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2D immunomic approach for the study of IgG autoantibodies in the experimental model of multiple sclerosis

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ABSTRACT

2D-immunomics may be useful in the identification of autoantigens in neurological autoimmune diseases, but its application may be limited by denaturation of target proteins. Here we compared the capacity of a single or multiple antigens to elicit autoantibodies targeting multiple neural autoantigens by ELISA and 2D-immunomics. We induced experimental autoimmune encephalomyelitis (EAE) with MBP peptide$_{89-104}$, total MBP or spinal cord homogenate. Both techniques showed anti-MBP IgG only after immunization with total MBP. In addition, 2D-immunomics revealed the presence in EAE mice of autoantibodies targeting other neural proteins, some displaying partial sequence homology with MBP. The present finding by 2D-immunomics of multiple neural proteins targeted by autoantibodies generated by a single antigen may help to explain the complex autoimmune response observed in multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease where the pathogenic cascade probably includes a process of molecular mimicry leading to the activation of T and B cell clones, some of which cross-react with self antigens (Hohlfeld and Wekerle, 2001; Hueber et al., 2002).

A major goal of neuroimmunology is the identification of autoantigens in autoimmune diseases, which may help the identification of the foreign antigens and the design of vaccines. As far as MS is concerned, extensive efforts have been spent in the last decades, with no conclusive results (Cross and Stark, 2005; Ziemssen and Ziemssen, 2005; Fraussen et al., 2009). In most of these cases, autoreactivity to pre-selected, recombinant proteins/peptides was assessed by using ELISA or radio-immunoassay techniques; although autoreactivity to a number of neural proteins has been reported, the specificity of autoreactive IgG remains unclear (for review, see Fraussen et al., 2009). Such methodological approaches have been validated in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, which can be induced upon immunization with myelin proteins or peptides (Furlan et al., 2009).

We have recently applied a 2D-immunomic approach in MS (Lovato et al., 2008) and Hashimoto's encephalopathy (HE) (Gini et al., 2008), two autoimmune conditions with production of autoantibodies towards unknown autoantigens. After 2D separation of human neural proteins and transfer to nitrocellulose membranes, sera and cerebro-spinal fluid (CSF) have been applied and the autoreactive spots identified. This approach led to the identification of few neural antigens recognized by IgG in the CSF of HE patients (Gini et al., 2008), whereas the situation in MS was highly complex, with a wide array of neural proteins recognized by autoantibodies both in serum and in CSF (Lovato et al., 2008).

A great advantage of such technique is represented by the opportunity to use the (almost complete) human neural proteome, with proteins present in all their isoforms with post-translational modifications. We have found evidence that these modifications play an important role both in MS and HE, since single isoforms were specifically recognized by autoreactive IgG, while others were targeted also by controls (Lovato et al., 2008). A major limitation of such approach may be represented by the denaturing conditions of the technique with loss of conformational structure; as a consequence, IgG directed to conformational epitopes would probably be under-estimated, when tested against denatured proteins.

Here we compared the ability of 2D-immunomic and ELISA techniques to detect the presence of autoantibodies in the serum of

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EAE mice. In addition, we assessed by 2D-immunomic approach the differential recognition of neural proteins following the induction of EAE with increasing complexity of the immunogen: a single peptide of the myelin basic protein (MBP), the total recombinant MBP or spinal cord homogenate (SCH).

2. Materials and methods

2.1. Animals and induction of EAE

Female SJL/j mice at 6–8 wk of age were purchased from Charles River (Calco, Milan, Italy) and housed in pathogen-free conditions. All procedures involving animals were performed according to the guidelines of the San Raffaele Scientific Institute Institutional Animal Care and Use Committee. For EAE induction, lyophilized antigens from SCH, total bovine MBP (Sigma-Aldrich) and bovine MBP_{89-104} peptide (Espikem, Florence, Italy) were re-suspended in saline to a final concentration of 4 mg/mouse for SCH and 200 μg/mouse for MBP peptide or protein and injected subcutaneously with 1:1 CFA (Difco) (Furlan et al., 2009; Levine and Sowinski, 1973; Miller et al., 2010; Vanderlugt et al., 2000). Each immunization (5 animals per group) was followed by two intra-peritoneal injections of 500 ng of pertussis toxin (List Biological Laboratories) the day of immunization and 48 h later. As controls, immunization was performed with CFA alone (5 mice). Spinal cord homogenate has been prepared as described elsewhere (Furlan et al., 2009). Briefly, spinal cord was homogenized through a 70 μm cell strainer, suspended 4:1 (w/v) with saline, lyophilized and stored at −80 °C. Mice were weighed and scored daily. All EAE animals showed clinical signs of the disease with scores at peak between 2.5 and 3.5. Blood was collected at day 30 post-immunization (5 animals for each EAE sub-groups and control groups) and serum was stored at −20 °C until use. Before use for ELISA and 2D-immunomics, serum samples were normalized to the amount of IgG by commercial ELISA.

2.2. ELISA assay

$MBP\ peptide_{89-104}$ or total MBP protein (10 μg/ml of PBS) were coated onto 96-well plates (Microtest™ Flat-bottomed Plate, Falcon) overnight at 4 °C. The plates were then washed with PBS and blocked with 3% BSA solution. After washing with PBS-0.1% Tween-20 buffer, serum from each immunized and control mouse was incubated (1:100) for 2 h at 37 °C. To remove the excess of unbound serum, 4 washes with PBS-0,1% Tween-20 were performed and anti-mouse IgG HRP-conjugated (Millipore) was incubated for 1 h at 37 °C. The plates were washed with PBS-0.1% Tween-20, treated with TMB substrate solution (Sigma) for 45 min and read with a Microplate Elisa reader (Bio-Rad) at 450 nm. The OD background values were determined as the mean of OD values of wells coated with peptides or whole MBP protein without serum.

2.3. 2D-PAGE analysis and immunoblotting

2D immunomics was performed as previously described with minor modifications (Lovato et al., 2008). Normal SJL mice white matter brain samples were homogenized in lysis buffer (7 M Urea, 2 M Thiourea, 0.4% CHAPS, 0.1% DTT, 0.5% Triton X-100) and purified by centrifugation at 13,000 g for 5 min. Protein concentration was determined by a commercial protein assay (Sigma), with BSA as standard. Samples were solubilized in 2-D sample buffer (7 M Urea, 2 M Thiourea, 0.4% CHAPS, 0.1% DTT and 0.5% Triton X-100) to a final concentration of 120 μg/ml protein, followed by 0.5% Ampholine solution and Bromophenol blue. The 18-cm long IPG strips pH 3-11 NL (GE Healthcare) were rehydrated with 350 μl of protein solution for 1 h passively and 7 h at 30 V constant. Isoelectric focusing was carried out with a Ettan IPGphor 3 (GE Healthcare) in a sequence of constant and linear voltage gradient from 300 to 3500 V for 5 h total; the voltage was then increased to 8000 V and kept at such value until reaching 90,000 Vh total. For the second dimension, the IPGs strips were equilibrated in a solution containing 6 M Urea, 2% SDS, 30% Glycerol, 0.130 M DTT and 0.5 M Tris–HCl (pH 6.8). The alkylation reaction was performed by adding 0.136 M IAA to the solution for 5 min. The IPG strips were then loaded on an 9–16% polyacrylamide gradient SDS-PAGE with 0.8% agarose in the cathode buffer (192 mM Glycine, 0.1% SDS and 25 mM Tris). The electrophoretic run was performed with a Protean Multi-cell 2D sistem (Bio-Rad) by setting a current of 40 mA/gel. Proteins were then transferred onto 0.2 μm nitrocellulose membrane (Bio-Rad) in the transfer buffer (Tris-Glycine and 20% Methanol) at 10 V constant at 4 °C overnight. For 2D immunoblotting, membranes were blocked with 10% BSA or 10% no-fat Milk and 0.1% Tween-20 in TBS (pH 7.4) for 2 h and incubated overnight with serum from each animal (1:10,000) or antibodies anti-MBP (1:1000; Dako, Milan, Italy), myelin-oligodendrocyte glycoprotein (MOG) (1:1000; Sigma, Milan, Italy), 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (1:20,000), actin (1:25,000), α-tubulin (1:30,000; Abcam). After washing, membranes were incubated with appropriate secondary IgG HRP-conjugated (1:8000) and developed with Advanced ECL (GE Healthcare), whereas the reference 2D gel was stained with the silver or Sypro-Ruby stainings. The reference 2D-gel and autoradiographs were analyzed with the Image Master 2D Platinum v6.0 software (Amersham). By the same software, auto-reactive spots were matched with the reference gel; the threshold of intensity of each spot was arbitrarily set at 50,000.

2.4. In-gel digestion and peptide sequencing by nano RP-HPLC-ESI-MS/MS

In gel digestion and peptide sequencing by nano RP-HPLC-ESI-MS/MS were performed as previously described (Cecconi et al., 2009). The mass spectrometry analyses were conducted by using a nanoflow-HPLC system (Agilent 1200 series) coupled with an ion trap (Esquire 6000 Bruker-Daltonik, Germany). The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information non-redundant database (NCBInr) using the Mascot program (Matrix Sciences, London, UK). The following parameters were adopted for database searches: complete carbamidomethyl formation of cysteines and partial oxidation of methionines, N-acetylation, peptide mass tolerance ±1 Da, fragment mass tolerance ±0.9 Da and missed cleavages of 1. For positive identification, the score of the result of $[-10\times\mathrm{Log}(P)]$ had to be over the significance threshold level ($p<0.05$). Relevant spots identified by mass spectrometry are indicated in Fig. 2 and identification parameters are summarized in Supplementary Table 1.

3. Results

3.1. Autoantibody response following different EAE immunization protocols revealed by ELISA

To characterize the immune response in EAE, immunization was performed with different encephalitogenic myelin antigens: total MBP protein, MBP_{89-104} peptide and SCH; Since two-dimensional electrophoresis implicates strong denaturating conditions to separate proteins, we first evaluated anti-MBP autoantibodies in different EAE immunization protocols by ELISA, an assay in which autoreactivity is tested against non-denatured antigens, i.e. MBP_{89-104} peptide and total MBP. The comparison of the reactivity by ELISA with those obtained with the 2D immunomic approach enabled us to assess whether the use of reducing agents modified the protein conformation and reduced/abolished the epitope binding. As shown in Fig. 1A, high levels of IgG recognizing total MBP protein were detected only in mice inoculated with the same antigen, but not after immunization