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Analysis of *hsp70* gene polymorphism in allergic asthma

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Key words: allergy/asthma; gene polymorphism; heat-shock proteins; human leukocyte antigens; inflammation; major histocompatibility complex.

Background: Allergic asthma is a multifactorial and probably multigenic inflammatory disease of the upper airways, and has been associated with the HLA class II alleles DR4 and DR7. Here we investigated possible associations with other polymorphic susceptibility/resistance genes located within the major histocompatibility complex, i.e., the genes coding the major 70-kDa heat-shock proteins (HSP; Hsp70) *hsp70-1*, *hsp70-2*, and *hsp70-HOM*, whose products are overexpressed in the bronchi of asthmatic patients.

Methods: Genomic DNA was extracted from peripheral blood lymphocytes or buccal epithelial cells of 48 patients with allergic asthma and 31 selected nonatopic control subjects, in whom we previously reported a strong association of atopy with DR4/DR7 alleles.

Results: No evidence was found for an independent role of *hsp70* gene polymorphism in susceptibility to allergic asthma. However, *hsp70* alleles might be involved in extended haplotypes of HLA markers.

Conclusions: Our data suggest that Hsp70 overexpression in asthma results from complex interactions between environmental exposures and genetic background rather than from specific genetic variations in *hsp70* genes.

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Allergic asthma is a multifactorial and probably multigenic inflammatory disease of the upper airways, arising from complex interactions between genetic and environmental factors. We have previously reported that allergic asthma is associated with HLA class II alleles DR4 and DR7 (1, 2). Susceptibility or resistance genes other than HLA are also

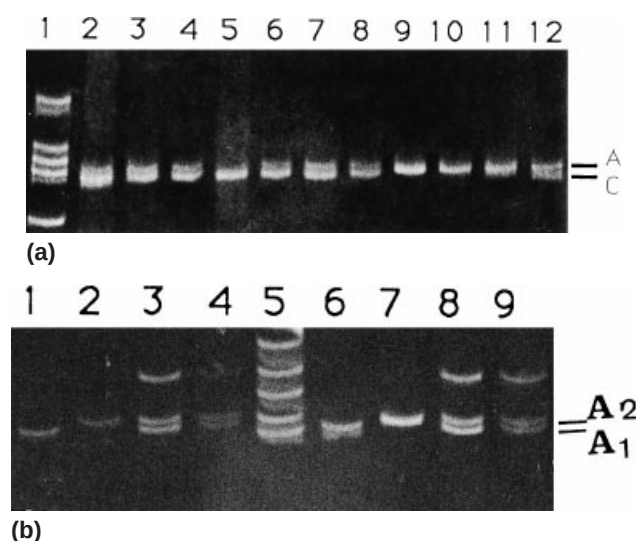


Figure 1a. Polyacrylamide gel electrophoresis patterns of PCR products from *hsp70-1* 5' regulatory region, in 11 patients. Lane 1) DNA markers; lane 5) patient homozygous for allele C; lanes 9–11) patients homozygous for allele A; lanes 2–4, 6, 7, 8, 12) patients heterozygous for alleles A and C.

Figure 1b. Polyacrylamide gel electrophoresis patterns of PCR products from *hsp70-2* 3' noncoding region in eight patients. Lane 1) patients homozygous for allele A1; lanes 2, 7) patients homozygous for allele A2; lanes 3, 4, 6, 8, 9) heterozygous patients.

located within the major histocompatibility complex (MHC), including tumor necrosis factor (TNF) genes, complement genes, and three genes coding the major 70-kDa heat-shock proteins (HSP; Hsp70), i.e., *hsp70-1*, *hsp70-2*, and *hsp70-HOM*, located within the HLA class III region, between the TNF and the C2 genes (3, 4). *hsp70* genes, like HLA genes, are polymorphic, although for the former, this polymorphism is more restricted. The genes coding for Hsp70 were the subject of the present study.

The chromosomal mapping of *hsp70* genes within the MHC and the linkage disequilibrium with other relevant HLA genes has led a number of authors to investigate the possibility that *hsp70* genes are susceptibility genes in chronic inflammatory or autoimmune diseases (reviewed in Ref. 5). A restriction fragment length polymorphism (RFLP), within the coding region of *hsp70-2*, has been described with a 8.5-kb PstI *hsp70* allele (allele L), leading to a silent mutation (3–5). Allele L has been found to be included in HLA haplotypes carrying known susceptibility markers of Graves's disease, celiac disease, and insulin-dependent diabetes mellitus (IDDM) (5). Polymorphism has also been investigated within the promoter region of the *hsp70* genes. A three-allele polymorphism (A, B, C) has been found within the 5' flanking regions of the *hsp70-1* gene (6, 7). Single

nucleotide differences at –110 and +120 were responsible for changes in the electrophoretic mobility of the three alleles (A: slow; B: fast; C: intermediate). The presence of some of these polymorphic variations was shown to be associated with specific HLA class II DR haplotypes (7). Furthermore, a pentanucleotide tandem duplication polymorphism in the 3' untranslated region of the *hsp70-2* gene has been described; i.e., alleles A1 and A2 (8).

hsp70 genes encode a group of both constitutively expressed and stress-inducible proteins that function as molecular chaperones, and are essential for cell survival under stressful conditions. In particular, Hsp70 protects cells and tissues against TNF- α , reactive oxygen species (ROS), and apoptosis, both *in vitro* and *in vivo* (9, 10). Several studies have shown that Hsp70 is overexpressed in the bronchi and alveolar macrophages of patients with asthma, such expression correlating with intrapulmonary eosinophilia and with the severity of the disease (11, 12). The possibility that Hsp70 expression plays an autoprotective role in asthma has thus been suggested (12), but, more recently, it has been proposed that Hsp70 overexpression might also play a deleterious role in asthma, possibly contributing to chronic inflammation (13, 14).

Here we investigated a possible association between these polymorphisms and allergic asthma, in the same groups of patients and selected nonatopic control subjects in whom we previously reported a strong association with DR4/DR7 alleles. Our data argue against an independent role of *hsp70* gene polymorphism in susceptibility to allergic asthma.

Material and methods

This study was carried out according to the principles of the Declaration of Helsinki, with written consent and institutional approval.

Patient and control selection

All subjects were European-born, unrelated whites living in France, in the Paris area. Forty-eight unrelated white patients (mean age: 43 ± 2.3 years) were admitted to the study. They were administered a standardized questionnaire by physicians with extensive experience in allergic diseases. Phenotype characterization included medical history, asthma symptoms (shortness of breath, wheezing, coughing, and chest tightness), and bronchial hyperresponsiveness to methacholine challenge. The mean of forced expiratory volume(s) was $86\% \pm 3$ of the predicted values. Subjects were

Table 1. Distribution of *hsp70-1* and *-2* genotypes and allelic frequencies in patients and controls

		<i>hsp70-1</i> genotypes			Allelic frequencies		
		A/A	A/C (+AB)	C/C (+CB)	A	B	C
Patients	<i>n</i>	15	30 (+3)	3	60	3	33
	<i>n</i> = 48	% 31.25	62.5	6.25	62.5	3.1	34.4
Controls	<i>n</i>	14	13	4 (+2)	41	2	19
	<i>n</i> = 31	% 45.2	41.9	12.9	66.2	3.2	30.6

		<i>hsp70-2</i> genotypes			Allelic frequencies	
		A1/A1	A1/A2	A2/A2	A1	A2
Patients	<i>n</i>	16	30	2	62	34
	<i>n</i> = 48	% 33.3	62.5	4.2	64.6	35.4
Controls	<i>n</i>	8	15	2	31	19
	<i>n</i> = 25	% 32.0	60.0	8.0	62.0	38.0

defined as atopic/asthmatic if they met one or more of the following criteria:

- 1) positive skin-prick tests carried out with 17 common allergens consisting of *Dermatophagoides pteronyssinus* and *D. farinae*, grass and tree pollens, animal danders, and common molds (one negative control and two positive controls were also applied)
- 2) a raised total IgE (two standard deviations above the predicted mean for age)
- 3) bronchial hyperresponsiveness.

We also studied 31 unrelated white selected nonatopic control subjects (mean age = 41.0 ± 2.6 years) from the same

geographic area. They answered the same questionnaire as the patient group, and the criteria for admission to the study included the absence of any personal and familial history of atopy through three generations. Skin tests and bronchial reactivity challenges were also performed on control volunteers, yielding consistent negative results. Subjects were included in the study only if they had no history of cancer, diabetes, cardiovascular disease, or any other major disease.

DNA preparation and HLA typing

Genomic DNA was extracted from peripheral blood lymphocytes or buccal epithelial cells as previously described (1, 2). A panel of genomic DNA samples, previously obtained (2) and typed for HLA class II DRB1, DQA1, DQB1, and DPB1 alleles and haplotypes by PCR-RFLP (15), was used. For DNA amplification of the *hsp70-1* regulatory region, we used PCR as previously described (6), with few modifications: the first denaturation step was at 95°C instead of 97°C, and a final elongation step at 72°C for 3 min was carried out at the end of the amplification cycles. The sequences of the primers were as follows: upstream primer: 5' CGCCATGGAGACCAACACCC 3'; downstream primer: 5' GCGGTTCCCTGCTCTCTGTC 3'. An amount of 500 ng of genomic DNA was amplified in a Hybaid thermal reactor (Teddington, Middlesex, UK). PCR products were controlled in a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The amplification products were then analyzed by vertical

Table 2. Association of A and C *hsp70-1* alleles with A1 and A2 *hsp70-2* alleles in patients and controls

<i>hsp70-1</i>	<i>hsp70-2</i> associated allele	<i>n</i>	OF	EF	Δ	χ^2	<i>P</i>
Patients <i>n</i> = 48							
A	A1	44	0.917	0.88	0.037	–	NS
	A2	30	0.625	0.61	0.015	–	NS
C	A1	29	0.604	0.59	0.01	–	NS
	A2	28	0.583	0.41	0.17	25.6	4.2 10 ⁻⁷
Controls <i>n</i> = 25							
A	A1	21	0.840	0.80	0.04	–	NS
	A2	14	0.560	0.59	–0.03	–	NS
C	A1	13	0.520	0.49	0.03	–	NS
	A2	12	0.48	0.36	0.12	6.5	1.0 10 ⁻²

OF: observed frequency of haplotype carrying two alleles; EF: expected frequency of haplotype carrying two alleles; Δ : linkage disequilibrium coefficient; χ^2 : chi-square test; NS: not significant.

polyacrylamide gel electrophoresis, as previously described (6). In order to confirm that the bands of different mobility corresponded to the alleles A, B, and C, DNA samples derived from three homozygous cell lines, A/A, B/B, and C/C, obtained from the International Histocompatibility Workshop, were used as controls. Furthermore, because the difference in mobility observed between alleles B and C was small, an additional assay was carried out to detect the presence of allele B. A volume of 6 µl of amplified product was digested with 4 U of the endonuclease Nci I (Boehringer-Mannheim, Mannheim, Germany), which recognizes a unique restriction site at position +120, and size-analyzed in a 1.8% agarose gel. This experimental procedure was used in all cases not homozygous for A. For DNA amplification of the 3' untranslated region of *hsp70-2*, the protocol and the sequence of the primers were the same as those described by Dressel & Günther (8); the forward 5' GCTTCAGCTCTTTGCTGCTTCACT 3' and the reverse primers 5' ACAAACTCGTACAGAAGGTGGCAG 3'. A two-allele polymorphism was detectable by size separation of the PCR products; according to Milner & Campbell (4), the shorter band (allele A1) contained 183 bp and the longer one, 188 bp (allele A2), with a tandem duplication of the five nucleotides AAGTT (positions 2257–2261).

Sequence analysis

The amplification products from one control subject and two patients, representative of different sets of alleles, were sequenced by an automatic method (Gene-Express, Grenoble, France). The sequences did not differ from those described by Cascino et al. (6).

Statistical analysis

Allele frequency was calculated by the gene-counting method of maximum likelihood (16). Significant associations were evaluated by either the chi-square test (with Yates' correction, where adequate) or Fisher's exact test with 2×2 contingency tables. Linkage disequilibrium was calculated from phenotypes according to Mattiuz et al. (17).

Results

Clinical and functional data

Both the patients and the selected controls used for this study have been previously described in detail (2). Briefly, all

patients had a documented history of allergic symptoms; among the 24 asthmatic subjects who underwent a bronchial challenge with methacholine, a 20% fall in FEV₁ was observed in 21 of them based on a group average of methacholine (PD₂₀FEV₁) of 580±170 µg. In the other patients, the FEV was too low to permit the methacholine challenge to be performed safely. As many as 91% of patients presented skin reactivity to at least one allergen. Only 34% of patients had elevated total IgE. The lack of personal and familial history of allergy in our healthy controls was confirmed in a subgroup of them by negative prick tests (*n*=14) and negative nonspecific inhalation challenge (*n*=6).

Polyacrylamide gel electrophoresis patterns in the *hsp70-1* promoter region and *hsp70-2* 3' untranslated region

Amplification of the regulatory region of the *hsp70-1* gene and the 3' untranslated region of the *hsp70-2* gene detected the three allelic *hsp70-1* forms, namely, A, B, and C, and the two-allelic *hsp70-2* forms A1 and A2. In both patients and controls, *hsp70-1* A/A and A/C were predominant (Fig. 1a), and among all subjects tested, only three heterozygous patients and two heterozygous controls carried allele B. For *hsp70-2*, homozygous subjects (A1/A1 or A2/A2) exhibited one band, while two bands were seen in heterozygous subjects (Fig. 1b).

Distribution of *hsp70-1* and *hsp70-2* alleles in patient and control groups

Frequencies for *hsp70-1* A, B, and C alleles was estimated from the phenotypes of our 48 patients and 31 controls, while the frequencies for *hsp70-2* A1 and A2 alleles were estimated from the phenotypes of the same 48 patients and 25 controls. No significant differences were observed between patients and controls, either in the distribution of the *hsp70-1*, A, B, and C alleles, or in the distribution of the *hsp70-2* alleles (Table 1). In contrast, a significant linkage disequilibrium was observed between allele *hsp70-1* C and *hsp70-2* A2, in both patients and controls (Table 2). The distribution of the three *hsp70-1* alleles was also analyzed in patients and in controls, in relation to the previously typed HLA class II alleles DRB1, DPB1, DQA1, and DQB1. No significant linkage disequilibrium was observed between any of the HLA class II and the *hsp70-1* A, B, or C alleles (data not shown).

Discussion

MHC genes are likely candidates for controlling the immune response, and different HLA class II genes may represent risk factors for, or protective factors against, the development of asthma and allergy (2, 15). Among the MHC-encoded genes are *hsp70* genes, the products of which are overexpressed in the airways of patients with severe asthma (11). Thus, we tested the hypothesis of a specific association between *hsp70* regulatory regions gene polymorphism and asthma, using two groups of unrelated, European-born, white, adult, allergic patients, with or without asthma, and healthy asymptomatic control subjects.

The chromosomal mapping of three members of the *hsp70* gene family in the HLA class III region (4), and the linkage disequilibrium with other relevant HLA genes previously described (6, 7), has led many groups to investigate the role of *hsp70* genes as susceptibility genes in chronic inflammatory diseases (5). However, Cascino et al. (18) observed no independent contribution of *hsp70-1* genes to three autoimmune diseases; namely, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis. In allergic asthma, we found that the overall frequency of the three *hsp70-1* alleles A, B, and C, and of the two *hsp70-2* alleles, A1 and A2, was similar within both patient and control groups. In both groups, allele *hsp70-1* C was significantly associated with allele *hsp70-2* A2.

Our results argue against an independent association between *hsp70* polymorphism and allergic asthma. Although a possible association between HLA DR4 and *hsp70-1* A could not be analyzed (since DR4 is nearly excluded by selecting for the lack of atopy/allergy), the HLA DR4/DR7 alleles, which are implicated in the susceptibility to allergic asthma (2), might contribute to an extended haplotype including *hsp70* alleles, as additive susceptibility markers. Alternatively, polymorphic variations of other *hsp*

genes possibly involved in asthma, could determine inter-individual variations in asthma phenotype (19). Furthermore, functional alterations of HSP, or aberrant expression of *hsp70* genes due to sequence differences in the regulatory regions, could influence the outcome inflammatory disorders.

The inclusion of the *hsp70-2* Pst I restriction site alleles from the coding region in some risk haplotypes for autoimmune diseases, might be due to a linkage disequilibrium between the former alleles and risk alleles for the disease; however, the association with the disease might also result from the presence of extended haplotypes. We also analyzed the polymorphism of the Pst I restriction site in the coding region of the *hsp70-2* gene in 46 atopic/asthmatic patients (unpublished results). The frequency of atopic patients found to be homozygous for Pst I was very low (4%), as compared to the percentage (about 30%) observed in certain autoimmune diseases (20).

In conclusion, our data suggest that in asthma and allergy, overexpression of Hsp70 – and other HSP such as Hsp27 (19, Vignola et al., unpublished) – results from the complex interactions between distinct environmental exposures and the patient's genetic background, rather than from specific genetic variations in *hsp* genes. Although we provide no evidence for an independent association between *hsp* gene polymorphism and allergic asthma, the possibility that differential induction and/or function of these protective proteins modulates the susceptibility to asthma remains to be examined.

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