Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis

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Multiple sclerosis (MS) is a chronic relapsing disease of the central nervous system (CNS) in which immune processes are believed to play a major role. To date, there is no reliable method by which to characterize the immune processes and their changes associated with different forms of MS and disease progression. We performed antigen microarray analysis to characterize patterns of antibody reactivity in MS serum against a panel of CNS protein and lipid autoantigens and heat shock proteins. Informatic analysis consisted of a training set that was validated on a blinded test set. The results were further validated on an independent cohort of relapsing–remitting (RRMS) samples. We found unique autoantibody patterns that distinguished RRMS, secondary progressive (SPMS), and primary progressive (PPMS) MS from both healthy controls and other neurologic or autoimmune driven diseases including Alzheimer's disease, adrenoleukodystropy, and lupus erythematosus. RRMS was characterized by autoantibodies to heat shock proteins that were not observed in PPMS or SPMS. In addition, RRMS, SPMS, and PPMS were characterized by unique patterns of reactivity to CNS antigens. Furthermore, we examined sera from patients with different immunopathologic patterns of MS as determined by brain biopsy, and we identified unique antibody patterns to lipids and CNS-derived peptides that were linked to each type of pathology. The demonstration of unique serum immune signatures linked to different stages and pathologic processes in MS provides an avenue to monitor MS and to characterize immunopathogenic mechanisms and therapeutic targets in the disease.

antibodies | autoimmunity | biomarker

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) of presumed autoimmune etiology (1). Approximately 85–90% of patients begin with a relapsing–remitting (RRMS) course and 40% eventually become progressive (secondary progressive MS, SPMS); in 10%, MS presents a primary progressive course (PPMS). MS is also heterogeneous in its immunopathological patterns of active infiltrating inflammatory cells and demyelination (2).

There is evidence that immune processes play a major role in disease pathogenesis and progression (1); however, to date there has been no reliable method by which to identify and characterize immune processes in the serum that are unique to MS. Antigen microarrays are newly developed tools for the high-throughput characterization of the immune response (3), and have been used to analyze immune responses in vaccination and in autoimmune disorders (3–6). Our interest in investigating autoreactivity to arrays of self-antigens in MS is based on the hypothesis that patterns of multiple reactivities may be more revealing than single antigen–antibody relationships (7, 8) as shown in our previous analysis of autoimmune repertoires of mice (5, 9) and humans (10, 11) in health and disease. Thus, autoantibody repertoires have the potential to provide insights into the pathogenesis of the disease and to serve as immune biomarkers (12) of the disease process.

Results

Conditions to Detect Specific Microarray Autoantibodies in MS. We constructed antigen microarrays using 362 myelin and inflammation-related antigens [supporting information (SI) Table S1] that encompassed CNS antigens associated with MS, CNS antigens associated with other neurological diseases and heat shock proteins (HSP). Antigens were spotted on epoxy glass slides as described (5).

We compared the sensitivity of the antigen-microarray technique with that of a standard ELISA technique using commercially available monoclonal and polyclonal antibodies directed against CNS, HSP and lipid antigens. The antigen microarray detected antigen reactivities at $\log_{10}$ dilutions that were 1–2 logs greater than the reactivities detected by using the ELISA method (Table S2). Thus, the antigen microarray appear to be more sensitive than a standard ELISA. These results are consistent with published reports (3).

To determine which serum dilution was optimal to investigate immune signatures in MS, we analyzed the reactivity of healthy controls (HC) and RRMS subjects at dilutions of 1:10, 1:100 and 1:1,000 for both IgG and IgM antibodies. In MS the mean IgG antibody reactivity to CNS antigens, lipids and heat shock proteins was highest at 1:10 as compared with 1:100 and 1:1,000 where minimal reactivity was observed ($P < 0.0001$, 2-way ANOVA, Fig. S1A). The mean IgG reactivity was also highest at a 1:10 dilution in HC ($P < 0.0001$, 2-way ANOVA), but this reactivity was less than that manifested in MS subjects ($P < 0.001$, $P < 0.001$ and $P < 0.05$ for CNS antigens, lipids and heat shock proteins respectively, 2-way ANOVA); indeed, at dilutions of 1:100 and 1:1,000, there were no differences between the magnitude of IgG reactivity in MS compared with HC. The IgM reactivities in controls were as high, if not higher than in MS subjects. This is consistent with the observation that healthy humans are born with IgM autoantibodies to myelin antigens and heat shock proteins (11). Because MS subjects manifested significantly elevated serum IgG autoantibodies at a 1:10 dilution, we investigated serum antibody patterns with antigen microarrays by using this dilution.

To establish that the reactivity detected at a 1:10 dilution was specific, we carried out inhibition experiments that demonstrated that reactivity to PLP$_{261-277}$ on the antigen array could be inhibited

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by preincubation of the serum with excess, unbound PLP$_{261-277}$, but not with a control peptide, HSP60$_{1-20}$ (Fig. S1*B*).

Autoantibody Pattern Analysis Identifies an Immune Signature for RRMS. To investigate whether we could identify unique antibody signatures in RRMS we studied the antibody repertoire in 38 patients with RRMS and 30 HC subjects. We allocated samples into a training set (24 RRMS and 20 controls) and a randomly selected test set (14 RRMS and 10 controls). The training set was used to determine whether we could identify patterns of antibody reactivity that could discriminate RRMS from control samples. If such patterns were found, they were then validated on the test set. The training set was analyzed by using the Wilcoxon–Mann–Whitney test; we controlled for the false discovery rate using the method of Benjamini and Hochberg (13). The clinical characteristics of the patients and HC are listed in Table S3.

As shown in the heat map in Fig. 1*A*, we identified a pattern of reactivity that distinguished RRMS from HC ($P < 0.0001$, Fisher's exact test). This pattern consisted of 94 antibody reactivities (Table S4). Of the 94 reactivities, 90 were up-regulated and 4 were down-regulated in MS versus controls. Thus, RRMS was associated with both a gain and a loss of particular autoreactivities. Of the up-regulated reactivities, 50% were IgM antibodies binding to peptides of CNS antigens and 49% were IgM antibodies binding to peptides of heat shock proteins. The ability to distinguish MS vs. controls was not observed at dilutions of 1:100 or 1:1,000 (data not shown).

To validate the discriminating pattern shown in Fig. 1*A*, we performed a leave-1-out cross-validation analysis (LOOCV) in the training set (13) and then validated the results on the test set. For the LOOCV in the training set, the number of true (correct) and false (incorrect) classifications was computed to estimate the success rate, positive predictive value (PPV), negative predictive value (NPV) in the training set. The LOOCV revealed a positive predictive value (PPV)—defined as the fraction of RRMS patients identified as RRMS by their antigen microarray reactivity—of 0.75 and a negative predictive value (NPV)—defined as the fraction of HC identified as HC by their antigen microarray reactivity—of 0.90; the success rate was 0.83 ($P < 0.0001$). The most rigorous validation is to test the patterns identified in the training set to determine whether they can differentiate MS subjects from HC in the test set. We found that the pattern identified in the training set was able to classify the test set of samples with a PPV of 0.85 and a NPV of 0.80, and with an success rate of 0.83 ($P = 0.004$, Fisher's exact test).

To further validate our findings, we analyzed 51 untreated RRMS obtained from the University of Seville to determine whether we could distinguish RRMS from HC using an independent cohort of samples from another institution and geographic area. We were able to discriminate RRMS from HC in this independent cohort with a success rate of 0.69 with a PPV of 0.73 and a NPV of 0.58 ($P = 0.01$, Fisher's exact test).

As a specificity control for the patterns detected in MS, we investigated sera from patients with systemic lupus erythematosus (SLE), adrenoleukodystrophy (ALD) and Alzheimer's disease (AD). SLE is a chronic autoimmune disease characterized by circulating antibodies to a broad range of self-antigens (14). ALD is a degenerative disorder characterized by the accumulation of very long-chain fatty acids and a CNS neuroinflammatory process that shares features with MS (15). AD is not considered an autoimmune disease; however, immune responses to β-amyloid-derived peptides have been reported (reviewed in ref. 16). We found that antibody patterns detected on antigen microarrays discriminated RRMS from SLE, ALD and AD samples ($P < 0.0001$, Fisher's exact test).

Autoantibody Pattern Analysis Identifies an Immune Signature for PPMS. PPMS has a different clinical course than RRMS, and it has been suggested that PPMS may involve disease mechanisms dif-
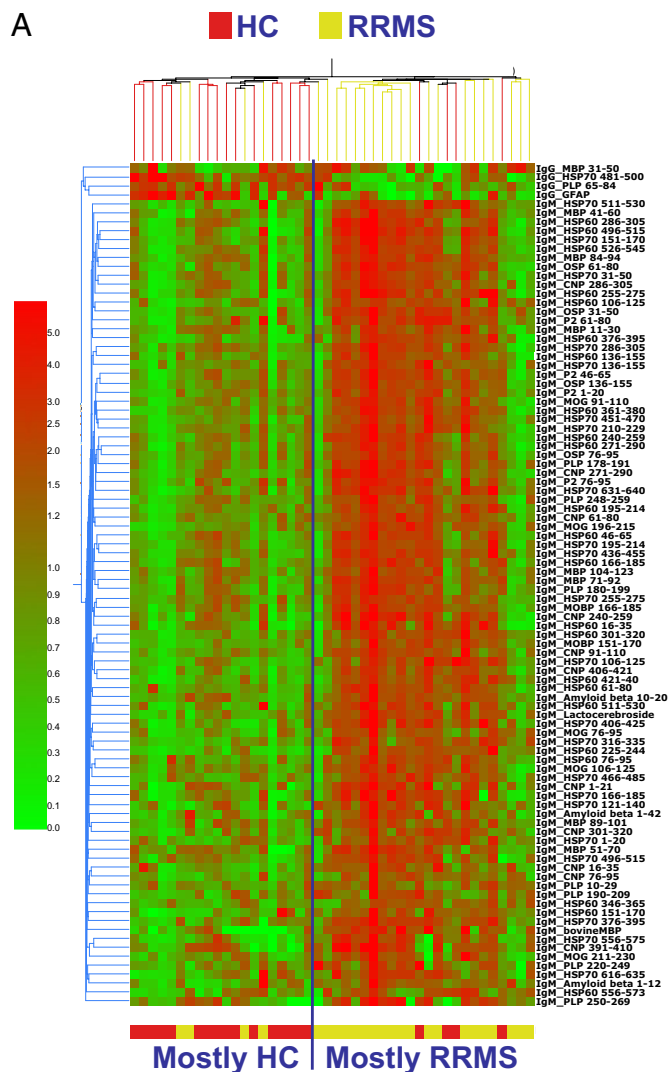

Fig. 1. Serum antibody reactivity in RRMS and PPMS. (*A* and *B*) Antibody reactivities discriminating RRMS (*A*) and PPMS (*B*). Shown is a heat map depicting the antibody reactivity in RRMS (*A*), PPMS (*B*) or HC samples. The antibody reactivities included in these heat maps are listed in Table S4 (RRMS) and Table S5 (PPMS). (*C*) Antigen specificity in RRMS and PPMS, shown as the relative contribution of CNS, HSP, and lipid antigens (percentage relative to total number of discriminating antigens) found to be up- or down-regulated in MS relative to HC. (*D*) Diagram summarizing the immune signatures associated with RRMS, SPMS, and PPMS. (*E*) Heat map depicting the antibody reactivities in SPMS and RRMS samples. The antibody reactivities included in this heat map are listed in Table S8. (*F*) Antigen specificity in SPMS, shown as the relative contribution of CNS, HSP, and lipid antigens (percentage relative to total number of discriminating antigens) found to be up- or down-regulated in SPMS relative to RRMS.

ferent from those in RRMS (17). We studied 24 PPMS and 25 age- and gender- matched HC in a training set, and 13 PPMS and 12 controls in a test set of samples. The heat map in Fig. 1b shows the antibody reactivities that passed significance tests and could discriminate PPMS and HC both in the training set ($P < 0.0001$, Fisher's exact test) and the test set ($P < 0.01$, Fisher's exact test). The LOOCV on the learning set revealed an overall efficiency of 86%, with PPV = 0.87 and NPV = 0.85. The efficiency for the test set was 72%; the PPV = 0.79 and the NPV = 0.75. As with RRMS, antigen microarrays were able to discriminate PPMS from control subjects at a 1:10 dilution but not at dilutions of 1:100 or 1:1,000. Furthermore, as with RRMS and SPMS, the antigen microarray analysis discriminated between PPMS and other diseases (SLE, ALD, AD; $P < 0.001$, Fisher's exact test).

B

HC   PPMS

Mostly HC   Mostly PPMS

IgM_PLP 215-232
IgG_PLP 215-232
IgM_mMBP
IgG_HSP70 195-214
IgM_smLPS
IgM_HSP70 210-229
IgM_Chondritin 4-Sulfate
IgG_HSP70 166-185
IgG_bovineMBP
IgM_bovineMBP
IgG_PLP 137-150
IgG_MOG 46-65
IgG_CNP 406-421
IgG_P2 31-50
IgG_CNP 1-20
IgG_MOG 16-35
IgG_P2 76-95
IgM_Neurofilament 68kDa
IgM_Beta Amyloid
IgG_HSP70 466-485
IgM_AB 1-40
IgM_PLP 161-180
IgM_PLP 40-59
IgM_PLP 137-150
IgG_HSP60 76-95
IgG_MOG 151-170
IgG_P2 1-20
IgG_OSP 61-80
IgM_Secreted APPalpha
IgG_PLP 178-191
IgM_gpMBP
IgG_HSP70 16-35
IgG_MBP 104-123
IgM_SOD
IgM_CNP 1-20
IgM_ecLPS
IgG_HSP70 121-140
IgM_MOBP 61-80
IgG_OSP 1-20

C

Up-regulated MS vs. HC

% of antibodies (relative to number of discriminating antibodies)

CNS   HSP   Lipid

RRMS   PPMS

Down-regulated MS vs. HC

% of antibodies (relative to number of discriminating antibodies)

RRMS   PPMS

D

Healthy subjects

CNS[1]
↑HSP

CNS[2]

RRMS → CNS[3] → SPMS   PPMS
      ↓HSP

E

RRMS   SPMS

Mostly SPMS   Mostly RRMS

IgM_MOG 61-80
IgM_HSP60 376-395
IgM_MOG 31-50
IgM_CNP 361-380
IgM_Amyloid beta 1-23
IgM_CNP 346-365
IgM_HSP60 496-515
IgM_OSP 1-20
IgM_HSP60 511-530
IgM_OSP 61-80
IgM_HSP60 286-305
IgM_CNP 240-259
IgM_HSP70 601-620
IgM_HSP60 210-229
IgM_HSP60 451-470
IgM_MOBP 166-185
IgM_HSP60 166-185
IgM_MBP 138-147
IgM_CNP 195-214
IgM_HSP70 286-305
IgM_P2 1-20
IgM_HSP60 526-545
IgM_MBP 155-178
IgM_P2 46-65
IgM_HSP60 195-214
IgM_P2 31-50
IgM_HSP60 271-290
IgM_HSP60 136-155
IgM_CNP 286-305
IgM_HSP70 210-229
IgM_HSP70 136-155
IgM_PLP 150-163
IgM_HSP70 166-185
IgM_HSP60 255-275
IgM_HSP60 16-35
IgM_bovineMBP
IgM_CNP 181-199
IgM_CNP 121-140
IgM_Asialoganglioside-GM2
IgM_Amyloid beta 1-12
IgM_OSP 121-140
IgM_Secreted APPbeta
IgM_Cardiolipin
IgM_HSP70 406-425
IgG_HSP60 361-380
IgG_Amyloid beta 17-40
IgG_Cholesterol
IgG_Amyloid 1-42
IgG_PLP 80-99
IgG_PLP 65-84
IgG_PLP 40-59
IgM_PLP 1-19
IgG_PLP 1-19
IgG_PLP 151-173
IgG_HSP70 421-440
IgG_huMBP
IgG_MOBP 16-35
IgG_CNP 16-35
IgG_RBP
IgG_HSP70 331-350
IgG_OSP 121-140
IgG_MBP 113-132
IgG_beta Crystallin
IgG_CNP 240-259
IgG_PLP 178-191

Disease

F

Up-regulated RRMS vs. SPMS

% of antibodies (relative to number of discriminating antibodies)

CNS   HSP   Lipid

IgM   IgG

Down-regulated RRMS vs. SPMS

% of antibodies (relative to number of discriminating antibodies)

IgM   IgG

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Fig. 1. (continued)

The discriminating reactivities in PPMS were IgG (51%) and IgM (49%) and were mainly directed against CNS antigens (Fig. 1 *B* and *C* and Table S5). The CNS antigens in the PPMS immune signature were different from those in the RRMS signature. We have termed the RRMS CNS signature CNS[1] and the PPMS CNS signature CNS[2] (Fig. 1*D* and Table S6). When we further compared RRMS and PPMS we found a pronounced reactivity against HSP60 or HSP70 in RRMS that was not observed in PPMS (Fig. 1*A*–*D* and Table S7). Furthermore, 46% of the discriminating reactivities in PPMS consisted of antibodies that were decreased in PPMS compared with HC, whereas in RRMS only 4% of the discriminating antibodies were decreased compared with HC (Figs. 1 *B* and *C*). There was only a minor overlap between the reactivities that discriminated PPMS and those that discriminated RRMS compared with HC (Table S6 and Table S7). This finding is compatible with the view that different immune processes occur in these 2 forms of MS (17).

Autoantibody Pattern Analysis Identifies an Immune Signature for SPMS. Approximately 50% of the RRMS patients become progressive (SPMS) (18). Although there is no consensus on the mechanisms involved in the transition to SPMS, several studies suggest changes in the nature of the inflammatory response and the emergence of neurodegenerative processes occur in the secondary progressive phase of MS (18). Having identified an autoantibody signature in RRMS that consisted of increased reactivity to HSP and a unique pattern of reactivity to CNS antigens (CNS[1]) we studied the antibody signature associated with SPMS by comparing antibody reactivity in 37 RRMS vs. 30 SPMS samples (Fig. 1*E*).

We found that SPMS could be discriminated from RRMS with a success rate of 71% ($P = 0.0073$). SPMS was characterized by a decrease in the IgM antibodies to HSP60 and HSP70 that we found in RRMS (Table S7 and Table S8 and Fig. 1*E*). Thus, SPMS and PPMS are similar in that both have only minimal reactivity to HSP. When we examined the CNS reactivity in SPMS we found a decrease in CNS IgM antibodies that were up-regulated in RRMS, and an increase in CNS-reactive IgG antibodies. The CNS signature for SPMS differed from both RRMS and PPMS and we have termed it CNS[3] (Figs. 1 *D*–*F* and Table S6).

Autoantibody Patterns Distinguish Pathologic Subtypes of MS. Lucchinetti, Bruck and Lassman have defined four immunopathologic patterns of MS (2). The pattern of active demyelination is identical among multiple active lesions examined from a given MS patient, yet heterogeneous between patients, suggesting pathogenic heterogeneity. Pattern I is characterized by T cell/macrophage-mediated demyelination. Pattern II is characterized by antibody/complement-associated demyelination. Pattern III is defined by a distal oligodendrogliopathy, and pattern IV is characterized by oligodendrocyte degeneration in the periplaque white matter; to date pattern IV has only been identified in autopsy cases. Patterns I and II lesions show the typical perivenous distribution and sharp borders that are the pathological hallmarks of MS lesions and are thought to result from classical autoimmune mechanisms (2). We investigated serum taken at the time of brain biopsy from 15 Pattern I and 30 Pattern II subjects. As shown in Fig. 2, we were able to discriminate pattern I from pattern II ($P = 0.0082$, Fisher's exact test). To validate this finding, we analyzed a blinded set of samples that contained the 15 pattern I that we used for analysis above mixed randomly with 23 new pattern II samples. In this validation test, we were also able to distinguish pattern I from pattern II ($P = 0.0017$, Fisher's exact test). The LOOCV on the learning set revealed a success rate of 0.78, with PPV = 0.78 and NPV = 0.67, the success rate for the test set was 0.78; the PPV = 0.82 and the NPV = 0.73.

The immune signature that distinguished pattern I from pattern II consisted of 13 IgG and 1 IgM reactivities against lipids, HSP and CNS antigens (Fig. 2). Pattern II subjects showed increased IgG reactivity to HSP60, MOG, OSP and PLP peptide epitopes. Note-
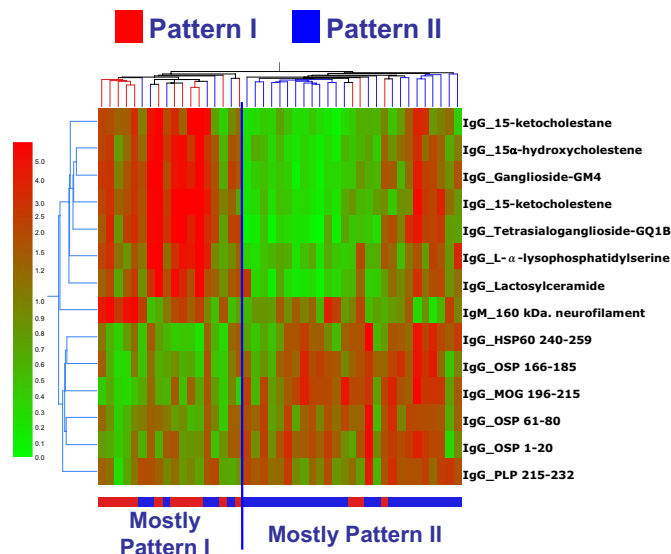

Pattern I   **Pattern II**

IgG_15-ketocholestane
IgG_15α-hydroxycholestene
IgG_Ganglioside-GM4
IgG_15-ketocholestane
IgG_Tetrasialoganglioside-GQ1B
IgG_L-α-lysophosphatidylserine
IgG_Lactosylceramide
IgM_160 kDa. neurofilament
IgG_HSP60 240-259
IgG_OSP 166-185
IgG_MOG 196-215
IgG_OSP 61-80
IgG_OSP 1-20
IgG_PLP 215-232

Mostly Pattern I   **Mostly Pattern II**

Fig. 2. Antibody reactivity associated with brain pathology. Shown is a heat map depicting the antibody reactivity in Pattern I and Pattern II samples according to the colorimetric scale shown on the left. The antibody reactivities used to construct this heat map are listed in Table S9.

worthy, the up-regulated reactivities in pattern I subjects were IgG antibodies to 7 lipids; 3 of these lipids were oxidized derivatives of cholesterol (15-ketocholestene, 15-ketocholestane and 15a-hydroxycholestene).

Cholesterol Derivatives Worsen Experimental Autoimmune Encephalomyelitis (EAE). To explore the relationship between autoantibodies to oxidized cholesterol derivatives (oxChol) and disease pathology, we examined the effect of these lipids on EAE, which serves as an immune model of MS. We administered 15-ketocholestene, 15-ketocholestane and 15α-hydroxycholestene to C57BL/6 mice at the time of EAE induction with MOG_{35-55} and on days 4, 7 and 10 after induction. Administration of oxChol enhanced EAE as measured clinically (Fig. S2*A*, $P < 0.0001$, 2-way ANOVA), and augmented inflammatory infiltrates ($P < 0.05$, 1-way ANOVA), demyelination ($P < 0.01$, 1-way ANOVA) and axonal loss ($P < 0.001$, 1-way ANOVA) (Fig. S2*B*).

It has been postulated that the oxidized derivative of cholesterol, 7-ketocholesterol, contributes to MS pathology by activating microglial cells via a poly (ADP-ribose)-polymerase-1 enzyme (PARP)-dependent pathway (19). To investigate whether the effect of oxChol on EAE we observed was mediated by PARP we used the PARP inhibitor, 5-Aminoisoquinolinone (AIQ) (20). We found that AIQ abrogated the worsening of EAE caused by oxChol both clinically ($P < 0.0001$, 2-way ANOVA) and histopathologically ($P < 0.001$, 1-way ANOVA) (Fig. S2) but did not affect T cell responses to MOG_{35-55} as measured by cytokines (IFN-γ and IL-17) or proliferation (data not shown). In addition, transfer of serum from oxChol-treated mice did not enhance EAE. Taken together, these results suggest that the effect of oxChol on EAE is due to the effect of oxChol through PARP and not through the induction of anti-lipid antibodies or affecting adaptive T cell responses to MOG_{35-55}.

Discussion

We report here that antigen-microarray analysis of autoantibodies can identify serum autoantibody signatures associated with different clinical forms and pathologic subtypes of MS; the signatures were based on collective autoantibody patterns, not single autoantibody reactivities. These informative patterns emerged from autoantibodies that bound peptides of myelin molecules and HSP,

proteins and lipids; the informative autoantibodies were detectable at 1:10, but not at higher dilutions. Moreover, the informative patterns included decreases as well as increases of autoantibody reactivities relative to those found in healthy subjects. Antigen microarrays have been used to characterize serum autoantibodies in SLE (21) and rheumatoid arthritis (22), but have not been successfully applied to MS. In the CSF, antigen microarrays in MS have detected antibodies to lipid (6) and αB-crystallin (23) and of note, the αB-crystallin reactive antibodies were of low affinity, detectable at 1:20 dilution (23). High-affinity autoreactive antibodies in the serum have not been consistently found in MS (24–28).

We found that unique autoantibody signatures characterize RRMS, SPMS and PPMS based on reactivity to CNS antigens and HSP. Each of the different clinical forms of MS had a unique antibody signature directed against CNS antigens that we have termed CNS[1] (RRMS), CNS[2] (PPMS) and CNS[3] (PPMS). CNS[1] was characterized by a broad IgM reactivity to CNS antigens, CNS[2] by a more restricted IgM and IgG reactivity and CNS[3] by an increase in IgG reactivity to CNS antigens. In addition to the reactivity to CNS antigens, RRMS was notable for a pronounced antibody response to HSP. Strikingly, antibody responses to HSP were markedly decreased in both SPMS and PPMS. HSP are augmented in response to inflammation, and HSP up-regulation has been reported in MS lesions (29). Thus, the decrease of reactivity to HSP in progressive forms of MS is consistent with the less inflammatory nature of progressive MS and its relative lack of response to immunomodulatory therapy (17, 18). Indeed, we have found increased serum levels of HSP60 and HSP70 in RRMS patients (F.J.Q. and H.L.W., unpublished work).

In addition, HSP-specific immunity (30–32) and HSP60 itself (33) have been reported to be immunoregulatory. Thus, it is also possible that the intermittent attacks and recovery that characterize RRMS reflect HSP-linked immunomodulation.

We found that unique serum antibody patterns were associated with different patterns of MS pathology. Pattern II MS pathology was associated with increased IgG antibodies to HSP60, OSP, MOG and PLP peptide epitopes, whereas increased antibody reactivity to lactosylceramide and L-α-lysophosphatidylserine was linked to pattern I, and these antibodies have been described in the CSF of MS patients (6). Pattern I serum samples also contained antibodies to oxidized cholesterol derivatives. Increased levels of 7-ketocholesterol, a related oxidized derivative of cholesterol, have been found in the CSF of MS patients and may contribute to MS pathology by activating microglial cells via a PARP-1 dependent pathway (19). Consistent with this, we found that the administration of oxidized cholesterol derivatives worsened EAE by activating PARP-1 in microglia and CNS macrophages.

Pattern I pathology in MS is considered to result from macrophage/microglia-mediated demyelination, whereas Pattern II is thought to involve complement activation and other antibody-dependent mechanisms (34). Our results support a pathogenic role for oxidized derivatives of cholesterol and identify PARP as a target for therapeutic intervention in pattern I MS pathology. Furthermore, from a clinical standpoint patients with pattern II, but not I, have been reported to respond to plasmapheresis (35). Thus, the antibody signatures we have identified may be useful for identifying patients that would be responsive to treatment with plasmapheresis.

Although cell-mediated immunity against myelin antigens is felt to play a major role in MS (1), B cells and autoantibodies also appear to contribute to disease pathogenesis (36). How do our results relate to the role antibodies vs. T cells in the MS disease process? Most of the CNS antigen-reactive autoantibodies detected with our antigen microarrays are directed at linear epitopes. Antibodies to linear epitopes in MBP and MOG have been purified from active CNS lesions of MS patients (37) and may trigger the deposition of complement and other disease-amplifying mechanisms (38). In this regard, we detected antibodies to linear epitopes in CNS antigens in serum samples from MS patients with pattern

II lesions, which are characterized by antibody deposition and complement activation (2). In addition, it has been shown that in MS patients there is an overlap between linear B and T cell epitopes targeted on MBP (39). Thus, the serum autoantibodies to linear epitopes might reflect the specificity of an ongoing T cell response. Note, however, that low-affinity autoantibodies are present in healthy individuals, and changes in the reactivity of these autoantibodies have been described in several autoimmune disorders including MS (40, 41). It is now recognized that MS is a complex heterogeneous immunologic disease (1). We believe that the serum autoantibody immune signatures we have identified reflect this complex process, not a single autoantibody that plays a dominant role in the disease such as would be the case for antibodies to the acetylcholine receptor in myasthenia gravis (42). Thus, the differences in the fine antibody specificity between MS and HC are not likely to identify pathogenic antibodies causing MS.

Taken together, how do the immune signatures we have identified relate to the disease process? As described above, the reactivity to HSP in RRMS appears related to the more inflammatory nature of MS in the initial relapsing stages. We believe that the different antibody signatures against CNS antigens also reflect the ongoing inflammatory process in the brain due to the traffic of immune cells, antibodies and/or antigen between the brain and periphery (36, 43). This is supported by our finding of unique serum patterns linked to type I or type II pathology as measured by brain biopsy. We have also studied antibody reactivity in paired CSF and serum samples. Our data suggest unique CSF antibody patterns in MS compared with other neurologic diseases and a partial overlap of the antibody response in the CSF compared with serum in MS (F.J.Q., M.F.F., G.I., M.L., I.R.C., and H.L.W., unpublished work).

In summary, our findings provide an avenue for the study of immune mechanisms in MS. The demonstration that serum microarray antibody patterns are linked to disease stage and pathologic subtype suggests that they may be used to monitor disease progression and aid in decisions regarding therapy. Furthermore, because the antibody signature appears to reflect immune processes in the CNS, antigen arrays provide a tool to identify new immunopathogenic mechanisms and therapeutic targets.

Materials and Methods

Antigens, Antibodies, and ELISA. The antigens used in the construction of antigen microarrays are listed in Table S1, they were purchased from Sigma, Abnova, Matreya, Avanti Polar Lipids, Calbiochem, Chemicon, GeneTex, Novus Biologicals, Assay Designs, ProSci, EMD Biosciences, Cayman Chemical, HyTest, Meridian Life Science, and Biodesign International. The peptides were synthesized at Harvard Medical School. The antibodies were purchased from Abcam, Matreya, Abnova, Calbiochem, and Jackson ImmunoResearch. ELISA was performed as described (11).

Samples. Serum samples from untreated RRMS during clinical remission, PPMS patients or HC were collected at the Partners MS Center; the patients did not present with other autoimmune disorders. Paired CSF and serum samples were collected at the University Hospital, School of Medicine, University of Seville from RRMS patients and controls. Sixty-two patients with biopsy-proven CNS inflammatory demyelinating disease were identified from the CNS biopsy cases belonging to the MS Lesion Project (MSLP). The MSLP database consists of a unique collection of biopsy-proven CNS cases with detailed pathological, clinical, imaging and serological material (NMSS RG3184-B-3-02). Active demyelinating lesions were classified into either pattern I or II based on published criteria (2). The clinical characteristics of the patients, pathological cohorts and healthy controls are listed in Table S3.

Antigen Microarray Production, Development, and Data Analysis. The antigens were spotted as described (5, 10). The microarrays were blocked for 1 h at 37 °C with 1% BSA, and incubated for 2 h at 37 °C with the sample in blocking buffer. The arrays were then washed and incubated for 45 min at 37 °C with a 1:500 dilution of goat anti-human IgG Cy3-conjugated and goat anti-human IgM Cy5-conjugated detection antibodies (Jackson ImmunoResearch). The data were analyzed with the nonparametric Wilcoxon–Mann–Whitney test, by using the

Benjamini and Hochberg method with a false discovery rate of 0.05 or 0.2 (for immunopathology pattern I and II samples) (3). The samples were classified by using a support vector machine constructed by using the antibody reactivities identified to be discriminatory on the training set (13).

EAE Induction. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Harvard Medical School. EAE was induced and scored as described (44). AIQ (Sigma) was administered daily at 3 mg/kg, i.p. Axonal loss and demyelination were assessed at day 19 after EAE induction (44).

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