

Prevalence of heat shock protein in patients with Meniere's disease and allergy

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OBJECTIVE: The study purpose was to investigate the prevalence of elevated heat shock protein 70 (HSP-70) in patients with Meniere's disease who have milk allergy compared with those who are not allergic to milk.

METHODS: Fifty-five patients with Meniere's disease and allergy in whom milk allergy had been confirmed by intradermal progressive dilutional food testing or skin testing to milk antigen were included. Blood serum was tested for HSP-70 elevation with a Western blot assay using bovine renal extract. The 29 women and 26 men ranged in age from 29 to 76 years (mean age 52.8 years). Forty percent of the patients had bilateral Meniere's disease.

RESULTS: Overall prevalence of HSP-70 elevation was 29.1%. This was higher in bilateral patients (50%) than unilateral patients (15%) ($P \leq 0.007$). The effect was based primarily on elevated HSP-70 in bilateral (62.5%) and unilateral (7.7%) patients in those with no milk allergy ($P \leq 0.006$). The prevalence of HSP-70 elevation was actually lower in those with milk allergy (19.2%) than in those with no milk allergy (37.9%). This difference was statistically significant only in the subset of patients who were <50 years old (0% vs 33.3%, respectively; $P \leq 0.04$).

CONCLUSIONS: We found no relationship between the presence of antibodies to HSP-70 and allergy to milk. The significance of elevated HSP-70 in patients with Meniere's disease has yet to be defined. (Otolaryngol Head Neck Surg 2002;126:677-682.)

Since Prosper Meniere first described the symptom complex that bears his name, its etiology has been ascribed to many possible causes, including underlying cochlear vascular pathology,^{1,2} abnormalities in endolymph production or absorption,^{3,4}

abnormal ion homeostasis,^{5,6} and allergic,⁷ autoimmune or inflammatory,⁸⁻¹⁰ and viral^{11,12} causes.

In 1990, Harris and Sharp¹³ first described the presence of a serum antibody against a 68-kD protein in Western blot assays against bovine inner ear antigen. Antibody activity in the form of a single or double band migrating at a molecular mass of 68 kD was found in the sera of 35% of patients with a rapidly progressive sensorineural hearing loss thought to be consistent with autoimmune inner ear disease (AIED), whereas only 7% of the sera of normal patients had such a finding. This antibody was also found in the sera of guinea pigs immunized with cochlear protein extract that developed subsequent hearing loss. Moscicki et al¹⁴ reported in 1994 that IgG antibodies to the 68-kD protein were usually present in patients with active clinical symptoms of suspected AIED and appeared to predict a positive response to treatment with steroids. The 68-kD antibody was subsequently identified by both Billings et al¹⁵ and Bloch et al¹⁶ as heat shock protein 70 (HSP-70). Although there appears to be a strong relationship between the presence of HSP-70 and AIED, its precise role, as either a possible antigen or epiphenomenon, has not been established.

In 1995, Gottschlich et al¹⁷ reported that the sera of 30% of patients with Meniere's disease contain antibodies to the 68-kD inner ear antigen compared with 5% of control patients. Subsequent studies by Shin et al¹⁸ and Rauch et al¹⁹ found anti-HSP-70 proteins in 33% and 47%, respectively, of patients with Meniere's disease.

The technique involved in the Western blot test to detect antibodies against inner ear antigens involves an overnight incubation with casein, a protein derived from cow's milk.¹⁴ Derebery and Berliner²⁰ recently reported a prevalence rate of allergy in 40% of patients with Meniere's disease compared with 27% of patients with other otologic conditions and 20% of the normal population. Cow's milk is reported as the most prevalent food allergen worldwide.²¹ In the present study, we investigated whether the prevalence of an elevated

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Table 1. Demographic characteristics of HSP-70 study subjects

Characteristic	n (%)
Gender	
Male	26 (47.3)
Female	29 (52.7)
Race/ethnicity	
Non-Latino white	50 (90.9)
Asian	2 (3.6)
Black	1 (1.8)
Hispanic	1 (1.8)
American Indian/Alaskan	1 (1.8)
Ear(s) with Meniere's disease	
Right	15 (27.3)
Left	18 (32.7)
Both	22 (40.0)

Age at study was 51.3 years (median; range, 30 to 76 years).

level of HSP-70 is different in allergic patients with Meniere's disease who have milk allergy compared with those not allergic to milk.

METHODS

Adult patients meeting the American Academy of Otolaryngology-Head and Neck Surgery criteria for the diagnosis of Meniere's disease were eligible for inclusion in this study.²² Patients with a history of thermal burns, diabetes mellitus, Alzheimer's disease, cancer, or recent influenza, fever, or ulcerative colitis were excluded because these conditions are associated with increased levels of HSP-70. Those who recently used medications such as steroids that may affect HSP-70 serum values were also excluded. Sixty patients were enrolled and tested for the presence of milk allergy by either intradermal progressive dilutional food testing (IDPFT)²³ or intradermal skin testing to milk antigen, and they provided information on their milk products consumption. In addition, study subjects had blood serum tested for HSP-70 elevation with an immunoassay previously described (see later).^{14,19} The outside testing laboratory was blinded regarding milk allergy and milk consumption status. Results of HSP-70 testing were not available for 5 patients due to laboratory problems.

Thus, the final subject population included 55 patients who met the inclusion/exclusion criteria and for whom both HSP and milk allergy data were obtained. Table 1 provides a demographic description of the study population. There were a

similar number of males and females. Mean age at the time of study was 52.8 years (SD 12.2 years, range 29.5 to 76.1 years). Forty percent of the subjects had bilateral Meniere's disease.

Immunoassay Technique

The technique used has been described in detail but is summarized here.^{14,19} Polyvinylidene difluoride membranes (Immobilon; Millipore Corp, Bedford, MA) were used instead of nitrocellulose membranes. All sera were initially screened at a dilution of 1:10 using bovine renal extract (which has been shown to be equivalent to inner ear extracts) as antigen for detection of antibody to the 68-kD protein associated with idiopathic, progressive, bilateral sensorineural hearing loss. Serum was incubated with the membrane for 4 hours at 20°C, followed by washing and incubation for 1 hour at 20°C with antihuman IgG-specific anti-serum conjugated to alkaline phosphatase. After further washing, the membrane was incubated with substrate for 4 minutes at 20°C.

Stress-inducible recombinant human HSP-70 (rhHSP-70) was obtained commercially (Stress-Gen Biotechnologies Corp, Vancouver, British Columbia, Canada). According to the manufacturer, the protein was prepared in *Escherichia coli* and purified to remove *E coli* proteins. Stress-inducible recombinant bovine HSP-70 (rbHSP-70) was prepared in *E coli* from the complementary DNA (cDNA) encoding bHSP-70; it was also purified to remove *E coli* protein. All serum samples were tested for immuno-reactivity against bovine renal extract and rbHSP-70. Sera containing antibodies to either of the bovine antigens were also tested for reactivity against rhHSP-70. Recombinant proteins were tested with serum diluted 1:50. Positive control serum containing antibody to HSP-70 and capable of reacting with renal extract, rbHSP-70, and rhHSP-70 was included in each assay.

Data Analysis

A Fisher exact test was used to determine whether milk allergy status was associated with HSP elevation status, both for the overall group of 55 subjects and for various subgroups based on stratification by current milk product consumption, ears involved (unilateral or bilateral), age, and gender. A logistic regression was also used to control for possible con-

Table 2. Relationship between elevation in HSP-70 levels and milk allergy overall and in subsets based on ear involvement

	Elevated HSP-70 levels				<i>P</i>
	Milk allergy		No milk allergy		
	Total	n (%)	Total	n (%)	
Overall	26	5 (19.2)	29	11 (37.9)	NS
Ear					
Unilateral	20	4 (20.0)	13	1 (7.7)	
Bilateral	6	1 (16.7)	16	10 (62.5)	
<i>P</i>		NS		≥0.006	
Current consumption*	21	5 (23.8)	25	9 (36.0)	NS
Current consumption–ear*					
Unilateral	17	4 (23.5)	13	1 (7.7)	
Bilateral	4	1 (25.0)	12	8 (66.7)	
<i>P</i>		NS		≤0.004	

*Current, regular consumption of milk products, at least once every fourth day.

founding of factors. Criterion for statistical significance was set at $P \leq 0.05$, 2-tailed.

RESULTS

The overall prevalence of HSP-70 elevation was 29.1% (16 of 55). The overall prevalence of milk allergy was 47.3% (26 of 55). The relationship between HSP-70 elevation and milk allergy is summarized in Table 2. The prevalence of HSP-70 elevation was actually lower in those with milk allergy (19.2%) than in those with no milk allergy (37.9%), although this difference did not achieve statistical significance.

A subset of the patients no longer had current, regular milk product consumption. Current, regular milk product consumption was defined as having milk products at least once every fourth day. Forty-six of the 55 subjects currently consumed milk on a regular basis, whereas 7 no longer consumed milk products. The prevalence of HSP-70 elevation was 30.4% (14 of 46) in those with current, regular milk product consumption and 14.3% (1 of 7) in those without current, regular milk product consumption. This difference was not statistically significant, perhaps due to the small number of subjects in the one group. To eliminate possible confounding due to milk product consumption, the relationship between HSP-70 and milk allergy was reexamined in the 46 subjects with reported current, regular milk product consumption. These results are also shown in Table 2. The prevalence of HSP-70 elevation was 23.8% in

the milk allergy group and 36.0% in those without milk allergy. Again, this difference was not statistically significant.

Forty percent of the patients had bilateral disease. The prevalence of HSP-70 elevation was significantly higher in these patients (50%) than in those with unilateral disease (15.2%) ($P \leq 0.007$). Table 2 shows the relationships between ear involvement and HSP-70 for those with and without milk allergy. There were no significant differences in the prevalence of elevated HSP-70 between those with and without milk allergy, although within the bilateral group there was a tendency for higher prevalence in the group without a milk allergy. Within the group without a milk allergy, there was a significant difference in HSP-70 findings between the unilateral and bilateral cases, with bilateral patients having a significantly higher prevalence of elevated HSP-70. Findings were similar when evaluating only those with current, regular milk consumption.

The relationship between milk allergy and HSP-70 was also stratified by age and gender (Table 3). The prevalence of HSP-70 elevation in the milk allergy group was substantially lower in younger (<50 years) relative to older patients (≥50 years). None of the 13 younger patients with milk allergy had elevated HSP-70 levels, whereas 38.5% of the older patients with milk allergy did have elevated HSP-70 levels. This difference was statistically significant ($P \leq 0.04$). Within the younger group, there was a significant difference

Table 3. Relationship between elevation in HSP-70 levels and milk allergy in subsets of the study population based on age and gender

	Elevated HSP-70 levels				<i>P</i>
	Milk allergy		No milk allergy		
	Total	n (%)	Total	n (%)	
Age (y)					
<50	13	0 (0.0)	12	4 (33.3)	≥0.04
≥50	13	5 (38.5)	17	7 (41.2)	NS
Current consumption–age*		≤0.04		NS	
<50 y	11	0 (0.0)	11	4 (36.4)	
≥50 y	10	5 (50.0)	14	5 (35.7)	
<i>P</i>		≤0.01		NS	
Gender					
Male	13	2 (15.4)	13	5 (38.5)	NS
Female	13	3 (23.1)	16	6 (37.5)	NS
<i>P</i>		NS		NS	

*Current, regular consumption of milk products, at least once every fourth day.

in the prevalence of elevated HSP-70 levels between those with and without milk allergy; those without milk allergy had a higher prevalence. This difference did not appear within the older age group. When considering just the 21 patients with milk allergy and current milk product consumption, results were similar.

Age category was highly related to ear involvement, with bilateral patients also being more likely to be older patients. Thus, much of the age effect may likely be related to ear involvement. A logistic regression entering ear involvement first showed no additional effect of age group.

The prevalence of HSP-70 elevation did not appear to be related to gender.

DISCUSSION

The Western blot immunoassay shows the presence of circulating antibodies that react with both bovine and inner ear antigens of molecular mass 68 kD, which have subsequently been shown to be HSP-70, in a significant percentage of patients with both suspected AIED and Meniere's disease. Kumar and Rajadhyaksha²⁴ reported 2 bands around molecular mass 70 kD on Western blot that show evidence of immunoreactivity: 70 and 72 kD. Hirose et al²⁵ concluded that the Western blot for HSP-70 shows the best predictive value for corticosteroid responsiveness in patients with sensorineural hearing loss. Bloch et al²⁶ suggested that the stimulus for anti-HSP-70 antibody pro-

duction in Meniere's disease and suspected AIED might be an entirely unrelated, unknown protein that shares epitopes with HSP-70. They postulated that an inner ear-specific HSP-70 might exist, becoming immunogenic to the host because of its overexpression secondary to inflammation caused by another agent.

Heat shock or heat stress proteins are a family of proteins whose synthesis is upregulated in response to environmental stressors such as hyperthermia. The proteins resulting in the heat shock response to environmental stressors assist in the folding and reassembly of denatured proteins, protecting the cell from damage. Members of the HSP-70 family serve as molecular "chaperones" involved in the controlled folding of proteins. Their actions serve to maintain the appropriate configuration of proteins to permit their translocation out of the endoplasmic reticulum and mitochondria, as well as to inhibit nonproductive folding of proteins or their inappropriate aggregation.²⁷ Pasteurized cow's milk, which has obviously been heated during the pasteurization process, serves as the source of casein used in the Western blot assay for HSP-70 and would share epitopes with HSP-70. If milk sensitivity served as the inflammatory stimulus, we hypothesized that we might find a greater prevalence of HSP-70 in those patients with Meniere's disease who also have known immune reactivity to milk in comparison with those who are not milk allergic.

Our prevalence rate of HSP-70 in 29% of patients with Meniere's disease is consistent with previously reported studies. Our lower prevalence of antibodies to HSP-70 in patients with milk allergy compared with those without milk allergy was unexpected. Because the treatment of food allergy is elimination of the reactive food from the diet, which would likely result in an eventual lowering of sensitivity and antibody formation to the food, we further analyzed whether the current consumption of milk ("cheating") in those patients previously diagnosed with milk allergy would have an impact on their HSP-70 antibody response compared with those who had no previously diagnosed reaction to milk. Again, there was no significant relationship.

We found a higher prevalence of elevated HSP-70 in patients with bilateral disease, as have others.^{14,19} Bilateral disease can be thought of as one marker of disease severity. However, this difference between bilateral and unilateral patients occurred within the group without milk allergy. Of patients with milk allergy, there was a statistically significant higher prevalence of an elevated antibody to HSP-70 in those relatively older (≥ 50 years) compared with the younger cohort. This might suggest a confounding variable of age: the older the subject and presumably the longer the exposure to milk antigen, the greater the likelihood of antibody formation. However, the younger patients without milk allergy were found to have a statistically significantly higher prevalence of antibodies than those with milk allergy. Thus, one might wonder if the severity of symptoms or duration of disease was related to the HSP-70 results independent of the milk allergy issue. We did not obtain data from our patients specifically regarding duration or severity of disease or steroid responsiveness of symptoms, which have been shown to positively relate to the presence of antibodies to HSP-70. Our results suggest the possibility of a relationship of HSP-70 to disease duration and/or severity, if severity is defined by bilaterality. However, Rauch et al¹⁹ did not find a relationship between vestibular or auditory symptoms and the status of antibodies to HSP-70.

We previously reported the most common food allergen found in patients with Meniere's disease is wheat (68.2%), followed by milk in 59.6%.²⁰ It

is possible that our diagnosis of the presence or absence of milk allergy was flawed. Inhalant allergies largely trigger an immediate Type I Gel and Coombs hypersensitivity reaction, whereas food antigens have been shown to stimulate all 4 initially described Gel and Coombs reactions. The double-blind, placebo-controlled oral challenge food test has been described as the "gold standard" of food allergy testing. It is possible that the diagnosis of food allergy by IDPFT, as was done in many of these subjects, or any skin testing technique lacks the specificity and sensitivity of inhalant allergy diagnosis. However, King²³ has shown an excellent correlation between the IDPFT and oral challenge testing.

The significance of the presence of antibodies to HSP-70 in patients with Meniere's disease or AIED is not known. All eukaryotic and prokaryotic cells contain stress proteins, which are necessary for normal cell function. Heat shock proteins, so named because of their first discovery in heat-stressed *Drosophila*, were initially named because of their increased synthesis after stress but have subsequently been found to be consistently present at some levels in normal, nonstressed tissue. Because HSP-70 is an endogenous protein found throughout the body, it has not been identified as a target antigen. It has been suggested in other studies that antibodies formed against the stress proteins of infecting pathogens such as bacteria may in turn recognize the host human stress proteins and result in an autoimmune reaction.²⁸ Several authors have hypothesized that the mechanism for the observed hearing loss is that antibodies directed against foreign HSP-70 may also recognize host HSP-70 on inner ear cells, thus causing cochlear dysfunction through an autoimmune reaction.^{15,16,19} However, Trune et al²⁸ found that mice injected with foreign HSP-70, while developing antibodies against it, showed no evidence of either abnormal immune complex formation, systemic autoimmune or hypersensitivity reaction, or change in auditory function.

Alternatively, the presence of elevated antibodies may serve as a nonspecific epiphenomenon such as an elevated erythrocyte sedimentation rate. Shinnick²⁹ noted that bacterial stress proteins are upregulated during the attack of the host immune system on invading bacteria. When the bacteria

are eventually lysed, a large amount of HSP-70 is dumped into the host circulation, generating antibody formation.²⁹ Subsequent symptom production such as hearing loss would result from the actual systemic infection rather than from the antibody production against foreign HSP-70. An elevation in antibody would simply serve as a coincident marker rather than as a cause of hearing loss.

CONCLUSION

Although we found some evidence that HSP-70 might have a relationship to the severity of disease in patients with Meniere's disease, we found no relationship between the presence of antibodies to HSP-70 and allergy to milk. The significance of elevated HSP-70 in patients with Meniere's disease has yet to be defined.

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