

Linkage analysis of *Dermatophagoides pteronyssinus*-specific IgE responsiveness with polymorphic markers on chromosome 6p21 (*HLA-D* region) in Caucasian families by the transmission/disequilibrium test

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Background: Recently, we have obtained evidence for linkage between Der p 1-specific IgE antibodies and markers on chromosome 6p21 (*HLA-D* region) in a genome-wide screening in Caucasian families recruited as a part of the Collaborative Study on the Genetics of Asthma (CSGA).

Objective: Specific IgE antibodies toward different *Dermatophagoides pteronyssinus* (Der p) polypeptides were detected by immunoblotting analysis, and the transmission/disequilibrium test (TDT) was performed between specific IgE responsiveness toward each different Der p polypeptide and markers on chromosome 6p21 to better clarify the genetic contribution of *HLA-D* genes.

Methods: We studied 299 individuals in 45 Caucasian families participating in the CSGA. Serum samples from 137 individuals that showed elevated specific IgE antibodies toward the Der p crude allergen (> -0.5 log IU/mL) by ACCESS immunoassay were subjected to immunoblotting analysis. TDT was conducted between the presence of specific IgE antibodies toward each of 12 different Der p polypeptides and 4 polymorphic markers on chromosome 6p21.

Results: The 196-bp allele of *D6S1281* and the 104-bp allele of *DQCAR* showed significant excess transmission to specific IgE responders toward a particular Der p polypeptide (120 kd, 55

kd, 45 kd, or 37 kd). In contrast, the 200-bp allele of *D6S1281* and the 204-bp allele of *D6S291* showed significantly decreased transmission to specific IgE responders toward a particular Der p polypeptide (120 kd, 90 kd, 52 kd, or 45 kd). Deviation from the expected 50% transmission in heterozygous parents was statistically significant after correcting for multiple comparisons.

Conclusion: This study supported our previous findings that genes on chromosome 6p21 (*HLA-D* region) may influence the expression of Der p-specific IgE responsiveness in this Caucasian population. Our results, however, reveal the complexity of genetic regulations of Der p-specific IgE responsiveness by *HLA-D* genes, suggesting the strong influence of non-*HLA* loci and perhaps environmental factors for the development of Der p-specific IgE responsiveness. (J Allergy Clin Immunol 1998;102:443-8.)

Key words: *Dermatophagoides pteronyssinus*-specific IgE responsiveness, immunoblotting analysis, *HLA-D* genes, transmission/disequilibrium test

Genetic studies of specific IgE responsiveness to allergens have preferentially been performed on *HLA-D* genes. Over the past several years, numerous studies showed association between *HLA-D* genes and several allergens, including Amb a 5, Bet v 1, Lol p 1, and Ole e 1.¹⁻⁴ Although the degree of association with specific allergens tends to decrease as the molecular weight of the study allergen increases, some studies have demonstrated *HLA-D* restriction in antigen-specific T cells, including responses to complex allergens such as Der p 1 and Der p 2.^{5,6} Previous attempts to identify genetic contributions of *HLA-D* genes to specific IgE responsiveness toward the Der p allergens by means of linkage analysis or population-based studies^{7,8} were generally inconclusive, partly because high molecular weight allergens presumably produce much more peptides compatible with available *HLA-D* molecules than do low molecular weight allergens.

Using a genome-wide search, we have recently obtained evidence for linkage of Der p 1-specific IgE

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† Professor David G. Marsh was actively involved in the design and implementation of this study. He passed away on March 29, 1998, after a heroic battle against brain cancer. The authors wish to dedicate this paper to his memory.

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TABLE I. Summary of immunoblotting analysis in 137 Caucasian subjects with Der p-specific IgE antibodies ($\log[\text{Der p crude-specific}] > -0.5 \log \text{IU/mL}$)

	Size of peptides	No. of subjects with specific IgE response (%)*	No. of informative probands for TDT			
			D6S1281	D6S273	DQCAR	D6S291
1	160 kd†	65 (21.7)	34	44	47	39
2	140 kd	55 (18.4)				
3	120 kd†	73 (24.4)	43	54	58	47
4	115 kd†	64 (21.4)	34	51	54	41
5	95 kd†	64 (21.4)	29	42	44	37
6	90 kd†	88 (29.4)	46	61	64	55
7	76 kd	36 (12.0)				
8	64 kd	33 (11.0)				
9	60 kd	17 (5.69)				
10	58 kd	54 (18.1)				
11	55 kd†	80 (26.8)	34	54	56	45
12	52 kd†	88 (29.4)	41	57	62	53
13	45 kd†	81 (27.1)	43	58	61	52
14	40 kd	15 (5.0)				
15	37 kd†	106 (35.5)	51	75	76	63
16	32 kd†	77 (25.8)	36	51	55	49
17	30 kd†	123 (41.1)	54	83	88	72
18	28 kd	59 (19.7)				
19	25 kd	14 (4.7)				
20	22 kd	17 (5.7)				
21	18 kd	10 (3.3)				
22	16 kd	21 (7.0)				
23	14 kd†	105 (35.1)	50	75	79	64
24	6 kd	6 (2.0)				

*Numbers in parentheses indicate the percentage of subjects with specific IgE responses toward each Der p polypeptide among 299 white subjects in this study.

†These peptides had 20% or more responders in this population and were selected for TDT.

Abbreviations used

CSGA: Collaborative Study on the Genetics of Asthma

Der p: *Dermatophagoides pteronyssinus*

TDT: Transmission/disequilibrium test

responsiveness with markers on chromosome 6p21 (*HLA-D* region) in a Caucasian population recruited as part of the Collaborative Study on the Genetics of Asthma (CSGA) (N. Hizawa et al, manuscript submitted). Although there are, at present, 10 different *Dermatophagoides pteronyssinus* (Der p) allergens purified and characterized,⁹ immunoblotting or crossed-radio immunoelectrophoresis has identified many other IgE-binding components.¹⁰ Exposure to a broad array of different Der p polypeptides renders it difficult to define the genetic contribution of *HLA-D* genes to the expression of Der p-specific IgE responsiveness. In this study, to better clarify the genetic contribution of *HLA-D* regions, Der p-specific IgE responsiveness was evaluated by immunoblotting analysis and studied for genetic linkage analysis by means of the transmission/disequilibrium test (TDT). Although detailed information of amino acid sequence or protein structure of some Der p polypeptides is currently not available, immunoblotting analysis allowed us to identify the overall IgE responsiveness toward the Der p allergens, as well as the pres-

ence of specific IgE antibodies to a panel of the constituent polypeptides.

METHODS

Population

We studied 299 individuals (150 men and 149 women) in 45 Caucasian families (14 extended and 31 nuclear families) participating in the CSGA. The detailed study design and protocol for the CSGA have been described elsewhere.¹¹⁻¹³ Briefly, families were chosen at the Johns Hopkins University or Howard University through a pair of asthmatic sibs, and both probands and family were interviewed, underwent pulmonary function and skin prick testing, and provided blood for both genetic analysis and measurement of total IgE levels and specific IgE levels to Der p crude, Der p 1, and Der p 2 allergens. Asthma was diagnosed by the following criteria: (1) presence of at least 2 symptoms (cough, wheezing, or dyspnea); (2) either a fall in baseline FEV₁ of 20% or greater at 25 mg/mL or greater methacholine or a 15% or greater increase in baseline FEV₁ after bronchodilator use; (3) nonsmoking subject; (4) current age of 6 years or greater; and (5) no conflicting pulmonary disease.^{11,12} One hundred thirty-seven individuals who had specific IgE antibodies to Der p crude ($\log[\text{Der p crude-specific IgE}] > -0.5 \log \text{IU/mL}$) by ACCESS immunoassay¹³ were subjected to immunoblotting analysis in this study.

SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed according to a standard Laemmli protocol¹⁴ under reducing conditions. The purified crude extract of Der p was obtained from Greer Laboratories, Inc (Lot no. XPB70-D19-8.7). The Der p crude protein was separated by SDS-PAGE in

a 4% to 20% gradient gel in a Mini-Protein II cell (Bio-Rad) and transferred to a nitrocellulose membrane (0.45 micron, Bio-Rad). Each nitrocellulose strip was incubated for 1 hour with serum from a Der p responder (undiluted or diluted from 1:1 to 1:200). The degree of serum dilution was defined according to Der p crude-specific IgE antibody levels measured by the ACCESS immunoassay system as previously described (Beckman Instruments, Inc).¹³ Strips were then incubated with monoclonal anti-IgE antibodies (mouse IgM and IgG) for 1 hour. Binding of Der p-specific IgE antibodies was visualized by using an enhanced chemiluminescent detection system (Amersham) with Hyperfilm-ECL (Amersham). These visualized bands were scanned by a densitometer (UltraScan XL Enhanced Laser Densitometer, LKB/Pharmacia) to derive a relative quantification of specific IgE antibodies to these different Der p polypeptides. Molecular weight of each polypeptide was obtained on the basis of protein standards (Mark12, Novel Experimental Technology) by using the GelScan XL software program version 2.1 (LKB/Pharmacia).

Genotype

DNA was extracted from venous blood by standard techniques. Four polymorphic markers on chromosome 6p21 were studied. *D6S273*, *DQCAR*, and *D6S291* were genotyped as described previously.¹⁵ The *D6S1281* marker was genotyped at the Mammalian Genotyping Service (Marshfield, Wis) by using a fluorescent-based detection system (<http://www.marshmed.org/genetics>).

The map orders and genetic distances obtained for *D6S1281*-*HLAA-HLAE-D6S273*-*HLAC-HLAB-TNF-HLADR-HLADQ* (*DQCAR*)-*TAP1/2-HLADP-D6S291* are 2.2, 0.4, 0.1, 1.8, 0.2, 0.1, 0.5, 0.02, 0.2, 0.1, and 0.3 cM, respectively, according to the Genetic Location Database (http://cedar.genetics.soton.ac.uk/public_html/).

Statistical methods

The presence of specific IgE responses toward a particular Der p polypeptide was defined as the presence of a detectable band on immunoblots by densitometric analysis. Der p 2-specific IgE antibody levels measured by the ACCESS immunoassay system¹³ were compared with Der p 2-specific IgE antibody levels estimated by immunoblotting analysis with the Pearson correlation coefficient (SYSTAT, SYSTAT Inc). For this comparison, individuals with no detectable 14-kd bands as determined by immunoblotting were classified as having a minimum detectable value of Der p 2-specific IgE antibody levels. Relationship between the number of detectable bands on immunoblots and serum total IgE levels or asthma was also examined by using the Pearson correlation coefficient or the Mann-Whitney U test, respectively (SYSTAT, SYSTAT Inc).

TDT

Specific IgE responsiveness toward 12 of 24 different polypeptides detected by immunoblotting analysis was highly prevalent (>20%) in this Caucasian population (Table I). Among 137 responders toward Der p crude, 133 (97%) showed specific IgE antibodies to at least 1 of these 12 polypeptides. Therefore the phenotype of specific IgE responses toward each of these 12 different polypeptides was further studied by TDT with 4 polymorphic markers on chromosome 6p21. The number of times a certain allele is transmitted or not transmitted from a heterozygous parent to an affected offspring has an expected ratio of 1:1 under the null hypothesis of no linkage, and significant deviations from this indicate the presence of linkage between the marker and susceptibility locus.¹⁶ The TDT was evaluated by using the program SIB-PAIR.¹⁷ Linkage analysis was also performed for the phenotype of multiple responsiveness toward the Der p allergens (the presence of specific IgE

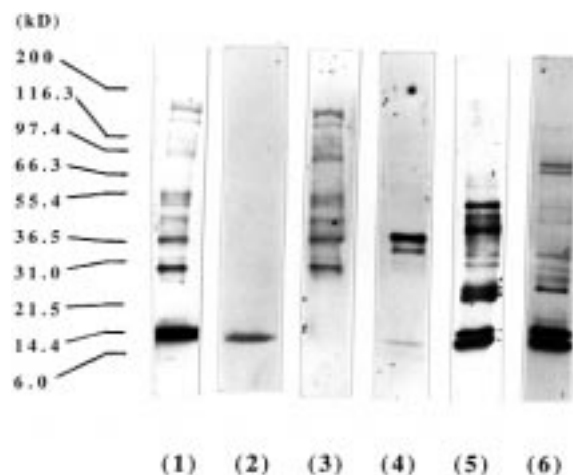


FIG 1. SDS-PAGE and immunoblotting analysis of purified Der p crude allergen. Representative IgE immunoblots of the Der p allergens. Individuals 1, 2, 3, 4, 5, and 6 (lanes 1, 2, 3, 4, 5 and 6) showed detectable IgE antibodies specific to 15, 1, 11, 3, 13, and 12 different polypeptides, respectively. All individuals shown here are unrelated. Individuals 1, 2, 5, and 6 responded to more than 5 Der p allergens (multiple Der p responsiveness).

responsiveness toward 5 or more of 24 Der p polypeptides) with 4 polymorphic markers on chromosome 6p21. Statistical significance was adjusted for multiple comparisons by using the Bonferroni correction.¹⁸

RESULTS

We identified a total of 24 Der p polypeptides of different sizes (range, 6 to 160 kd) in this Caucasian population (Table I and Figs 1 and 2) by means of immunoblotting analysis. Der p 2-specific IgE antibody levels measured by the ACCESS immunoassay system¹³ were strongly correlated with specific IgE antibody levels to the 14-kd polypeptide (Der p 2) as assessed by immunoblotting analysis, validating the accuracy of the immunoblotting approach (Pearson correlation coefficient = 0.93, $P < .0001$, $n = 137$).

Among 137 responders toward the Der p allergens, 107 individuals (78%) showed specific IgE responsiveness toward 5 or more of 24 Der p polypeptides. The number of detectable bands on immunoblots was significantly correlated with total serum IgE levels (Pearson correlation coefficient = 0.543, $P < .0001$, $n = 299$). Furthermore, 124 subjects with asthma responded to an average of 7.1 ± 6.5 (mean $\pm$ SD) of these 24 Der p polypeptides, and 175 subjects without asthma responded to an average of 2.8 ± 5.2 of these 24 Der p polypeptides (Mann-Whitney U test, $P < .0001$) in this population.

TDT

The 196-bp allele of *D6S1281* showed significant excess transmission to offspring with specific IgE responses toward the 120-kd polypeptide and to offspring with specific IgE responses toward the 37-kd polypeptide. The 104-bp allele of *DQCAR* showed significant excess transmission to offspring with IgE

TABLE II. TDT results for IgE responsiveness to 12 different Der p polypeptides

Marker	<i>D6S1281</i>				<i>DQCAR</i>		<i>D6S291</i>	
Allele	200 bp T/TN*	<i>P</i> value	196 bp T/TN*	<i>P</i> value	104 bp T/TN*	<i>P</i> value	204 bp T/TN*	<i>P</i> value
p160 kd	11/21	.077	17/8	.071	23/18	.43	3/9	.083
p120 kd	12/29	.0079	24/7	.0023†	28/15	.047	3/12	.02
p115 kd	12/19	.21	18/8	.049	35/16	.0078	4/10	.11
p95 kd	11/20	.1	20/8	.023	23/13	.095	3/10	.052
p90 kd	14/32	.0080	26/12	.023	34/18	.026	4/16	.0073†
p55 kd	8/18	.05	17/11	.25	35/14	.0027†	5/11	.13
p52 kd	14/29	.022	26/12	.023	30/21	.02	4/16	.0073†
p45 kd	11/29	.0044†	26/10	.0077	35/15	.0047†	5/16	.016
p37 kd	17/33	.023	30/12	.0055†	41/24	.035	5/16	.016
p32 kd	11/26	.013	19/8	.034	30/17	.057	4/14	.018
p30 kd	17/35	.012	30/15	.025	46/28	.036	5/17	.01
p14 kd	16/34	.01	25/14	.078	41/27	.089	4/15	.011
≥5 Der p polypeptides	18/34	.026	28/15	.047	41/25	.049	5/16	.056

Gene frequencies of 200-bp and 196-bp alleles (*D6S1281*), 104-bp allele (*DQCAR*), and 204-bp allele (*D6S291*) are 36.9%, 24.8%, 35.0%, and 5.6%, respectively. No alleles of marker *D6S273* showed deviated transmissions to specific IgE responders to any of the Der p polypeptides.

*Ratio of transmitted/nontransmitted alleles.

†Significance level reached 0.05 after Bonferroni correction for multiple comparisons.

responses toward the 55-kd polypeptide and to offspring with specific IgE responses toward the 45-kd polypeptide (Table II). In contrast, the 200-bp allele of *D6S1281* showed significantly decreased transmission to offspring with specific IgE responses toward the 45-kd polypeptide. The 204-bp allele of *D6S291* showed significantly decreased transmission to offspring with specific IgE responses toward the 90-kd polypeptide and to offspring with specific IgE responses toward the 52-kd polypeptide (Table II). Conversely, there was no deviation in transmission of these marker alleles to offspring without specific IgE response toward these Der p peptides compared with the expected value ($P > .05$, data not shown), which ruled out the possibility of a generalized segregation distortion at these markers.

DISCUSSION

Studies of specific IgE immune responsiveness have provided an excellent model for our understanding of the complex molecular genetics of human immune responsiveness.¹⁹ The HLA system consists of a cluster of linked genes controlling highly polymorphic proteins involved in the presentation of antigenic peptides to the T-cell receptor. Moreover, several studies suggest that allelic variation of class II molecules could influence the T_{H1} - or T_{H2} -like phenotype.^{20,21} Indeed, a substantial amount of evidence now exists on the association of specific HLA types with specific IgE responsiveness to several allergens.¹⁻⁴ The existence of *HLA*-linked genes controlling IgE responses toward mite allergens has been suggested by a study of 20 families with a history of asthma caused by mite allergy.²² An association was also reported between Der p 2-specific IgE antibody levels and *HLA-DRB1** 1501 alleles.²³

This study stems from our previous evidence for linkage between Der p 1-specific IgE antibodies and chromosome 6p21 in a genome-wide screening for genes

influencing Der p-specific IgE responsiveness.¹³ We extended our genetic study by evaluating specific IgE immune responses toward the Der p allergens by means of immunoblotting analysis. This approach allowed us to identify the presence of specific IgE antibodies to a panel of polypeptides without knowing the detail structures of these allergens or without having mAbs specific to these allergens. We used a dichotomized phenotype for TDT because less than 50% of individuals have detectable IgE antibodies to any one polypeptide, whereas a quantitative linkage method assumes a normal distribution of the phenotype. Given the complexity of the genetics of HLA, we used TDT, which is recognized to be a more sensitive method than conventional linkage tests such as lod scores or affected sib-pair analysis, which often fail to detect linkage when genetic effects of disease genes are minor or moderate.²⁴

We identified two alleles (the 196-bp allele of *D6S1281* and the 104-bp allele of *DQCAR*) that showed increased transmission to Der p responders, supporting our previous findings that the *HLA-D* region on chromosome 6p21 may influence expression of Der p-specific IgE responsiveness in this Caucasian population. Furthermore, 2 other alleles (the 200-bp allele of *D6S1281* and the 204-bp allele of *D6S291*) showed significantly decreased transmission to Der p responders. The important effects of untransmitted alleles on the biologic properties of transmitted alleles have been implicated in the genetic study of insulin-dependent diabetes mellitus.²⁵ The complexity and multigenic nature of the immune regulation by the HLA system was reported in a study that showed epistatic interaction between *HLA-DQ* and *HLA-DR* in controlling the immune response to schistosomal antigen in humans.²⁶ Several different alleles at *DR*, *DP*, and *DQ* genes may contribute independently with some alleles showing positive effects and with others showing negative effects. Also, genes encoding TNF

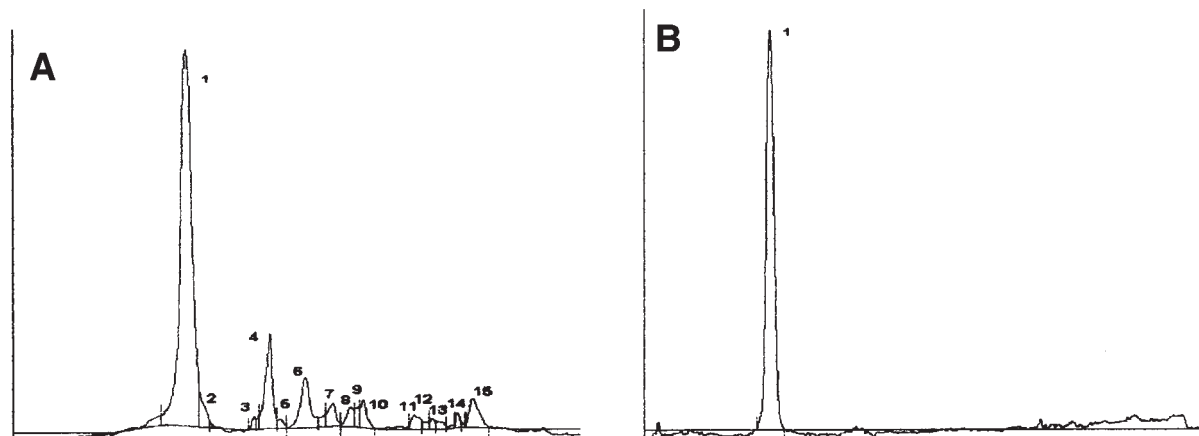


FIG 2. Densitometric analysis of immunoblots. Scanned images of *lane 1* and *2* of Fig 1 are shown. Intensity of each peak was automatically calculated by GelScan XL software program version 2.1 (LKB/Pharmacia) and used to estimate relative amount of specific IgE antibodies to each polypeptide. Intensity of peak 1 (corresponding to 14 kd) was 0.992 for subject 1 (Der p 2-specific IgE antibodies, 1.447 log IU/mL) (**A**) and 1.57 for subject 2 (Der p 2-specific IgE antibodies, 1.254 log IU/mL) (**B**).

or transporter antigen peptide-1, which have recently been implicated in the pathogenesis of atopy or asthma,^{27,28} are located on a chromosome 6p21. Moffatt et al²⁷ recently showed that it is feasible to distinguish genetic effects of TNF from those of *HLA-D* by using a large number of unrelated families (413 subjects from 88 nuclear families) by studying detailed genotype of *HLA-DRB1* and *TNF*-308 polymorphism and *LTa*NcoI polymorphism. Four alleles (196-bp and 200-bp alleles of *D6S1281*, 104-bp allele of *DQCAR*, and 204-bp allele of *D6S291*) identified in this study could be in tight linkage disequilibrium in the Caucasian families, and each of the *TNF*, *TAP*, and *HLA* genes could contribute to the development of Der p-specific IgE responsiveness. Therefore we believe that association or linkage disequilibrium analysis examining polymorphisms at each gene, including detailed genotyping of *HLA-DR*, *DQ*, and *DP* in a much larger population will be necessary to clarify the complex genetic regulation of Der p-specific IgE responsiveness by *HLA-D* genes.

Different polypeptides showed evidence for linkage with the same alleles (Table II). This may indicate that these polypeptides have common antigenic sites of similar amino acid sequences or may indicate that *HLA-D* restrictions toward these Der p polypeptides are loose because these structures are still complex. The phenotype evaluated by immunoblotting analysis allowed us to examine the overall IgE responsiveness toward the Der p allergens. The number of detectable bands on immunoblots was significantly correlated with serum total IgE levels and was significantly associated with asthma. These findings suggested that the higher the concentration of total IgE, the more the peptides (or epitopes) are recognized and that IgE-mediated sensitivity to multiple Der p polypeptides contribute to the development of asthma in this Caucasian population. No alleles, however, showed significantly deviated transmission to offspring with multiple Der p responsiveness (the pres-

ence of specific IgE antibodies to 5 or more different Der p polypeptides) from the expected value (50%). Therefore it is most likely that non-*HLA* IgE-regulating loci and environmental factors also contribute to the expression of overall IgE responsiveness toward the Der p allergens¹³ in addition to *HLA-D* genes.

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Correction

The following correction applies to the article by Sarpong and Karrison entitled "Season of birth and cockroach allergen sensitization in children with asthma," which appeared in volume 101, pp 566-568, 1998, of The Journal.

The first sentence of the second paragraph under "Methods" reads "Immediate wheal and flare skin tests were performed by the prick-puncture method with bifurcated needle and glycerinated full-strength mixed (German and American) cockroach allergen extract (Bayer, Spokane, Wash.). This sentence should read "Immediate wheal and flare skin tests were performed by the prick method using Multi-test (Lincoln Diagnostics, Inc., Decatur, Ill.) and full-strength mixed (German and American) cockroach allergen extract (Bayer, Spokane, Wash.)."