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Chaperone Activity of α B-Crystallin Is Responsible for Its Incorrect Assignment as an Autoantigen in Multiple Sclerosis

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For 15 y, α B-crystallin (heat shock protein [Hsp] B5) has been labeled an autoantigen in multiple sclerosis (MS) based on humoral and cellular responses found in humans and animal models. However, there have been several scientific inconsistencies with this assignment, ranging from studies demonstrating small differences in anticrystallin responses between patients and healthy individuals to the inability of crystallin-specific T cells to induce symptoms of experimental allergic encephalomyelitis in animal models. Experiments in this article demonstrate that the putative anti-HspB5 Abs from 23 MS patients cross-react with 7 other members of the human small Hsp family and were equally present in normal plasma. Biolayer interferometry demonstrates that the binding was temperature dependent, and that the calculated K_a increased as the concentration of the sHsp decreased. These two patterns are characteristic of multiple binding sites with varying affinities, the composition of which changes with temperature, supporting the hypothesis that HspB5 bound the Ab and not the reverse. HspB5 also precipitated Ig heavy and L chains from sera from patients with MS. These results establish that small Hsps bind Igs with high affinity and refute much of the serological data used to assign α B-crystallin as an autoantigen. *The Journal of Immunology*, **2011, 186: 4263–4268.**

The association of α B-crystallin (heat shock protein [Hsp] B5) with multiple sclerosis (MS) has been a puzzling story (1). Van Noort and colleagues (2, 3) first proposed the protein to be an autoantigen in 1995 based on the reactivity of PBMCs from MS patients and healthy control subjects to proliferate in response to a fraction of myelin from MS brains containing α B-crystallin. Subsequently, several groups reported detection of Abs to the protein using Western blotting, enzyme-linked immunoassays, or multiplex Ab arrays in sera and spinal fluid from patients with MS, Guillain–Barré syndrome, neuro-Behçet's disease, and other inflammatory neurologic disorders (4–7). However, in several of the reports, cellular and humoral responses against the protein also were seen in normal plasma (3, 4, 8). A further confusing aspect of the story was that $CD4^+$ T lymphocytes specific for HspB5 were found both in mice with experimental autoimmune encephalomyelitis (EAE) and MS patients, but unlike $CD4^+$ T cells specific for myelin components, the HspB5-specific T cells did not induce EAE in mice (9), which is inconsistent with a definition of a disease-associated autoantigen.

Independent of these studies, our group established that HspB5 was both the most prominent protein in plaques of MS patients (10) and the gene whose expression was most induced in the MS plaques compared with normal tissue (11). Further support for the importance of HspB5 in MS was the observation that mice lacking the HspB5 exhibited more severe symptoms of EAE compared with wild type mice (6). Most surprisingly, i.v. injection of HspB5 dramatically reduced inflammatory symptoms of EAE (6). Analysis of the lymphocytes and macrophages of the animals receiving multiple HspB5 injections revealed that inflammatory response of each cell population was modulated significantly, and the amount of apoptosis in neurologic tissue was greatly limited. Collectively, these experiments emphasized that the protein exhibited relatively fast-acting, broad anti-inflammatory activity, the kinetics of which were inconsistent with the induction of Ag-specific tolerance, and questioned the assignment of the protein as an autoantigen. Nevertheless, experiments in our laboratories, using protein and peptide arrays, confirmed that plasma of mice with EAE and MS patients apparently contained significant amounts of HspB5-specific Abs (6).

HspB5 is a member of the small Hsp (sHsp) family, expressed in a wide spectrum of long-lived cells including the lens, muscle, and neuronal tissue (12, 13). Like many other Hsps, the expression of HspB5 is induced by the heat shock factor 1 when cells are stressed by elevation of temperature, infection, or hypoxia. The protein lacks a leader peptide and has been shown to be cytoprotective both by binding partially unfolded proteins and by inhibiting aggregation, and apparently has more specific interactions with proteins such as p53 and bax to inhibit apoptosis (14–18). The crystal structures of sHsps from wheat, pea, tape worm, and mycobacteria revealed the family of proteins form a variety of

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; Hsp, heat shock protein; MS, multiple sclerosis; sHsp, small heat shock protein.

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dynamic oligomers with different stoichiometry, but in all cases, the unit cell was a dimer with the monomer folding as conserved β barrel with amino- and carboxyl-terminal extensions (19). The extensions are integral in the formation and stabilization dimers and aggregates of dimers (20). Recent resolution of the β barrel of human HspB5 has shown the dimer contacts are between the edge strands (20, 21). A groove, which is postulated to be one of the principal hydrophobic binding sites, is formed between the two subunits because the register of the strands is not symmetrical. A conserved structural feature of the groove is the presence of salt bridges at both ends formed between a conserved arginine at residue 120 of one domain with a conserved aspartic acid at residue 110 in the adjacent subunit (21). A naturally occurring mutation of this arginine to a glycine has been shown to compromise the structural integrity of the groove and the chaperone activity of the protein (22). Most importantly, the mutation has been shown to be physiologically relevant being linked to a human desmin myopathy (23, 24).

HspB5 is one of a family of 10 sHsps in humans, all of which are closely related in structure and function (12). They are molecular chaperones, capable of binding partially unfolded proteins principally through hydrophobic contacts limiting the formation of aggregates (25). Functional studies of the family have established they have multiple binding sites, and most importantly, the chaperone activity increases with temperature. The recent crystal structures have provided a model for this effect in which the C terminus of a subunit occupies the principle groove at ambient temperature but dissociates at temperatures greater than 37˚C, exposing both the groove and the conserved hydrophobic residues of the terminus (20, 21). Such a model would be consistent with greater binding capacity of the molecules with increasing temperature and the observed dynamic nature of the molecular aggregate.

Abs are prone to aggregate by forming extended β pleated sheets at high local concentrations, leading to problems in both their handling in the laboratory and, more importantly, in vivo pathology leading to systemic amyloidosis (26, 27). Clearly, Abs specific for HspB5 will present a significant complication if the protein were to be used as a human therapeutic. Because HspB5 was therapeutically effective in animals with EAE, the Abs either were directed at determinants that did not inhibit the anti-inflammatory activity of the protein or there was an alternative explanation for the presence of the humoral response. The alternative we propose in this article is that as molecular chaperones, the sHsps bind the Igs at multiple sites, which could help explain the varying experimental inconsistencies in the story in the literature.

Materials and Methods

Cloning, expression, and purification of T7-human sHspB1-8

The full-length clones of human sHspB1-8 were obtained from Open Biosystems. An EcoR1, an ATG site, a HindII, and stop site were introduced into the gene encoding each of the sHsps using PCR. The resulting sHsp PCR fragments were ligated into the EcoR1-HindIII restriction site of pET21b (+) (Novagen) in-frame with the amino-terminal T7-tag. One-shot TOP10 cells (Invitrogen) were transformed with the resulting plasmid. Several of the resulting colonies were selected, expanded, and the insertion was verified by restriction digest with EcoR1 and HindIII, and sequencing. The proteins were produced in small scale by transforming BL21 Codon Plus cells (Stratagene) for protein expression. Larger scale production and purification of T7-HspB1-8 was accomplished by growing selected colonies in 250–1000 ml Luria-Bertani broth with carbenicillin, induced with isopropyl β-D-thiogalactoside, and isolating the bacteria 4–12 h later. The cells were lysed with a bacterial protein extraction buffer (Thermo Scientific) with sonication while being cooled on ice, and the supernatant collected after centrifugation; saturated ammonium sulfate was added to 20% v/v, and the mixture was centrifuged. Sufficient saturated ammonium sulfate was added to the supernatant to increase the concentration of the solution to 45% v/v. After centrifugation, the pellet containing HspB1-8 was resuspended in 50 mM NaCl and 50 mM Tris pH 8.0. Additional sHsp was recovered by extracting the initial pellet from the bacterial lysate with 6 M guanidine hydrochloride, 100 mM Tris pH 8.0, and dialysis against 50 mM NaCl and 50 mM Tris pH 8.0. The dialyzed mixture was spun and the supernatant combined with the resuspended pellet from the 50% ammonium sulfate precipitation and applied to DEAE fast flow column to remove DNA and negatively charged glycosaminoglycans. The flow through was concentrated and applied to a Sephacryl S-300 column. The fractions corresponding to the large M_r sHsp (~400 kDa) were pooled and concentrated, and finally applied to an anti-T7 column, and the T7-sHsps were eluted with glycine buffer pH 3.0. The eluate was neutralized with 1 M Tris pH 8.0, concentrated. The purity of the protein was established using Coomassie-stained SDS-PAGE gels, and the structure was confirmed by mass spectrometry. The quaternary structure was established using gel filtration on Sephacryl S-300 and by dynamic light scattering.

Enzyme-linked immunoassays

Solutions of recombinant T7-sHspB1-8 in PBS pH 7.4 were coated on 96-well ELISA plates (MaxiSorp; Nunc, Rochester, NY) overnight at 4˚C at a concentration of 20 μg/ml. Subsequent incubations and washes were performed at room temperature. The plates were blocked with 3% BSA in PBS with 0.05% Tween 20 (Sigma, St. Louis, MO) for 1 h, washed, and incubated for 2 h with serum samples diluted 1:200 from either patients with active MS or healthy individuals. The plates were washed, and bound Abs in sera were detected using HRP-conjugated secondary reagents specific for human IgG (H chain) Abs diluted to 1:10,000 (Jackson ImmunoResearch, West Grove, PA). The colorimetric reaction was developed with 3,3′,5,5′-tetramethylbenzidine (1-step Ultra TMB; Thermo Scientific), stopped with 1 M sulfuric acid, and quantitated using a SpectraMax spectrometer (Molecular Devices).

Biolayer interferometry

The Octet QK biosensor (Fortebio, Menlo Park, CA) uses biolayer interferometry, a label-free, optical-based technology capable of measuring interactions between sensor-immobilized and solution-phase compounds. Each of the small Hsps was titrated from 10 μM to 123 nM in PBS pH 7.2 containing 0.1% BSA and 0.002% Tween 20 in black 96-well polystyrene plates (E&K Scientific Products, Santa Clara, CA). Kinetic analyses of the binding of the sHsps to commercially available biosensors coated with anti-human IgG Fc were performed on an Octet QK system (ForteBio, Menlo Park, CA) under orbital shaking conditions (1000 rpm) following details recommended by the manufacturer at 40, 37, or 23˚C depending on the assay.

The association and dissociation curves were fit using a single-phase exponential model, with the individual rate constants being calculated using Origin software provided by the manufacturer (ForteBio). Data from maximum wavelength (nm) shift under equilibrium binding conditions at increasing sHsp concentrations were plotted and fit to a single exponential association curve, $Y = Y_0 + A(1 - \exp[-k_{obs}*t])$, where k_{obs} is [sHsp], Y_0 is wavelength shift at $t = 0$ s, A is maximum wavelength shift (time dependent), and k_{obs} is observed first-order rate constant. Maximum wavelength shift at each concentration was determined by the summation of Y_0 and A. Maximum wavelength shift at a each concentration of sHsp was plotted and fitted to one site binding model (hyperbola), $S = \text{Max}*[\text{sHsp}]/(K_d + [\text{sHsp}])$, where S is wavelength shift at equilibrium, Max is maximum wavelength shift, and [sHsp] is sHsp concentration.

Mass spectral analyses

T7-HspB5, 10 μg, was added to 300 μl MS plasma or normal plasma and incubated at 42˚C for 2 h, after which 50 μl anti-T7 Sepharose beads was added and the mixture was incubated an additional 2 h at 42˚C. The resin was separated from the plasma by centrifugation, washed multiple times with PBS pH 7.4, eluted with 100 μl of 100 mM glycine pH 1.8. Aliquots of samples were separated on 12% Tris PAGE, and the gel was stained with Coomassie brilliant blue. Gel pieces corresponding to the five prominent bands were subjected to trypsin (Sigma) digestion and identified by liquid chromatography tandem mass spectrometry using the Agilent 1100 LC system and the Agilent XCT plus Ion Trap (Agilent Technologies, Santa Clara, CA) as previously described (28). The tandem mass spectrometry spectra were scanned against the SwissProt database using the SpectrumMill software (Agilent); a minimum of two peptides was required for protein identification, with $p = 0.05$ for each peptide identified.

Results

Interactions between sHsps and Igs when measured using solid-phase assays

To confirm that MS patient sera contain Abs specific for HspB5 and to explore whether there was reactivity to any of the other sHsps, we coated 96-well plates with HspB1-8 and treated them with serial dilutions of plasma from 23 different MS patients. The amount of Ig bound was quantified by treating the wells with an HRP-conjugated mouse anti-human Ig, washing, adding a colorimetric HRP substrate, and measuring the OD of each well. Surprisingly, strong signals were seen for each sHsp, with HspB5 not even being the strongest signal (Fig. 1*A*). A possible explanation was that the high homology among the family members led to highly cross-reactive Ab specificity. However, if the Abs were generated against HspB5, the relatively low response to HspB5 was inconsistent with this hypothesis. An alternative explanation became apparent when sera from 17 healthy individuals, together with sera from 4 additional MS patients, were assayed (Fig. 1*B*). As with the patients' sera, strong signals were seen for each of the sHsps, but equivalent pattern of reactivity, within error, was observed for both normal and patient sera. Clearly, these data are inconsistent with the patients having a humoral response against HspB5, but does argue there is a biologically significant interaction between the sHsps and Igs. The sHsps appear to be binding the Igs, not the reverse.

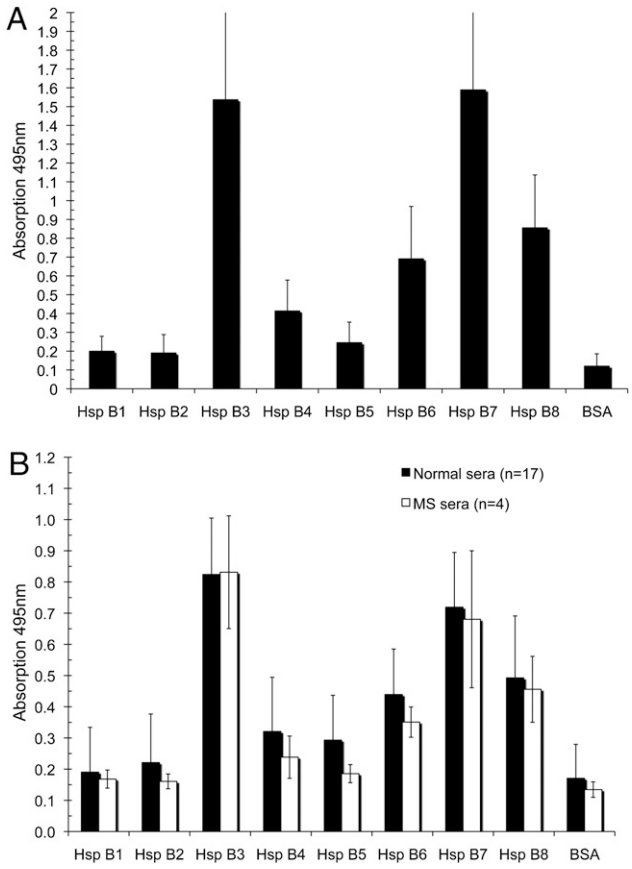

FIGURE 1. Average binding of Igs in MS patients' and normal sera to each of the recombinant human sHsps. *A*, Differential binding of Igs to the family of human sHsps from sera of 23 MS patients; (*B*) comparison of the relative signals from sera from 13 healthy individuals with that of sera from an additional 4 MS patients. Error bars represent SD of both the variation between the individual samples and the small differences from four separate readings.

Biolayer interferometry

Support for this hypothesis came from biolayer interferometry, in which the rate of binding and dissociation of the eight sHsps to a biotinylated polyclonal murine anti-human Fc Ig attached to a sensor on a surface of a tip compatible with a 96-well plate is measured (Fig. 2*A*). The system was chosen both because of its ease and simplicity, and because the attached Ab configuration was commercially available and well studied. The design is used to attach a second Ab of desired specificity and measure its binding kinetics to the relevant Ag. For this study, the murine anti-human Fc represents an Ig with known specificity completely unrelated to sHsps. In interferometry, binding of sHsp in solution to a sensor-immobilized Ab changes the interference pattern of white light reflected from the sensor surface relative to a reference surface. The change in interference pattern is a direct measure of the change in thickness of the biological layer. The binding signal, measured in units of nanometer shift, is proportional to the number of sHsp molecules bound to the Ab.

Ab binding to an Ag through a single combining site with high specificity exhibits defined rates of association and dissociation that do not differ significantly with temperature or the concentration of the ligand. In contrast, because sHsps have multiple binding sites, each with different affinities for the ligand, the binding will be temperature dependent, and because of the multiple binding sites, the rates of association and dissociation might vary with the concentration of the sHsp (25). When HspB5 was incubated with the Ab-coated probe, biologically relevant rates of association were observed with varying concentrations of the sHsp from 10 μM to 13 nM, with significantly lower rates of dissociation, consistent with tight binding between the Hsp to an Ab with an irrelevant specificity (kinetic details are provided in the Supplemental Material). Supporting that the sHsps bound the Ab and not the reverse was that both the rate and amount of complex formation was temperature dependent (Fig. 2*B*). After 4000 s, there was a clear and significant difference in the amount of complexes formed as the temperature increased from 23 to 40°C (Fig. 2*B*). Increasing the temperature from 23 to 37°C resulted in a 36% increase in complex formation, whereas increasing the temperature to 40°C increased binding an additional 42%. Such a pattern of temperature dependence is consistent with the expected behavior of a sHsp and is not characteristic of Ig–Ag interactions.

At a constant temperature, 40°C, analysis of the binding data revealed another characteristic consistent with the Hsp binding the Ig. The rate of association (k_a) increased as the concentration of the sHsp decreased, whereas the rate of dissociation (k_d) remained approximately constant (Supplemental Tables I, II). Consequently, the observed rate constant (k_a) and the calculated K_D decreased over two orders of magnitude as the concentration of the sHsp decreased (Fig. 2*C*, Table I). Variations of K_D with the concentration of an Ag are not characteristic of Ab–Ag interactions. Although there is not a published precedent for kinetic analysis of sHsp binding clients under relatively native conditions as in this assay, the pattern can be rationalized for the sHsp binding the Ab on the sensor. A relatively conserved, slow rate of dissociation, which does not significantly vary with concentration, would be expected from an oligomer, in which multiple binding sites in close proximity would limit the rate of dissociation. An increase in the rate of association with decreasing concentration of the "ligand" would be consistent with a protein with multiple binding sites, each with a different K_D. At low concentrations, relative to the Ab concentration on the sensor, the majority of the binding will be at the highest affinity sites. As the relative concentration of the Hsp increases, the rate of association becomes an average of

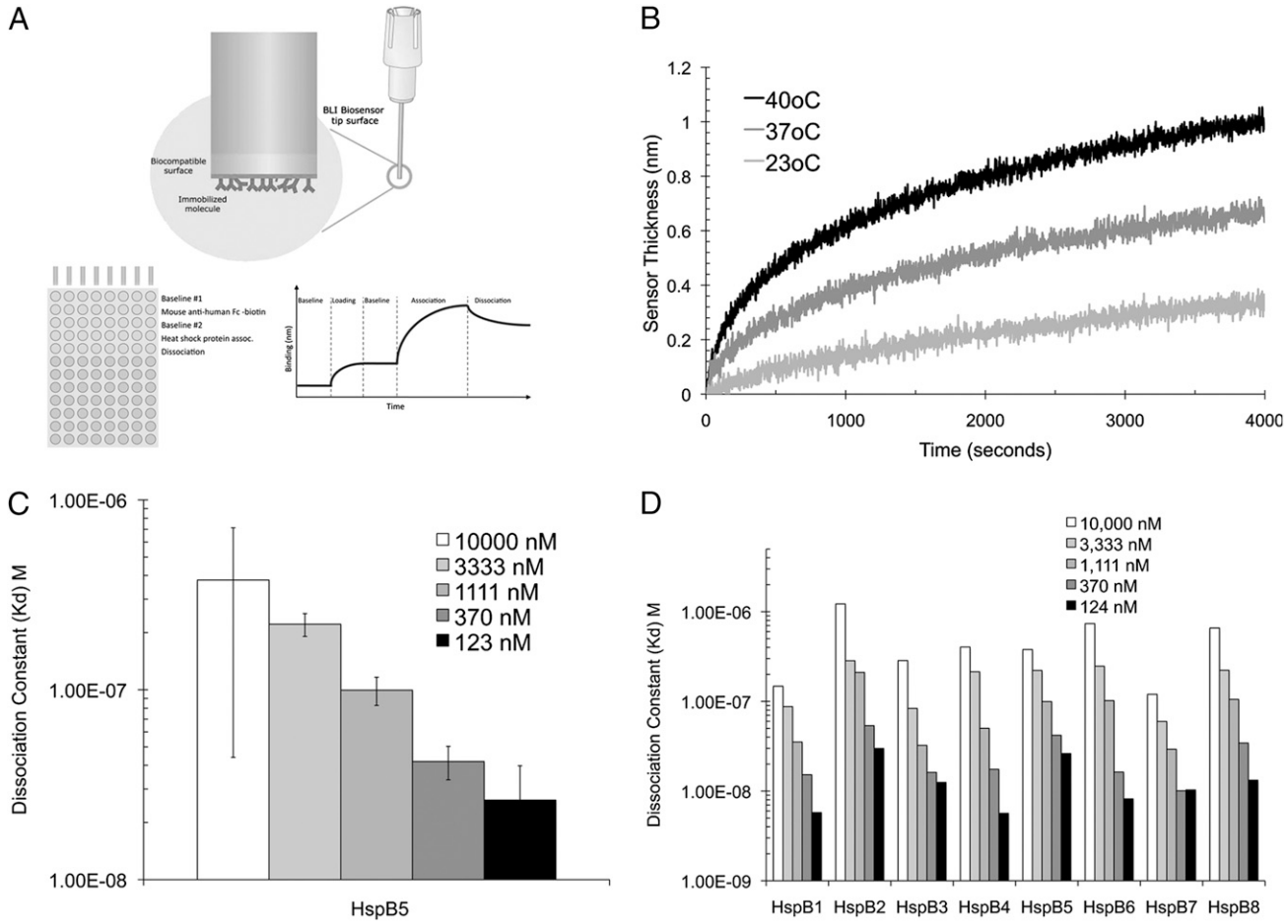

FIGURE 2. Binding kinetics of sHsps to polyclonal murine anti-human Fc Ab using biolayer interferometry. Schematic diagram displaying the design of the probe and how the rates of association and dissociation are measured by moving eight probes into different rows of a 96-well plate (*A*). Temperature dependence of HspB5 binding to anti-Fc Abs demonstrated by analyzing the rate of 10 μM HspB5 binding to the Ab-coated probes at 23, 37, and 40°C (*B*). The calculated dissociation constants of HspB5 (*C*) and HspB1-B8 (*D*) with the Ab-coated surface over a range of concentrations from 10 μM to 123 nM in PBS at 40°C decrease as the concentration of the Hsp decreased. Each assay was done a minimum of two times.

the rate of binding to multiple sites, with the high-affinity sites being less important and the association rates decreasing. The observed temperature-dependent interaction between HspB5 and an irrelevant Ab supports the hypothesis that the sHsp is binding the Ab. The observed variation of the association rates with the concentration of the Hsp provides even stronger evidence supporting the direction of the interaction.

A similar pattern of variation in the rates of association, and in the dissociation constants, was observed between the Ab-coated probe and seven other human sHsp family members (Fig. 2*D*, Supplemental Table II), consistent with the earlier ELISA result that each of the proteins bound the Ig. The different sHsps bound the polyclonal Ab with a range of K_D values from approximately micromolar to high nanomolar, which was consistent with a specific and biologically relevant interaction. The only caveat of this interpretation of the results is that the murine anti-Fc Ab attached to the tip was polyclonal. Consequently, a similar argument using the varying affinities of the Abs can be made to rationalize the observed variation in the association rates, but such an argument does not explain how an anti-Fc Ab binds not just one, but eight sHsps with apparent equivalent affinity. Such an interaction also would not be expected to exhibit the observed temperature depen-

Table I. Observed kinetic rate constants and calculated K_D of the interaction between HspB5 and a murine anti-Fc Ab when measured using bilayer interferometry

Molar Concentration (M)	k_{obs} (1/s)	Error in k_{obs}	k_d (1/s)	Error in k_d	k_a (1/Ms)[a]	K_D (M)[a]	Associated R^2
1.00E-05	8.47E-04	5.48E-06	1.19E-05	4.20E-07	8.36E+01	1.42E-07	0.9321
3.33E-06	8.07E-04	6.30E-06	5.65E-05	6.66E-07	2.25E+02	2.51E-07	0.9018
1.11E-06	1.14E-03	1.02E-05	1.10E-04	1.75E-06	9.27E+02	1.19E-07	0.8577
3.70E-07	8.25E-04	5.71E-06	7.67E-05	1.04E-06	2.02E+03	3.79E-08	0.9309
1.23E-07	1.52E-03	2.21E-05	3.48E-04	6.18E-06	9.55E+03	3.65E-08	0.7114
4.10E-08	8.51E-04	6.87E-06	8.07E-05	1.24E-06	1.88E+04	4.30E-09	0.9056

[a]Increases occur in the measured rates of association, and consequently a decrease in the calculated K_d values, as the concentration of the sHsp is reduced.

dence. More likely, the polyclonality of the Ig contributes to the complexity of the binding kinetics of the sHsps.

Immunoprecipitation and identification of interactions using mass spectrometry

If sHsps bind Igs with micromolar-nanomolar affinity/avidity in vitro, the interaction should be detected in human serum. To test this hypothesis, we incubated 5 μg HspB5 with 300 μl aliquots of sera from MS patients and healthy individuals at 42˚C for 4 h, after which time we added anti–T7-resin (recognizing the HspB5 epitope tag) and incubated the mixture at 42˚C for another 4 h. The beads were separated from the sera by centrifugation, and the HspB5 and the mixture of bound proteins were eluted with glycine buffer pH 1.8. There was approximately three times more protein eluted from patients than normal sera at this step based on absorption at 280 nm. The eluted proteins were concentrated, and the resultant client proteins and HspB5 were separated by SDS-PAGE. The Coomassie-stained bands were excised, the proteins lysed with trypsin, and the resultant peptides analyzed by mass spectrometry.

The principal bands precipitated from MS sera in addition to HspB5 corresponded to IgM, IgG1, and IgG4 H chains, λ and κ L chains, and albumin, whereas albumin, IgG1 H chain, and λ and κ L chains were observed in samples from normal sera (see Supplemental Material). The ability of HspB5 to precipitate Ig from patients' and normal sera is the third independent confirmation that sHsps can bind Igs.

Discussion

Capturing Abs from patients' sera using microtiter plates or microchips coated with HspB5 was the basis for assigning the protein as a serological autoantigen in each literature citation we were able to identify (3–7, 29). In this article, the interaction between HspB5 and Igs was confirmed using this assay; however, by extending the investigation to multiple members of the sHsp family and by studying the kinetics of binding, the sHsps were shown to be receptors of, and not Ags for, Igs. By demonstrating that sHsps bind Igs, the puzzle of how the sHsps were historically assigned as autoantigens, even though they were therapeutically beneficial, can be understood. Because the sHsps can bind Abs, the standard method of analyzing whether Abs in plasma can bind the putative Ag when adhered to a solid support leads to problems of interpretation because the assay is fundamentally ambiguous. Whether there is a humoral response to HspB5 in MS or other neurologic disorders will be difficult to establish because the design of most immunoassays relies on the specificity of the Ab–Ag interaction and does not consider the possibility of an Ag binding the Ab.

The experiments in this report also revealed a number of important features of sHsps, previously unappreciated. The first was that the entire family of sHsps is able to bind Igs in a temperature-dependent fashion because of their shared function as chaperones. The binding of Igs in vivo also could be a factor in the therapeutic role of sHsp in reducing inflammation (6). The doses of the protein used in the therapeutic experiments were too low to modulate anti-myelin–specific Igs from sera (6), but sHsps could modulate Ig concentration in cerebrospinal fluid. Reduction of Abs to myelin correlated with a positive therapeutic outcome in experiments on Ag-specific tolerance to myelin proteins (30). The temperature dependence of binding was expected from an Hsp; nevertheless, the differences in the amount of protein bound were impressive. What was unexpected was the rate of association of sHsps with an Ig as their concentration decreased without significant changes in the rate of dissociation, resulting in significant lower dissociation constants. Such a result can be rationalized because of the multiple, divergent binding sites on the Hsp, but nevertheless was remarkable and could explain how a "nonspecific" Hsp could exhibit selectivity in binding proteins at sites of inflammation and not act systemically. This important hypothesis is being explored further in a separate study (J.B. Rothbard, M. Kurnellas, C. Addams, L. Su, C.G. Fathman, and L. Steinman, manuscript in preparation).

Where on the Ig molecule the sHsps bind remains to be determined, but as chaperones, they likely will interact with the edge strands, which are known to be susceptible to partial unfolding and responsible for the formation of extended β-pleated sheets characteristic of L chain amyloid formation (26, 27, 31). Reduced folding stability appears to be a unifying property of amyloidogenic L chains (27).

The assignment that α B-crystallin was an autoantigen was primarily based on the presence of specific Abs against the protein. Data in this article establish that the association arises from the sHsp binding the Ab and not the reverse, which also is consistent with similar apparent responses between MS patients and healthy control subjects. When the lack of clear data that there is a humoral response to the protein is taken together with the inability of T cells specific for HspB5 to transfer symptoms of EAE (9, 32), the argument that this protein is a pathogenic autoantigen is weak and perhaps without foundation.

Disclosures

The authors have no financial conflicts of interest.

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