

Immunity to Heat Shock Proteins and Neurological Disorders of Women

G. Birnbaum* and L. Kotilinek

Department of Neurology, University of Minnesota, School of Medicine, Minneapolis, MN

KEY WORDS

heat shock proteins; multiple sclerosis; myelin protein; experimental autoimmune encephalomyelitis

Autoimmune diseases are generally more common in women. One of these is multiple sclerosis (MS), a disease that has a female:male ratio of about 2–3:1. The etiology of MS is not known, but there is considerable circumstantial evidence that an immune response to central nervous system (CNS) myelin proteins is important in pathogenesis.

Multiple sclerosis is a multifocal inflammatory disease affecting only the CNS. The target of the inflammatory response is CNS myelin and the myelin-producing cells, oligodendrocytes. The myelin is destroyed in patches, resulting in the formation of astrocytic scars called plaques. Areas of inflammation are characterized by accumulations of large numbers of lymphocytes (predominantly T cells) and macrophages. Increased concentrations of cytokines and chemokines also are present in regions of inflammation, as are antibodies to myelin proteins.^{1–5}

The course of MS is highly variable, ranging in severity from an incidental finding at autopsy to severe disability and death within months to years of onset. The variables responsible for these differences in clinical course are not known, but at least two events have been associated with changes in disease activity. These are infections, especially viral infections, and the postpartum period.

While there are several possible mechanisms to explain the association between infections and attacks or relapses of MS, we proposed that immune responses to an infectious agent's heat shock pro-

tein (hsp) cross-reacted with endogenous hsp present at sites of CNS inflammation that are the hallmark of the disease process in MS. Recruitment of anti-hsp responsive lymphocytes to such regions, with resulting release of toxic cytokines, could be a significant factor in either amplifying or perpetuating the MS disease process. We initiated a series of studies to support this hypothesis.

STRESS PROTEINS AS TARGETS OF IMMUNE RESPONSES

There are several characteristics of hsps that increase their potential to act as antigens in the development of autoimmune diseases.⁶ First, hsps are phylogenetically conserved. Thus, there is greater than 50% sequence homology between certain prokaryotic hsps and that of mammalian cells.^{6–9} Second, hsps are the immunodominant antigens for many infectious agents, including bacteria, mycobacteria, and parasites.^{10,11} Third, hsps are expressed at sites of acute and chronic inflammation.^{12,13} Thus, exposure to an infectious agent in a genetically susceptible host at the appropriate time during the host's development may result in an immune response to the infecting agent's hsp that either cross-reacts with normal host hsp or cross-reacts with organ-specific proteins, resulting in an autoimmune disease.

HEAT SHOCK PROTEINS AND MULTIPLE SCLEROSIS

The evidence that MS is an autoimmune disease, or for that matter, an immunologically mediated

*Correspondence to: Gary Birnbaum, MD, Department of Neurology, Box 295, UMHC, Minneapolis, MN 55455. E-mail: birnb001@tc.umn.edu

disease, is circumstantial. Numerous attempts to find immune responses to unique antigens in persons with MS or to find immune responses to myelin and other antigens not present in normal individuals have not succeeded to date. Persons with MS do have increased immune responses to a variety of myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin/oligodendrocyte glycoprotein (MOG),¹⁴⁻²⁰ and increased numbers of activated T cells responsive to these antigens.^{14,21} In addition, many of the myelin antigens recognized by MS (and normal) T cells are potent autoantigens capable of inducing experimental autoimmune encephalomyelitis (EAE), an animal model for MS.²² The presence of increased numbers of myelin reactive cells in persons with MS clearly indicates sensitization to such proteins. However, it is not clear whether the heightened immune responses to these antigens are primary or secondary to the disease process, nor is it known what role these responses play either in disease initiation or perpetuation.

As noted above, there is a well documented association between MS disease activity and antecedent infections.^{23,24} The study by Sibley and associates showed a clear association between infections, usually viral, and attacks or worsening of disease. While preexistent symptoms of MS will worsen during the acute phase of an infection, especially if there is an associated fever, true disease exacerbations occur days to weeks after recovery.

Several mechanisms could explain the association between infection and the worsening of an organ-restricted, inflammatory, possibly autoimmune disease:

1. Infections result in increased concentrations of circulating cytokines. Some of these, such as tumor necrosis factor (TNF) or interferon (INF)-gamma, in addition to having direct effects on oligodendrocytes, could alter the blood-brain barrier, allowing easier entry of myelin-specific immunocompetent cells and/or antibodies. Cytokines could also nonspecifically activate anti-myelin T cells resident within the CNS of MS patients.
2. Infections result in immune responses to the infectious organism's hsps. These immune responses could cross-react with phylogenetically

conserved hsp expressed by oligodendrocytes in areas of inflammation.²⁵

3. Immune responses to an infectious agent's hsp could cross-react with myelin antigens, resulting in myelin destruction.
4. A combination of the above effects may be important.

A variety of approaches have been used to determine whether hsps, or immune responses to hsps, are involved in the pathogenesis of MS. These include studies of the following areas:

- a. The expression of hsp in the brains of persons with MS
- b. Immune responses to hsp in MS patients
- c. Cross-reactivity between hsp and CNS myelin.

In addition, several laboratories studied the distribution of gamma/delta T cells in MS brains and spinal fluids.²⁶⁻²⁹ While most T cells that respond to hsp express the alpha/beta receptor complex, a high proportion ($\geq 50\%$) of T cells expressing gamma/delta receptors respond to hsp. Results of these studies revealed that there were increased numbers of gamma/delta T cells around areas of demyelination as well as in the spinal fluids during acute exacerbations and that greater numbers of gamma/delta cells were found in the cerebrospinal fluid (CSF) of individuals with shorter duration of disease.²⁷ These data provide circumstantial support for the hypothesis that immune responses to hsps may play a role in MS pathogenesis.

Expression of Heat Shock Proteins in Multiple Sclerosis Central Nervous System

Several laboratories studied expression of hsps in areas of MS demyelination. Selmaj and his associates^{28,30} demonstrated expression of Hsp65 in immature oligodendrocytes at the edges of MS plaques. There also was constitutive expression of Hsp72 in MS and non-MS brain, especially in astrocytes. Wucherpfennig et al.²⁶ demonstrated expression of Hsp60 in foamy macrophages at the edges of acute plaques and expression of Hsp90 in reactive astrocytes. In our studies, we utilized a panel of 20 monoclonal and polyvalent antibodies to human and mycobacterial hsp to immunocytochemically stain fixed, frozen sections of normal and MS brains.³¹ Patterns of staining varied with

different antibodies. Some antibodies stained cell bodies of neurons and astrocytes. Others stained neurofilaments. A small number of antibodies (discussed below) stained normal myelin. In sections of spinal cord, staining of both central and peripheral myelin was observed. We could not detect increased staining at the edges of either acute or chronic plaques. Indeed, demyelinated areas did not stain at all. Thus, there are epitopes on normal myelin that are recognized by monoclonal antibodies to mycobacterial hsp.

Using a different approach, a number of laboratories studied the expression of hsp by glial cells in vitro. Again, results varied with the techniques and antibodies used for the assays. Selmaj et al.,³⁰ Freedman et al.,³² and Satoh et al.³³ identified oligodendrocytes as cells expressing hsp. Selmaj et al. detected constitutive Hsp65 expression in oligodendrocytes but not astrocytes, yet noted some Hsp70/72 expression in astrocytes. Satoh et al. detected constitutive expression of Hsp60 in cultured murine oligodendrocytes and "marginally detectable" levels in most astrocytes. Expression of Hsp60 was increased in oligodendrocytes following heat stress. Freedman et al. detected constitutive expression of Hsp60 and Hsp70/72 expression in oligodendrocytes with increased expression of Hsp70/72 following heat stress. No astrocytes expressed Hsp70/72, and only a small percentage of astrocytes expressed Hsp60. In contrast, Marini and coworkers,³⁴ using tissue cultures of rat astrocytes and neurons, demonstrated heat inducible expression of Hsp70 predominantly in astrocytes, but oligodendrocytes may have been absent from these cultures. Using purified cultures of rat astrocytes, Dwyer et al.³⁵ demonstrated that such cells synthesized hsps of 30–34, 68, 70, 89, and 97 kD and that exposure to heat readily induced expression of Hsp65.

Since MS is an inflammatory disease, concentrations of cytokines are increased in MS brain.^{4,5} D'Souza et al.²⁵ studied the effects of cytokines on the expression of hsp in mixed cultures of human glial cells.^{31,32} A mixture of cytokines induced expression of Hsp72, predominantly in oligodendrocytes. The specific cytokines involved in this induction were interleukin (IL)-1, INF-gamma, and TNF-alpha. In unpublished studies, we prepared cultures of purified murine astrocytes and exposed them to heat shock (43°C) with or without expo-

sure to a mixture of cytokines. Cytokines alone induced small amounts of Hsp70/72. However the combination of heat shock and cytokine exposure augmented expression of Hsp70/72 two- to 11-fold. The cytokines responsible for this phenomenon were INF-gamma and TNF-alpha.

The above data show that results can vary widely, depending on the specificities of the antibodies used and the particular methods used for study. Nevertheless, there is good evidence that some antibodies to hsp bind to normal myelin while others bind to oligodendrocytes or astrocytes. In addition, the inflammatory milieu within MS brains increases the expression of hsp within glia, increasing the possibility that immune responses to infectious agents' hsp could cross-react with their human hsp homologues or cross-reactive myelin epitopes.

Immune Responses to Heat Shock Proteins in Multiple Sclerosis Patients and Controls

Work in this area can be divided into studies involving cellular immune responses and those involving humoral responses.

Two groups described cellular immune responses to hsps in persons with MS and other neurologic disorders: Salvetti et al.³⁶ and our laboratory.³⁷ Salvetti et al. studied peripheral blood T-cell proliferative responses to recombinant Hsp65 and Hsp70 from *Mycobacterium bovis* in 31 persons with MS, 19 individuals with other neurologic disorders, and 19 normal controls. Proliferative responses to Hsp70 were significantly more frequent in persons with MS compared to those with other neurologic disorders and healthy controls. Responses to Hsp65 were equivalent in the three groups. Lines of T cells were established from 10 MS patients and 12 healthy controls, using purified protein derivative as antigen. Again, Hsp70 reactive lines were significantly more common in MS patients than in healthy controls. Interestingly, cytofluorometric analyses of purified protein derivative responsive lines revealed that only a minority of responding cells expressed gamma/delta T-cell receptors. Our laboratory studied T-cell proliferative responses to *Mycobacterium tuberculosis*, tetanus toxoid, and recombinant Hsp65 from *Mycobacterium leprae*. T cells were concurrently collected from the peripheral bloods and spinal fluids of 20 persons with MS and nine persons with inflammatory neu-

rologic diseases other than MS. Cells were cultured in vitro and stimulated with the above antigens. Significantly increased spinal fluid lymphocyte proliferative responses to mycobacterial sonicate, relative to responses from paired peripheral blood lymphocytes, were present in 14 of the 20 specimens from patients with MS, compared to two of nine specimens from patients with other neurological diseases ($P < 0.025$). Spinal fluid lymphocytes also responded to tetanus toxoid, but differences between blood and spinal fluid were not statistically significant. Lymphocytes from one patient with MS responded only to recombinant Hsp65. When MS patients were classified according to duration of disease, nine of 10 with duration less than 2 years had spinal fluid T cells responding to *M. tuberculosis*, compared with five out of 10 with disease longer than 2 years ($P < 0.012$). These data supplement the observations of Shimonkevitz et al.²⁷ that described increased numbers of activated gamma/delta cells predominantly in spinal fluids from persons with recent onset MS.

Studies of humoral immune responses to hsp in MS are mainly the work of our laboratory^{38,39} and that of Freedman and his associates.⁴⁰ We used immunoblots to detect antibodies to native and recombinant mycobacterial and bacterial hsp in paired spinal fluids and sera from persons with MS and other neurologic diseases. Antibodies to many hsps, including those of the 60kD and 70kD families, were present in CSF and sera from all patient groups. Patterns of antibodies varied between CSF and sera, and between patients, but no disease-distinctive patterns were seen. When anti-hsp antibodies were analyzed for isotypes, patients with MS had higher concentrations of anti-hsp immunoglobulin (Ig) A antibodies than did patients with other neurologic diseases. This suggested an in situ synthesis of such antibodies within the CNS in persons with MS. Prabhakar et al.⁴⁰ studied antibody concentrations to recombinant Hsp 60 using enzyme-linked immunosorbent assay (ELISA). Titers of antibodies in MS spinal fluids were significantly higher than those seen in persons with other neurologic diseases, and the higher titers correlated with the presence of oligoclonal bands in MS CSF but not CSF of those with other neurologic diseases. In another recent study, Gao et al.⁴¹ demonstrated the presence of antibodies to mycobacterial Hsp65 and human Hsp60 in spinal fluids of persons

with MS as well as those with other neurologic illnesses. In this study, immune responses to hsp were not disease specific.

The presence of immune responses to hsp in spinal fluids of persons with a variety of chronic inflammatory CNS diseases is not unexpected, since exposure to cytokines and inflammatory cells increases expression of these proteins and could result in an accumulation of anti-hsp T cells and antibodies in this anatomic compartment. However, since all of the above studies used large protein molecules as antigens, immune responses to particular hsp epitopes could not be detected, nor were differences in cytokine secretion studied. Patterns of immune responses to particular hsp peptides vary among individuals, depending on differences in their major histocompatibility complex (MHC) genes and their different illnesses.⁴² Such differences in patterns of immune response, especially those related to individual MHC genotypes, may be important in determining susceptibility to autoimmune diseases such as MS.

HEAT SHOCK PROTEINS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

While there is no completely comparable animal model for MS, EAE, an autoimmune CNS disease induced by immunization with myelin proteins or peptides, has served as a useful tool in studying potential disease processes in human CNS autoimmune diseases. Several approaches, similar to those used in studies with patients with MS, have yielded persuasive data for a role of immune responses to hsp in animals with EAE.

Responsiveness to Heat Shock Proteins in the Central Nervous System of Rats With Experimental Autoimmune Encephalomyelitis

Mor et al.⁴³ were among the first to present data in support of the hypothesis that immune responses to hsps play a role in autoimmune demyelination. These investigators prepared T cells from the spinal cords, blood, spleen, and lymph nodes of rats during the acute phase of EAE or during recovery from EAE. Using limiting dilution analyses, they determined the frequency of T-cell responses to the myelin protein, MBP, and to recombinant Hsp65 and Hsp70. As expected, responses to MBP were enriched in the spinal cords of rats during and

after acute EAE. However, there was also enrichment of T cells responsive to Hsp65. T-cell lines established from spinal cord lymphocytes responded to MBP, Hsp65, and Hsp70. When EAE was induced with an anti-MBP responsive T-cell line, similar patterns of enrichment for MBP and Hsp65 reactive T cells were noted, indicating that responses to hsp occurred in the absence of exposure to adjuvant mycobacteria.

Modulation of Experimental Autoimmune Encephalomyelitis by Immunization With Heat Shock Proteins

While studies with MS patient materials suggested that immune responses to hsp may play a role in pathogenesis, the best evidence for such a phenomenon comes from studies of EAE.

As noted above, we found that certain anti-hsp antibodies stain normal myelin. We extended these observations by performing immunoblots with normal human myelin as antigen.³¹ A panel of 20 anti-stress protein antibodies was assayed for their ability to bind to normal human myelin proteins separated on SDS-PAGE. Three monoclonal antibodies, specific for either mycobacterial or human hsp, stained bands of normal myelin proteins. One murine monoclonal to *M. leprae* Hsp65, IIH9, stained a 44–46 kD doublet. This doublet was the same size as the myelin protein 2', 3', cyclic 3' nucleotide phosphodiesterase (CNP). To study this observation further, purified CNP was used in immunoblots. IIH9 bound to CNP in a pattern identical to that seen with whole myelin. A non-peptide region of sequence homology was identified between the epitope of Hsp65 recognized by IIH9 and CNP. This peptide (hsp-CNP peptide) was synthesized and used in immunoblots. IIH9 strongly bound to this peptide, proving that this region of sequence homology was responsible for the observed cross-reactivity.

To determine whether immune responses to this peptide had biologic consequences, we immunized rats with hsp-CNP peptide, using either complete or incomplete Freund's adjuvant, the only difference between the two adjuvants being the presence of heat-killed *M. tuberculosis* in the complete Freund's adjuvant (CFA). No clinical disease or histologic changes in the CNS were observed. Four weeks later, animals were challenged with guinea pig spinal cord in CFA. Those animals

that originally received peptide in CFA developed accelerated and enhanced EAE. Those animals previously immunized with peptide in incomplete Freund's adjuvant (IFA) either remained healthy (40%) or developed very mild EAE. Differences between the two groups were statistically significant. Thus, an immune response to a hsp peptide having sequence homology with a myelin protein modulated the course of EAE.

In an attempt to determine the mechanisms involved in the protection offered by peptide immunization, we measured spleen-cell proliferative responses of rats immunized with hsp-CNP peptide upon stimulation with either recombinant, mycobacterial Hsp65, hsp-CNP peptide, or no antigen. Cells were collected at two time points, 12 days and 31 days after peptide immunization. As expected, strong proliferative responses were seen at both time points with cells from animals given peptide in CFA, that is, adjuvant containing mycobacteria. In contrast, day-12 spleen cells from rats given hsp-CNP peptide in IFA did not respond to Hsp65. This too was expected, since IFA contains no mycobacteria. However, by day 31 there was a dramatic change. Spleen cells from rats given peptide in IFA proliferated to Hsp65 stimulation as strongly as cells from rats given peptide in CFA. No proliferative responses were seen in spleen cells from any group when stimulated by hsp-CNP peptide alone. This latter observation is not unexpected since hsp-CNP peptide is a B-cell determinant. We interpret these observations to indicate that epitope spreading may have occurred following immunization with hsp-CNP peptide in IFA. In other words, even though there were no proliferative response to peptide alone, sufficient T-cell stimulation was induced to allow diversification of the response to other epitopes of Hsp65. By 31 days after immunization, numbers of these Hsp65 responsive cells could be detected in our proliferative assays. The phenomenon of epitope spreading or diversification of immune responses initiated by restricted peptide epitopes is an important one that has significant implications in terms of determining patterns of immune response in any chronic inflammatory process, including MS.^{44,45}

We next measured antibodies to Hsp65 and hsp-CNP peptide in sera from rats immunized 12 and 31 days previously with hsp-CNP peptide in either CFA or IFA. Antibodies to peptide were present in

all animal groups. Only one of three rats immunized with peptide in CFA had antibodies to Hsp65 at 12 or 31 days after immunization. In contrast, antibodies to Hsp65 were seen in rats given peptide in IFA, both 12 days previously (one of three rats) and 31 days previously (four of four rats). These results support our hypothesis that, as a result of immunization with hsp-CNP peptide, epitope spreading occurred such that antibodies to other determinants of Hsp65 were evoked.

Immune responses can be categorized by the patterns of cytokines secreted by activated T cells. Th1 responses are those in which T cells secrete IL-2 and INF-gamma. Th2 responses are those in which T cells secrete IL-4 and IL-5.^{42,46} Th1 responses have been implicated in the pathogenesis of cell mediated diseases such as MS and EAE.⁴⁷ We wished to determine the patterns of immune responses to hsp-CNP peptide in terms of Th1 versus Th2. To do this we determined the isotypes of the IgG anti-peptide antibodies. Immunoglobulin G2a antibodies require secretion of INF-gamma. Thus, their presence indicates a Th1 pattern of response.⁴⁸ Immunoglobulin G1 antibodies arise in the presence of IL-4 and are an indicator of a Th2 pattern of T-cell response.⁴⁸ Anti-peptide antibodies from peptide-CFA immunized rats were a mixture of IgG1 and IgG2a isotypes on day 12 after immunization. By day 31, anti-peptide antibodies were almost exclusively IgG2a, indicating the presence of a strong Th1 response. In contrast, anti-peptide antibodies in the IFA immunized group were mainly IgG1 at both time points, though some IgG2a isotypes were detected on day 31. Thus, protection against EAE was associated with a Th2 pattern of response. These data are in agreement with other observations showing that Th1 responses are responsible for the development of EAE,^{47,49,50} and that Th2 cytokines, such as transforming growth factor-beta, protect against EAE.⁵¹

Lewis rats are susceptible to acute EAE but rarely go on to have relapses. To study a model of EAE more closely resembling MS, we induced EAE in the SJL mouse by immunization with an encephalitogenic peptide of proteolipid protein, PLP139-151, emulsified in CFA. This results in a chronic relapsing form of EAE that has many similarities to MS. Following immunization with peptide, animals develop an acute illness from which

almost all recover completely over a period of 10–14 days. One to three weeks after recovery, mice begin to have spontaneous relapses of disease. Recovery from these attacks is often incomplete, and some animals develop chronic progressive disability. To study the role of hsp in this model mice were injected intravenously with Hsp60 coupled to syngeneic spleen cells. Controls received either bovine serum albumin (BSA) or PLP139-151. One week later, animals were immunized with PLP139-151 in CFA to induce EAE. All animals became ill with EAE, though as expected, fewer mice receiving intravenous PLP139-151 developed EAE, and those that did had very mild disease. Mice were then carefully monitored over the ensuing 3 months. Mice given intravenous BSA recovered from their acute illness and went on to have mild-to-moderate relapses with good recovery between attacks. The few mice that became ill in the intravenous-PLP139-151 group recovered completely and never experienced relapses. Mice pretreated with Hsp60 had disease as severe as BSA-pretreated mice, indicating that their acute EAE was not modified by exposure to Hsp60. However, over the ensuing 3 months, Hsp60-treated animals experienced a significant increase in the severity and frequency of their relapses, as well as sustaining incomplete recovery between attacks. While the reasons for this effect remain to be elucidated, it is clear in this model that immune responses to hsps play an important role not in the acute phase of EAE induced by peptide, but in the relapses that occur subsequent to recovery. An understanding of the mechanisms involved in this phenomenon may shed light on the causes of relapses in persons with MS.

Environmental Infections Influence the Course of Experimental Autoimmune Encephalomyelitis

Since infections in humans are associated with an increased frequency of MS relapses we studied the role of infections in mice immunized with PLP139-151 to induce relapsing EAE. We studied two groups of mice. One group was housed in a specific-pathogen-free (SPF) facility where frequencies of infections were greatly reduced and only normal commensal bacteria were present in the animals. The other group of mice was housed in a conventional facility where animals are routinely exposed to a large number of viral, bacterial, and

parasitic infectious agents. We wished to alter the normal pattern of immune responses to hsp in these animals, skewing them toward either a Th1 (presumably disease exacerbating) or Th2 (presumably disease ameliorating) bias. To do this, animals in both groups were injected intraperitoneally with either CFA or IFA. Complete Freund's adjuvant induces a Th1 pattern of immune response to Hsp60, whereas IFA induces a Th2 pattern of response to this protein.⁵² Our published results⁵³ showed the following. There were strong immune responses to hsp, particularly Hsp60, in all groups of animals, both SPF- and conventionally housed mice. Thus, as shown by others in humans and mice, immune responses to hsp are a normal phenomenon. Acute EAE was of equivalent severity and duration in both groups of animals. However, mice housed in the SPF facility had significantly more severe relapses than did mice housed in a conventional facility. In association with this was a skewing of immune responses to Hsp60 toward a Th1 phenotype. In contrast the strong immune responses to Hsp60 noted in conventionally housed animals were skewed toward a Th2 phenotype. Mice injected with CFA intraperitoneally had more severe disease, and this was seen in both SPF- and conventionally housed mice. We interpret these data to indicate that immune responses to hsp, induced by environmental exposure to infectious agents, play a role in relapses of EAE, and that, interestingly, fewer infectious exposures are associated with more severe disease. Multiple sclerosis is more common in individuals of higher socioeconomic background, presumably those with fewer environmental infectious exposures. Our data suggest a mechanism to explain this observation.

Tolerance to Alpha B-Crystallin, a Possible Autoantigen in Multiple Sclerosis, Alters the Course of Relapsing Experimental Autoimmune Encephalomyelitis

Recent data suggest that immune responses to the small (25kD) hsp alpha B-crystallin may play a role in MS. Immune responses to alpha B-crystallin are found in especially high frequency in persons with MS,⁵⁴ and alpha B-crystallin and Hsp27, another small hsp, are expressed in regions of acute inflammation in MS brains.^{55,56} We hypothesized that if immune responses to alpha B-crystallin were important in a chronic autoimmune CNS disease,

than tolerance to this protein should alter the course of disease. To study this hypothesis we studied the role of immune responses to alpha B-crystallin in mice with relapsing EAE.

Mice were made tolerant to alpha B-crystallin by injecting them intravenously with high doses of either alpha B-crystallin or a control protein (BSA or hen egg lysozyme [HEL]). Animals were then immunized with PLP139-151 to induce EAE. Mice made tolerant to alpha B-crystallin had significantly decreased acute disease and a significantly reduced frequency and severity of relapses. This was associated with a decreased response of CNS lymphocytes to the encephalitogenic peptide and a decreased expression of inflammatory cytokine messenger RNA (INF-gamma and TNF-alpha) in the CNS. When mice were made tolerant to alpha B-crystallin or HEL after the onset of acute EAE, that is, at the time of at peak disease, there was a significant decrease in the frequency of relapses of alpha B-crystallin-tolerant mice, but those mice that did experience relapses were, as a group, sicker than HEL-tolerant animals. Immune responses to PLP139-151 were significantly increased in the CNS of alpha B-crystallin-tolerant mice compared with controls, but this was not associated with any changes in patterns of cytokine secretion. No cross-reactivity could be demonstrated between alpha B-crystallin and PLP-139-151.

These data are the first to demonstrate that immune responses to a putative autoantigen in MS, alpha B-crystallin, are also able to alter the course of relapsing EAE, supporting a role for such responses in the pathogenesis of chronic, autoimmune CNS disease. Understanding the mechanisms behind these phenomena could shed light on disease processes in MS and may offer insights into new treatment opportunities.

SUMMARY

Stress or heat shock proteins are constitutively expressed in normal CNS tissues in a variety of cell types (oligodendrocytes, astrocytes, and neurons). Their presence may protect cells from various stresses, such as hypoxia, anoxia, and excessive excitatory stimulation. Increased amounts of hsp are expressed in various cells of the CNS during acute toxic-metabolic states and in chronic degenerative and inflammatory diseases. Increased expression of hsp may lead to immune responses to these proteins.

Antibodies to mycobacterial hsp bind to normal human myelin and to oligodendrocytes in regions of MS demyelination. Cellular immune responses to hsp occur with increased frequency and magnitude in persons with MS, especially those with recent onset of disease. In addition, there are populations of T cells expressing gamma/delta T cells in the brains and spinal fluids of persons with MS, suggesting an in situ immune response to hsps. Humoral immune responses to hsp are found in CSF, but no disease specificity has been documented. Some myelin proteins have sequence homology with particular hsps. One instance is the homology between a peptide of mycobacterial Hsp65 and the myelin protein CNP. Our data on EAE suggest that immune responses to either cross-reactive hsp epitopes or whole hsp can modify the course of both acute and chronic relapsing EAE. In addition, the severity and frequency of environmental exposure to infectious agents can modify the course of EAE, possibly by altering the patterns of immune response to hsp. Finally, tolerance to the small hsp, alpha B-crystallin, a putative autoantigen in persons with MS, alters the course of relapsing EAE, supporting its role in chronic, autoimmune CNS disease.

Modifying immune responses to hsp may be a potential new treatment option for persons with MS.

REFERENCES

1. Kermode AG, Thompson AJ, Tofts P, et al. Blood-brain barrier abnormalities in longstanding multiple sclerosis lesions. An immunohistochemical study. *J Neuropathol Exp Neurol* 1994;53:625-636.
2. Warren KG, Catz I, Steinman L. Fine specificity of the antibody response to myelin basic protein in the central nervous system in multiple sclerosis: the minimal B-cell epitope and a model of its features. *Proc Natl Acad Sci USA* 1995;92:11061-11065.
3. Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* 1995;37:424-435.
4. Hofman FM, Hinton DR, Johnson K, Merrill JE. Tumor necrosis factor identified in multiple sclerosis brain. *J Exp Med* 1989;170:607-612.
5. Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J Clin Invest* 1991;87:949-954.
6. Jones DB, Coulson AF, Duff GW. Sequence homologies between hsp60 and autoantigens. *Immunol Today* 1993;14:115-118.
7. Lamb JR, Bal V, Mendez-Samperio P, et al. Stress proteins may provide a link between the immune response to infection and autoimmunity. *Int Immunol* 1989;1:191-196.
8. Quayle AJ, Wilson KB, Li SG, et al. Peptide recognition, T cell receptor usage and HLA restriction elements of human heat-shock protein (hsp) 60 and mycobacterial 65-kDa hsp-reactive T cell clones from rheumatoid synovial fluid. *Eur J Immunol* 1992;22:1315-1322.
9. Steinhoff U, Schoel B, Kaufmann SH. Lysis of interferon-gamma activated Schwann cell by cross-reactive CD8+ alpha/beta T cells with specificity for the mycobacterial 65 kd heat shock protein. *Int Immunol* 1990;2:279-284.
10. Morimoto RI, Tissières A, Georgopoulos C, eds. *Stress Proteins in Biology and Medicine*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990.
11. Kaufmann SHE. Heat-shock proteins: A link between rheumatoid arthritis and infection? *Curr Opin Rheum* 1990;2:430-435.
12. Res PCM, Telgt D, van Laar JM, Pool MO, Breedveld FC, De Vries RRP. High antigen reactivity in mononuclear cells from sites of chronic inflammation. *Lancet* 1990;336:1406-1408.
13. Kantengwa S, Donati YR, Clerget M, et al. Heat shock proteins: an autoprotective mechanism for inflammatory cells? *Semin Immunol* 1991;3:49-56.
14. Allegratta M, Nicklas JA, Sriram S, Albertini RJ. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* 1990;247:718-721.
15. Baxevanis CN, Reclos GJ, Servis C, et al. Peptides of myelin basic protein stimulate T lymphocytes from patients with multiple sclerosis. *J Neuroimmunol* 1989;22:23-30.
16. Hafler DA, Benjamin DS, Burks J, Weiner HL. Myelin basic protein and proteolipid protein reactivity of brain- and cerebrospinal fluid-derived T cell clones in multiple sclerosis and postinfectious encephalomyelitis. *J Immunol* 1987;139:68-72.
17. Johnson D, Hafler DA, Fallis RJ, et al. Cell-mediated immunity to myelin-associated glycoprotein, proteolipid protein, and myelin basic protein in multiple sclerosis. *J Neuroimmunol* 1986;13:99-108.
18. Liblau R, Tournier LE, Maciazek J, et al. T cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects. *Eur J Immunol* 1991;21:1391-1395.
19. Lisak RP, Zweiman B. In vitro cell-mediated immunity of cerebrospinal-fluid lymphocytes to myelin basic protein in primary demyelinating diseases. *N Engl J Med* 1977;297:850-853.
20. Pette M, Fujita K, Kitze B, et al. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurol* 1990;40:1770-1776.
21. Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* 1994;179:973-984.

22. Tuohy VK, Sobel RA, Lees MB. Myelin proteolipid protein-induced experimental allergic encephalomyelitis. Variations of disease expression in different strains of mice. *J Immunol* 1988;140:1868–1873.
23. Sibley WA, Bamford CR, Clark K. Clinical viral infections and multiple sclerosis. *Lancet* 1985;1:1313–1315.
24. Panitch HS. Influence of infection on exacerbations of multiple sclerosis. *Ann Neurol* 1994;36(Suppl):S25–S28.
25. D'Souza SD, Antel JP, Freedman MS. Cytokine induction of heat shock protein expression in human oligodendrocytes: an interleukin-1-mediated mechanism. *J Neuroimmunol* 1994;50:17–24.
26. Wucherpfennig KW, Newcombe J, Li H, Keddy C, Cuzner ML, Hafler DA. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci USA* 1992;89:4588–4592.
27. Shimonkevitz R, Colburn C, Burnham JA, Murray RS, Kotzin BL. Clonal expansions of activated gamma/delta T cells in recent-onset multiple sclerosis. *Proc Natl Acad Sci USA* 1993;90:923–927.
28. Selmaj K, Brosnan CF, Raine CS. Colocalization of lymphocytes bearing gamma delta T-cell receptor and heat shock protein hsp65+ oligodendrocytes in multiple sclerosis. *Proc Natl Acad Sci USA* 1991;88:6452–6456.
29. Hvas J, Oksenberg JR, Fernando R, Steinman L, Bernard CC. Gamma delta T cell receptor repertoire in brain lesions of patients with multiple sclerosis. *J Neuroimmunol* 1993;46:225–234.
30. Selmaj K, Brosnan CF, Raine CS. Expression of heat shock protein-65 by oligodendrocytes in vivo and in vitro: implications for multiple sclerosis. *Neurology* 1992;42:795–800.
31. Birnbaum G, Kotilinek L, Schlievert P, et al. Heat shock proteins and experimental autoimmune encephalomyelitis (EAE): I. Immunization with a peptide of the myelin protein 2'3' cyclic nucleotide 3' phosphodiesterase that is cross-reactive with a heat shock protein alters the course of EAE. *J Neurosci Res* 1996;44:381–396.
32. Freedman MS, Buu NN, Ruijs TC, Williams K, Antel JP. Differential expression of heat shock proteins by human glial cells. *J Neuroimmunol* 1992;41:231–238.
33. Satoh J, Nomaguchi H, Tabira T. Constitutive expression of 65-kDa heat shock protein (HSP65)-like immunoreactivity in cultured mouse oligodendrocytes. *Brain Res* 1992;595:281–290.
34. Marini AM, Kozuka M, Lipsky RH, Nowak TSJ. 70-kilodalton heat shock protein induction in cerebellar astrocytes and cerebellar granule cells in vitro: comparison with immunocytochemical localization after hyperthermia in vivo. *J Neurochem* 1990;54:1509–1516.
35. Dwyer BE, Nishimura RN, de Vellis J, Clegg KB. Regulation of heat shock protein synthesis in rat astrocytes. *J Neurosci Res* 1991;28:352–358.
36. Salvetti M, Buttinelli C, Ristori G, et al. T-lymphocyte reactivity to the recombinant mycobacterial 65- and 70-kDa heat shock proteins in multiple sclerosis. *J Autoimmun* 1992;5:691–702.
37. Birnbaum G, Kotilinek L, Albrecht L. Spinal fluid lymphocytes from a subgroup of multiple sclerosis patients respond to mycobacterial antigens. *Ann Neurol* 1993;34:18–24.
38. Birnbaum G, Schlievert P. Antibodies to mycobacterial antigens in the spinal fluids and blood from patients with multiple sclerosis and other neurologic diseases. *Neurology* 1992;42(suppl 3):247.
39. Birnbaum G, Kotilinek L. Antibodies to 70-kd heat shock protein are present in CSF and sera from patients with multiple sclerosis. 45th Annual Meeting of the American Academy of Neurology, New York, NY, 1993.
40. Prabhakar S, Kurien E, Gupta RS, Zielinski S, Freedman MS. Heat shock protein immunoreactivity in CSF: correlation with oligoclonal banding and demyelinating disease. *Neurology* 1994;44:1644–1648.
41. Gao YL, Raine CS, Brosnan CF. Humoral response to hsp 65 in multiple sclerosis and other neurologic conditions. *Neurology* 1994;44:941–946.
42. Mutis T, Cornelisse YE, Datema G, van den Elsen PJ, Ottenhoff TH, de Vries RR. Definition of a human suppressor T-cell epitope. *Proc Natl Acad Sci USA* 1994;91:9456–9460.
43. Mor F, Cohen IR. T cells in the lesion of experimental autoimmune encephalomyelitis. Enrichment for reactivities to myelin basic protein and to heat shock proteins. *J Clin Invest* 1992;90:2447–2455.
44. Lehmann PV, Sercarz EE, Forsthuber T, Dayan CM, Gammon G. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol Today* 1993;14:203–208.
45. McRae BL, Vanderlugt CL, Dal Canto MC, Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med* 1995;182:75–85.
46. Mutis T, Kraakman EM, Cornelisse YE, et al. Analysis of cytokine production by *Mycobacterium*-reactive T cells: Failure to explain *Mycobacterium leprae*-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. *J Immunol* 1993;150:4641–4651.
47. van der Veen RC, Stohlman SA. Encephalitogenic Th1 cells are inhibited by Th2 cells with related peptide specificity: relative roles of interleukin (IL)-4 and IL-10. *J Neuroimmunol* 1993;48:213–220.
48. Lin MS, Chen YW. B cell differentiation. II. Isotype potential of a single B cell. *Cell Immunol* 1993;150:343–352.
49. van der Veen RC, Kapp JA, Trotter JL. Fine-specificity differences in the recognition of an encephalitogenic peptide by T helper 1 and 2 cells. *J Neuroimmunol* 1993;48:221–226.
50. Mustafa M, Vingsbo C, Olsson T, Ljungdahl A, Hojberg B, Holmdahl R. The major histocompatibility complex influences myelin basic protein 63-88-induced T cell cytokine profile and experimental autoimmune encephalomyelitis. *Eur J Immunol* 1993;23:3089–3095.
51. Stevens DB, Gould KE, Swanborg RH. Transforming

- growth factor-beta 1 inhibits tumor necrosis factor-alpha/lymphotoxin production and adoptive transfer of disease by effector cells of autoimmune encephalomyelitis. *J Neuroimmunol* 1994;51:77-83.
52. Anderton SM, van der Zee R, Goodacre JA. Inflammation activates self hsp60-specific T cells. *Eur J Immunol* 1993;23:33-38.
53. Birnbaum G, Kotilinek L, Miller SD, et al. Heat shock proteins and experimental autoimmune encephalomyelitis. II: environmental infection and extra-neuraxial inflammation alter the course of chronic relapsing encephalomyelitis. *J Neuroimmunol* 1998;90:149-161.
54. van Noort JM, van Sechel AC, Bajramovic JJ, et al. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. *Nature* 1995;375:798-801.
55. Aquino DA, Capello E, Weisstein J, et al. Multiple sclerosis: altered expression of 70- and 27-kDa heat shock proteins in lesions and myelin. *J Neuropathol Exp Neurol* 1997;56:664-672.
56. Bajramovic JJ, Lassmann H, van Noort JM. Expression of alphaB-crystallin in glia cells during lesional development in multiple sclerosis. *J Neuroimmunol* 1997;78:143-151.