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ADVERSE EFFECTS OF A SOD1-PEPTIDE IMMUNOTHERAPY ON SOD1^{G93A} MOUSE SLOW MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract—Previous reports from our lab had shown that some anti-purinergic receptor P2X4 antibodies cross-reacted with misfolded forms of mutant Cu/Zn superoxide dismutase 1 (SOD1), linked to amyotrophic lateral sclerosis (ALS). Cross-reactivity could be caused by the abnormal exposure of an epitope located in the inner hydrophobic region of SOD1 that shared structural homology with the P2X4-immunizing peptide. We had previously raised antibodies against human SOD1 epitope mimicked by the P2X4 immunizing peptide. One of these antibodies, called AJ10, was able to recognize mutant/misfolded forms of ALS-linked mutant SOD1. Here, we used the AJ10 antigen as a vaccine to target neurotoxic species of mutant SOD1 in a slow mouse model of ALS. However, the obtained results showed no improvement in life span, disease onset or weight loss in treated animals; we observed an increased microglial neuroinflammatory response and high amounts of misfolded SOD1 accumulated within spinal cord neurons after AJ10 immunization. An increase of immunoglobulin G deposits was also found due to the treatment. Finally, a significantly worse clinical evolution was displayed by an impairment on motor function as a consequence of AJ10 peptide immunization.

Key words: amyotrophic lateral sclerosis, immunotherapy, superoxide dismutase, conformational-specific antibody, microglia, neuroinflammation.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by the gradual loss of upper motoneurons (MNs) in the cerebral cortex and of lower MNs in the brain stem motor nuclei and spinal cord ventral horn. MN degeneration results in irreparable muscle paralysis, leading to death within a few years; there is no effective treatment for this condition. Most cases of ALS occur sporadically, but approximately 10% are inherited or familial amyotrophic lateral sclerosis (FALS). In approximately 20% of patients with FALS, the disease is caused by mutations in the gene encoding Cu/Zn-superoxide dismutase 1 (*SOD1*) (Deng et al., 1993; Rosen et al., 1993). Transgenic mice overexpressing FALS-linked SOD1 transgenes develop a phenotype resembling human ALS (Gurney et al., 1994; Turner and Talbot, 2008). Although the underlying mechanism why SOD1 mutations cause MN death is still unknown, there is now a general consensus that such mutations lead to an increase in the toxic function, which causes MN loss due to a mechanism involving non-neuronal cells (Clement et al., 2003). For example, the degeneration of mutant SOD1-expressing MNs is delayed when they are associated with wild-type (WT) non-neural cells. Chronic microglial neuroinflammation with increased production of neurotoxic substances is well-documented in ALS (Brites and Vaz, 2014). Investigations have identified multiple perturbations of cellular function in ALS MNs, implying excessive (or insufficient, see Saxena et al., 2013) excitatory tone, protein misfolding, impaired energy production, abnormal calcium metabolism, altered axonal transport and the activation of proteases and nucleases (Cleveland and Rothstein, 2001).

More than 100 different ALS-associated mutations in *SOD1* gene have been identified. Most of these mutations result in the substitution of single amino acids throughout the 153-residue SOD1 polypeptide, although some mutations lead to amino acid insertions, deletions and truncations of the C terminus (Shaw, 2005).

We have previously reported that degenerating SOD1^{G93A} ALS rodent MNs exhibit strongly positive immunoreactivity when examined with a commercially available anti-P2X4 ATP receptor subunit antibody (Casanovas et al., 2008). These degenerating neurons are often associated with microglial cells that display neuronophagic activity. We subsequently found that this positive immunoreaction was due to a cross-reactivity of

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Abbreviations: ALS, amyotrophic lateral sclerosis; CS, clinical score; ELISA, enzyme-linked immunosorbent assay; FALS, familial amyotrophic lateral sclerosis; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; MN, motoneuron; OFT, open-field test; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SEDI, SOD1-exposed dimmer interface; SOD1, superoxide dismutase 1; WT, wild type; α-syn, α-synuclein.

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the anti-P2X4 antibody with misfolded conformers of mutant SOD1 (Hernandez et al., 2010). An inner SOD1 epitope was abnormally exposed in misfolded SOD1, it shares homology with the P2X4 immunizing peptide, and it is responsible for this cross-reaction. We later raised a polyclonal antibody against the human SOD1 epitope mimicked by P2X4 immunizing peptide (AJ10) and demonstrated that the antibody recognizes misfolded conformers of ALS-linked mutant SOD1 sharing similar properties to those previously described with the anti-P2X4 antibody (Sábado et al., 2013).

Several laboratories have developed antibodies to specifically detect misfolded SOD1 (reviewed by Vande Velde et al., 2008). Variable clinical results were obtained on passive or active immunotherapeutic assays, using some of those antibodies or their antigenic proteins. Here, we focus on the conformational instability of mutant SOD1 that induces the formation of harmful aggregates that are immunoreactive for AJ10 antibody against misfolded SOD1. We performed a new vaccination assay applied after the administration of AJ10 peptide to SOD1^{G93A} low copy ALS mice. Our results showed that after immunization, there were an exacerbation of microglial reaction in spinal cord by an increase of immunoglobulin Gs (IgGs) and misfolded SOD1 depositions, and consequently worse clinical profile. We conclude that, under our circumstances, the presumable therapeutic effects of SOD1 peptide vaccination could have been masked by an adverse, and probably autoimmune, neuroinflammatory effect that should be taken into consideration for the future development of ALS therapies based on SOD1 immunological targeting.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

The transgenic animals used in this study were B6.Cg-Tg (SOD1*G93A)^{dl}1Gur/J (SOD1^{G93A}) mice obtained from Jackson Laboratory (Bar Harbor, ME, United States). This is a low copy model of the G93A mutation and once symptoms had developed, most of the animals died within 240–270 days. Adult male C57BL/6J mice were purchased from Charles Rivers Laboratory (Wilmington, MA, United States). For double labeling of anti-misfolded SOD1 using AJ10 and C4F6 antibodies, we used spinal cord sections from end-terminal transgenic SOD1^{G93A} rats processed in exactly the same way as described for mice (Sprague–Dawley NTac: SD-TgN(SOD1G93A)L26H, from Taconic Farms, Germantown, NY). Appropriate rules and procedures were followed (Generalitat de Catalunya DOGC 2073, 1995) and approved by the University of Lleida's ethical committee for animal testing.

Animals were deeply anesthetized with pentobarbital and transcardially perfused with physiological saline solution, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4, for light microscopy immunocytochemistry. After 24 h in PFA, the samples were transferred to 30% sucrose in 0.1 M PB and 0.02% sodium azide for cryoprotection and were then frozen for cryostat sectioning. Spinal nerve ventral roots (L4) were postfixed in 1% osmium tetroxide and embedded in Epon for semithin (1-μm thick) sections. After methylene blue staining, these sections were used for counting motor axons.

Immunohistochemical procedures

Dissected spinal cords were cut into 16-μm transverse sections on a cryostat, mounted on gelatinized slides and stored at −80 °C before use. The sections were pre-treated with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 1 h, blocked with 10% normal goat serum (NGS) for 1 h, and then incubated overnight at 4 °C with an appropriate primary antibody. Samples, previously washed with PBS were then incubated for 1 h with an appropriate fluorescent secondary antibody and labeled with one of the following fluorochromes: Alexa Fluor 488, Alexa Fluor 546, (Molecular Probes, Eugene, OR, United States), Cy3, Cy5, or DyLight 488 (Jackson Immuno Research Laboratories, West Grove, PA, United States). Nuclear counterstaining was performed by 4′,6-diamidino-2-pheny lindole dihydrochloride (DAPI; 50 ng/ml, Molecular Probes). Images were obtained using an Olympus BX51 (Hamburg, Germany) epifluorescence microscope, equipped with a cooled CCD camera (DP30BW).

The following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP) chicken polyclonal antibody (1:1000, Abcam, Cambridge, United Kingdom); anti-ionized calcium binding adaptor molecule (Iba1) polyclonal rabbit antibody (1:500, Wako Chemical, Neuss, Germany); anti-Mac2 rat monoclonal antibody (1:800, antibodies-online GMBH, Aachen, Germany). Finally, misfolded conformers of SOD1 were detected by AJ10 polyclonal rabbit antibody (1:1000, Sábado et al., 2013) and, in some cases, with C4F6 monoclonal antibody (1:100, Médimabs, Montreal Quebec, Canada). A FluoView 500 Olympus confocal laser-scanning microscope was also used. Digital images were analyzed with Visilog 6.3 software (Noesis, Orsay, France). Colocalization analysis of AJ10-C4F6 was performed by co-immunolabeling the appropiate plugin using ImageJ software (http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html).

AJ10 Peptide development and inoculation

The AJ10-immunizing peptide was synthetized by Antibody Bcn (Barcelona, Spain). Briefly, the peptide (VKVWGSIKGLTEGLHGFHVHEFGDNTAGC) was HPLC purified to >85% purity and was QC'd by nanospray mass spectrometry and HPLC analysis; the sequence was then confirmed using CID MS/MS. Our study was designed to analyze two different conditions, we used the peptide either alone or conjugated to keyhole limpet hemocyanin (KLH), using m-Maleimidobenzoyl-N-hydro xysuccinimide ester (MBS; a heterobifunctional agent that links a thiol group to an amino group at neutral pH) as immunizing agent. The mice were then inoculated by applying 2× 100 μl subcutaneous injections (SC), each of 0.25 μg/ml, at 5, 7, 9 and 16 weeks, and a booster dose at 24 weeks (the pre-symptomatic stage). The