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Presentation of αB-Crystallin to T Cells in Active Multiple Sclerosis Lesions: An Early Event Following Inflammatory Demyelination

Jeffrey J. Bajramović,* Arianne C. Plomp,* Annette van der Goes,† Cindy Koevoets,* Jia Newcombe,‡ M. Louise Cuzner,§ and Johannes M. van Noort‡*‡

In the development of multiple sclerosis (MS), (re)activation of infiltrating T cells by myelin-derived Ags is considered to be a crucial step. Previously, αB-crystallin has been shown to be an important myelin Ag to human T cells. Since αB-crystallin is an intracellular heat shock protein, the question arises at what stage, if any, during lesional development in MS this Ag becomes available for CD4+T cells. In 3 of 10 active MS lesions, αB-crystallin could be detected inside phagocytic vesicles of perivascular macrophages, colocalizing with myelin basic protein and myelin oligodendrocyte glycoprotein (MOG). Although the detectability of MOG in phagosomes is considered as a marker for very recent demyelination, MOG was detected in more macrophages and in more lesions than αB-crystallin. The disappearance of αB-crystallin from macrophages even before MOG was confirmed by in vitro studies; within 6 h after myelin-uptake αB-crystallin disappears from the phagosomes. αB-Crystallin-containing macrophages colocalized with infiltrating T cells and they were characterized by expression of MHC class II, CD40, and CD80. To examine functional presentation of myelin Ags to T cells, purified macrophages were pulsed in vitro with whole myelin membranes. These macrophages activated both myelin-primed and αB-crystallin-primed T cells in terms of proliferation and IFN-γ secretion. In addition, αB-crystallin-pulsed macrophages activated myelin-primed T cells to the same extent as myelin-pulsed macrophages, whereas myelin basic protein-pulsed macrophages triggered no response at all. These data indicate that, in active MS lesions, αB-crystallin is available for functional presentation to T cells early during inflammatory demyelination. The Journal of Immunology, 2000, 164: 4359–4366.

Multiple sclerosis (MS) is a chronic demyelinating disease of the CNS characterized by focal areas of demyelination (lesions or plaques). Although the exact etiology of MS is unknown, it is generally accepted that autoimmunity is involved and that the autoantigen(s) probably reside in CNS myelin, the target of the immune response (1, 2). Myelin consists of numerous candidate autoantigenic proteins among which are major myelin constituents like myelin basic protein (MBP) and proteolipid protein (PLP), and minor myelin constituents like myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase). In the complete collection of proteins extracted from MS-affected myelin, the dominant human Ag for CD4+T cells appears to be αB-crystallin (3), a small heat shock protein (4, 5).

αB-Crystallin is present at enhanced levels in the cytosol of oligodendrocytes and astrocytes in MS lesions, where it is upregulated already at the earliest stages of lesional formation (6). Elevated expression of αB-crystallin in the oligodendrocyte-myelin complex correlates strongly with the immunological activity of the lesion. To locally (re)activate T cells, a potential autoantigen must be presented to the cellular immune system. In immunologically active MS lesions, characterized by ongoing or recent demyelination and the presence of inflammatory infiltrates, several myelin proteins can be detected in macrophages (7), suggesting processing and presentation of these proteins to the cellular immune system.

Detectability of such myelin proteins in macrophages, along with activation markers of macrophages, can be used to assess lesional age (8). Detection of minor myelin constituents like MOG and/or CNPase in phagocytic vesicles inside macrophages indicate recent demyelination. Macrophages containing vesicles that show immunoreactivity for the major myelin constituents MBP and/or PLP, but not for MOG or CNPase, are indicative of more advanced myelin degradation. Inactive lesions may show infiltration of T cells and of macrophages containing either empty vacuoles or myelin protein-negative vesicles, whereas the most frequently found lesions in MS patients are even older and contain less, if any, immunological activity.

In addition to immunohistochemical analyses that do not provide information with respect to the functional relevance of (auto)Ag presentation, there has been extensive research on autoantigen-specific T cell responses in MS (9). Most of these in vitro studies have been performed with purified myelin proteins, assuming that during
demyelination these proteins will be liberated from the fatty myelin membrane and will subsequently be processed and presented by APC to T cells. The role of αβ-crystallin as a putative autoantigen has been studied with protein extracts of myelin in vitro (3), and αβ-crystallin is localized in the cytosol of oligodendrocytes and astrocytes (6) in MS lesions. So far it has thus remained unclear whether αβ-crystallin actually becomes presented to MHC class II-restricted T cells in MS lesions.

We therefore investigated whether presentation of αβ-crystallin to the cellular immune system occurs in MS lesions using a combination of in situ-in vitro approach. In this report, we show that in demyelinating MS lesions αβ-crystallin can be detected inside vesicles belonging to the endosomal/lysosomal pathway in a subset of myelin-phagocytosing macrophages. These perivascular macrophages express MHC class II molecules as well as CD80 and CD40 and they are found in close proximity of infiltrating T cells, suggesting presentation of myelin-derived αβ-crystallin to these T cells. Functional presentation of myelin-derived αβ-crystallin to T cells was demonstrated in vitro by proliferative responses and IFN-γ production of αβ-crystallin-primed T cells in response to macrophages fed with whole myelin membranes. The response of myelin-primed T cells to macrophages fed with whole myelin was similar to their response to macrophages fed with purified αβ-crystallin alone, showing that much of the human T cell response to APC fed with whole myelin is accounted for by the recognition of myelin-derived αβ-crystallin. The present study demonstrates that as an early event after myelin phagocytosis in MS lesions, αβ-crystallin becomes available to T cells, suggesting an important role of this autoantigen in the pathogenesis of MS.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed on snap-frozen brain sections (10 μm) from six patients with clinically definite MS and on two control cases (obtained from the Multiple Sclerosis Tissue Bank, Institute of Neurology, London, U.K.). Ten MS lesions were selected for the presence of active demyelination using Oil red O (ORO)-positive macrophage infiltration as a marker for myelin uptake. These active lesions were analyzed for cellular infiltrates and myelin-derived protein detectability in macrophages. Staining was performed as described previously. Sections were fixed in ice-cold acetone with 0.03% H2O2 (to block endogenous peroxidase) for 10 min. Following air-drying, the sections were incubated overnight at 4°C with 1% Tween 20. Primary Abs used for glial cell and myelin protein detection were rabbit polyclonal anti-γ-aminobutyric acid (α-GFAP; Zymed, San Francisco, CA), murine monoclonal α-MOG (clone 8-18CS, provided by Dr. Sarah Piddlesden, University of Melbourne, Melbourne, Australia), and murine monoclonal anti-human α-MBP (Boehringer Mannheim, Indianapolis, IN). To study markers of immunological relevance, the following primary Abs were used: murine monoclonal anti-MHC class II (α-HLA-D; NovoCastra, New Castle, U.K.), murine monoclonal anti-CD40 (provided by Dr. Mark de Boer, Tanox Pharma B.V., Amsterdam, The Netherlands), murine monoclonal anti-MHC class II (α-HLA-D; NovoCastra, New Castle, U.K.), murine monoclonal anti-CD68 (Dako, Carpinteria, CA), murine monoclonal anti-IAP (provided by Dr. Gerard Stege (University of Nijmegen, Nijmegen, The Netherlands). The secondary Abs used were biotinylated donkey anti-rabbit (Life Sciences, St. Petersburg, FL). They were incubated in 5% FCS as described previously (11). Human recombinant αβ-crystallin was prepared as follows. αβ-crystallin encoding mRNA derived from the astrocytoma cell line U373 was amplified by RT-PCR using primers, to which SacI and KpnI restriction sites were added at the 5’ end of the upstream primer and HindIII and PolⅡ restriction sites at the 3’ end of the downstream primer. Primer sequences were: upstream, 5’-GAGCTCGGGATCCGAGCTCGGATCCGGGCTC9 - and downstream, 5’-TGCGAGCTCTCGGGGCTC9 - . PCR products were cloned with KpnⅠ and HindⅢ and subcloned into the KpnⅠ/HindⅢ site of vector pQ30 (Qiagen, Chatsworth, CA). The sequence of the construct was confirmed by sequence analysis using the Applied Biosystems Prism automatic sequencing system (Perkin-Elmer, Norwalk, CT). Histidine-tagged recombinant protein was isolated using a Ni-NTA column, followed by reversed-phase-HPLC purification. Identity of the protein was confirmed by Western blot analysis using αβ-crystallin-specific mAb (3).

T cell proliferation assay

PBMC from healthy control subjects were cultured in RPMI 1640 medium (Dutch modification) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.4), and 5% NHS at 2 x 10^6 cells per 100 μl in round-bottom wells at 37°C and 5% CO2 in the presence of 10 μg/ml human recombinant αβ-crystallin or 25 μg/ml MS-affected myelin. On days 5 and 8, 10% Lymphocult-T (Biotest Seralc, Zaventem, Belgium) in culture medium was added as source of growth-promoting cytokines including IL-2. At day 10, the T cells were harvested and Ag specificity was determined in a standard T cell proliferation assay using purified monocytes/macrophages as APC.

To isolate monocytes/macrophages, 6-well plates were incubated overnight at room temperature with 250 μl (1 mg/ml) human plasma fibronectin (6 μg/ml; Sigma). PBMC were seeded at a concentration of 10^6 cells per well in culture medium and incubated for 2 h at 37°C and 5% CO2. Nonadherent cells were removed by washing the wells twice with RPMI 1640. After an additional 72-h incubation in culture medium at 37°C and

As an additional specificity assay for the αβ-crystallin staining, Abs were supplied with partly digested αβ-crystallin (incubation time, 120 min; see below) at a 10 μg/ml concentration and incubated for 1 h at room temperature before use in the above-mentioned immunohistochemical procedure.

In vitro myelin uptake assay

Twenty-four-well plates were incubated for 2 h at room temperature with 0.5 ml (2 mg/ml) human fibronectin (Central Laboratory of the Netherlands, Amsterdam, The Netherlands) per well. PBMC at a concentration of 2 x 10^6/ml in RPMI 1640 + 10% FCS + streptomycin (50 mg/ml), penicillin (100 IU/ml), and 1 mM glutamine was added per well and incubated for 48 h at 37°C and 5% CO2. The supernatant was discarded and the cells were washed twice with RPMI 1640. The adherent monocytes were incubated for 90 min with 0.5 ml RPMI 1640 + 4% NHS and fed with 20 μg MS patient-derived myelin. The supernatant was discarded and cells were washed twice with RPMI 1640. At various time points, cells were harvested using 0.5 ml RPMI 1640 + 5 mM EDTA/well. Cells were left on ice for 15–30 min before they were harvested and centrifuged for 5 min at 1500 rpm. Cells were resuspended in PBS + 0.1% BSA and used for cytospins at 500 rpm. Cytospins were analyzed for macrophage purity using the anti-CD68 mAb and found to contain >85% monocytes. Immunohistochemistry was performed as described above.

Western blotting and in vitro degradation of αβ-crystallin

Myelin samples were lyophilized and dissolved in sample buffer (60 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-ME, and 0.01% bromophenol blue). They were subjected to standard SDS–PAGE analysis using 15% (5% gradient) gel for myelin and 10% gel for human recombinant αβ-crystallin (Pharmacia LKB, Piscataway, NJ). Western blots were analyzed for the presence of αβ-crystallin using rabbit polyclonal anti-lap70 and alkaline phosphatase-labeled swine anti-rabbit Abs (Dako). Degradation of αβ-crystallin was performed in vitro by the addition of 50 μg/ml cathepsin B (28 U/mg) and 50 μg/ml cathepsin D (8.5 U/mg) to a 2 mg/ml solution of αβ-crystallin in 50 mM sodium acetate (pH 5.0) at 37°C. Catepsins B and D were preincubated in sodium acetate/acetic acid buffer supplied with 0.14 M 2-ME. Samples were taken at various time points and diluted 10-fold in 0.5 M Tris (pH 9.0) buffer to terminate proteolytic activity. Western blot analysis of these samples using the polyclonal Abs w3/13 and lap70 was performed as described above.

Ags

Whole myelin derived from an MS patient was isolated by density gradient centrifugation, as described (10). Human MBP was isolated from human myelin as described previously (11). Human recombinant αβ-crystallin was prepared as follows. αβ-crystallin encoding mRNA derived from the astrocytoma cell line U373 was amplified by RT-PCR using primers, to which SacI and KpnI restriction sites were added at the 5’ end of the upstream primer and HindIII and PolⅡ restriction sites at the 3’ end of the downstream primer. Primer sequences were: upstream, 5’-GAGCTCGGGATCCGAGCTCGGATCCGGGCTC9 - and downstream, 5’-TGCGAGCTCTCGGGGCTC9 - . PCR products were cloned with KpnⅠ and HindⅢ and subcloned into the KpnⅠ/HindⅢ site of vector pQ30 (Qiagen, Chatsworth, CA). The sequence of the construct was confirmed by sequence analysis using the Applied Biosystems Prism automatic sequencing system (Perkin-Elmer, Norwalk, CT). Histidine-tagged recombinant protein was isolated using a Ni-NTA column, followed by reversed-phase-HPLC purification. Identity of the protein was confirmed by Western blot analysis using αβ-crystallin-specific mAb (3).
5% CO₂, the wells were washed with RPMI 1640 and adherent monocytes/macrophages were trypsinized using 0.25% w/v porcine trypsin (Sigma, St. Louis, MO) in 0.12 M NaCl, 50 mM KCl, 10 mM NaHCO₃, 0.7 mM EDTA, 20 mM HEPES, and 0.1% w/v glucose. Purity of the monocyte/macrophage population was determined by direct immunofluorescence surface staining using mAbs directed against CD14, CD3, and CD19 conjugated with FITC or PE (Becton Dickinson, Mountain View, CA) and analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson). The population contained >85% monocytes/macrophages. Proliferation assays were performed by seeding 5 × 10⁵ T cells with varying doses of irradiated (30 Gy) monocytes/macrophages in the presence of 25 μg/ml human recombinant αβ-crystallin or MS-affected myelin in 200 μl culture medium in flat-bottom wells. After a 72-h incubation, 20 kBq [³H]thymidine (Amersham Life Sciences, Arlington Heights, IL) was added per well. After 16 h, [³H]thymidine incorporation was determined by using a beta plate counter (Canberra Packard, Meriden, CT).

**IFN-γ assay**

To determine IFN-γ release in response to the Ags examined in the proliferation assays, 100 μl of culture supernatant was harvested before the addition of [³H]thymidine. IFN-γ production was determined by ELISA using a commercially available detection kit (BioSource International, Camarillo, CA).

**Results**

**αB-Crystallin is present in phagocytic vesicles in a subset of myelin-phagocytosing macrophages in active MS lesions**

Lesions were selected on the basis of the presence of inflammatory infiltrates and active demyelination. Myelin uptake, as revealed by ORO-positive lipids and MBP-immunostaining inside macrophages, indicated that ORO and MBP were detectable in numerous macrophages distributed throughout these lesions. In agreement with our previous findings (6), such lesions contain oligodendrocytes and astrocytes with clearly elevated levels of αβ-crystallin (Fig. 1A). In three of these lesions αβ-crystallin could also be detected in phagocytic vesicles of perivascular macrophages. Co-localization of ORO, MBP, MOG, and αβ-crystallin in macrophages, as demonstrated by the use of serial sections (Fig. 1, B–E), revealed that all αβ-crystallin-containing macrophages were actively phagocytosing myelin. Moreover, αβ-crystallin- and MOG-containing macrophages were far less abundant than MBP- or ORO-positive macrophages. They were restricted to the perivascular spaces and represented a small subset of MBP-positive macrophages only. In addition, MBP-containing macrophages were detected in more lesions than MOG- and αβ-crystallin-containing macrophages and MOG was detected inside phagocytic vesicles in two lesions in which αβ-crystallin was undetectable in macrophages (Table I). The pattern of Ag-laden macrophages therefore suggested a temporal relationship in the disappearance of individual myelin Ags from phagocytic vesicles after myelin uptake in the following order: αβ-crystallin, MOG, and MBP.

**αβ-Crystallin-laden macrophages reflect very recent myelin phagocytosis**

In vitro experiments were performed to gain more insight into the detectability of individual myelin proteins within intracellular vesicles in macrophages. MS-affected myelin was fed to monocytes/macrophages freshly isolated from PBMC. This myelin contained small amounts of αβ-crystallin as confirmed by Western blotting (data not shown). By immunohistochemical criteria, macrophages pulsed in vitro with whole myelin were indistinguishable from myelin-laden macrophages in MS lesions (Fig. 2, A–C). Within 6 h after myelin uptake, αβ-crystallin disappeared from phagocytic vesicles, whereas MBP and MOG remained detectable well after this period (Fig. 2, D–F). The order in which individual myelin Ags disappear from macrophages following myelin uptake is a function of the sensitivity and specificity of the Abs used to detect the Ags, the relative concentration of each Ag in myelin at the time of phagocytosis, and the relative rate of degradation of each Ag in the endosomal pathway of macrophages. For the in vitro experiment in Fig. 2, these parameters are probably comparable to those that apply to Fig. 1. The Abs used to stain the macrophages were the same as those used to stain the tissue sections in Fig. 1, the myelin membranes used as an Ag source in Fig. 2 were extracted from MS brains, and in both cases phagocytosed myelin appeared to undergo degradation in the endolysosomal compartment. These data therefore confirm the suggested order of disappearance of individual myelin Ags from phagocytic vesicles after myelin uptake in MS lesions (Table I) and support the notion that the detectability of αβ-crystallin in phagocytosing macrophages reflects very recent myelin uptake.

**αβ-Crystallin enters a proteolytic pathway inside macrophages after myelin phagocytosis**

To verify that the vesicles in which αβ-crystallin was visible belong to the endosomal/lysosomal pathway, two antisera were used that discriminate between intact and cathepsin-degraded αβ-crystallin. Recognition of αβ-crystallin by the lap70 polyclonal antibody is dependent on the integrity of the C-terminal amino acid sequence. This sequence is highly susceptible to cleavage by both cathepsins B and D, two major endosomal/lysosomal proteases. In vitro degradation followed by amino acid analyses revealed that cathepsin B recognizes two major scissile bonds in the C terminus of αβ-crystallin, i.e., T₁₇₀–A₁₇₁ and K₁₇₄–P₁₇₅ (data not shown). As shown by Western blotting, only the w3/13 antisem recognizes αβ-crystallin degradation products generated by cathepsins B and D digestion, albeit for a limited period of time (Fig. 3). The lap70 antisem almost exclusively recognizes intact αβ-crystallin, indicating that the integrity of the C terminus of αβ-crystallin is indeed rapidly lost upon proteolytic degradation. Only w3/13 was able to detect αβ-crystallin in phagocytic vesicles inside macrophages, whereas both Abs proved able to recognize αβ-crystallin expressed in the cytosol of astrocytes and oligodendrocytes. This confirms that αβ-crystallin-positive vesicles indeed belong to the endosomal/lysosomal pathway. The pattern of recognition also renders it highly unlikely that the transient detection of αβ-crystallin in macrophages reflects endogenous production of αβ-crystallin rather than myelin uptake.

**Colocalization of T cells, costimulatory molecules, and macrophages that contain minor myelin proteins in perivascular infiltrates**

To examine where Ag presentation is most likely to take place, we analyzed the MS lesions for the presence of T cells, MHC class II molecules, and costimulatory molecules CD80 and CD40. MHC class II molecules were detected widely on macrophages, microglia, and astrocytes throughout the lesional area. In contrast, expression of the costimulatory molecules CD80 and CD40 on T cells and macrophages was found to be more restricted to the perivascular regions often localized at the lesion edge. Analysis of serial sections revealed that recent phagocytic activity, as shown by the presence of MOG in phagocytic vesicles of perivascular macrophages, colocalized with the presence of MHC class II and costimulatory molecules on macrophages as well as with the presence of T cells in the direct vicinity (Fig. 1, F–I). Ag presentation in these lesions is thus most likely to take place in the perivascular regions, colocalizing with recent myelin phagocytosis.
FIGURE 1. \(\alpha B\)-Crystallin is present in a subset of myelin-phagocytosing macrophages that express MHC class II molecules as well as CD40 and CD80 in actively demyelinating MS lesions. In 3 of 10 actively demyelinating MS lesions, \(\alpha B\)-crystallin (brown) could be detected in the cytoplasm of astrocytes (arrow) and oligodendrocytes (half-open arrowhead) as well as in intracellular vesicles (arrowheads) in macrophages using the W3/13 Ab (A). Serial sections showing colocalization of myelin-phagocytosing ORO\(^{+}\) macrophages (red; B) with MBP\(^{-}\) (C), MOG\(^{-}\) (D), and \(\alpha B\)-crystallin-positive (E) macrophages. Analysis of serial sections of the same tissue for the presence of co-stimulatory molecules and T cells showed the presence of MHC class II at the cell surface of macrophages, microglia, and astrocytes widespread throughout some lesions, but also on the perivascular macrophages that were found positive for myelin proteins (F). CD80 (G) and CD40 (H) expression was found to be restricted to the perivascular spaces where active myelin phagocytosis was also detectable. In addition, T cells were found to be present as confirmed by CD3 staining (I). Magnification: A, \(\times 100\); B–I, \(\times 400\); insets, \(\times 1000\). Nuclei were counterstained using hematoxylin (blue).
In vitro uptake of whole myelin by macrophages leads to presentation of aB-crystallin to T cells

Next, we investigated whether uptake of total myelin by monocytes/macrophages not only leads to protein degradation but also to presentation of aB-crystallin to T cells. Macrophages were fed in vitro with myelin membranes derived from MS-affected brain. As a readout for Ag presentation, proliferative responses and IFN-γ production were monitored of aB-crystallin- and myelin-primed T cells derived from the same donor as the macrophages. Proliferative responses of aB-crystallin-primed T cells were found against both aB-crystallin- and myelin-fed macrophages (Fig. 4A).

In addition, these T cells produce readily detectable amounts of IFN-γ, indicative of Ag-specific activation (Fig. 4C). Similarly, myelin-primed T cells also proliferated and produced IFN-γ in response to either myelin or aB-crystallin (Fig. 4, B and D). It is remarkable to note that the strength of the proliferative responses as well as the amount of IFN-γ produced by these T cells in response to aB-crystallin-fed macrophages are comparable to the responses measured against macrophages pulsed with total myelin. These results are in marked contrast to the lack of proliferation and IFN-γ production shown in response to macrophages fed with the major myelin constituent MBP. All T cell responses were Ag

Table I. Immunohistochemical analysis of myelin degradation products in actively demyelinating lesions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diagnosis</th>
<th>Age (yr)</th>
<th>Disease Duration (yr)</th>
<th>Cause of Death</th>
<th>Sample Type</th>
<th>Presence of ORO⁺ Mφ⁺</th>
<th>Presence of MBP⁺ Mφ⁺</th>
<th>Presence of MOG⁺ Mφ⁺</th>
<th>Presence of aB-Crystallin⁺ Mφ⁺</th>
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<td>+++</td>
<td>+++</td>
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<tr>
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* Mφ, macrophage.
* +, 5 positive perivascular infiltrates; ++, 10 positive perivascular infiltrates; ++++, >10 positive perivascular infiltrates; and ++++, widespread presence of positive macrophages, not restricted to perivascular spaces.

FIGURE 2. In vitro phagocytosis of myelin by macrophages mimic the appearance of macrophages in demyelinating lesions. Macrophages were fed whole MS-affected myelin and they were fixed and analyzed by immunocytochemistry at t = 2 h (A–C) and t = 6 h (D–F). Intracellular vesicular staining of macrophages could be found for MBP (brown, A and D), MOG (brown, B and E), and aB-crystallin (using the w3/13 Ab; brown, C and F). Magnification, ×1000. Nuclei were counterstained using hematoxylin (blue).
specific since tetanus toxoid-primed T cells did neither proliferate nor produce IFN-γ in response to MBP-, αB-crystallin-, or myelin-fed APC while showing marked responses to APC fed with tetanus toxoid (data not shown).

Discussion

We have examined whether αB-crystallin, a candidate autoantigen in MS, is presented to the cellular immune system in demyelinating MS lesions. Therefore, we performed a detailed immunohistochemical analysis of 10, difficult to obtain, active demyelinating MS lesions.

The immunohistochemical analysis confirmed the previously reported enhanced expression of αB-crystallin in the cytosol of oligodendrocytes and astrocytes already during early phases of lesion formation. In this study, we show that intracellular vesicles in a subset of myelin-digesting macrophages contain αB-crystallin in addition to other myelin proteins. The use of two different antisera that discriminate intact from partially degraded αB-crystallin showed that inside these macrophages αB-crystallin enters a proteolytic pathway, most likely resulting in MHC class II-restricted presentation of αB-crystallin-derived peptides to T cells.

The detectability of myelin proteins in phagocytosing macrophages in MS lesions using immunohistochemistry has been described in great detail and is used to estimate lesional age (8). Major myelin constituents like MBP remain detectable in phagocytic vesicles inside macrophages for a longer period of time than...
minor myelin constituents like CNPase or MOG. Lesions or lesional areas containing macrophages positive for minor myelin proteins reflect recent demyelination and are therefore classified as early active lesions (7, 8). Since αβ-crystallin is a minor myelin constituent, one would expect the detectability of this protein inside macrophages to be limited. The pattern of αβ-crystallin-positive macrophages in actively demyelinating lesions was consistent with this expectation. A small number of MBP-positive macrophages and a larger number of MOG-positive macrophages were also found to be positive for αβ-crystallin. Detectability of αβ-crystallin in macrophages is thus an even more selective feature than the detectability of MOG. In vitro assays in which macrophages were fed MS, brain-derived total myelin confirmed that the detectability of αβ-crystallin in phagocytosing macrophages is indeed limited to a very short period of time, i.e., <6 h, compared with MBP and MOG which were still present after 6 h (A. van der Goes, T. K. van der Berg, and C. D. Dijkstra, manuscript in preparation). Thus, detectability of αβ-crystallin inside macrophages can be used as a marker for very recent myelin uptake.

Macrophages that contained endosomal/lysosomal vesicles positive for MBP, MOG, and αβ-crystallin, expressed MHC class II as well as costimulatory molecules CD80 and CD40, and they were found in the close proximity of infiltrating T cells. This indicates that all factors required for productive (re)activation of T cells accumulate in the areas where MOG- and αβ-laden macrophages were observed. The strict perivascular localization of APC that are fully equipped to present Ag and deliver the necessary costimulatory signals is in concordance with earlier reports describing expression patterns of CD80 and CD40 in MS lesions (12–15). Experimental allergic encephalomyelitis (EAE) studies using mannosylated liposome-encapsulated dichloromethylene diphosphonate to deplete peripheral monocytes (16–18) have shown an important role for hematogenous perivascular macrophages in the induction of EAE. Also, bone marrow chimera studies showed that the inflammatory response in EAE can start, proceed, and end virtually in the absence of resident microglia that may also be able to present Ag in the right MHC class II context (19). Recently, hematogenous macrophages were shown to modulate local activation of T cells in the brain and subsequent migration of macrophages into the brain parenchyma in adoptively transferred EAE (20). Functional interaction between monocytes/macrophages and T cells is likely to play a key role in the early phases of MS lesion development (21, 22). The perivascular localization of the phagocytosing CD68-positive cells strongly suggests that these cells are infiltrating hematogenous macrophages and not resident microglia. Recently, there have been reports that local activation of resident microglia in normal-appearing white matter in MS brains is followed by myelin phagocytosis as an initiating or very early event in MS pathogenesis, followed by the recruitment of large numbers of hematogenous macrophages (23).

In our studies, minor myelin protein-positive microglia in unaffected white matter were not observed. The most recent myelin breakdown in our material, as assessed by the detectability of minor myelin proteins in macrophages, was found to occur in the perivascular spaces. Clustering of such blood vessels was often found at the edges of the lesion. The absence of dendritic cells in the CNS renders it highly unlikely that the interactions between APC and T cells in the perivascular spaces lead to activation of naïve T cells against myelin-derived Ags. Therefore, we would strongly favor the idea that, if these T cells are to play a role in the pathogenesis of MS, they must already have been primed against myelin Ags in the periphery. Several mechanisms have been described for such a peripheral priming against CNS-specific autoantigens, many of which hypothesize the involvement of infectious agents (8, 24–27). Recently, we described a novel mechanism for the activation of peripheral T cells to αβ-crystallin, involving virus-induced presentation of this Ag by EBV-infected B lymphocytes to peripheral T cells (28).

The presence of αβ-crystallin inside phagocytic vesicles in macrophages in the vicinity of infiltrating T cells strongly suggests functional Ag presentation to T cells. To confirm functional presentation of myelin-derived αβ-crystallin to T cells, we used peripheral monocytes/macrophages fed with total myelin membranes. Total myelin membranes contain 75% lipids, whereas of the remaining 25% protein fraction MBP makes up about 12% and αβ-crystallin only makes up 0.5%. Despite this low level of expression in whole myelin, we showed that much of the total T cell response to total myelin is accounted for by the responses to αβ-crystallin. This is consistent with the previously reported vigorous T cell responses to αβ-crystallin (3). Although we took great care in purifying monocytes from peripheral blood, we cannot rule out that small numbers of dendritic cells or B cells might have had a role in Ag presentation in addition to macrophages in these in vitro assays. Even if this were the case, however, our data still show that with MS-affected myelin as starting material, αβ-crystallin becomes available for T cell recognition and acts as a dominant Ag.

In summary, data presented here show that the presence of αβ-crystallin in myelin-phagocytosing macrophages can be used as a marker for very recent myelin uptake. In these macrophages, αβ-crystallin enters the endosomal/lysosomal compartment which generally leads to MHC class II-restricted Ag presentation. Functional presentation of total myelin-derived αβ-crystallin was demonstrated using hematogenous macrophages as APC in an in vitro approach. The localization and immunological properties of αβ-crystallin-containing macrophages strongly suggests that functional presentation of αβ-crystallin to T cells takes place in the perivascular spaces of active MS lesions. As an early event following myelin phagocytosis, αβ-crystallin becomes available to the cellular immune system. The vigorous response of T cells to this protein is likely to initiate (29) or to enhance the ongoing immune response via chemokine/cytokine-induced attraction of macrophages and modulation of the properties of the blood-brain barrier.

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References