No Independent Association Between *HSP70* Gene Polymorphism and IDDM

ALBERTO PUGLIESE, ZUHEIR L. AWDEH, ALDO GALLUZZO, EDMOND J. YUNIS, CHESTER A. ALPER, AND GEORGE S. EISENBARTH

A role for heat shock proteins (HSPs) in autoimmunity has recently been suggested by several authors. Autoantibodies against HSPs have been associated with such autoimmune diseases as systemic lupus erythematosus, polymyositis, and the NOD mouse model of diabetes. Moreover, genes for the major 70,000-M_r HSP (HSP70) are located within the MHC. To investigate a potential association of an *HSP70–2* gene polymorphism with insulin-dependent diabetes mellitus (IDDM), we analyzed restriction-fragment–length polymorphism (RFLP) of this gene in 29 families with one or more member affected by IDDM. With the enzyme *Pst*I, as reported previously, two *HSP70–2* alleles of 8.5- and 9.0-kb were found. The 8.5-kb allele was found more frequently on diabetic haplotypes compared with control haplotypes (41 of 66 [62%] vs. 20 of 46 [43%], $P = 0.03$). This association was due to the conservation of alleles on extended haplotypes we previously reported to be associated with diabetes on initial analysis of families. Twenty-three of 26 diabetic DR3 haplotypes and 3 of 3 normal DR3 haplotypes and all instances of [HLA-B8, SC01, DR3] and [HLA-B18, F1C30, DR3] had the 8.5-kb allele, whereas 0 of 9 normal DR2 haplotypes and 0 of 2 diabetic DR2 haplotypes had the 8.5-kb allele ($P = 8 \times 10^{-7}$ DR3 vs. DR2 haplotypes). The alleles were equally distributed among DR4 haplotypes. Our studies indicate that the 8.5-kb allele is part of [HLA-B8, SC01, DR3] and [HLA-B18, F1C30, DR3] haplotypes, and the 9.0-kb allele is associated with DR2 but no independent association (greater than DR association) is found between *HSP70–2* alleles and diabetes. *Diabetes* 41:788–91, 1992

From the Joslin Diabetes Center, Boston; Brigham and Womens Hospital, Boston; The Center for Blood Research, Harvard Medical School, Boston, Massachusetts; and the Clinica Medica B, University of Palermo, Palermo, Italy.

Address correspondence and reprint requests to George S. Eisenbarth, MD, PhD, Joslin Diabetes Center, Section on Immunology, One Joslin Place, Boston, MA 02215.

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Insulin-dependent diabetes mellitus (IDDM) appears to be a chronic autoimmune disease in which autoimmunity against β-cells is triggered in genetically susceptible individuals (1). DR3 and DR4 HLA alleles increase diabetes risk, and studies have reported that the DQw8 allele is closely associated with IDDM on DR4 haplotypes, particularly in DR3/DR4 heterozygous individuals (2).

We previously characterized and reported increased frequencies of a few extended haplotypes among diabetic haplotypes, demonstrating that these extended haplotypes, with relatively fixed alleles, are carriers of susceptibility genes for the disease and provide most of the individual high-risk alleles (3,4). Many aspects of the immunogenetics of β-cell destruction remain unclear and nonhistocompatibility genes within the MHC may increase the risk of developing IDDM or influence autoantibody production against several target antigens (insulin, glutamic acid decarboxylase, carboxypeptidase H) (5–7).

Several studies suggest a role for heat shock proteins (HSPs) in the pathogenesis of autoimmune disorders (8–11). A role for these molecules in antigen presentation has been reported (12). Moreover, autoantibodies against HSP70 and HSP90 have been found in patients affected by systemic lupus erythematosus and polymyositis (13,14), and it has been suggested that HSPs could also be a target antigen in IDDM (15). Elias et al. (16) reported autoantibodies against a 65,000-M_r HSP in the NOD mouse before IDDM onset (16), but no reactivity against HSPs was found in diabetic patients (17; unpublished observations). Three genes coding for a 70,000-M_r HSP (HSP70) map within the MHC (Fig. 1) located 92 kb telomeric to the C2 gene and 280 kb centromeric to the tumor necrosis factor–α gene: two intronless genes, *HSP70–1* and *HSP70–2*, encode an identical protein product of 641 amino acids. The *HSP70* duplicated loci

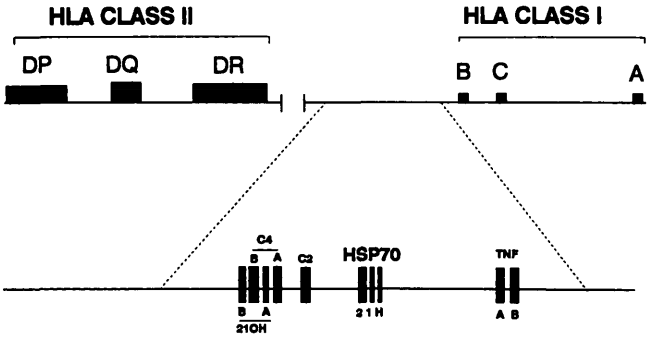

FIG. 1. MHC including the *HSP70* locus. Three *HSP70* genes (*HSP70–2, HSP70–1, HSP70-Hom*) are shown. TNF, tumor necrosis factor.

are 12 kb apart, and a third intronless gene (*HSP70-Hom*) is located ~4 kb telomeric to the *HSP70–1* gene (18). The amino acid sequences of the products of the *HSP70* genes within the MHC reveal a ≥90% homology (18). Restriction-fragment–length polymorphism (RFLP) analysis of the *HSP70* genes performed by Goate et al. (19) detected polymorphic variation with four enzymes (*Pst*I, *Pvu*II, *Bst*I, *Bgl*I). All RFLPs segregated independently. On the basis of cell hybrid data, only the *Pst*I RFLP has been assigned to chromosome 6 (19) and referred to the *HSP70–2* gene, which has only one *Pst*I restriction site. This study examined the association of this polymorphism with IDDM. Demaine et al. (20), in a cross-sectional study not analyzing complete families, reported that the *HSP70–2* 8.5-kb allele is increased in frequency in diabetic patients and often associated with a C4A deletion on the [HLA-A1,B8,DR3]-extended haplotype (20).

To more completely assess the association of *HSP70–2* alleles with diabetes-susceptibility and other MHC alleles, we performed RFLP analysis of the *HSP70–2* gene in 29 MHC-typed families in which one or more members were affected by IDDM.

RESEARCH DESIGN AND METHODS

We analyzed 29 families with 47 diabetic patients and 102 healthy nondiabetic relatives under clinical follow-up and already MHC typed. Families from the New England area were identified from two sources: *1*) random diabetic families and *2*) families identified through the screening for autoantibodies of first-degree relatives of diabetic patients. The age of onset of diabetes ranged from 5 to 35 yr. Fifteen families had 1 individual with diabetes, 12 families had 2, and 2 families had 3 individuals with diabetes. DR allele distribution among our (white) diabetic patients was similar to previously reported studies (21). Those haplotypes found among IDDM patients were defined as diabetic haplotypes, although they may also appear in nondiabetic family members. Those detected in nondiabetic relatives but not in patients represent control haplotypes (3). Because at least 30% of MHC haplotypes in nondiabetic whites consist of fixed or conserved stretches of DNA generating extended haplotypes defined by HLA-B, complotype, and DR allelic sets, if an MHC allele occurs on such a haplotype, it will be found with increased frequency among diabetic patients compared with nondiabetic individuals (4).

RFLP ANALYSIS OF THE *HSP70–2* GENE ON CHROMOSOME 6

Genomic DNA extraction. For each family member, DNA was extracted from peripheral blood mononuclear cells obtained by Ficoll-hypaque density gradient centrifugation or from Epstein-Barr virus cell lines. In brief, $3–5 \times 10^7$ peripheral blood mononuclear cells was incubated at 37°C in 2 ml TNE (10 mM Tris [pH 7.5], 10 mM NaCl, 1 mM EDTA) and 6 ml lysis buffer (0.2 M NaCl, 80 mM Tris, 0.4 M EDTA, 1% sodium dodecyl sulfate, 0.1 mg/ml proteinase K) for 16 h. Proteins and lipids were removed by phenol-chloroform extraction, and DNA was recovered by dialyzing extensively against TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8]). Ten micrograms of DNA for each sample was digested with the restriction enzyme *Pst*I (10 U/μg) at 37°C overnight. Ten percent vol 3 M NaAc and 2.5 vol cold ethanol (−20°C) were added to each digested sample, and the samples were incubated for 2 h at −20°C. DNA was collected by centrifugation (15,000 rpm for 15 min at 4°C) in a microfuge. The DNA was resuspended in 50 μl TE buffer (pH 8), incubated at 65°C for 10 min, and then electrophoresed on 1% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 35 V overnight. *Hin*dIII-digested bacteriophage-λ DNA was run in parallel on each gel for molecular weight markers.

Southern blotting. DNA was blotted onto nylon membranes (Oncor) that were incubated in hybridization solution (50% formamide, 6× sodium citrate–sodium chloride (SSC), 10% dextran sulfate, 10 μg/ml salmon sperm DNA, 0.1% Denhardt's solution) at 45°C for 16 h (22). The whole human *HSP70–1* gene (a *Bam*HI/*Hin*dIII 2.3-kb insert subcloned in *pAT153*) was used as a probe for our experiments and was kindly donated by Richard Morimoto (Northwestern Univ., Evanston, IL) (23). This gene is an uninterrupted sequence of 2.3 kb, which directs the synthesis of a 2.6-kb mRNA, which in turn is translated into the *HSP70* (23). The *Bam*HI/*Hin*dIII probe hybridizes to all three *HSP70* genes within the MHC and to other *HSP70* genes elsewhere in the genome. The probe was labeled with ^{32}P-dCTP with the random primer method (24). The radioactive probe was then incubated with the nylon membranes at 45°C for 16 h in hybridization solution (indicated above). Membranes were washed three times in 0.1× sodium citrate–sodium chloride, 0.1× sodium dodecyl sulfate at 45°C for 15 min and then at 52°C for 1 h before exposure with intensifying screen to Kodak XAR-5 film for 3–6 days at −70°C.

Statistical analysis. Comparison between groups was with Fisher's exact test.

RESULTS

With the restriction enzyme *Pst*I, two polymorphic bands (8.5- and 9.0-kb) were detected with the *HSP70* probe. The 8.5-kb allele was present on 62% (41 of 66) of diabetic haplotypes compared with 43% (20 of 46) of control haplotypes ($P = 0.03$, Fisher's exact test). The 47

TABLE 1
Heat shock protein alleles distribution on DR1, DR2, DR3, DR4, and DR5 control and diabetic haplotypes

	Diabetic	Control
DR1		
8.5-kb Allele	5 of 7 (71)	3 of 4 (75)
9.0-kb Allele	2 of 7 (28)	1 of 4 (25)
DR2		
8.5-kb Allele	0 of 2 (0)	0 of 9 (0)
9.0-kb Allele	2 of 2 (100)	9 of 9 (100)
DR3		
8.5-kb Allele	23 of 26 (88)	3 of 3 (100)
9.0-kb Allele	3 of 26 (12)	0 of 3 (0)
DR4		
8.5-kb Allele	7 of 20 (35)	5 of 11 (45)
9.0-kb Allele	13 of 20 (65)	6 of 11 (55)
DR5		
8.5-kb Allele	0 of 1 (0)	2 of 6 (33)
9.0-kb Allele	1 of 1 (100)	4 of 6 (77)

Values are *n* with percentages in parentheses.

diabetic patients analyzed were more often homozygous for the 8.5-kb allele than unaffected relatives (IDDM homozygous 8.5 = 47%, 22 of 47 vs. control homozygous 8.5 = 25%, 26 of 102; IDDM heterozygous 8.5/9.0 = 32%, 15/47, and control heterozygous 8.5/9.0 = 53%, 53/102; $P = 0.006$, Fisher's exact test). Analyzing only the 29 original IDDM probands, the observed distribution of homozygotes and heterozygotes was not significantly different from that expected from the Hardy-Weinberg equilibrium (8.5/9.0 = 11, expected = 12.8; 8.5/8.5 = 13, expected = 13.1; 9.0/9.0 = 5, expected = 3.1).

To better understand the association between *HSP* alleles and diabetic haplotypes, we analyzed haplotypes subdividing them by the serologically determined DR alleles (Table 1). For DR3 haplotypes (mostly [HLA-B8, SC01, DR3], $n = 18$), we found that only 3 of 29 DR3 haplotypes had the 9.0-kb allele. Thus, 90% of DR3 haplotypes carry the 8.5-kb allele. None of the 3 unusual DR3 diabetic haplotypes with the 9.0-kb allele were the extended haplotypes [HLA-B8, SC01, DR3] or [HLA-B18, F1C30, DR3] usually associated with the 8.5-kb allele and with diabetes susceptibility ($n = 0$ of 20 vs. 3 of 9 nonextended haplotypes, $P = 0.02$). Because the size of control (occurring only in nondiabetic family members) DR3 haplotypes in the families studied was small ($n = 3$), we studied three additional diabetic families with DR3 control haplotypes. Only a [HLA-B7, SC31, DR3] haplotype had the 9.0-kb allele. Thus, 5 of 6 DR3 control haplotypes and 28 of 31 diabetic haplotypes carried the 8.5-kb allele. Moreover, 28 control DR3 haplotypes from 14 independent homozygous Epstein-Barr virus–transformed lymphoblastoid cell lines with defined extended haplotypes [HLA-B8, SC01, DR3] and [HLA-B18, F1C30, DR3] were analyzed, and all were homozygous for the 8.5-kb allele. Thus, considering all the DR3 haplotypes analyzed, 0 of 49 of the above DR3 extended haplotypes carried the 9.0-kb allele versus 4 of 11 DR3 nonextended haplotypes ($P < 0.001$).

The 9-kb allele was the only *HSP70–2* allele present on nine DR2 control haplotypes and two DR2 diabetic haplotypes (DR3 vs. DR2 haplotypes, $P = 10^{-7}$). The frequency of the *HSP70–2* alleles was not significantly different on DR4 haplotypes from the overall distribution of *HSP70–2* alleles. The 9.0-kb allele was found on 55% (6 of 11) of normal and 65% (13 of 20) of diabetic DR4 haplotypes (Table 1).

***HSP70–2* polymorphism and autoantibody production.** We recently reported an association between DR4 and high levels of competitive insulin autoantibodies (5). We investigated whether there was an association of *HSP70–2* alleles with insulin-autoantibody levels in autoantibody-positive nondiabetic DR4$^+$ first-degree relatives of IDDM patients. No association with mean insulin-autoantibody levels was found for the two *HSP70–2* alleles.

DISCUSSION

The aim of this study was to investigate the potential contribution of polymorphism of the *HSP70–2* gene on chromosome 6 to diabetes susceptibility in 29 HLA-typed IDDM families. This gene is located between the C2 and tumor necrosis factor genes, of which alleles of both have been reported to be associated with increased diabetes risk (25). Confirming prior studies with the enzyme *Pst*I and the *HSP70* probe (20), two alleles with a molecular size of 8.5- and 9.0-kb were identified, and the 8.5-kb allele was slightly increased in frequency in diabetic patients (62%) compared with control haplotypes (43%).

Typing of complete families for haplotype assignments indicated that the association with diabetic haplotypes of the 8.5-kb allele was due to the presence, among our families, of highly conserved stretches of DNA generating extended haplotypes carrying the 8.5-kb allele on DR3 and the 9.0-kb allele on DR2 haplotypes. Indeed, 3 diabetic haplotypes contained DR3 and the 9.0-kb allele. None of these three haplotypes were the extended haplotypes [HLA-B8, SC01, DR3] or [HLA-B18, F1C30, DR3] usually associated with the 8.5-kb allele and with diabetes susceptibility ($n = 0$ of 20 vs. 3 of 9 nonextended haplotypes, $P = 0.02$). The association between these DR3-extended haplotypes and the 8.5-kb allele was confirmed by analyzing additional haplotypes containing DR3. Twenty-eight DR3-extended haplotypes came from control-independent homozygous cell lines, and three additional families were analyzed with a DR3 haplotype occurring only in nondiabetic family members. In total, none of the above 49 DR3-extended haplotypes carried the 9.0-kb allele compared with 4 of 11 nonextended haplotypes ($P < 0.001$).

A similar conservation of haplotypes including *HSP70–2* alleles was found on 11 DR2 haplotypes, of which all carried the 9.0-kb allele. Among these haplotypes were the extended haplotypes [HLA-B7, SC31, DR2] ($n = 6$) and [HLA-B18, S042, DR2] ($n = 2$).

Despite the linkage of the 9.0-kb allele with DR2 haplotypes, usually associated with resistance to diabetes, there were two diabetic DR2 haplotypes that carried the 9.0-kb allele. In a previous study, we reported the

sequences of the HLA class II genes from three siblings who carried one of these haplotypes (26). They had the [HLA-B7, SC31, DR2] haplotype with a unique DQB1*0402 allele (replacing the usual protection-associated DQB1*0602 allele). Thus, we believe the 9.0-kb allele is usually part of DR2-extended and nonextended haplotypes but is not itself associated with resistance to IDDM.

Because antibodies to a series of target antigens (especially enzymes contained in the secretory granules) have recently been associated with IDDM (insulin, glutamic acid decarboxylase, carboxypeptidase H), it is becoming clear that autoantibody production in IDDM patients is heterogeneous. The possible involvement of HSP70 in antigen presentation and its presence in the same vesicles as other autoantibody targets in diabetes and in other autoimmune diseases suggest that a polymorphism of its gene within the MHC could influence autoantibody production.

We reported a strong association between insulin autoantibody levels and DR4 in first-degree relatives of patients with IDDM (5). We investigated whether the *HSP70–2* alleles were associated with insulin autoantibody levels in $DR4^+$ first-degree relatives, but we did not find an association. It remains to be tested whether *HSP70–2* alleles are associated with glutamic acid decarboxylase or carboxypeptidase-H autoantibody production.

In conclusion, our data indicate an association of the 8.5-kb *HSP70–2* allele with diabetic haplotypes, which was also reported by Demaine et al. (20). Our analysis of families indicates that this association with diabetes is due to the presence of extended haplotypes that, in this study, always carried the 8.5-kb allele with DR3 and the 9.0-kb allele with DR2. Thus, the 8.5-kb allele is part of DR3-extended haplotypes [HLA-B8, SC01, DR3] and [HLA-B18, F1C30, DR3] associated with diabetes susceptibility, whereas the 9.0-kb allele is carried by the protective DR2-extended haplotypes [HLA-B7, SC31, DR2] and [HLA-B18, S042, DR2]. Because of such extended haplotypes, no evidence for an independent role for *HSP70–2* variants on diabetes susceptibility was found. Our study provides a further characterization of extended haplotypes associated with IDDM and indicates by analysis of families that the association of *HSP70–2 PstI* polymorphism with IDDM results from its association with diabetes-related extended haplotypes.

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