The proteasome is a major autoantigen in multiple sclerosis

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Summary
Multiple sclerosis seems to be an autoimmune disease of unknown aetiology affecting the white matter of the CNS. It is generally accepted that the autoimmune response is directed against specific components of myelin. We show here that proteasome, a ubiquitous protease complex composed of 14 different subunits, is a target for autoantibodies (IgG and IgM classes) present in the serum (66%, 73 out of 110) and in the CSF (61%, 16 out of 26) of patients with multiple sclerosis. Using recombinant proteasomal subunits we demonstrate the presence of specific autoantibodies against subunits C2, C8, C9 and C5 in multiple sclerosis patients. Recombinant C2 constructs allow us to localize an immunodominant autoepitope recognized by the sera of multiple sclerosis patients within the C-terminal of C2 proteasomal subunit (251-DEPAEKADEPMEH-263). In addition, two constructs of the recombinant proteasomal subunits C2 and C8 were also used to study the proliferation of peripheral blood mononuclear cells from multiple sclerosis patients; 12 out of 30 (40%) multiple sclerosis patients show positive proliferation with one or both of these recombinant subunits. The high prevalence of anti-proteasome autoantibodies in multiple sclerosis sera compared with sera from patients with other chronic inflammatory conditions: systemic lupus erythematosus (35%, 35 out of 100), primary Sjogren’s syndrome (16%, 5 out of 31), vasculitis (0 out of 20), sarcoidosis (7%, 1 out of 13) and Behcet’s disease (19%, 4 out of 21) suggest that humoral auto-reactivity to proteasome could be a useful test in multiple sclerosis patients that may be of help in the diagnosis and/or progression of this chronic inflammatory disease. Finally, these results suggest that some global abnormality in B and/or T cell function is also involved in the chronic inflammatory response observed in multiple sclerosis patients, as it is frequently observed in other human organ-specific autoimmune diseases.

Keywords: human autoantibodies; antigen epitopes; demyelinating diseases; cell-mediated autoimmunity

Abbreviations: GST = glutathione S-transferase; Ig = immunoglobulin; MBP = myelin basic protein; MCP = multicatalytic proteinase, proteosome; MOG = myelin oligodendrocyte glycoprotein; PBMC = peripheral blood mononuclear cells; PP = primary progressive; R/R = relapsing/remitting; SLE = systemic lupus erythematosus; SI = stimulation index; SP = secondary progressive; SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Introduction
Multiple sclerosis is an inflammatory disease of the CNS characterized by perivascular inflammation and demyelination in the white matter (Steinman, 1996; Noseworthy, 1999; Noseworthy et al., 2000). The presence of immunoglobulin deposits, macrophages, B and T cells in the lesions argues that demyelination is due to an immune response elicited by environmental factors in genetically predisposed individuals and perpetuated by an autoimmune mechanism (Steinman 1996; Noseworthy, 1999; Noseworthy et al., 2000).
The antigens driving the autoimmune response in multiple sclerosis are still not clear. In this context, myelin proteins have been studied as potential targets of autoimmune reactivity in the pathogenesis of multiple sclerosis. The importance of myelin-derived antigens [major constituents: myelin basic protein (MBP) and proteolipid protein; minor constituents: myelin associated glycoprotein and myelin oligodendrocyte glycoprotein (MOG)] have been demonstrated by their abilities to produce experimental allergic encephalomyelitis, a multiple sclerosis-like disease in various animal models (Steinman, 1999; Wong et al., 1999). In multiple sclerosis, response to these myelin antigens has been described and is probably implicated in the progression of the disease, but T-cell response to MBP, proteolipid protein and MOG can be found with similar frequencies in healthy subjects and in patients with multiple sclerosis (Warren et al., 1995; Wucherpfennig et al., 1997; Diaz-Villoslada et al., 1999).

Humoral response in multiple sclerosis patients, apart from its role in mediating demyelination (Steinman, 1996; Noseworthy, 1999; Noseworthy et al., 2000; Lassmann et al., 2001), provides a scientific rationale for the study of the T-cell response in multiple sclerosis patients. The presence of circulating autoantibodies against MBP in multiple sclerosis patients is rather controversial (Colombo et al., 1997), but they can be eluted from brain demyelinating lesions (Warren et al., 1995; Wucherpfennig et al., 1997). Antibody and T-cell mediated immune response against oligodendrocyte transaldolase (Banki et al., 1994; Colombo et al., 1997; Esposito et al., 1999) and against α B-crystallin (van Noort et al., 1995; Agius et al., 1999) have been described in multiple sclerosis patients. 2′-3′-Cyclic nucleotide 3′-phosphodiesterase, a protein present in myelin membranes, has also been described as a target of the humoral response in multiple sclerosis patients (Walsh and Murray, 1998). Nevertheless, the clinical utility of these autoantibodies remains to be fully established.

The proteasome is a 20S complex responsible for most non-lysosomal protein degradation in eukaryotes; structural homologues have been described in archaea and eubacteria (Coux et al., 1996; Bochtler et al., 1999). The overall structure of the proteasome is a hollow cylinder composed of four heptameric rings in the configuration α7β7β7α7. In eukaryotes, the proteasome is composed of seven different α and seven different β subunits (Groll et al., 1997; Bochtler et al., 1999). In vertebrates, the three active proteasome β-type subunits can be replaced by α-interferon-inducible subunits which improve the surface expression of major histo-compatibility complex class I-bound antigenic peptides and presentation (York et al., 1999). The first evidence that proteasomes are involved in autoimmune diseases was our report that sera from patients with systemic lupus erythematosus (SLE) contain specific autoantibodies directed against different polypeptide components of the proteasome (Arribas et al., 1991). This finding was later confirmed by other authors (Feist et al., 1996). Autoantibodies against proteasomes have also been reported to be present in the sera of patients with polymyositis–myositis and primary Sjögren’s syndrome (Feist et al., 1996, 1999). Subsequent work from our group demonstrated that proteasome autoantibodies are not present in several autoimmune diseases including rheumatoid arthritis, vasculitis, scleroderma, autoimmune thyroid diseases, primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis (Mayo et al., 2000). The present study demonstrates that proteasome is a major autoantigen for humoral and cell mediated response in multiple sclerosis patients.

Material and methods

Human sera

Patients included 102 with multiple sclerosis that were under no treatment prior to blood or CSF collection. All patients satisfied the criteria of definitive diagnosis of multiple sclerosis and were classified under three categories: relapsing/remitting (R/R), secondary progressive (SP) and primary progressive (PP) following established clinical consensus (Lublin and Reingold, 1996). Sera from 100 healthy subjects and 40 patients with other neurological diseases were used as controls. The disease diagnosis for patients with other neurological diseases was: four with cerebellar ataxia; five with CNS neoplasm; five with myasthenia gravis; six with cerebrovascular disease; five with peripheral neuropathies; two with viral meningitis; three with polynuropathy; and 10 with Guillain–Barré syndrome.

The anti-proteasome autoreactivity was also studied in sera of patients with other autoimmune diseases or conditions that resembled multiple sclerosis (Trojano and Paolicelli, 2001) including: 31 patients with primary Sjögren’s syndrome; 21 patients with Behcet’s disease; and 13 patients with sarcoidosis.

We have also studied CSF and sera from patients with multiple sclerosis kindly provided by Dr W. W. Tourtelotte from the National Neurological Specimen Bank, Veterans Administration Medical Center, Los Angeles, CA, USA (patient identification numbers: 7845, 7848, 6985, 10550, 8782, 9603, 8951 and 8691). The current investigation was approved by the ethical committees of the participating hospitals according to the principles of the Declaration of Helsinki and informed consent was obtained from all subjects.

Purification of proteasome and recombinant proteasomal subunits

Purified rat liver and human erythrocyte proteasome complexes were obtained as previously described (Arribas and Castaño, 1990; Ruiz de Mena et al., 1993). Purified recombinant subunits C2, C5, C8 and C9 were obtained as previously described (Arribas et al., 1994; Castaño et al., 1996; Rodriguez-Vilariño et al., 2000). The C2 constructs
used for epitope mapping have also been described (Arribas 

et al., 1994). The GST-C2 C-terminal construct was obtained 

by polymerase chain reaction (PCR) amplification of the 

coding sequence for the last 16 amino acids of the human C2 

subunit (QAADEPAEKADEPMEH) and subcloning into the 
pGEX-4T1 vector (Amersham Biosciences, Uppsala, 

Sweden). Glutathione S-transferase (GST) and GST-C2 C- 
terminal proteins were affinity-purified using glutathione–

Sepharose (Amersham Biosciences, Uppsala, Sweden). 

EClpP was purified as described previously (Arribas and 

Castano, 1993). The recombinant proteins used for prolifera-
tion assays were dialysed and concentrated against sterile 

phosphate-buffered saline (PBS) using Centrikon 30 

(Milipore, Bedford, MA, USA).

Western immunoblot analysis
Purified proteasome complex (1–2 μg) or the indicated 
recombinant proteasomal subunit (1 μg) was separated on 

14% sodium dodecyl sulfate–polyacrylamide gel electro-
phoresis (SDS–PAGE) as previously described (Arribas 

et al., 1991). Gels were either stained with Coomassie blue or 

transferred to nitrocellulose for immunoblotting. Nitro-
cellulose strips were blocked with blocking buffer 

TTBS (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% 

Tween-20) with 3% bovine serum albumin (BSA) for

3 h at room temperature with sera or CSF from human subjects 

(unless otherwise indicated) at 1/100 or 1/20 dilution, 

respectively (Arribas et al., 1991). The blots were developed 

with alkaline phosphatase labelled goat anti-human anti-

bodies (Bio-Rad, Hercules, CA, USA) at 1/1000 or a mix of 

anti-immunoglobulin (Ig) IgA, IgM and IgG (Sigma, St 

Louis, MO, USA) at 1/5000 dilution as previously described 

(Arribas et al., 1994). The Ig subclass was defined by 

developing immunoblots with alkaline phosphatase labelled 

antibodies against human IgA, IgM and IgG at 1/5000 
dilution.

Proliferation assays
Peripheral blood mononuclear cells (PBMC) were incubated 
in 200 μl serum-free AIM-V medium (Life Technologies, 
Gaithersburg, MD, USA) at 37°C in humidified air containing 

5% CO₂ at a density of 10⁶ cells/well in 96 well round bottom 
tissue culture plates (Diaz-Villoslada et al., 1999). The 

following reagents were added to triplicate wells: control, no 

addition; phytohaemagglutinin (PHA) (Sigma Chemical Co., 
MO, USA) 1 μg/ml; GST-C2 C-terminal (antigen) 10/25 μg/ 
ml, C8 (antigen) 10/25 μg/ml, GST (control) 10/25 μg/ml, 
EClpP (control) 10/25 μg/ml, MCP 2 μg/ml and recombinant 
rat MOG 10 μg/ml.

The recombinant proteins were purified from DH5α 

Escherichia coli (GST-C2Ct and GST) or from BL-21 
E.coli (C8, MOG and EClpP) (Castano et al., 1996). 
After 2 days, 0.5 μCi/well ³H thymidine (Amersham 
Biosciences, Uppsala, Sweden) was added. Cultures were 
harvested 18 h later and c.p.m. determined using a 
Beckman scintillation counter (Beckman-Coulter Inc., 
Fullerstone, CA, USA). Stimulation indices (SI) were 
calculated as the ratio of c.p.m. in the presence of antigen 
to c.p.m. in the absence of antigen (control). Positive 
proliferative responses were arbitrarily defined as SI 
values >2.0, which was 4SD above the mean of the 
control. The optimal concentrations of antigens for 
proliferation assays used in this study were established 
in preliminary experiments using a range of 2–50 μg/ml 
for each antigen. No significant proliferation (SI<2) was 
found for the proteasome antigens and control proteins 
with samples from 10 healthy subjects and from 10 
patients with multiple sclerosis unrelated diseases (clinical 
diagnoses: two with cerebellar ataxia; three with cephalae; 
three with peripheral polynuropathy; and two with 
cerebrovascular disease).

Statistical analysis
A crosstab analysis was used (χ² test) to evaluate intergroup 
differences in the percentage of positives (reactivity against 
antigens with SI >2.0). Results are presented as mean ± SD. 
All statistical analyses utilized SPSS 9.0 software (SPSS 
Science, Chicago, IL, USA).

Results
Detection of anti-proteasome antibodies in sera 
and CSF of multiple sclerosis patients
We analysed sera from 102 multiple sclerosis patients and 
100 healthy subjects by western immunoblotting with 
purified proteasome (1–2 μg); seropositivity was based on 
immunoreactivity with one or several proteasome poly-
peptides at serum dilutions of 1/100 or higher. Sixty-five sera from multiple sclerosis patients were positive and representative results are shown in Fig. 1 with titres 1/200–1/2000. Similar results were obtained with purified human erythrocyte proteasome (data not shown). No anti-proteasome seropositivity (serum dilutions 1/20–1/50) was detected in the sera of healthy subjects (n = 100) nor of patients with other neurological diseases (n = 40, see Material and methods for diagnosis). To demonstrate specificity, human antibodies were affinity-purified from proteasomes and tested by immunoblotting with total tissue extracts (Arribas et al., 1991); polypeptides with similar mass were detected in samples of purified proteasome and in total brain or liver extracts (data not shown). These results indicate that the proteasome immunoreactivity of multiple sclerosis sera is specific and not due to cross-reacting antibodies.

The majority of patients with multiple sclerosis have R/R multiple sclerosis but, in more that 50% of these patients, it eventually becomes SP multiple sclerosis. A minority, ~10% patients, have PP multiple sclerosis (Lublin and Reingold, 1996). Seropositivity against proteasome was found in 58% (34 out of 59) of patients with R/R multiple sclerosis, in 81% with SP multiple sclerosis (25 out of 31) and in 50% (6 out of 12) with PP multiple sclerosis. As a consequence, the presence of anti-proteasome antibodies is not restricted to a particular group of multiple sclerosis patients. To determine fluctuations in proteasome immunoreactivity, sera from 14 positive and 10 negative multiple sclerosis patients were tested on two to six different occasions, 6 months to 5 years apart. Sequential immunoblot analysis showed that the autoantibody titre did not change significantly (data not shown).

CSF samples from multiple sclerosis patients were also analysed for the presence of anti-proteasome antibodies. CSF from seronegative multiple sclerosis patients (n = 6) and from controls (n = 10) were negative at a 1/5 dilution (data not shown). In contrast, 66.7% (8 out of 12) CSF samples from seropositive multiple sclerosis patients were positive at 1/20 dilution (titre 1/40–1/200). In addition, 100% (8 out of 8) sera and their matched CSF samples from the National Neurological Research Bank (USA) were also positive. Fig. 2 shows the results obtained for one of these patients (10550); both serum and CSF of this patient contain antibodies to almost all the polypeptide components of the proteasome (similar to Patient 8 in Fig. 1). Fig. 2 also illustrates a common feature of proteasome autoreactivity of samples from multiple sclerosis patients—the concurrent presence of autoantibodies against different proteasomal subunits, albeit with substantially different titres. In general, the titre of proteasome antibodies was 5–10-fold lower in the CSF than in the serum of the same patient. Nevertheless, the concentration of proteasome antibodies based on the total Ig content was enriched at least 50-fold in the CSF.
SLE, Sjogren’s syndrome, vasculitis, Behcet’s disease and sarcoidosis may have clinical manifestations that can sometimes lead to misdiagnosis of multiple sclerosis patients. Therefore, it was interesting to study the seroreactivity to proteasome in these patients. We have already reported the presence of anti-proteasome seroreactivity in SLE patients (35 out of 100) (Arribas et al., 1991) and its absence in the sera of patients with vasculitis (0 out of 20) (Mayo et al., 2000). With respect to the other inflammatory conditions, we found seropositivity against proteasome in 16% (5 out of 31) of sera from patients with primary Sjogren’s syndrome (titre 1/100–1/200), in 19% (4 out of 21) of sera from patients with Behcet’s disease (titre 1/100–1/200) and in 7% (1 out of 13) of sera from patients with sarcoidosis (titre 1/200) with recognition of several polypeptide components of the proteasome complex (data not shown). These results clearly indicate that the prevalence of anti-proteasome seroreactivity in multiple sclerosis is significantly higher than in the other inflammatory conditions tested, even when compared with SLE patients (35%, n = 100) ($\chi^2 = 21.05$, $P < 0.001$).

**Autoantibodies to proteasome in multiple sclerosis are predominantly IgM and IgG in serum and CSF**

To determine the Ig class of anti-proteasome response, we analysed matched serum and CSF samples from multiple sclerosis patients with secondary antibodies specific for human IgA, IgG or IgM; representative results are shown in Fig. 3. The predominant response to proteasome was IgM and/or IgG for both serum and CSF in all the patients studied ($n = 15$). Note that for a given patient (Fig. 3), some polypeptides of the proteasome are predominantly recognized by IgM immunoreactivity while others by IgG immunoreactivity. Only one patient serum out of 30 sera analysed for immunoglobulin class shows a predominance of IgA anti-proteasome reactivity (data not shown).

The following facts suggest that the autoimmune response in multiple sclerosis patients is oligo or polyclonal:

(i) The presence in sera and CSF of multiple sclerosis patients of IgM and IgG antibodies against several proteasomal subunits with different titres.

(ii) The concordance of Ig class of anti-proteasome antibodies between CSF and serum.

(iii) The oligomeric structure of the proteasome.

It is noteworthy that the immunoglobulin subclass of positive anti-proteasome sera from patients with SLE ($n = 10$), Sjogren’s syndrome ($n = 5$), Behcet’s disease ($n = 6$) and sarcoidosis ($n = 1$) were also mainly IgM and IgG (data not shown).

**Detection of autoantibodies against proteasome with recombinant subunits**

Eukaryotic proteasomes are composed of seven different $\alpha$ and seven different $\beta$ subunits. As illustrated in Fig. 1 (Patient 8) and Fig. 2 (Patient 10550), some multiple sclerosis sera have antibodies against the whole set of proteasomal subunits, while other multiple sclerosis sera have antibodies to some but not all of the proteasomal subunits (Figs 1 and 3). The identification of the reactive subunits by simple SDS-PAGE is difficult and by two-dimensional analysis is time-consuming and may require micro-sequencing. Characterization of reactivities by the use of recombinant proteins is a general approach to the study of autoimmune diseases. To this end, recombinant C2, C8, C9 and C5 proteasomal subunits were used as substrates in immunoblotting with sera from multiple sclerosis patients. Sera of Patient 2 (Fig. 1) and Patient 15 (not shown) recognize proteasomal polypeptides of 32 and 28 kDa. Fig. 4 shows that Patient 2 serum has antibodies against the C2 (32 kDa) and both the C8 and C9 (co-migrating at 28 kDa) proteasomal subunits (Fig. 4), while Patient 15 contains antibodies to the C2 and C9 proteasomal subunits, but none to subunit C8 (Fig. 4). Serum of patient 5 reacted with a 28 kDa proteasome polypeptide (Fig. 1) and...

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**Fig. 4** Recombinant proteasomal subunits can be used for detection of proteasome seroreactivity in multiple sclerosis patients. Recombinant purified $\alpha$- (C2, C8, C9) and $\beta$- (C5) type proteasomal subunits were resolved by 14% SDS-PAGE and either stained (as indicated) or immunoblotted with sera of different multiple sclerosis patients (labelled Iblot). Iblot 2 corresponds to serum of Patient 2 shown in Fig. 1. Iblot 15 corresponds to serum of a patient that shows against whole proteasome—a pattern identical to Patient 2. Iblot 5 corresponds to serum of Patient 5 shown in Fig. 1. Iblot 6 corresponds to serum of Patient 6 shown in Fig. 1.
contains antibodies to subunits C8 and C9 (Fig. 4). Serum of Patient 6, recognizing a 23 kDa proteasome polypeptide (Fig. 1), contains antibodies against the \( \beta \)C5 subunit (Fig. 4).

These results demonstrate that recombinant proteasomal subunits produced in bacteria can be used to detect and characterize the antibody repertoire of multiple sclerosis patients and that the main epitopes recognized by these autoantibodies are present in the primary sequence of the proteasomal subunits. At present, we have not found any correlation between the pattern of reactivity to individual proteasomal subunits and either disease type or duration.

**Epitope mapping of anti-C2 antibodies from multiple sclerosis patient sera**

Another advantage of recombinant antigens is their use for epitope mapping. Using a full-length recombinant C2 subunit, seropositivity against C2 by immunoblotting was found in 14 out of 32 proteasome-seropositive multiple sclerosis patients (at 1/100 dilution). To define the regions of proteasomal subunit C2 targeted by the C2 autoantibodies present in sera of multiple sclerosis patients, deletion constructs of full-length C2 cDNA were used. Representative results from four of these C2-seropositive multiple sclerosis patients are shown in Fig. 5. Sera of multiple sclerosis patients recognize the C2 subunit present in the proteasome complex and the full-length recombinant subunit C2 (Fig. 5, lanes 1 and 2). A C-terminal deletion construct, mC2.24, that eliminates the last 13 amino acids (Fig. 5, lane 3) is not recognized by the antibodies present in the sera of multiple sclerosis patients. Extended deletions from the C-terminal (protein constructs mC2.23 and 22; Fig. 5 lanes 4 and 5) are also not recognized by the multiple sclerosis patient sera. Restoration into a truncated form of the C2 subunit (construct mC2.23) of the DNA encoding the C-terminal of subunit C2 by in-frame ligation (construct mC2.26) restores recognition by sera of multiple sclerosis patients (Fig. 5, lane 6). Furthermore, a fusion protein of GST and the last 13 amino acids of the C2 C-terminal is also recognized by the multiple sclerosis sera (data not shown). Similar results were obtained with 10 different sera from multiple sclerosis patients that were seropositive to the C2 subunit. These results clearly demonstrate that the last 13 amino acids of the C-terminal of subunit C2 (DEPAEKADEPMEH) is a major epitope targeted by multiple sclerosis autoantibodies in the C2 subunit.

**Proliferation of PBMC with proteasomal antigens**

To investigate whether proteasome may be a target of autoreactive T-cells, its effect on the proliferation of PBMC was evaluated. Highly purified recombinant fusion GST-C2 C-terminal (containing the main epitope recognized by multiple sclerosis autoantibodies against C2) and C8 proteasomal subunit proteins, whole proteasome and MOG were used in these studies. We have arbitrarily taken an SI > 2 as positive proliferative response (see Material and methods).
Fig. 6 shows a plot of the SI of individual samples versus the different proteins used in the proliferation assays. Proliferative response to phytohaemagglutinin was observed in all the samples studied (Fig. 6). We also observed positive proliferation with MOG ($P = 0.022$ with respect to controls), a known T-antigen for PBMC from multiple sclerosis patients (Fig. 6). With proteasomal antigens, we observed a modest, but clearly significant, proliferative response. The percent of positive proliferative response by intergroup analysis was significantly higher with GST-C2 C-terminal (GST C2b) 25 μg/ml ($P = 0.02$), C8 (C8b) 25 μg/ml ($P = 0.001$) and proteasome ($P = 0.018$) when compared with controls (Fig. 6).

Table 1 summarizes the clinical data and antibody response for the multiple sclerosis patients analysed in proliferation assays. From a total of 30 multiple sclerosis patients, 40% (12) show an SI $>2$ using the fusion protein GST-C2 C-terminal and/or the recombinant C8 subunit. It is also noteworthy, that 100% (8 out of 8) multiple sclerosis patients who were proteasome seronegative were also negative in proliferative assays. Proliferation of PBMC of healthy subjects was not stimulated significantly by any of the proteins.

Table 1 Disease data of multiple sclerosis patients analysed for proliferative response to proteasomal antigens

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Age initial symptoms (years)</th>
<th>Dx duration (years)</th>
<th>Dx type</th>
<th>Proteasome antibody</th>
<th>C2 antibody</th>
<th>C8 antibody</th>
<th>SI recombinant GSTC2 C-terminal</th>
<th>SI recombinant C8</th>
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<tr>
<td>8</td>
<td>F</td>
<td>16</td>
<td>7</td>
<td>R/R</td>
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<td>++</td>
<td>++</td>
<td>1.5</td>
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<td>37</td>
<td>M</td>
<td>45</td>
<td>24</td>
<td>PP</td>
<td>+++</td>
<td>+</td>
<td>+/±</td>
<td>2.6 ± 0.3*</td>
<td>3.8 ± 0.5*</td>
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<tr>
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<td>18</td>
<td>7</td>
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<td>3.4 ± 0.4*</td>
<td>2.2 ± 0.2*</td>
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<td>F</td>
<td>42</td>
<td>5</td>
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<td>40</td>
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<td>+</td>
<td>+/±</td>
<td>3.1 ± 0.4*</td>
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<td>41</td>
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*aSerum antibody titre: +++ = >1/1000; ++ = 1/400–1/1000; + = 1/200–1/400; +/- = 1/100–1/200. *Positive proliferation index (≥2) ± SD (n = 3), assayed at 25 μg/ml of the indicated recombinant protein with respect to control proteins (GST, ECIP). n.d. = not determined.
recombinant proteins (SI<2, n = 10) or purified proteasome (data not shown). These results suggest that proteasome is also an antigenic target to cell-mediated response in multiple sclerosis patients.

Discussion
The overall data presented in this study (including eight patients from the USA) show a prevalence of anti-proteasome antibodies of 66% (65 + 8 out of 102 + 8 = 73 out of 110) in serum samples. With the caveat that differences could be due to geographical differences, this prevalence is higher than the prevalence reported for autoantibodies against human transaldolase (30.8%; 29 out of 94) and similar to that reported for autoantibodies against 2,3’-cyclic nucleotide 3’-phosphodiesterase (74%; 52 out of 70) in multiple sclerosis patients, respectively (Colombo et al., 1997; Walsh and Murray, 1998). Anti-proteasome antibodies were found in 80% (8 + 8 out of 12 + 8 = 16 out of 20) of CSF samples from proteasome-positive multiple sclerosis patients. This prevalence of anti-proteasome antibodies in CSF is similar to the one reported for autoantibodies against transaldolase (Colombo et al., 1997) in CSF samples from transaldolase-seropositive multiple sclerosis patients (76.4%, 13 out of 17). The prevalence of proteasome-seropositivity with respect to the clinical form of multiple sclerosis [58% in R/R multiple sclerosis (n = 59), 50% in PP multiple sclerosis (n = 12) and 80% in SP multiple sclerosis (n = 31)] indicates that the presence of anti-proteasome antibodies was not restricted to a particular group of multiple sclerosis patients. It is also noteworthy that 66.7% (8 out of 12) multiple sclerosis patients were seropositive at the time of their first attack (before definitive clinical diagnosis), indicating that anti-proteasome antibodies are present very early in the course of multiple sclerosis (data not shown). These results, together with unchanged titre of anti-proteasome antibodies during prolonged periods of the disease course, may indicate that autoreactivity to proteasome reflects a sensitization to the antigen and could serve as a stable marker along the course of the disease.

We have also shown that recombinant proteasomal subunits can be used to characterize the anti-proteasome repertoire of multiple sclerosis sera, demonstrating that the main epitopes recognized by these autoantibodies are present in the primary sequence of the proteasomal subunits. The use of recombinant subunits allowed us to map the epitopes of the anti-C2 antibodies present in the sera of multiple sclerosis patients, demonstrating the presence of an immunodominant B-cell epitope within the last 13 amino acids of the C-terminal of the C2 α subunit. The evidence of cell-mediated immunoreactivity to proteasomal antigens in multiple sclerosis patients was demonstrated by the specific stimulation of proliferation of PBMC with two soluble recombinant proteasomal antigens (a fusion protein of GST with the C-terminal of proteasomal subunit C2 and subunit C8). The total prevalence of positive proliferation (40%, 12 out of 30) suggests that proteasome is also a target of cell-mediated immunity in multiple sclerosis patients, while whole proteasome under these assays conditions is less effective than the recombinant proteasomal subunits probably due to its high native molecular weight (670 kDa). All the above considerations suggest that both T and B cell response against the proteasome is present in the same patient and both may contribute to the chronic inflammatory response that characterizes multiple sclerosis.

Autoantigens relevant to multiple sclerosis, as with other organ-specific autoimmune diseases (insulin dependent diabetes mellitus Type I, Graves disease and myasthenia gravis), are thought to specifically reside in myelin of the CNS—the target of the disease (Steinman, 1996; Noseworthy, 1999; Noseworthy et al., 2000). Here we have shown that multiple sclerosis patients present B and T cell autoreactivity against the proteasome—a ubiquitous cell antigen also present in glial and neuronal cells (Mengual et al., 1996). The anti-proteasome response found in multiple sclerosis is similar to that described for SLE patients (Arribas et al., 1991), a prototype of systemic autoimmune diseases. It is also present in patients with Sjögren’s syndrome, Behcet disease and sarcoidosis—albeit with much lower prevalence (present study). Other authors have described a higher prevalence of anti-proteasome antibodies in SLE patients (58%, 30 out of 52), polymyositis and dermatomyositis (47%, 16 out of 34) and Sjögren’s syndrome (39%, 17 out of 43) (Feist et al., 1996, 1999) than we found in our screening by western immunoblotting: SLE (35%, 35 out of 100); polymyositis and dermatomyositis (19%, 4 out of 21) (Arribas et al., 1991; Mayo et al., 2000); and Sjögren’s syndrome (16%, 5 out of 31) (present study). These differences could be attributed in part to the geographical origin of patients, but more likely reflect methodological differences. We have used western immunoblotting followed by development with chromogenic substrates (alkaline phosphatase), while the other authors used immunoblotting followed by detection by the enhanced chemiluminescence (ECL) method (Feist et al., 1996). Due to the higher sensitivity of the ECL method, they could detect low-affinity or low-titre anti-proteasome autoantibodies that will be negative under our assay conditions.

Intriguingly, proteasome is a shared antigenic target in multiple sclerosis and several systemic inflammatory conditions. These differences are not only from the clinical point of view, organ-specific versus systemic, but also from the point of view of the major immunological mechanism responsible for the disease—aberrant response to particular antigens versus alteration in selection, regulation or survival of T and B cells (Davidson and Diamond, 2001). In this context, there are some patients with SLE that present clinical features between both diseases (multiple sclerosis and SLE) that are termed ‘lupoid sclerosis’ and are actually classified as having demyelinating syndromes associated with lupus (Tola et al., 1992; Kovacs et al., 2000). Similarly patients with Sjögren’s syndrome (Fox et al., 2000), Behcet’s disease (Siva and Fresko, 2000) and sarcoidosis (Gullapalli and Phillips, 2002)
can present involvement of the CNS and, in some cases, may lead to misdiagnosis of multiple sclerosis—especially in the case of chronic sarcoidosis and CNS–neuro–Behcet syndrome.

As we have shown here, the prevalence of anti-proteasome antibodies in multiple sclerosis patients is significantly higher than in those chronic inflammatory conditions and, at present, we can not find any correlation of anti-proteasome seropositivity and neurological involvement in those chronic inflammatory diseases. The above discussion suggests that some global abnormality in B and/or T cell function is also involved in the chronic inflammatory response observed in multiple sclerosis patients, as it is frequently observed in other human organ-specific autoimmune diseases (Davidson and Diamond, 2001). Clearly further studies are required to understand the exact role of proteasome autoreactivity in the pathogenesis of multiple sclerosis and other chronic inflammatory conditions, including the mechanisms involved in loss of self-tolerance to this ubiquitous antigen.

In conclusion, autoantibodies to proteasome are new markers for multiple sclerosis disease. The high prevalence and persistence of the autoreactivity to proteasome in multiple sclerosis patients compared with patients with other inflammatory conditions (SLE, Sjogren’s syndrome, vasculitis, sarcoidosis and Behcet’s disease) suggest its possible clinical utility as a new marker for the heterogenous pathogenetic components involved in this chronic inflammatory disease.

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