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Heat shock protein 70 (HSP70) and complement C4 genotypes in patients with hyperthyroid Graves' disease

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SUMMARY

The genetic polymorphisms of the heat shock protein 70 (HSP70) and complement component C4 were investigated in 90 patients with hyperthyroid Graves' disease and 92 normal control subjects. The 8·5-kb *Pst*I HSP70 allele was strongly associated with Graves' disease when compared with controls ($P<0.001$). The presence of the 8·5-kb *Pst*I HSP70 allele was strongly associated with a deletion of the C4A gene in both patients and controls ($P<0.0003$ and $P<0.00005$ respectively). However, in the absence of C4A gene deletion, the frequency of the 8·5-kb *Pst*I HSP70 allele was still significantly higher in patients when compared with controls ($P<0.04$). These results suggest that the HSP70 locus may have an immunological role to play in autoimmune hyperthyroid Graves' disease.

Keywords heat shock protein complement C4 Graves' disease restriction fragment length polymorphism

INTRODUCTION

The human MHC comprises three classes of genes on the short arm of chromosome 6 (6p21·3). The MHC class I and class II regions lie approximately 1100 kbp apart and contain genes that encode for cell membrane glycoproteins. Separating the class I and class II regions is the class III region which contains at least 29 genes. Lying approximately 350 kbp from the HLA-D region in a 120-kb segment of DNA is the cluster of genes encoding serum complement components, C2, Bf, C4A and C4B, as well as two genes encoding the cytochrome P450 21-hydroxylase enzymes, CYP21-OHA and CYP21-OHB, respectively (Carroll, Campbell & Porter, 1985a). More recently, genes encoding the cytokines, tumour necrosis factors (TNF-α and TNF-β) and three members of the heat shock protein 70 (HSP70) family, HSP70-1, HSP70-2, HSP70-Hom, and a cluster of expressed genes of unknown function, have been mapped between the HLA-B and complement genes (Carroll *et al.*, 1987; Dunham *et al.*, 1987; Sargent *et al.*, 1989a; Sargent, Dunham & Campbell, 1989b; Milner & Campbell, 1990; Spies *et al.*, 1989, respectively).

Graves' disease is an organ-specific autoimmune disease characterized by hyperthyroidism due to the presence of autoantibodies which by binding to the thyrotrophin (TSH) receptor stimulate thyroid function. The association of Graves' disease amongst Caucasians with HLA-DRw17 (DR3) is not absolute, but has been confirmed at both the phenotypic (serological typing) and genotypic (DNA-RFLP) level (Ratanachaiyavong *et al.*, 1990). Although the polymorphic structure of the MHC class II antigen molecules is likely to be important in determining genetic susceptibility towards the development of autoimmune diseases (Benacerraf, 1981; Todd *et al.*, 1988), these polymorphic structures are also present in the normal population. No unique sequence of the MHC class II antigen which might be responsible for the development of autoimmune disease has been identified. Therefore, it is likely that other additional genetic and/or environmental factors confer susceptibility to a particular autoimmune disease.

Recent studies have also demonstrated the importance of the induction of the MHC expression in the target tissue in the development of autoimmune disease. The tissue-specific increase in MHC class II expression in susceptible animals with experimental allergic encephalomyelitis was shown to be correlated with the development of the disease (Massa, Meulen & Fontana, 1987). In contrast, the decreased production of the cytokine, TNF-α, in HLA-DR2/DQw1 patients with systemic lupus erythematosus is associated with an increased incidence of lupus nephritis (Jacob *et al.*, 1990). More recently, it has been proposed that the products of the HSP70 gene family have an important role in immune modulation, particularly in (i) protein translocation and degradation (Flynn, Chappell & Rothman, 1989; Chiang *et al.*, 1989) which is pertinent for antigen processing; (ii) protein anchoring to the cell surface, i.e. antigen presentation (Lakey, Margoliash & Pierce, 1987; VanBuskirk

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et al., 1989); and (iii) binding of the immunoglobulin heavy chain and the assembly of immunoglobulin molecules (Hass & Meo, 1988). It seemed of interest to know more about the HSP70 loci and their relation to neighbouring genes. In this report we have investigated the genetic polymorphism of the MHC-associated HSP70 loci and their relationship to C4 polymorphisms in patients with Graves' disease and normal controls using probes derived from the HSP70-2 and C4 genes.

MATERIALS AND METHODS

Population study

EDTA blood samples were obtained from a British Caucasoid population consisting of 90 unrelated patients (69 women and 21 men) with a clinical and biochemical diagnosis of hyperthyroid Graves' disease and 92 healthy individuals with no personal and family history of autoimmune disease.

DNA extraction and hybridization

Genomic DNA was extracted from EDTA blood samples as described previously (Ratanachaiyavong, Campbell & McGregor, 1989a). Separate aliquots of 5 μg of high molecular weight DNA were digested with both *Pst*I and *Taq*I restriction endonucleases under the conditions recommended by the manufacturer. Digested DNA samples were sized-fractionated in 0·7% agarose gel electrophoresis, capillary transferred (Southern, 1975) to nylon membranes (Hybond-N, Amersham International, Amersham, UK) and UV cross-linked for 3–5 min. Filters were prehybridized for at least 3 h before hybridization at 65°C, overnight in hybridization buffer (6 × SSC/5 × Denhardt's solution/0·5% SDS/50 μg/ml of sonicated salmon sperm DNA) containing 10% dextran sulphate and heat denatured ^{32}P-labelled probes. Post-hybridization washing was carried out under high stringency. Filters were first washed with 2 × SSC/1% SDS at room temperature for 10 min, washed twice for 20 min each with 2 × SSC/1% SDS at 65°C, followed by 30 min with 0·2 × SSC/1% SDS at 65°C. Autoradiography was performed between two intensifying screens at −70°C for 4–7 days.

DNA probes

The HSP70-2 probe is a 0·9-kb *ClaI/BamHI* fragment derived from the middle of the HSP70-2 gene (Sargent *et al.*, 1989a), and the C4 probe is a full-length cDNA probe as previously described (Belt, Carroll & Porter, 1984). Both probes were purified using LMP agarose, ^{32}P-dCTP labelled by random hexanucleotide priming (Feinberg & Vogelstein, 1984) with specific activity > 10^7 ct/min/μg of DNA.

Restriction fragment length polymorphism (RFLP)

The HSP70-2 probe also hybridizes to other members of the HSP70 family. In conjunction with the restriction endonuclease *Pst*I, a biallelic polymorphism of 9·0- and 8·5-kb fragments (Fig. 1a) have been described (Goate *et al.*, 1987).

The *Taq*I restriction endonuclease in conjunction with the full-length C4 cDNA probe detects four different fragments of size 7·0, 6·0, 5·4 and 6·4 kb (Fig. 1b). These represent the 5′ end of C4A, C4B-long, C4B-short and C4A gene deletion in association with an apparently short C4B gene respectively (Schneider *et al.*, 1986).

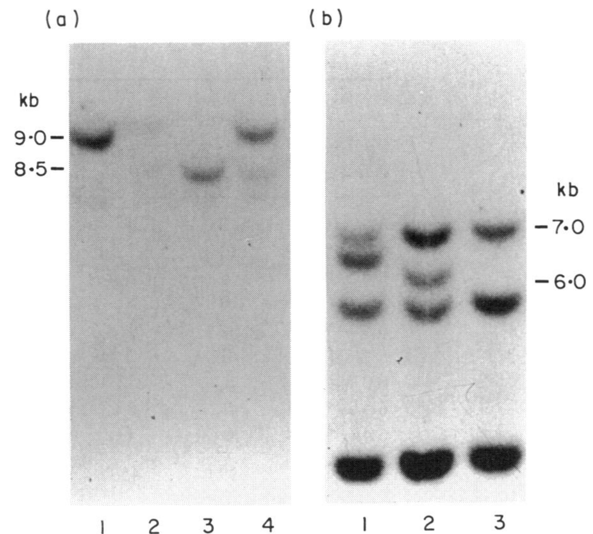

Fig. 1. (a) The HSP70 probe in conjunction with the restriction endonuclease *Pst*I detects two allelic fragments of either 9·0 or 8·5 kb. Lanes 1 and 3 demonstrate two individuals who were homozygous for the 9·0 and 8·5 kb, respectively. Lanes 2 and 4 are individuals who were heterozygous for the 9·0 and 8·5 kb. The difference in intensity of the 9·0-and the 8·5-kb fragments in the heterozygous individuals was due to the differences in hybridization signal. In the presence of the *Pst*I site, a DNA fragment containing a 700-bp complementary region to this probe is cleaved off. (b) The C4 probe in conjunction with the restriction endonuclease *Taq*I detects allelic fragments of 7·0, 6·4, 6·0 and 5·4 kb.

Table 1. Distribution of HSP70 genotypes and their allelic frequencies in 90 patients with Graves' disease and 92 control subjects

	*Pst*I/HSP70 genotypes			Allelic frequency	
	9·0/9·0	9·0/8·5	8·5/8·5	9·0	8·5
Graves' disease	19	45	26	83	97
(n = 90)	21·1%	50·0%	28·9%	0·461	0·539
Healthy control	39	40	13	118	66
(n = 92)	42·4%	43·5%	14·1%	0·641	0·359

The differences in the distribution of HSP70 genotypes and the allelic frequencies between patients and controls were determined by χ^2-test using 3 × 2 (χ^2 = 11·5, P < 0·005) and 2 × 2 (χ^2 = 11·9, P < 0·001) tables, respectively.

Statistical analysis

The χ^2-test was applied for statistical comparison using 3 × 2 or 2 × 2 contingency tables.

RESULTS

The *Pst*I polymorphism of the HSP70 locus gave three possible genotypes of 9·0/9·0, 9·0/8·5 and 8·5/8·5 kb. The distribution of HSP70 genotypes and its allelic frequencies in 90 patients with Graves' disease and 92 healthy controls is shown in Table 1. There was a significant difference in the frequency of the HSP70 genotypes between patients and controls (χ^2 = 11·52, d.f. = 2,

Table 2. The relation between C4B and HSP70 genotypes in 90 patients with Graves' disease and 67 healthy control subjects

*Taq*I/C4B genotypes	Graves' disease *Pst*I/HSP70 genotypes				Healthy control *Pst*I/HSP70 genotypes			
	9·0/9·0	9·0/8·5	8·5/8·5	Total	9·0/9·0	9·0/8·5	8·5/8·5	Total
6·0/6·0	12	4	0	16	24	6	1	31
6·0/—*	0	1	0	1	0	0	0	0
6·0/5·4	3	8	4	15	6	11	1	18
5·4/5·4	0	4	1	5	1	4	0	5
5·4/—*	0	1	0	1	1	0	0	1
5·4/6·4	1	2	11	14	0	4	3	7
6·0/6·4	3	21	5	29	0	3	2	5
6·4/—*	0	3	0	3	0	0	0	0
6·4/6·4	0	1	5	6	0	0	0	0
Total	19	45	26	90	32	28	7	67

* The absence of the second C4B gene was defined by decreased intensity of the C4B fragment when compared with the 7·0-kb *Taq*I fragment of C4A gene. The C4B gene deletion was also supported by deletion of either 21OH-A or 21OH-B using 21OH probe.

Table 3. The association between the HSP70 alleles and the C4A gene deletion defined by the 6·4-kb *Taq*I fragment in 90 patients with Graves' disease and 67 control subjects

	*Taq*I/C4A 6·4 kb fragment	*Pst*I/HSP70 alleles 9·0 kb	8·5 kb	χ^2	*P* value
Graves' disease	+	35	69	15·4	<0·0003
	$n=52$	(0·337)	(0·663)		
	+	48	28		
	$n=38$	(0·632)	(0·368)*		
Control subjects	+	7	17	21·2	<0·00005
	$n=12$	(0·292)	(0·708)		
	−	85	25		
	$n=55$	(0·773)	(0·227)*		

The allelic frequency is shown in parentheses.
* Statistically significant difference ($\chi^2=4{\cdot}4$, $P<0{\cdot}04$).

$P<0{\cdot}005$). The frequency of the 8·5-kb *Pst*I allele was also significantly higher in patients when compared with controls (0·539 versus 0·359, $\chi^2=11{\cdot}23$, d.f. $=1$, $P<0{\cdot}002$).

The three different sizes of C4B *Taq*I restriction fragments detected by the C4 probe revealed nine possible genotypes. The distribution of the C4B and HSP70 genotypes in 90 patients and 67 control subjects is shown in Table 2. The presence of the 6·0-kb *Taq*I fragment of the C4B-long gene was strongly associated with the presence of the 9·0-kb *Pst*I HSP70 allele, while the presence of the 6·4- and 5·4-kb *Taq*I fragments were associated with the 8·5-kb *Pst*I HSP70 allele. Table 3 shows the frequency of the *Pst*I HSP70 alleles in the presence or absence of C4A gene deletions in patients and controls. The 8·5-kb *Pst*I HSP70 allele was strongly associated with the 6·4-kb *Taq*I fragment which corresponds to a C4A gene deletion in both patients and controls (0·663 *versus* 0·368, $\chi^2=15{\cdot}4$, $P<0{\cdot}0003$ and 0·708 *versus* 0·227, $\chi^2=21{\cdot}2$, $P<0{\cdot}00005$, respectively). However, in those patients and controls with no C4A gene deletion, the frequency of the 8·5-kb *Pst*I HSP70 allele was still significantly higher in the patient group (0·368 *versus* 0·227, $\chi^2=4{\cdot}4$, $P<0{\cdot}04$).

Though C4A gene deletion is strongly associated with the 8·5-kb *Pst*I HSP70 allele, this allele is not always associated with C4A gene deletion. As shown in Table 2, the presence of a C4A gene deletion (6·4-kb *Taq*I fragment) was always accompanied by the presence of the 8·5-kb *Pst*I HSP70 allele in controls and in 91·4% (53/58) of the patients. In contrast, only 54·6% (53/97) and 28·6% (12/42) of the 8·5-kb *Pst*I HSP70 allele were associated with the C4A gene deletion in patients and controls, respectively. The difference in the frequencies of this association between patients and controls was statistically significant ($\chi^2=8{\cdot}0$, $P<0{\cdot}004$).

A further question has been raised as to whether the increased association of the 8·5-kb *Pst*I HSP70 allele with Graves' disease in the absence of C4A gene deletion has any relation with the structural polymorphisms of the C4B gene. In order to exclude any epistatic effect of the C4A gene deletion on the C4B-short gene which appears as a 6·4-kb *Taq*I fragment on this haplotype, all the C4B-short genes identified as a 6·4-kb *Taq*I fragment were excluded from further analysis. As shown in Table 4, the number of C4B genes was determined by direct gene counting. Due to lack of family information, individuals who were heterozygous at both C4 and HSP70 loci were excluded from the analysis. After exclusion of all the C4B-short genes of the C4A gene deletion haplotypes (represented by the 6·4-kb fragment), there was no difference in the frequencies of the C4B-short (5·4-kb *Taq*I fragment) and -long (6·0-kb *Taq*I fragment) genes between patients and controls, 0·342 *versus* 0·298 for the C4B-short and 0·658 *versus* 0·702 for the C4B-long genes, respectively. It is intriguing that in patients with Graves' disease, the 5·4-kb *Taq*I fragment of the C4B-short gene was also strongly associated with the 8·5-kb *Pst*I HSP70 allele when compared with the 6·0-kb *Taq*I fragment of the C4B-long gene (0·724 *versus* 0·277, $\chi^2=14{\cdot}5$, $P<0{\cdot}0004$). A similar but weaker association was also observed in the control group (0·380 *versus*

Table 4. The association of the C4B gene and HSP70 alleles in patients with Graves' disease and control subjects

Graves' disease	Sizes of C4B genes	Total number of genes*	Number of gene exclusion†	HSP70 alleles (kb) 9·0	8·5	Total
	6·0 kb C4B-long	77 (0·658)	30	34 (0·723)	13 (0·277)	47
	5·4 kb C4B-short	40 (0·342)	11	8 (0·276)	21 (0·724)‡	29
Control subjects	6·0 kb C4B-long	85 (0·702)	14	60 (0·845)	11 (0·155)	71
	5·4 kb C4B-short	36 (0·298)	15	13 (0·620)	8 (0·380)‡	21

* The total number of genes was determined by direct gene counting.

† Individuals who were heterozygous at both C4 and HSP70 loci were excluded. The allelic frequency is shown in parentheses. The 5·4-kb *Taq*I fragment of the C4B-short gene is strongly associated with the 8·5-kb *Pst*I fragment of HSP70 when compared with the C4B-long gene in both patients ($\chi^2 = 14{\cdot}5$, $P < 0{\cdot}0004$) and controls ($\chi^2 = 5{\cdot}1$, $P < 0{\cdot}03$).

‡ The frequency of the 8·5-kb *Pst*I HSP70 allele in patients with a C4B-short gene is significantly higher than in controls ($\chi^2 = 5{\cdot}9$, $P < 0{\cdot}02$).

0·155, $\chi^2 = 5{\cdot}1$, $P < 0{\cdot}03$). As shown in Table 4, 72% of the 5·4-kb *Taq*I fragment of the C4B-short genes were associated with the 8·5-kb *Pst*I HSP70 allele in the patients but only 38% in the controls ($\chi^2 = 5{\cdot}9$, $P < 0{\cdot}02$).

DISCUSSION

Graves' disease is one of the organ-specific autoimmune diseases which has been shown to be associated with HLA-B8/DR3 (DRw17) in the Caucasoid population (Tiwari & Terasaki, 1985). HLA-B8/DR3 is not only associated with Graves' disease, but also with many other autoimmune disorders, e.g. insulin-dependent diabetes mellitus (IDDM), subgroups of myasthenia gravis, chronic active hepatitis, systemic lupus erythematosus (SLE) and coeliac disease (Tiwari & Terasaki, 1985). One interesting immunogenetic aspect of this haplotype is its strong association with a C4A gene deletion in both normal control (Carroll *et al.*, 1985b; Schneider *et al.*, 1986; Partanen *et al.*, 1987) and disease populations such as SLE (Fronek *et al.*, 1988; Goldstein *et al.*, 1988) and Graves' disease (Ratanachaiyavong, Lloyd & McGregor, 1989b) suggesting a strong linkage disequilibrium of this haplotype.

HSP70 genes were mapped 92 kb from the C2 gene (Sargent *et al.*, 1989b; Milner & Campbell, 1990), i.e. approximately 150 kb from C4A gene. The possibility was raised as to whether the association of the 8·5-kb *Pst*I HSP70 allele with Graves' disease was due to its linkage disequilibrium with the C4A gene deletion. Our data suggest that this is the case, since there was a strong association between the C4A gene deletion and the 8·5-kb *Pst*I HSP70 allele both in patients and controls. A similar association was also observed in patients with IDDM (Caplen *et al.*, 1990). However, in the absence of C4A gene deletion, the frequency of the 8·5-kb *Pst*I HSP70 allele was still significantly higher in patients when compared with controls. This suggests that the association is not entirely due to its linkage disequilibrium with the C4A gene deletion. Though there was a significant association between the 8·5-kb *Pst*I HSP70 allele and the 5·4-kb *Taq*I fragment of the C4B-short gene in both patient and control groups, the association was also significantly stronger in the patient group. As mentioned earlier there was no difference in the frequencies of the C4B-short and C4B-long genes between the two groups. The increased association of the 8·5-kb *Pst*I HSP70 allele in patients with the C4B-short gene is unlikely to be due to the increased frequency of the C4B-short gene, but may well reflect the preferential association of the 8·5-kb *Pst*I HSP70 allele with Graves' disease.

There is growing evidence that the products of the HSP60 (60/65 kD) family of several micro-organisms (e.g. *Mycobacterium tuberculosis*, *M. leprae*, *Escherichia coli* and *Streptococcus*) are the major antigenic epitopes recognized by B and T cells from patients who have recovered from infections or suffer from rheumatoid arthritis. The HSP products could therefore be a major target for autoimmune attack in patients with rheumatoid arthritis via the mechanism of molecular mimicry (Dubois, 1989; Kaufmann, 1990; Young, 1990). The HSP70 family encodes several highly conserved proteins with over 60% of amino acid sequence identity between eukaryotes and nearly 50% identity between humans and *E. coli* (Hunt & Morimoto, 1985). The *Pst*I polymorphism of the HSP70-2 gene is due to the silent G–A transition at position 1267 and results in loss of *Pst*I restriction site which corresponds with the 9·0-kb HSP70 allele (Milner & Campbell, 1990). Our results indicate that the presence of this *Pst*I restriction site is strongly associated with Graves' disease. Whether or not this RFLP of the HSP70-2 gene can be used as a genetic marker to detect any differences in terms of the regulatory function of the HSP70 gene products which may be relevant to the development of autoimmune disease is the subject of future studies. Until we learn and understand more about the immunological role of the HSP70 gene products, it is difficult at this stage to speculate on its association with autoimmune thyroid disease.

In conclusion, the 8·5-kb *Pst*I HSP70 allele is strongly associated with Graves' disease, and correlates with the presence of both the C4A gene deletion and the C4B-short gene. This association could be either primary or secondary to its linkage disequilibrium with the MHC class II and III regions. Further studies to understand the immunoregulatory function of the HSP70 gene products in both physiological and pathological conditions may elucidate the significance of this association.

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