Induction of EAE by T cells specific for alpha B-crystallin depends on prior viral infection in the CNS

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Abstract

While myelin-reactive T cells are widely believed to play a pathogenic role in multiple sclerosis (MS), no substantial differences appear to exist in T-cell responses to myelin antigens between MS patients and healthy subjects. As an example, indistinguishable peripheral T-cell responses and serum antibody levels have been found in MS patients and healthy controls to alpha B-crystallin, a dominant antigen in MS-affected brain myelin. This suggests that additional factors are relevant in allowing myelin-reactive T cells to become pathogenic. In this study, we examined whether the inflammatory state of the CNS is relevant to the pathogenicity of alpha B-crystallin-specific T cells in mice. In normal mice, T-cell responses against alpha B-crystallin are limited by robust immunological tolerance. Reactive T cells were therefore generated in alpha B-crystallin-deficient mice, and these T cells were transferred into C57BL/6 recipients. While such a transfer in itself never induced any clinical signs of experimental autoimmune encephalomyelitis (EAE) in healthy recipient mice, acute EAE could be induced in animals that had been infected 7 days before with the avirulent A7(74) strain of Semliki Forest virus (SFV). SFV infection alone did not induce clinical disease, nor did it alter the expression levels of the target antigen. Our findings indicate that at least in mice, alpha B-crystallin-specific T cells can trigger EAE but only when prior viral infection has induced an inflammatory state in the CNS that helps recruit and activate T cells.

Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative disease characterized by foci of inflammation throughout the CNS, predominantly in white matter. At these inflamed sites, immune responses are believed to be directed against myelin proteins, a notion that is supported by a wealth of animal model data (1, 2). While this autoimmune paradigm of MS remains well accepted, no substantial differences have been documented so far between peripheral myelin-reactive T-cell repertoires in MS patients and healthy control subjects (1). As an example, the human T-cell response to myelin from MS-affected brains is predominantly directed against alpha B-crystallin, reflecting a significant pro-inflammatory memory T-cell repertoire against this protein in humans (3-5). Yet, both T-cell and antibody responses to this protein are very similar in patients and controls (3, 6). The apparent lack of any difference in the myelin-reactive T-cell repertoire in MS patients as compared with healthy controls appears to be at odds with the widely held notion that such cells are the major pathogenic factor in disease.

One possible explanation for this apparent dilemma has been offered by the idea of determinant spreading (7). In this concept, continuously changing pathogenic autoimmune specificities are considered to be the driving force behind chronic autoimmune disease. Based on this concept, one would not necessarily expect antigen-specific differences to emerge from cross-sectional studies between groups of MS patients and controls. After all, the pathogenic antigen or epitope may be continuously different among patients, with obvious consequences for potential antigen-specific therapies (8). However suitable this may seem to explain the above dilemma, the direct evidence for pathogenic determinant spreading of myelin-directed T-cell responses in chronic MS patients is still weak (9). This contrasts with the clear documentation of the determinant spreading phenomenon in animal model studies that equally use peripheral T-cell reactivity as the primary read-out. Also, recent data indicate that spreading responses may not necessarily be pathogenic even in traditional models of experimental autoimmune
Induction of EAE by alpha B-crystallin-specific T cells

Bethesda, MD, USA (20) and bred at TNO. Biozzi ABH mice obtained from Janvier (Bioservices, Schuijk, The Netherlands). All animals were kept under pathogen-free conditions and used between the ages of 8–14 weeks. All animals were used with the formal approval of the local Ethical committee.

SVF A7(74) propagation and titration

SVF A7(74) titers were determined by plaque assays using a monolayer of baby hamster kidney-21 (BHK-21) cells. First, BHK-21 cells were seeded in six-well plates at a concentration of 4 × 10^5 cells ml^-1 (5 ml per well) in RPMI-1640 supplemented with 100 U ml^-1 penicillin, 100 μg ml^-1 streptomycin, 10 mM glutamax, 2% (v/v) FCS (BHK culture medium). After 2 days, the sub-confluent layer was washed and serial dilutions of SVF in 200 μl in BHK culture medium were supplemented with 20 mM HEPES buffer pH 7.4. After 30 min at room temperature, virus was removed and the cells were overlaid with 1% agar in BHK culture medium. After 7 days, the agar was removed and cells were fixed with 10% formaldehyde for 10 min at room temperature and stained with 0.1% toluidine blue to reveal plaques. Virus titers of the stock used in the present study were 1.5 × 10^8 plaque-forming units (PFUs) per ml.

Induction of EAE

Active EAE in Biozzi mice was induced by immunizing mice subcutaneously with 1 mg murine total spinal cord homogenate (SCH) in CFA on days 0 and 7. On the day of immunization and again 24 h later, mice were injected intravenously with 20 μg pertussis toxin (Sigma, Poole, UK).

Passive induction of EAE was conducted by transfer of 40 × 10^6 mixed splenocytes and lymphocytes directed against alpha B-crystallin or proteolipid protein (PLP)139–151. T cells against alpha B-crystallin were generated in alpha B-crystallin−/− mice, since normal rodents are fully tolerant for the antigen. T cells react to a peptide representing amino acids 139–151 of PLP were generated in SJL mice. Mice were immunized subcutaneously with 100 μg recombinant mouse alpha B-crystallin or PLP139–151 emulsified in CFA containing 1 mg ml^-1 Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). Ten days after immunization, spleen, auxiliary and inguinal lymph nodes were collected and cultured in RPMI-1640 (BioWhittaker, Verviers, Belgium) supplemented with 100 U ml^-1 penicillin, 100 μg ml^-1 streptomycin, 50 μM 2-mercaptoethanol, 10 mM glutamax, 5% (v/v) FCS (BioWhittaker) and 10 μg ml^-1 recombinant mouse alpha B-crystallin or PLP139–151. Total splenocytes and lymphocytes were cultured for 96 h before transfer into recipient mice. Control T cells against sperm whale myoglobin (Serva, Heidelberg, Germany) were obtained in the same way as alpha B-crystallin-reactive T cells. We used alpha B-crystallin-deficient mice (H2b) as donors of alpha B-crystallin-reactive T cells and wild-type C57BL6/H2b mice as recipient mice.

As controls for the transfer studies, we used SJL/J mice as both donor and recipients of PLP(139–151)-specific T cells. Mice were examined daily for clinical signs of EAE and scored as follows: grade 0, no clinical signs; grade 0.5,
partial tail paralysis; grade 1, complete tail paralysis; grade 2, limb weakness or spastic limbs; grade 3, complete hind or front limb paralysis, and grade 5, death.

T-cell transfer into SFV-infected animals

Prior to transfer of alpha B-crystallin specific T cells, C57BL/6 mice were infected by intra-peritoneal administration of 5000–10 000 PFU of the avirulent strain A7(74) of SFV suspended in PBS. On days 3, 7, 10, 14 or 21 after SFV infection, 40 × 10⁶ alpha B-crystallin-reactive T cells were transferred intravenously into recipient C57BL/6 mice. Mice that served as controls received either PBS or myoglobin-reactive T cells intravenously. Mice were examined daily for clinical signs of EAE.

T-cell response assays

To test the reactivity of T cells isolated from spleen or auxiliary and inguinal lymph nodes, T cells were seeded at 1 × 10⁵ cells per well in the presence of 2 × 10⁵ syngeneic irradiated (30 Gy) splenocytes from naive mice in 200 µl RPMI-1640 culture medium supplemented with 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 50 µM 2-mercaptoethanol, 10 mM glutamax and 5% (v/v) FCS. Varying doses of recombinant mouse alpha B-crystallin or PLP(139–151) were added at the beginning of the culture. After 72 h of culture, 100 µl of supernatant was removed for cytokine analysis and the cells were cultured for another 18 h in the presence of 20 Kboq [³H]-thymidine ([³H]Tdr). Incorporation of [³H]Tdr was measured using a betaplate counter (PerkinElmer, Turku, Finland). IFN-gamma in culture supernatants was determined using a commercially available ELISA kit (PharMingen, San Diego, CA, USA) according to the protocol recommended by the manufacturer.

Western blot analysis

Brains and spinal cords were isolated from mice, homogenized in milliQ-water and lyophilized. Myelin proteins were delipidated as previously described (3) and subjected to standard SDS–PAGE by loading 80 µg of each sample onto a 15% (w/v) gradient polyacrylamide gel and blotted onto nitrocellulose filters. For detection of alpha B-crystallin in the samples, the murine mAb JAM01 was used (3).

Results

Transfer of alpha B-crystallin-specific T cells into healthy C57BL/6 mice

To generate T cells for adoptive transfer, we used alpha B-crystallin-deficient mice that lack any form of central or peripheral tolerance to alpha B-crystallin which is routinely observed in normal rodents. Upon immunization with the antigen in CFA, alpha B-crystallin⁻/⁻ mice develop marked antigen-specific proliferative T-cell responses accompanied by production of IFN-gamma, which is considered a key mediator for encephalitogenic T cells (Fig. 1). In quantitative terms, proliferation of lymph node-derived or spleen-derived T cells reached stimulation indices of 15–20, and released IFN-gamma into the culture medium at levels of 500 pg ml⁻¹. As a comparison, we immunized SJL mice with the PLP peptide 139–151, a widely used method to induce EAE or to generate encephalitogenic T cells for adoptive transfer. Levels of proliferation and IFN-gamma production by PLP(139–151)-reactive T cells in SJL mice were fully comparable to those of the alpha B-crystallin-reactive T cells (Fig. 1). Thus, the proliferative strength and quality of the T-cell response to alpha B-crystallin obtained in knockout mice appears to be consistent with an ability to induce EAE. However, transfer of 40 × 10⁶ activated alpha B-crystallin-specific T cells into MHC-compatible C57BL/6 mice did not induce any clinical signs of EAE over an observation period of 22 days (Fig. 2). As a control, we examined the effects of an identical transfer protocol but using 40 × 10⁶ PLP(139–151)-specific T cells transferred into SJL mice. In this case, all mice developed clinical signs of EAE 7 days after transfer (Fig 2).

It is of obvious relevance whether or not the target antigen is present in the brain and spinal cord of C57BL/6 mice at levels potentially sufficient to reactive T cells. Also, given the next experiment in which the effects of infection with SFV were examined, we evaluated by western blotting the expression levels of alpha B-crystallin in brains and spinal cords of recipient C57BL/6 mice both before and during SFV infection. The result, represented in Fig. 3, showed that alpha B-crystallin was expressed in normal mice at levels representing about 0.03% of total protein. While these levels are about 10 times lower than what we previously found in human brains (2), they do not provide an obvious explanation for the lack of disease induction. As an important part
of the result given in Fig. 3, SFV infection did not lead to any visible change in expression levels of alpha B-crystallin over a 36-day period.

Transfer of alpha B-crystallin-specific T cells into SFV-infected C57BL/6 mice

Before studying adoptive transfer of alpha B-crystallin-specific T cells in combination with a prior SFV infection, the effects of SFV infection alone were studied. Infection with SFV using up to \textbf{10}^6 PFU did not induce any clinical signs of EAE over an observation period of 21 days in any of 75 mice, either C57BL/6 or 129Sv/J wild-type mice (data not shown). Even though several mice did show mild signs of viral infection such as a ruffled fur, they never developed any of the clinical signs distinctive of EAE, notably including limb weakness or spasticity. Thus, when performed separately, neither SFV infection nor transfer of alpha B-crystallin-specific T cells alone resulted in clinical signs of EAE in C57BL/6 mice.

We next studied transfer of alpha B-crystallin-specific T cells at different days after SFV infection. The days of transfer were chosen based on the well-known pathology that results from SFV infection in C57BL/6 and Biozzi ABH mice (12–17). SFV-induced infiltration of leukocytes is first detected on day 3 and reaches maximum levels on day 7. This is followed by demyelination starting around day 10 and reaching its maximum between days 14 and 21. Transfer of alpha B-crystallin-reactive T cells 3 days after SFV infection resulted in clinical signs of EAE in 60% of the animals (Fig. 4A). The most severe clinical signs observed included partial tail paralysis (score 1) that emerged 3 days after transfer of T cells. These clinical signs generally lasted for 3 days. Transfer of T cells 7 days after SFV infection resulted in acute development of clinical signs varying from partial tail paralysis (score 1) to paralyzed hind limbs (score 2) or spastic hind limbs (score 2) (Fig. 5). EAE incidence following transfer reached maximum levels of 88%. The clinical signs became apparent within 24 h after transfer and often lasted for over 10 days. Yet, we observed a marked variation in the development of clinical signs between different experiments, as illustrated in Fig. 5, possibly related to the very low levels of expression of the target antigen. Clinical signs of EAE were also detected after transfer of T cells 10 days after SFV infection, but this time in only one of the two experiments (Fig. 6A). However, in the second experiment, no clinical signs were detected at all. Transfer of T cells at later stages on days 14 and 21 after SFV infection did not induce any EAE (data not shown).

Control mice were also infected with SFV and subsequently received PBS on the days T-cell transfers were performed. Overall, clinical signs were observed in <8% of the
animals (Figs. 4B, 5B and 6B). In all cases, clinical signs were either lower than grade 0.5 or they had already developed before injection of PBS, in which case these signs should be attributed to SFV infection only. As an additional control, transfer of myoglobin-specific T cells was performed on days 7 and 10 after infection. Like alpha B-crystallin, myoglobin is expressed at high levels inside muscle cells, but in contrast to alpha B-crystallin, it is not found anywhere in the CNS. Thus, myoglobin serves as a suitable control for possible anti-muscle autoimmune phenomena that could easily have interfered with some of the murine motor functions that are used to score EAE. In these control experiments, only a single mouse out of 24 SFV-infected mice developed mild clinical signs (score <0.5) similar to EAE. These clinical signs started 3 days after transfer of myoglobin-specific T cells, which was performed at day 7 after SFV infection, and lasted for 4 days (data not shown).

Immunization with alpha B-crystallin in ongoing EAE or in SFV-infected Biozzi ABH mice

In our experiments using active immunization, we used Biozzi ABH mice since this particular strain is only partially
tolerant for alpha B-crystallin. Immunization of Biozzi mice with complete SCH is well known to induce chronic, relapsing EAE, and to lead to spreading T-cell responses to alpha B-crystallin after two relapses (19). To examine the pathogenicity of alpha B-crystallin-specific T-cell responses in a background of ongoing EAE, we first induced chronic-relapsing EAE and challenged Biozzi mice with alpha B-crystallin in CFA after the second relapse, at a time between days 42 and 64, dependent on the course of disease in individual animals. As controls, mice were immunized with ovalbumin peptide (OVA) in CFA at the same time. We found no apparent differences in clinical signs between the animals challenged with either alpha B-crystallin or OVA, and all mice showed the normal continued course of chronic-relapsing EAE (Table 1).

Finally, the effects of active immunization were examined in a background on ongoing SFV infection, which in Biozzi ABH mice developed in ways very similar to C57BL/6 (15, 16). Immunization with alpha B-crystallin in CFA was performed at the time of SFV infection, and again 7 days later. As a control, OVA immunization was performed. In the group of 11 mice that were infected with SFV and subsequently immunized with alpha B-crystallin in CFA, one mouse died on day 11. None of the remaining mice developed the traditional clinical signs of EAE but other signs of disease were observed in four animals, including a hunched appearance, abnormal gait and increased urine production as was evident in three out of the four boxes in which the animals were kept, a common sign of either EAE or high viral titers (15) (S. Amor, unpublished data). The two control groups of mice that were only infected with SFV or infected with SFV and immunized with OVA did not develop any of these signs (Table 2). Histological examination of the brains and spinal cords from alpha B-crystallin immunized mice revealed increased cellular density in perivascular cuffs and enhanced frequency of these cuffs as compared with controls groups

**Table 1. Immunization in ongoing EAE in Biozzi ABH mice**

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>After challenge</th>
<th>Day</th>
<th>Antigen</th>
<th>Score</th>
<th>Maximum score</th>
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<tr>
<td>1</td>
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<td>64</td>
<td>aBC</td>
<td>0.5</td>
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<tr>
<td>2</td>
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<td>1.5</td>
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</tr>
<tr>
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<td>OVA</td>
<td>0</td>
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<tr>
<td>7</td>
<td></td>
<td>42</td>
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<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>42</td>
<td>OVA</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*aMice were challenged after the second relapse, at a time between days 42 and 64.

*babc: recombinant murine alpha B-crystallin.

*cClinical score 12 days after challenge.

**Table 2. Immunization of SFV-infected Biozzi ABH mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>wet box</th>
<th>disease incidence</th>
<th>histology score</th>
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</thead>
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<td>SFV + aBC</td>
<td>3/4</td>
<td>4/11</td>
<td>2.5</td>
</tr>
<tr>
<td>SFV + OVA</td>
<td>0/3</td>
<td>0/3</td>
<td>1.4</td>
</tr>
<tr>
<td>SFV</td>
<td>0/7</td>
<td>0/10</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*aNumber of boxes that were visibly wet due to increased urine production.

*bIncidence of clinical signs of disease including ruffled ur and hunched back.

*cMean number of infiltrates scored per microscopic section, 4 sections were evaluated from brain as well as spinal cord.

and the presence of activated microglia at the inflamed sites (Table 2 and Fig. 7A). These characteristics generally typify infiltrating autoreactive T cells as seen during EAE, as opposed to the less frequent and scattered appearance of T cells as the result of viral infection alone (Fig. 7B).
Discussion

In this study, we addressed the apparent discrepancy between the idea that myelin-reactive T cells are pathogenic in MS while known myelin-specific T-cell repertoires in patients appear to be not essentially different from those in healthy control subjects. We chose alpha B-crystallin as the experimental antigen of choice since this protein has previously been documented as a dominant antigen for human T cells in the context of the inflamed tissue of MS patients (3), and both T-cell and antibody repertoires against alpha B-crystallin are very similar in patients and control subjects (3, 5, 6). As the main finding of this study, our data indicate that peripheral alpha B-crystallin-specific T cells are not pathogenic in healthy mice but can trigger acute clinical signs of EAE in animals with an ongoing subclinical SFV infection in the CNS. Thus, the inflamed state of the target organ is critical for disease induction in this case.

Previous studies have clarified that virus infection in the murine CNS, including gammaherpesvirus-68 and SFV, can exacerbate EAE induced with traditional encephalitogens such as SCRs, MBP or PLP (21–26). In our study, SFV infection in the CNS was shown to allow pathogenicity to develop of T cells that are not pathogenic in normal healthy animals. Since no marked changes in the expression levels of alpha B-crystallin protein were observed in brain and spinal cord over the entire monitoring period, other virus-associated factors are more likely to explain our data. As has been well documented (12–17), during the normal course of SFV infection the first mononuclear cell infiltrates can be detected after 3 days and they reach maximum levels 7 days after infection. Around day 7, the blood–brain barrier is maximally activated and expresses peak levels of several adhesion molecules including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Also at day 7, MHC accumulates along with co-stimulatory molecules and pro-inflammatory cytokines including tumor necrosis factor-alpha, IL-1-alpha and IL-6 in the infected CNS parenchyma. Demyelination first starts on day 10, when the virus starts to become cleared from the CNS, and reaches its maximum between days 14 and 21. The consequences of adoptively transferring alpha B-crystallin-specific T cells appear to be closely linked to these stages in SFV infection. Transfer on day 3 after infection resulted in clinical signs that developed only after another 3 days, which is relatively late as compared with the effects seen on day 7 or 10. Transfer on days 7 and 10 resulted in EAE that invariably became apparent already within 24 h after transfer. Also, the highest disease incidence of 88% was recorded in our study when transfer was performed on day 7. No clinical disease could be triggered any longer after 10 days post-infection, when virus becomes cleared from the CNS, especially since expression of adhesion molecules wanes.

Alpha B-crystallin-reactive T cells have been show to develop in ongoing EAE in Biozzi mice (19). Challenging these alpha B-crystallin-reactive T cells in ongoing EAE induction did not augment EAE. This is in line with a recent study of Wang et al. (27) that showed that mice are resistant to active EAE induction and that transfer of alpha B-crystallin-reactive T cells neither induced passive EAE nor augmented ongoing EAE. Active EAE induction with alpha B-crystallin induced mild signs of disease only during ongoing neurotropic SFV infection, as shown in the present study. This emphasizes the importance of a microbial infection for alpha B-crystallin-reactive T cells to become encephalitogenic. Together, our
data suggest that the main factor in EAE induction in our study is virus-enhanced infiltration into the CNS of alpha B-crystallin-specific T cells. In addition, local activation of infiltrated T cells will also be promoted by SFV infection. The virus induces elevated expression of MHC class II and co-stimulatory molecules on microglia. Given that alpha B-crystallin-specific T cells secrete IFN-gamma (Fig. 1), which will further activate local antigen-presenting cell functions, these factors together will likely facilitate enhanced local presentation of myelin-derived alpha B-crystallin.

The current findings appear to be in line with other reports on the effects of viral infections in experimental autoimmunity, for example, in type I diabetes, despite the fact that the role of viruses in human autoimmune diseases is still largely unresolved and may well involve effects at different levels (11). The significance of viral infections for MS has similarly remained the subject of a long-standing debate. To date, the strongest link between MS and microbial infections appears to be that with EBV. It is particularly striking that essentially all MS patients are seropositive for the virus, and that antiviral antibody levels closely correlate with the risk for disease (28). The target antigen in our study, alpha B-crystallin, is in turn associated with EBV infection since, at least in vitro, EBV infection of human B cells triggers de novo expression of alpha B-crystallin and its MHC class II-associated presentation to T cells (4). In view of the fact that the human immune system is naturally non-tolerant towards alpha B-crystallin, common EBV infection could conceivably explain the establishment of a peripheral memory T-cell and IgG antibody repertoire as generally seen in normal human adults (4–6). Our data show that at least in mice such an autoimmune repertoire can very well remain silent in healthy individuals. Yet, it will pose risks upon development of CNS inflammation that triggers recruitment and local activation of the potentially reactive peripheral T-cell repertoire. No evidence was found in the current study that SFV infection of mice leads to any elevated local expression of the target antigen alpha B-crystallin, despite it being a heat shock protein. In humans, however, where basal levels of the protein in brains are already much higher, enhanced expression of alpha B-crystallin has been demonstrated in human oligodendrocytes and myelin at the earliest stages of an MS lesion [3, 5]. The trigger for this induction remains unknown but may well include a second infectious event, or other factors such as primary oligodendrocyte apoptosis (29), which has recently been shown to occur as an early event in at least in some MS cases (30). When taken together, our current data indicate that while a peripheral autoimmune T-cell repertoire could well be a stable factor among patients and healthy controls, local pro-inflammatory factors in the CNS such as those induced in mice by SFV may be equally crucial in allowing clinical autoimmune disease to develop.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
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<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>[3H]Tdr</td>
<td>[3H]-thymidine</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin peptide</td>
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<tr>
<td>PPU</td>
<td>plaque-pitting unit</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
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<td>SCH</td>
<td>spinal cord homogenate</td>
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<td>SFV</td>
<td>Semliki Forest virus</td>
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</table>

References

alphaB-crystallin in Biozzi ABH (H-2A(g7)) mice. J. Neuroimmunol. 104:47.


