This view is consistent with data showing that TCR-positive thymocytes can induce the development of $CD4^+8^+$ cells when injected into rearrangement-deficient mice[14,15] and explains the absence of $CD4^+8^+$ thymocytes in rearrangement-deficient[6,7], TCR-negative[6] as well as CD3-negative mice (B. Malissen, personal communication). Differentiation through the $CD4^-8^-25^-$ subset requires cell-autonomous signals delivered by the pre-TCR, and for that reason in normal mice almost all $CD4^+8^+$ T cells contain productive TCR-β genes[16], whereas in the absence of the pre-TCR, only ~2% of TCRαβ-positive $CD4^+8^+$ precursors are generated by some aberrant differentiation. Nevertheless, positive selection will operate on these cells and generate mature αβ T cells whose number is regulated by homeostasis independent of the pre-TCR[17]. □

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The small heat-shock protein αB-crystallin as candidate autoantigen in multiple sclerosis

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THE identification of key antigens in human autoimmune diseases is a crucial step towards the development of specific intervention. The autoantigen(s) relevant to multiple sclerosis (MS) probably reside in myelin of the central nervous system, the target of the disease[1]. Here we examine proliferative responses of human peripheral blood T cells to the complete collection of myelin proteins fractionated by reversed-phase high-performance liquid chromatography. Myelin isolated from MS-affected brain contained a single protein fraction to which T cells from MS patients and from healthy controls showed dominant responses. This highly immunogenic protein was identified as αB-crystallin, a small heat-shock protein. Immunohistochemical examination of MS lesions revealed the presence of oligodendrocytes and astrocytes with raised αB-crystallin expression, which were not found in unaffected myelin. Our findings indicate that αB-crystallin serves as immunodominant myelin antigen to human T cells when expressed at the elevated levels found in active MS lesions.

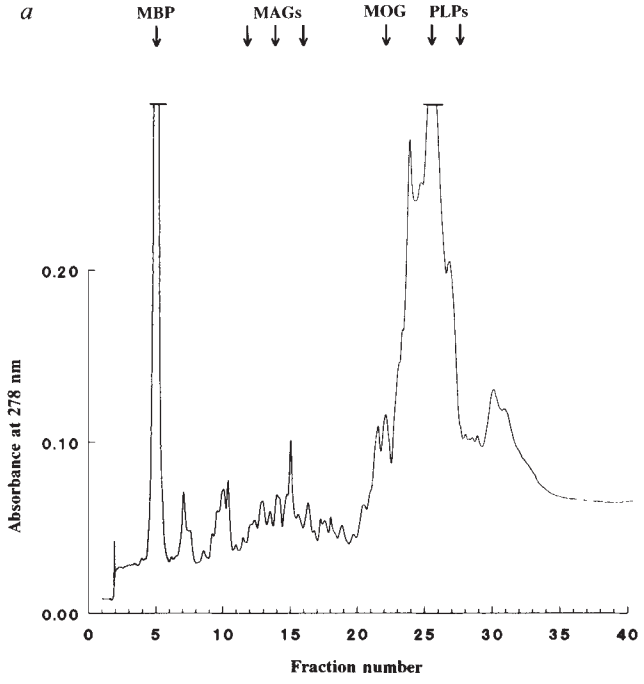
To examine responses of human T cells to the complete collection of myelin proteins from the central nervous system (CNS), short-term cultures were raised *in vitro* by priming peripheral blood mononuclear cells (PBMC) from 24 HLA-typed donors with total myelin protein. Proteins used for this purpose were prepared from either healthy control myelin or from myelin purified from MS-affected white matter. Five PBMC donors were clinically defined MS patients and the other nineteen were healthy control subjects. After one cycle of restimulation with total myelin protein, T cells were collected and tested for proliferative responses to each of the forty reversed-phase high-performance liquid chromatography (RP-HPLC) fractions prepared from the same protein preparation as that used for priming. Figure 1*a* shows the RP-HPLC profile of fractionated total myelin protein from MS-affected brain, which is virtually identical to that from control material. Data from the proliferation assays were essentially the same in all cases, irrespective of HLA-type or clinical status of the donor. A representative set of data from five donors is shown in Fig. 1*b*.

The data in Fig. 1 illustrate two points. First, bulk T-cell responses after priming with all CNS myelin proteins were always directed primarily at the various minor myelin proteins contained in HPLC fractions 7–20. In contrast, responses to the major components proteolipid protein or myelin basic protein were barely significant. These findings extend our previous observations that the relative immunogenicity of individual CNS myelin proteins to human T cells bears no apparent relationship to their relative abundance in myelin[2]. Second, the data in Fig. 1 reveal a distinct difference between responses to proteins derived from either healthy myelin or MS-affected tissue. In the latter case, a consistently predominant response was detected against the contents of RP-HPLC fraction 8. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) showed that this fraction contained only one detectable protein, with an apparent relative molecular mass (M_r) of about 23K (Fig. 2). The same protein was detected as the single component in the corresponding fraction derived from the control preparation but in smaller amounts.

To identify the 23K protein in fraction 8, the protein was purified from MS-affected white matter by RP-HPLC. Direct amino-acid sequencing of the purified preparation was unproductive, indicative of an N-terminal modification. Thus, the 23K protein was trypsinized and a selection of the resulting fragments was purified by RP-HPLC and sequenced. As a result, four internal sequences were identified that were identical to sequences of human αB-crystallin. The amino-acid composition of the purified 23K protein was consistent with the full sequence of human αB-crystallin. In line with this the behaviour of purified human 23K protein and bovine αB-crystallin was identical in RP-HPLC and in SDS–PAGE. Furthermore, polyclonal rabbit antibodies

raised against the purified 23K human protein were crossreactive with bovine αB-crystallin upon western blotting (Fig. 2).

To verify that αB-crystallin is indeed the immunogenic component in HPLC fraction 8, responses of myelin-primed T cells to fraction 8 were compared with those against αB-crystallin purified from bovine eye lens, which is identical to the human homologue at all but four of the 175 amino-acid positions. Figure 3 illustrates that responses of myelin-primed T cells to HPLC fraction 8 and purified αB-crystallin were comparable in proliferation as well as in their release of the pro-inflammatory cytokines interferon-γ and interleukin-2. Conversely, bulk T-cell cultures primed with purified bovine αB-crystallin were strongly crossreactive to total myelin protein as well as to the contents of HPLC fraction 8. Earlier reports identifying αB-crystallin as a small heat-shock protein[3,4] prompted us to test whether quantitative differences in the local expression of αB-crystallin could explain our findings (Fig. 1*b*). Expression of αB-crystallin was examined by immunohistochemistry of 34 MS plaques corresponding to different developmental stages from 28 MS patients and five control white-matter samples with no neuropathological condition. In all but two inactive plaques, αB-crystallin-expressing glial cells were found grouped within or at the edge of the lesional area. No, or very few, glial cells with αB-crystallin expression could be detected in control white matter or unaffected white matter from MS brains (data not shown). To identify the cellular origin of αB-crystallin in MS lesions, double staining was performed with myelin/oligodendrocyte glycoprotein and myelin-associated glycoprotein as a marker for oligodendrocytes, and glial fibrillary acidic protein as a marker for astrocytes (Fig. 4)[5,6]. αB-crystallin colocalized with oligoden-



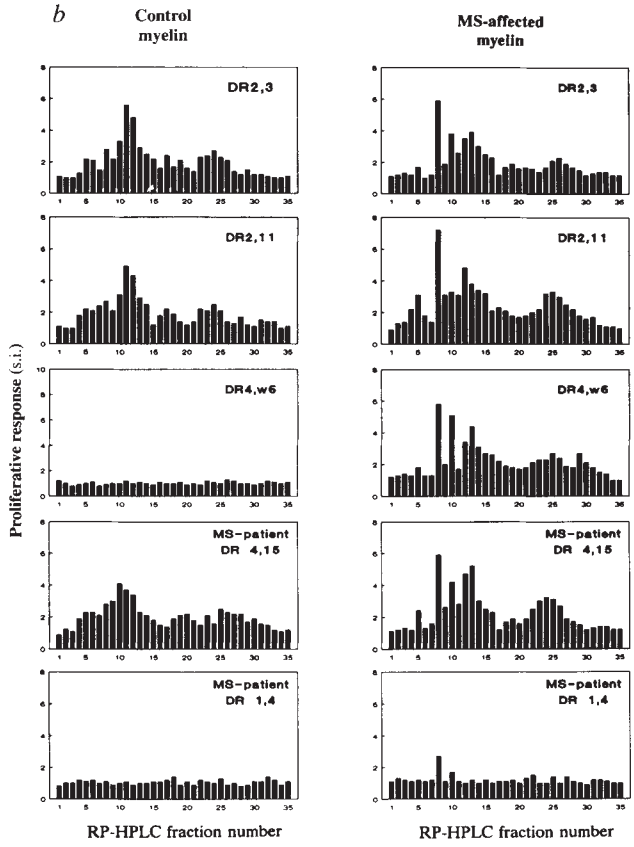


FIG. 1 *a*, RP-HPLC fractionation of the complete collection of CNS myelin proteins derived from MS-affected white matter[20]. As previously shown, many myelin proteins elute as sharp single protein peaks, like myelin basic protein (MBP) (fractions 5/6) and myelin/oligodendrocyte glycoprotein (MOG) (fraction 22). Others, such as proteolipid protein (PLP) or myelin-associated glycoprotein (MAG), exist as differentially modified species with varying hydrophobicities and elute over a series of adjacent fractions. In this way, MAG is recovered in fractions 10–15 and PLP in fractions 22–32. *b*, Proliferative responses of myelin-primed T-cell cultures to HPLC-fractionated CNS myelin proteins from either control myelin (left panels) or MS-affected myelin (right panels). s.i., Stimulation index, defined as (response with antigen)/(response without antigen).

METHODS. CNS myelin was purified by density-gradient centrifugation[21] from human white matter and collected as two distinct pools of samples. One pool contained myelin from eleven clinically definite MS patients; all samples include MS plaques, as confirmed by magnetic resonance imaging and histopathological examination. Control myelin was prepared from white matter from 21 control brains. Total myelin protein was extracted and delipidated as described[20]. Before using the total protein extract as antigen, lyophilized protein was dissolved into 2-chloroethanol/0.1% triofluoroacetic acid and dialysed extensively against water. Protein concentrations were determined by amino-acid analysis and equal amounts of proteins derived from either myelin preparation were used throughout all experiments. Peripheral blood mononuclear cells (PBMC) from each donor were cultured in RPMI1640 (Dutch modification; supplied with 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES pH 7.4 and 10% pooled human serum) at 2×10^5 cells per 200 μl at 37 °C in a humidified stove containing 5% CO_2. Total myelin protein was added as priming antigen at 25 μg ml^{-1}. After 7 days, growing T cells were restimulated with 10^5 irradiated (30 Gy) autologous PBMC per 200 μl and fresh antigen (25 μg ml^{-1}). After another 4 days, recombinant human IL-2 was added to a final concentration of 50 U ml^{-1}. At day 14, T cells were collected, pooled and tested for proliferative responses to various antigens by seeding 5×10^4 T cells together with 5×10^4 irradiated autologous PBMC and antigen in 200 μl of fresh culture medium. After 3 days, [^{3}H]thymidine was added (20 kBq per well) and after another 24 h of culture, thymidine incorporation was determined using a β-plate counter. For use as a test antigen, HPLC-fractionated proteins were lyophilized, redissolved in 2-chloroethanol/0.1% trifluoroacetic acid and dialysed against water. Protein concentrations were determined by amino-acid analysis. A fixed proportion of the contents of each fraction was added as antigen to give a final protein concentration of ~50 μg ml^{-1} for the fractions containing the largest amounts of protein (fractions 5/6 and 24–27). Varying the dose of antigen yielded data consistent with the altered dose, indicating that the low responses to MBP or PLP were not due to inappropriate dosage (data not shown).

droglial as well as with astroglial markers in both acute and chronic MS plaques. Intense staining of oligodendrocytes for αB-crystallin was frequent in early lesions whereas astrocytic staining predominated in older lesions. Some staining of myelin itself was also distinguishable, but was close to background. There was no colocalization of αB-crystallin with markers specific for infiltrated T cells, B cells or macrophages. As previously reported[7–10], enhanced αB-crystallin expression in some glial cells was also evident in white matter samples from donors affected by other neuropathological diseases such as Alzheimer's, Parkinson's, Huntington's, Pick's and Lewy body diseases (data not shown), but staining of glial cells was generally less intense than in MS lesions and was lost more rapidly at higher serum dilutions.

Other heat-shock proteins, including cognates of the Hsp60, Hsp70 and Hsp90 families, have been detected in glial cells in MS lesions[11–13], but the factors inducing these larger heat-shock proteins are probably different from those that trigger expression

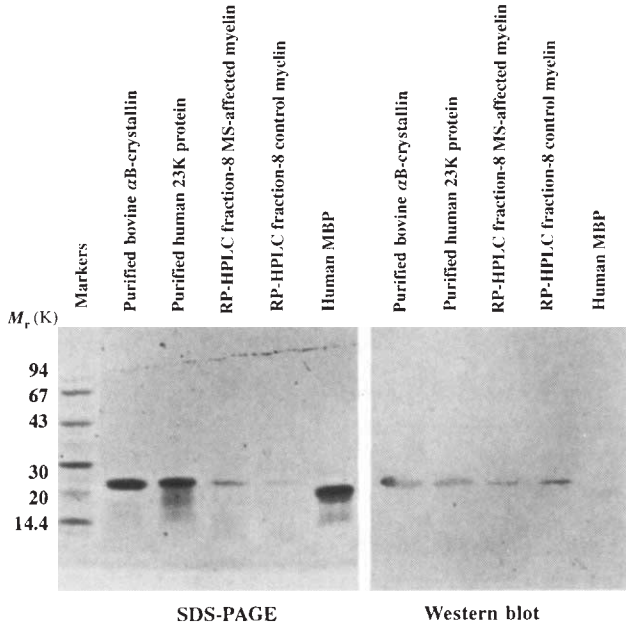


FIG. 2 SDS–PAGE and western blot analysis of the contents of HPLC fractions 8, compared to bovine αB-crystallin, purified 23K protein and human MBP, showing comigration between purified bovine αB-crystallin and the 23K protein contained in HPLC fractions 8. Western blotting reveals crossreactivity of a polyclonal rabbit serum raised against purified 23K protein towards bovine αB-crystallin. Sequence analysis of tryptic peptides from the 23K protein yielded the following sequences: LFDQFR (human αB-crystallin residues 23–28), YLR (residues 48–50), APSWFDTGLSEMR (residues 57–69) and IPADVDP(L) (residues 124–131).
METHODS. Bovine αB-crystallin was purified by RP-HPLC from a commercial preparation of α-crystallin (Sigma). Standard SDS–PAGE analysis was done using an 8–25% gradient polyacrylamide gel (Pharmacia LKB). Equal samples of the contents of HPLC fraction 8 derived from either control myelin or MS-affected myelin were used for comparison. For western blotting a 1:25 dilution of rabbit serum raised against purified 23K protein from MS-affected myelin was used.

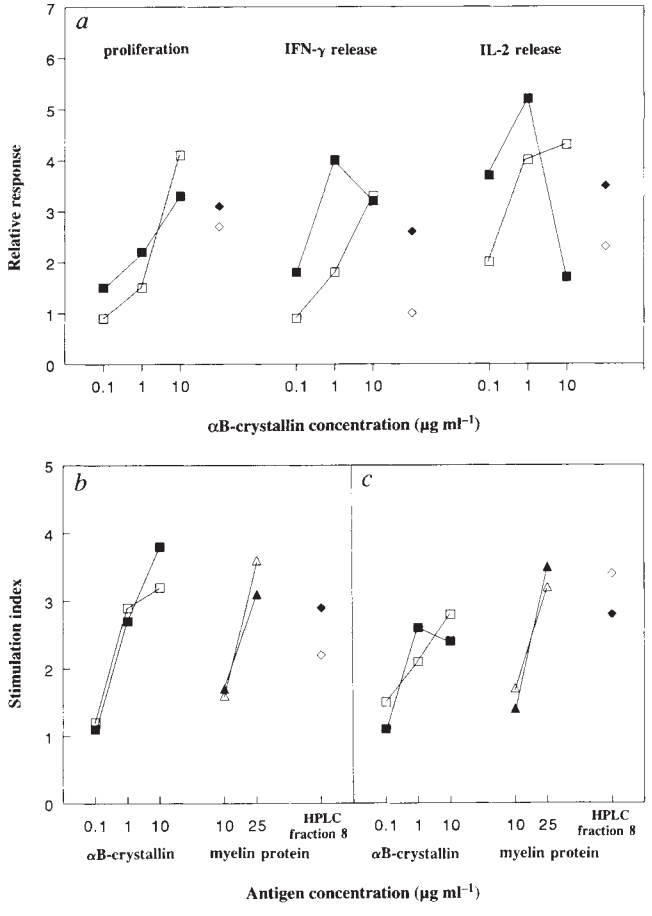


FIG. 3 Human T cells show full crossreactivity between total myelin protein, the contents of HPLC fraction 8, and αB-crystallin purified from bovine eye lens, identifying αB-crystallin as the immunogenic component in HPLC fraction 8. *a*, T cells primed with total myelin protein respond to purified αB-crystallin equally as well as they do to the contents of HPLC fraction 8. T cells from two donors (open symbols: DR2,4; closed symbols: MS patient DR4,15) were primed against total myelin protein. After 2 weeks, bulk responses against the contents of HPLC fraction 8 derived from MS-affected myelin (diamonds) were compared to those against varying amounts of purified αB-crystallin (squares). Proliferative responses as well as release of the pro-inflammatory cytokines IFN-γ and IL-2 are expressed relative to the levels found in the absence of any antigen. *b*, T cells primed against αB-crystallin crossreact with total myelin protein and the contents of HPLC fraction 8. *c*, T cells primed with the contents of HPLC fraction 8 crossreact with both αB-crystallin and total myelin protein. For *b* and *c*, PBMC were used from two other donors (open symbols: MS patients DR2,4; closed symbols: control donor DR 11,14). PBMC were primed with purified αB-crystallin at 1 μg ml^{-1} or with the contents of HPLC fraction 8 according to the protocol described in the legend to Fig. 2. After two weeks of culture, T cells were collected and assayed for proliferative responses to the antigens indicated. At the dosage of fraction 8 used in all experiments, the concentration of αB-crystallin as a component of this fraction was estimated by SDS–PAGE and western blotting at ~1 μg ml^{-1}.
METHODS. T cell priming and assays for proliferative responses are described in Fig. 2 legend. Release of IFN-γ and IL-2 were determined using culture supernatants drawn following a 2-day culture with the antigen indicated. Concentrations of IFN-γ were determined by ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam) and of IL-2 using the CTLL-L assay. Briefly, 5×10^3 CTLL-L cells were exposed to culture supernatant for 24 h before being pulsed with 20 kBq [^{3}H]thymidine. Thymidine incorporation was determined as for Fig. 2.

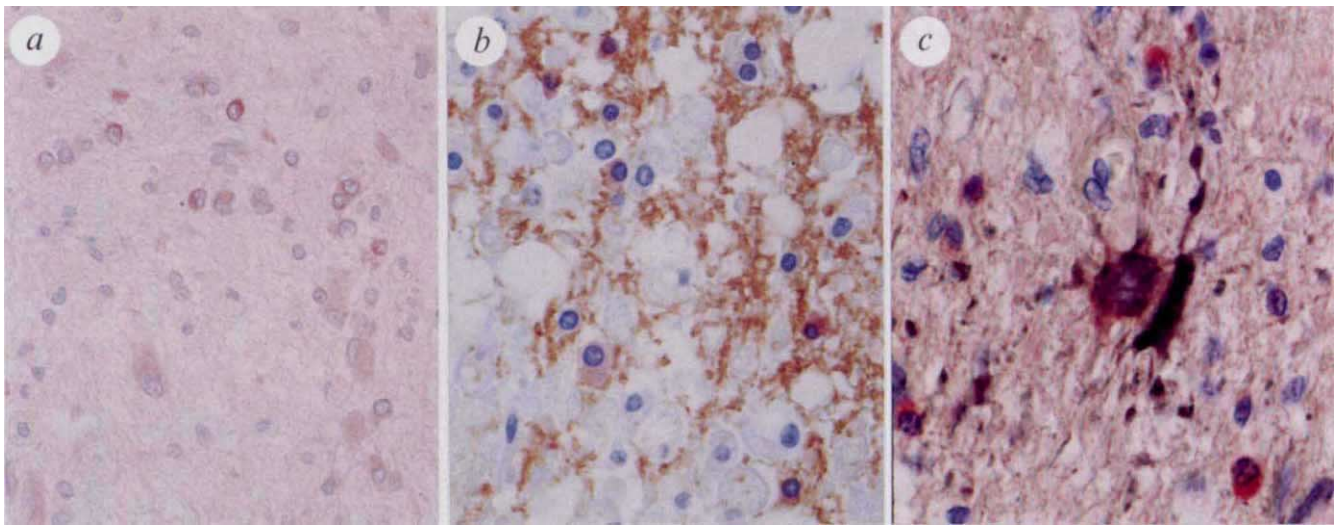


FIG. 4 Both oligodendrocytes and astrocytes in MS lesions show enhanced expression of αB-crystallin. Sections of formalin-fixed paraffin-embedded white-matter samples from active MS lesions were stained for human αB-crystallin, MOG as a surface marker for oligodendrocytes, or glial fibrillary acidic protein (GFAP) as a marker for astrocytes. *a*, Single staining of an acute MS lesion for αB-crystallin (red) showing high intracellular expression in some oligodendrocytes and moderate expression in astrocytes (**100**×). *b*, Double staining for αB-crystallin (red) and MOG (brown) identifying oligodendrocytes as αB-crystallin-producing cells in active MS lesions (**133**×). *c*, Double staining for αB-crystallin (red) and GFAP (dark blue) revealing αB-crystallin expression also in astrocytes (**250**×). A double-stained astrocyte is seen surrounded by single-stained oligodendrocytes.

METHODS. Formalin-fixed paraffin-embedded human white-matter sections (5 μm) were deparaffinized in xylene and hydrated in ethanol. Following blocking of endogenous peroxidase activity and a 10-min incubation in 10% FCS, sections were stained for the different markers. Staining for αB-crystallin was done with a 1:1,500 dilution in 10% (vol/vol) FCS of polyclonal rabbit antibodies raised against αB-crystallin purified from MS-affected human myelin. After rinsing, binding was visualized using biotinylated donkey anti-rabbit antibodies and, subsequently, avidin-labelled alkaline phosphatase and fast red (Sigma). Specificity of the αB-crystallin staining was confirmed by the observation that preincubating polyclonal rabbit antiserum with RP-HPLC-purified bovine αB-crystallin from eye lens at 5 μg ml^{-1} completely blocked the staining observed. No blocking was observed when the serum was preincubated with a 5 μg ml^{-1} purified bovine αA-crystallin, which shares about 60% sequence identity with αB-crystallin. MOG staining was performed with a murine anti-MOG monoclonal antibody, followed by peroxidase-conjugated goat anti-mouse antibody (Dianova Jackson) and 3'-diaminobenzidine. GFAP was visualized using a murine monoclonal antibody against GFAP (Boehringer Mannheim); secondary steps were the same as for anti-MOG staining except for the use of chloronaphthol as a chromogen. Counterstaining was performed using haematoxilin.

of the small Hsp αB-crystallin[14,15]. Also, rather than triggering responses in T cells with αβ T-cell receptors, Hsp60 and Hsp70 may help mount responses by γδ-bearing T cells[11,13]. Flow cytometry of short-term T-cell cultures raised against αB-crystallin provided no evidence for recruitment of γδ T cells by αB-crystallin *in vitro* (data not shown).

Our key finding is that αB-crystallin acts as immunodominant myelin antigen to human T cells when expressed at the increased levels found within and immediately around active MS lesions in the human brain. Stress-induced expression of αB-crystallin in human disease has so far been found primarily in CNS glial cells and much less—if at all—in other types of cells or tissues[7,10,16]. Neurodegenerative diseases, neurotropic infection and oncogene expression are among the factors found so far to be associated with elevated αB-crystallin expression in the CNS. Constitutive expression occurs in several other tissues, including eye lens, cardiac muscle and kidney epithelial cells[7,17,18]. Together, these data indicate that the expression of αB-crystallin is unlikely on its own to trigger demyelinating autoimmunity in MS, but may contribute to the amplification of local inflammatory responses in parallel with disturbance of the blood–brain barrier, cytokine production and expression of major histocompatibility antigens, and of co-stimulatory and adhesion molecules. Our data indicate that peripheral blood T cells from MS patients and healthy controls respond similarly to αB-crystallin, as they do to other self antigens[1,19], which suggests that local antigen-presenting features of the target tissue itself, rather than any aberrance of the peripheral T-cell repertoire, are crucial to the development of autoimmunity in MS. The fact that the immunodominant myelin antigen identified here is expressed at locally regulated levels is in line with this view. MS lesions may develop if pro-inflammatory factors—including the autoantigen itself—accumulate locally beyond a threshold of control as a result of stress-producing events such as local immune responses to viral antigens. We propose that αB-crystallin in this context serves as key myelin antigen in the development of MS. □

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