplotype analyses, an expectation-maximization (EM) algorithm that maximizes the likelihood of phased haplotype frequencies based on all observed genotypes in the nuclear family was used. All analyses with FBAT were first conducted under a general genotype model and subsequently under an additive or recessive model, as suggested by the general model.

Multiple comparisons resulting from sliding-window haplotype tests is of concern, and the significance of haplotype tests was evaluated by using permutations to deal with this issue. Specifically, empirical $P$ values are calculated through permutation analysis by using Monte Carlo methods under the null hypothesis of complete independence between haplotypes and the observed phenotypes. The FBAT sampling procedure stops when at least 100 Monte Carlo-based test statistic values are generated, whichever comes first.

**RESULTS**

Clinical characteristics of subjects and distribution of $CD14(-260)C>T$ genotypes are presented in Table E1 (available in the Online Repository at www.jacionline.org). We previously reported a significant association between the TT genotype at this $CD14$ marker and lower asthma severity scores ($P = .002$) in these families from Barbados. The frequency of CC homozygotes among African Caribbean families (46%) is nearly twice that reported in non-African populations, and the frequency of TT homozygotes (8%) is substantially lower.

Distributions of tIgE, serum IL-13, serum IFN-γ, and sCD14 concentrations were similar to those described before. Age and sex showed no effect on log[lgE] and sCD14, and sCD14 concentrations were highly correlated with each other ($r = 0.6037$, $P < .0001$). When IL-13 and IFN-γ levels were analyzed as the log[IL-13]/log[IFN-γ] ratio, there was no difference in means by asthma status, and values were not correlated with log[lgE] levels.

**Single-marker analyses**

The 28 AOAH SNPs spanning 313,068 bp are summarized in Table E2 (available in the Online Repository at www.jacionline.org). All 28 SNPs were in Hardy-Weinberg equilibrium among founders (n = 269). Fig 1 shows overall there was low LD in this gene, reflecting its large size and an average of 11.6 kb between neighboring SNPs. However, we did observe high LD for 13 of 27 pairings of contiguous SNPs (D’ ranging from 0.84-1.0). This was reflected in three 2-SNP blocks, according to the definition of Gabriel et al., comprised of markers rs3935953 and rs10275462 (block 1), rs4720210 and rs11770435 (block 2), and rs2727833 and rs2727831 (block 3), with a D’ of 0.96, 1.0, and 0.94, respectively ($r^2 = 0.69$, 0.25, and 0.37, respectively).

All single SNP analyses were first performed by using the robust genotype model (data not shown), and the strongest association signals suggested a recessive model was most likely; therefore all results are presented using a recessive model. All results of the 2-point tests for linkage and association between 28 AOAH markers and asthma and log[lgE], log[IL-13]/log[IFN-γ], and sCD14 concentrations with FBAT statistics are presented in Table E3 (available at www.jacionline.org).

Eight SNPs provide evidence for significant association across the 4 phenotypes, 5 of which showed evidence for association with more than one phenotype. The most compelling associations were for the 2 markers in LD block 3 in intron 12: (1) rs2727833, with a strong negative association (ie, minor allele associated with lower levels) for log[lgE] and log[IL-13]/log[IFN-γ] ($P = .008$ and $P = .008$, respectively, and (2) rs2727831, with a similar negative association for log[lgE], log[IL-13]/log[IFN-γ], and sCD14, and log[IL-13]/log[IFN-γ] ($P = .006$, $P = .041$, and $P = .023$, respectively). Additional regions of the AOAH gene highlighted by single-marker analyses included (1) the promoter region (rs1592497, $P = .032$ for sCD14), (2) introns 3 and 4 (rs11978395, $P = .014$ for sCD14; rs4720210, $P = .022$ for log[lgE] and $P = .023$ for asthma; rs11979472, $P = .012$ for log[IL-13]/log[IFN-γ], and (3) introns 18 and 19 at the 3’ end of the gene (rs1986468, $P = .027$ for log[lgE] and $P = .002$ for log[IL-13]/log[IFN-γ]; rs3801297, $P = .017$ for asthma and $P = .010$ for log[IL-13]/log[IFN-γ]).

**Haplotype analyses**

Restricting haplotype tests within observed LD blocks would have disregarded most of the data, and therefore a systematic sliding window approach was implemented, considering windows of 2 to 4 SNPs per window beginning with the first (5’) marker and working across the gene one marker at a time. Haplotype tests revealed multiple association signals in 3 specific regions (defined as signals of association across contiguous SNPs with little to no LD between signals) that overlapped with the single-SNP results described above.

Significant evidence for linkage and association was observed for all 4 phenotypes in the promoter region.
The strongest signals for each phenotype were as follows: 4-, 3-, and 2-SNP haplotypes, all beginning with marker rs2006882, for asthma \( (P = 0.016) \), log\[IgE\] \( (P = 0.002) \), and log\[IL-13\]/log\[IFN-\(\gamma\)] \( (P = 0.045) \), respectively. The only association in this region for sCD14 occurred with the marker rs1592497 \( (P = 0.032) \), as described above.

Significant associations for all 4 phenotypes were observed in the second region (rs10275462-rs2727822). Associations for asthma, log\[IgE\], and log\[IL-13\]/log\[IFN-\(\gamma\)] were centered between markers rs472010 to rs11979472; 3- and 4-SNP windows provided the strongest signals for these 3 traits \( (P = 0.020, P = 0.029, \text{ and } P = 0.044, \text{ respectively}) \). The 4-SNP window providing the strongest signal for sCD14 \( (P = 0.014) \) was 5’ to this marker but overlapped with signals for the other 3 phenotypes.

The third and most compelling region was the 3’ end of AOAH and included marker rs2727831. All 4 phenotypes demonstrated significant associations in this region flanked by markers rs2727833 and rs10951482. The most consistent signal was for log\[IgE\], with 7 of 15 haplotype tests \( (P < 0.05) \), including the 2-SNP haplotype of rs2727833 and rs2727831 \( (P = 0.006) \). Modest associations were observed for asthma, log\[IL-13\]/log\[IFN-\(\gamma\)]], and sCD14 \( (P = 0.022, P = 0.012, \text{ and } P = 0.038, \text{ respectively}) \).

Analyses performed using the additive model (data not shown) supported these results from a recessive model without revealing any additional signals.

### AOOAH expression according to AOOAH genotype

RNA samples from PBMCs were collected from 30 individuals stratified by the marker providing the most compelling evidence for association (rs2727831) to determine whether the C>T variant affected transcriptional levels of AOOAH. Subjects were also stratified according to 4 other AOOAH markers showing significant associations to more than one phenotype (rs4720210, rs2727833, rs1986468, and rs3801297). Comparison of cDNA expression of AOOAH according to the 3 genotypes at each AOOAH marker was performed for each of the 5 markers individually. There was no difference in AOOAH expression levels by genotype at any of the AOOAH markers tested.

### Evidence for gene-gene interaction between CD14(-260)C>T and AOOAH marker rs2727831

We evaluated evidence for gene-gene interaction between AOOAH marker rs2727831 and the CD14(C-260) C>T polymorphism because both variants were associated with lower IgE concentrations in this Barbados population. On the basis of the above results, we used a recessive model for both SNPs and accounted for dependence within families. The CC and CT genotypes were combined into a comparison group for both polymorphisms (ie, subjects with CC/CT at AOOAH rs2727831 and CC/CT at CD14(-260)C>T were the reference
Fig 3 depicts odds ratios (ORs) and P values for asthma, contrasting various genotypic groups with this group, and suggests that the presence of the TT genotype at either loci (but not both) reduced the risk for asthma (ORs of <1.0) and conferred lower IgE levels (data not shown) compared with those in the reference group, but the presence of the TT genotype at both loci increased risk (ORs >1), suggesting the presence of a qualitative interaction. When modeling interactions, the interaction coefficient was significant for both asthma (OR for interaction = 5.28, P = .036) and IgE (P = .006). When sCD14 or log[IL-13]/log[IFN-γ] was examined, there was no significant evidence of interaction.

**DISCUSSION**

This is the first report of association between variants in AOAH and human allergic disease. We have presented evidence for linkage and association among 8 markers in AOAH and the phenotypes of asthma, logIgE, sCD14, and log[IL-13]/log[IFN-γ]. The most compelling signal was for 2 SNPs flanking exon 13 and low IgE concentrations.

The precise relationship between AOAH and the Th2/Th1 lymphocyte-cytokine milieu in the context of asthma and allergic disease remains unclear. The first evidence for in vivo function for AOAH was recently presented by Lu et al, who showed that deacylation of LPS affected longer-term immune responses, specifically B-cell proliferation and polyclonal antibody production in response to gram-negative infection in mice. AOAH is an attractive candidate gene for asthma because it is localized on chromosome 7p14-p12, where evidence for linkage to asthma, bronchial hyperreactivity, and IgE concentrations has been reported from separate genome screens. Interestingly, linkage has also been observed at this locus for other inflammatory diseases, including multiple sclerosis, autoimmune thyroiditis, inflammatory bowel disease, and systemic lupus erythematosus. In unpublished gene-profiling studies to test for the effects of the CD14 (-260)C>T genotype on AOAH expression, we compared cDNA expression from PBMCs of asthmatic subjects stratified by genotype (CC, CT, or TT) at CD14 (-260). AOAH was consistently differentially expressed, and by using real-time RT-PCR, we validated these findings and determined that the expression of AOAH was significantly higher among asthmatic subjects with CD14 (-260) CC and CT genotypes compared with those with the TT genotype (1.46- to 1.48-fold increase, 1-sided P value = .045–.014; unpublished data). These findings suggested that AOAH might play an important role in the CD14–MD-2–TLR4 signaling pathway.
LPS recognition is essential for effective host defense, and the primary mechanism is through the CD14–MD-2–TLR4 signaling complex, which in turn initiates an inflammatory response by producing Th1 cytokines, chemokines, and other mediators. Soluble CD14 further enhances LPS-stimulated responses by mediating the response of cells lacking membrane-bound CD14. The agonistic effect of sCD14 on endothelial cells has been attributed to overactivation of nonspecific immune response to LPS, which underlies conditions such as septic shock. Alternatively, sCD14 is recognized as being important in neutralizing LPS and minimizing the damaging effects of endotoxin-induced cell activation.

Decaylation of LPS by AOAH diminishes stimulation of cells through this signaling complex, and in vitro murine studies in renal cortical tubule cells suggest that decaylation by AOAH limits inflammatory reactions to gram-negative bacteria. Both sCD14 and AOAH seem to function for a common end point: to deactivate LPS and regulate the inflammatory cascade associated with endotoxin-mediated host response.

Because the CD14(−260C>T) promoter substitution results in decreased affinity of Sp protein binding and enhanced transcriptional activity, there has been a great deal of focus on the effects of this variant on high sCD14 levels. Soluble CD14 has been shown to be correlated with development of recurrent wheezing in the first year of life and other markers of atopy, such as tIgE ratio) and several AOAH markers, suggesting that the AOAH gene plays a role in the Th2 milieu in addition to its effect on sCD14 levels. We speculate that AOAH activities are downstream of CD14 in the LPS signaling pathway, given that the conversion of LPS to deacylated LPS (resulting in approximately 0.2% to 1% as much activity) might take many hours or even days, whereas CD14 initiates a rapid response that normally begins within minutes of LPS exposure. Thus it is possible that abnormalities in AOAH expression might contribute to persistent inflammation in diseases such as asthma.

Taken together, these studies suggest an interaction between CD14 and AOAH. An evaluation of distributions of AOAH and CD14 genotypes among asthmatic subjects raises the possibility of gene-gene interaction or epistasis (Fig 3). These observed CD14 and AOAH markers might combine to control sCD14 levels, which in turn would affect the Th1/Th2 milieu (eg, tIgE and cytokine concentrations). Consequently, increased concentrations of sCD14 associated with CD14(−260)TT status could cause lower AOAH expression, either through reducing LPS-induced activation through competitive binding between sCD14 and membrane-bound CD14 or by enhancing the rate of LPS efflux (eg, transferring cell-bound LPS to plasma proteins).

In this family sample from Barbados, both the AOAH CT haplotype and the single marker rs2727831 variant influenced sCD14, suggesting that high sCD14 concentration is conferred, in part, by a wild-type AOAH allele. Alternatively, an epistatic mechanism for these 2 candidate genes might be through their effect on tIgE concentration. Homozygotes for the variant at both CD14(−260) and rs2727831 had lower tIgE concentrations than carriers of wild-type alleles. Alterations in AOAH might affect decaylation and subsequently result in sustained concentrations of circulating serum LPS, which in turn could modulate IgE regulation by favoring Th1 differentiation, suppressing Th2-dependent IgE responses, or both. Direct effects of sCD14 on tIgE concentrations have also been described in which sCD14 inhibits IgE production by interfering with CD40 signaling in B cells, increasing CD40 ligand expression on T cells, and inhibiting IL-2, IL-4, and IL-6. Our sample is unique in that very high levels of circulating sCD14 were observed among predominately asthmatic subjects (mean, 6710 ± 3243 ng/mL), but there was no inverse relationship between sCD14 and tIgE concentrations. It is possible that this sample lacked sufficient power to detect any association between this variant and sCD14 because of low frequency (8.2%).

Another explanation is environmental: we previously reported exceptionally high household concentrations of endotoxin in these Caribbean families enriched for allergic disease. Because increased sCD14 concentrations have been reported for in vitro models of chronic exposure to trace amounts of endotoxin, domestic exposure could account for the high sCD14 concentrations seen here. Similarly, AOAH activity can be induced after LPS exposure in murine dendritic cells and macrophages. Perhaps only subjects with either or both of these variants would have increased tIgE concentrations.

**FIG 3.** Interaction of CD14(C−260T) and AOAH rs2727831(C/T) genotypes for asthma. Bars indicate the ORs between the different combinations of genotypes for asthma. Under the recessive model, the CC and CT genotypes were collapsed, and the CC/CT group at both genes was used as the baseline comparison group.
In the current study we examined 28 SNPs throughout the AOAH gene, including 4 SNPs within approximately 5 kb of each other in intron 12, flanking exon 13. It is possible that one or more causal variants lie within or flank the TA/CT haplotype for markers rs2727833 and rs2727831 (P = .006), which showed the strongest association with serum IgE levels (Fig 2). AOAH is a highly polymorphic gene (211 kb), with large introns (n = 20; mean size, 10.5 kb), relatively small exons (n = 21; mean size, 107 bp) and very few SNPs in coding regions (5 synonymous and 5 non synonymous), raising the possibility of splicing variants playing a major role. These 28 AOAH markers averaged more than 11 kb apart, distances not typically found with strong LD. Conservative estimates suggest more than 150 haplotype-tagging AOAH SNPs would be necessary to fully cover AOAH in a population of African descent. Given the limited coverage of markers across AOAH, plus the fact that none of these variants are known to be functional, it is premature to draw conclusions about the functional relevance of the associations reported here. Analysis of AOAH expression from RNA in subjects of different AOAH genotypes did not support the hypothesis that these AOAH variants affected transcriptional levels of AOAH in peripheral blood, despite our previous unpublished studies showing significant differences in AOAH expression between TT and C genotypes at the functional CD14(−260) variant. After fine mapping of AOAH in this dataset and refinement of the linkage and association loci, appropriate future functional studies might be conducted.

In summary, CD14 and AOAH are 2 innate immunity candidate genes that might control risk to allergic diseases and quantitative traits associated with asthma in an interactive manner. Replication of these findings in other populations of African descent, as well as in non-African samples, will be needed to confirm these associations. With more extensive genotyping, causal variants might ultimately be identified.

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