ADVERSE EFFECTS OF A SOD1-PEPTIDE IMMUNOTHERAPY ON SOD1G93A 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 SOD1G93A 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 increase in the toxic function, which causes MN loss due to a mechanism involving non-neuronal 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).

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-neuronal 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 SOD1G93A 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...
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 SOD1G93A 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 deposits, 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(SOD1G93A)21Gur/J (SOD1G93A) 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 SOD1G93A 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 transectadially 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-phenylindole 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 appropriate plugin using ImageJ software (http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html).

AJ10 Peptide development and inoculation
The AJ10-immunizing peptide was synthesized by Antibody Bcn (Barcelona, Spain). Briefly, the peptide (VKWGSIKGTLTEGHLFHVHEFGDNTAGC) 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-hydroxysuccinimide 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
peptide was delivered mixed with an adjuvant (Sigma Adjuvant System Ribi, Sigma Aldrich, St. Louis, MO, United States) to potentiate immunostimulation. Each Ribi vial was reconstituted with sterile milliQ water (2 ml per vial) and vigorously mixed for 2–3 min to form an emulsion; the antigen was then added to obtain the desired concentration.

**Antibody titer and blood extraction**

To test the response of the mice to immunization, we extracted blood from tails at the end point. One or two cuts were made on the tail, an infrared light was used to promote blood flow, and samples were collected on Microvette CB300 heparinized vials (Sarstedt AG&Co., Nümbrecht, Germany). The samples were then centrifuged at 2000g for 5 min to isolate the plasma fractions; these were then stored at −80 °C until enzyme-linked immunosorbent assay (ELISA) was performed.

**Western blot (WBs)**

We performed WBs of spinal cord extracts with sera from AJ10-immunized mice and with anti-P2X4 antibody in order to assay its possible cross-reactivity to P2X4. We have previously observed (Sábado et al. 2013) that “the mutant SOD1 cross reactivity of commercially available anti-P2X4 antibody was irregular and varied from batch to batch (of anti P2X4 antibody)”. For the current study, we used an anti-P2X4 antibody from a batch (Alomone Lot AN-11), which does not cross-react with SOD1. Animals were killed using lethal doses of pentobarbital. Spinal cords were rapidly dissected, frozen in liquid N2 and stored at −80 °C until use. Samples were homogenized in 10 volumes of ice-cold buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, containing the protease inhibitor cocktail, Sigma P8340) and homogenized using 10–15 strokes of a motor-driven glass-Teflon homogenizer. Protein concentrations were measured using Bradford method. Samples were separated according to size through denaturing 11% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were electro-transferred to a nitrocellulose membrane in Tris–glycine–methanol buffer. The membrane was blocked for 1 h at room temperature in a blocking solution mixture of 5% non-fat dry milk, 0.1% Tween 20, and TBS. The membrane was then incubated for 2 h at room temperature with primary antibody. The membrane was rinsed once with TBS-Tween 20 for 15 min, washed twice with blocking solution for 5 min, and then incubated for 1 h at room temperature with peroxidase-labeled secondary antibodies (Amersham, Buckinghamshire, United Kingdom). The blot was washed once for 15 min, 10 min and three times for 5 min and then processed for an analysis using a Supersignal chemiluminiscence detection kit (Pierce, Rockford, IL, United States), as described by the manufacturer.

**Phenotypical score and behavior analysis**

We performed behavioral tests in order to assess the effectiveness of the immunotherapy. “clinical score” (CS) test is a visual observation of the phenotypical condition of the animal. When the animal is not visibly affected, it receives a CS of 4. When paralysis of the first hind limb appears, the CS is 3. A CS of 2 is assigned when second limb paralysis occurs. Finally, when the animal shows signs of symmetrical paralysis, the CS is 0. A CS of 3 is considered the onset of disease.

CatWalk ST System was used to assess the motor profile. CatWalk XT (Noldus, Wageningen, Netherlands; similar to the Paw Print Test) is a system used to quantify footfalls and gait in rats and mice. As the software showed some limitations for the study of later phases of a neurodegenerative disease like ALS, the interpodal distance was measured from 16 weeks on, but only pre-terminal stage data were analyzed. The pre-terminal stage was considered to represent the last point at which the animal was able to complete the test.

Open field test (OFT) was performed from 16 weeks of age until the end of the terminal stage. The OFT (Walsh and Cummins, 1976) provides measurements of locomotion, exploration and anxiety. Mice were placed in the middle of the apparatus and then allowed to freely explore the apparatus for 5 min. After each test, the mice were returned to their home cages and the field was cleaned with 70% ethanol and let it dry out before the next animal was tested. Latency to cross from the center to the periphery, total distance traveled, average speed, time spent in zones, number of crossings, rearing activity, and grooming behavior were collected and analyzed.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM) calculated from the respective standard deviations. Statistical significance analysis was carried out using an analysis of variance (ANOVA) followed by either Bonferroni’s post hoc test or Student’s t-test. In all cases, differences were considered to be statistically significant if \( p < 0.05 \).

**RESULTS**

Survival and disease onset did not improve following immunotherapy

Three imperative factors were measured and analyzed: the effectiveness of the treatment, improvements in life span, and delays in the onset of disease. Firstly, blood from terminal mice was analyzed by ELISA in order to assess the antibody response after immunization. As shown in Fig. 1A, almost every mouse that had been vaccinated with the unconjugated peptide developed an immune response (7 out of 10 animals; \( p < 0.05 \)); while the whole population of treated animals with the peptide conjugated to KLH showed much more homogeneous response after injection. In order to assay the possible cross-reactivity of the sera from AJ10-immunized animals, they were probed against spinal cord extracts by WB. The results obtained showed that immune sera recognized human SOD1 in transgenic animals but did not display any immunoreactive bands at the level of P2X4. In these experiments P2X4 was detected using a lot of anti-P2X4 antibody that did not cross-react with SOD1 (Fig. 1B).
We studied the effects of AJ10 immunotherapy on mouse lifespan and the onset of disease. Only one out of 10 studied animals showed a significant increase in lifespan after receiving the unconjugated peptide vaccination. In the case of vaccination with the AJ10 peptide linked to KLH, two out of nine animals tested showed signs of increased survival rate (Fig. 2). The analysis of Kaplan–Meier plots did not show any significant increment in the life span (p = 0.66 for AJ10 peptide; p = 0.53 for AJ10-KLH). Moreover, we found no relationship between the antibody titer and increased survival.

The onset of disease was measured using CS test, which takes a value of 3 (beginning of hind limb paralysis) as a reference point of the disease onset. Only a small percentage of the treated animals (Fig. 3A) displayed a delay in the onset of disease and, overall, the data obtained showed no significant differences between groups. Regression analysis demonstrated the absence of a correlation between the antibody titer and the onset of disease (Fig. 3B, C).

**Animal weight loss was not altered by the treatment**

Another important parameter that we assessed was whether animals were susceptible to loss weight after treatment, for that “global loss weight” parameter was calculated. “Global weight loss” is defined as the average weight along animal life and the weight at terminal point [(average weight – final weight)/average weight] * 100. As shown in the graph (Fig. 4), the treated animals displayed a non-significant tendency to lose weight compared with the controls.

**Increased neuroinflammatory response and neuronal degeneration after AJ10 SOD1-peptide vaccination assay**

One of the most important features of ALS is the neuroinflammatory environment that encloses the disease. We previously reported that AJ10 immunoreactive material was often found closely related to activated microglial (but not astroglial) cells, particularly in the case of those involved in neuronophagic processes surrounding degenerating neurons and dendrites (Sábado et al., 2013). Changes in the expression of common glial markers were analyzed after AJ10 vaccination. No changes in astroglia were revealed by GFAP immunostaining or observed after treatment with either peptide alone, or KLH-conjugated (Figs. 5A–D and 6A–D). In some cases, misfolded SOD1 was also detected by the commercially available C4F6 monoclonal antibody (Urushitani et al., 2007); when C4F6 antibody was combined with the rabbit AJ10 antibody in double labeling experiments, a close overlap between two signals was observed (Fig. 7). To prevent the visualization of endogenous deposits of IgG in mouse tissues during C4F6 immunolabeling, samples from transgenic rat spinal cord were used. It is interesting to note that the peptide residues used to generate C4F6
and AJ10 antibodies considerably differ in their SOD1 mapping sequences. C4F6 corresponds to 80–118 a.a. sequence encoded by exon 4, whereas AJ10 was raised using 30–58 a.a. sequence encoded by exons 2–3 in SOD1 (Pickles and Vande Velde, 2012). AJ10 peptide was identified in the basis of anti-SOD1 cross-immunoreactivity of some batches of anti-P2X4 ATP receptor antibody. Although we did not detect the cross-reactivity of any sera from AJ10 vaccinated animals with P2X4 by western blot, we cannot discard the possibility that these sera might have a low degree of affinity for P2X4 protein. Whatever the case, the fact that the pattern of immunolabeling displayed by AJ10 and by an antibody unrelated to P2X4 such as C4F6 is highly overlapping, indicates that the major protein target for AJ10-immunized animals is an emerging epitope in misfolded SOD1.

We combined two microglial markers: Iba-1, a generic microglial marker and Mac2, which is only present in a particular subset of microglia activated cells. Mac2 immunostaining has been used to evaluate neuroinflammatory activity in therapeutic assays in SOD1 ALS mice (Kriz et al., 2002). Mac2 is not expressed by intact Iba-1+ microglia but is highly upregulated on CNS injury (Venkatesan et al., 2010). We found that after treatment animals displayed a higher Mac2 immunoreactivity compared to control group (Figs. 5E–L and 6E–L). This seems to indicate that the neuroinflammatory response was exacerbated by the AJ10-peptide vaccination.

Substantial focal IgG deposits have been described in the spinal cord of SOD1 ALS mice; this is indicative of the breakdown of the blood–brain barrier (Zhong et al., 2008) and/or of an immunological reaction. IgG deposits were studied in lumbar spinal cord from AJ10-peptide vaccinated mSOD1 mice, where we found a significant increase in IgG deposits in ALS mice after immunization with AJ10-peptide (Figs. 5M–P and 6M–P).

Accumulation of misfolded SOD1 is indicative of the severity of disease course (Saxena et al., 2013). As previously described (Sábado et al., 2013), neurons containing misfolded SOD1 displaying strongly positive AJ10 immunostaining are often associated with activated microglia exhibiting neuronophagic activity. In our current study, vaccination with AJ10-peptide produced an
increased accumulation of mutant/misfolded neurotoxic conformers of SOD1 revealed by AJ10 antibody suggesting an acceleration of the disease progression (Figs. 5Q–T and 6Q–T). Semithin sections of L4 ventral nerve roots were used to evaluated MN loss by counting apparently healthy myelinated motor axons. A large number of degenerating axons were shown in nerves from end-terminal SOD1 G93A slow animals treated with vehicle or AJ10-peptide. The loss of motor axons was not modified by AJ10 vaccination (Fig 8).

Behavioral analysis showed increased motor deterioration in treated ALS mice

Gait analysis was performed using the CatWalk XT analysis system. This test, which is similar to traditional paw print analysis, consists of an enclosed walkway on a glass plate that is traversed by a rodent from one side of the walkway to the other. Data showing the gait pattern are acquired using an electronic device and analyzed with appropriate software. In our case, the interpodal distance (defined as the distance between the front and hind limbs on the same side of the body) was measured for each animal. Paw distance measurements were taken from both sides of the body. As previously commented, the animals only immunized with the peptide did not give a homogeneous response than the carrier group. The results were segmented according to their respective antibody titers (Fig. 9A). The interpodal distance was measured during the terminal stages of the disease. As shown in the graph, the WT group scored normal values, exhibiting a reduced interpodal distance with respect to transgenic animals. Comparison of the remaining groups (control and animals immunized with low or high antibody titers) showed that mice with low antibody titer exhibited similar interpodal distance to the control group. The higher interpodal distances were recorded in animals that had received
KLH-conjugated peptide (Fig. 9B). All of the vaccinated groups displayed significant differences with respect to WT group. However, treated animals had a tendency to exhibit increased interpodal distances, the differences observed were not statistically significant.

The OFT is a common way to measure exploratory behavior and general motor activity in mice. Two main parameters were analyzed: the number of crossings (number of times that the animal crosses an established grid on the floor of the OFT) and the total distance traversed.
traveled (total distance traveled during 5 min). In this model, symptoms started to become apparent from 7 to 8.5 months of age. The treated group showed a significant reduction in the number of crossings and total distance traveled; this suggested locomotive deterioration in concordance with the data that we had obtained from...
the CatWalk test (Fig. 9C, D). From 8.5 months onward, few mice remained able to complete the test and the limited number of animals that could continue with the test was insufficient to produce statistically significant results.

DISCUSSION

In the recent years, immunotherapeutic approaches to treat neurodegenerative diseases have emerged as a new and affordable procedure for neutralizing the harmful effects of misfolded/toxic protein deposition, which is a common pathological hallmark shared by all these disorders (Brody and Holtzman, 2008). This approach was initiated in the context of Alzheimer disease (AD). In a mouse model of AD, amyloid-beta (Aβ) deposition and behavioral abnormalities were both reduced by active vaccination with aggregated Aβ (Schenk et al., 1999). Evidence of the phagocytosis of Aβ deposits by activated microglial cells was seen in conjunction with high antibody titers. After these encouraging results, several clinical trials based on immunotherapeutic strategies started at phase II, but clinical trials were halted after a small percentage of patients developed acute meningoencephalitis (Nicoll et al., 2003; Orgogozo et al., 2003). Recent phase III trials involving passive immunotherapy using humanized anti-amyloid-beta monoclonal antibodies have not shown any advantages over clinical outcomes, even though some biomarkers did

**Fig. 7.** Double immunolabeling for comparative detection of misfolded SOD1 accumulation in the ventral horn of spinal cord using two separate conformational-specific anti-SOD1 antibodies (AJ10 and C4F6). Samples were obtained from end-terminal SOD1<sup>G93A</sup> transgenic rat. Co-immunostaining using rabbit AJ10 (red in A) and mouse monoclonal C4F6 (green in B) antibodies showed that a very similar pattern to misfolded SOD1 accumulation, which was revealed by both antibodies. The combined image is shown in (C). Image analysis demonstrates a high degree of colocalization between the red and green channels; colocalized pixels (white) are superimposed on a merged image in (D). Dashed lines indicate the delimitation of the ventral horn: Scale bar in C = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 8.** Semithin sections of L4 ventral roots from end-terminal SOD1<sup>G93A</sup> slow mice display similar massive degenerative changes in control-treated and AJ10-vaccinated animals when compared with WT. Counts of apparently healthy motor axons that reflect the number of spinal cord MNs did not show any significant changes in axonal loss as a consequence of AJ10-peptide vaccination. Data were obtained from four different animals for each condition: Scale bar = 50 μm.
show reduced levels of Aβ deposition (Doody et al., 2014; Salloway et al., 2014).

In relation to Parkinson’s disease (PD), immunization with α-synuclein (α-syn)-derived peptides resulted in ameliorating PD-like neuropathology and improved motor functions in mouse models for this disease; α-syn antibodies also blocked the propagation of α-syn from neuron to neuron in vitro (Games et al., 2014). Since, there is a growing evidence that supports the hypothesis of ‘prion-like’ manner spread of altered proteins in neurodegenerative diseases, it could be possible that specific antibodies may also exert a therapeutic effect by interfering with this mechanism (Aguzzi and Rajendran, 2009; Frost et al., 2009).

Extracellular mutant SOD1 could also be a therapeutic target for vaccination or passive antibody therapy against misfolded/neurotoxic SOD1 in ALS. Although SOD1 is a cytosolic protein, which does not contain a translocation signal for entering secretory pathways, there is evidence that SOD1 can be secreted to extracellular spaces after reaching the secretory pathway (Urushitani et al., 2006; Urushitani et al., 2008). It has also been shown that extracellular mutant SOD1 promotes microglial activation and neurotoxicity (Urushitani et al., 2007; Hernandez et al., 2010; Sábado et al., 2013). Reducing or neutralizing neurotoxic extracellular SOD1 by means of vaccination with recombinant human SOD1G93A conferred protection to an ALS mouse strain with a moderate overexpression of...
SOD1G37R but failed to ameliorate the mice overexpressing higher amounts of SOD1G93A (Urushitani et al., 2007).

This immunization was associated with an increased neuroinflammatory response in spinal cord with Mac2-positive microglial cells that also contained endogenous IgG. This pathological fact, but not the clinical outcome, was in line with the results that we obtained with the SOD1 peptide AJ10. Using the conformational-specific anti SOD1 antibody AJ10, we found that the content in misfolded, presumably neurotoxic, SOD1 increased after vaccination. The misfolded SOD1 mainly accumulates in neurons rather than in glial cells, as previously described (Sábado et al., 2013). In contrast, Urushitani et al. (2007) reported that when they used C4F6 monoclonal antibody to detect misfolded SOD1, they noted a reduction in these species that was consistent with a beneficial effect of vaccination. There is therefore a good correlation between the accumulation of misfolded SOD1 and the progression of disease during the immunotherapeutic assay.

Another set of experiments, which tested an active immunization strategy with a SOD1-derived peptide, has also been reported (Liu et al., 2012). In this case, the used peptide corresponded to a SOD1 epitope located between amino acid residues 145–151, at the SOD1-exposed dimer interface (SEDI) of this protein. An antibody that specifically recognizes this region had previously been obtained (Rakhit et al., 2007; Kerman et al., 2010) and it was used in a similar manner to our AJ10 (Sábado et al., 2013). SEDI antibody detects misfolded SOD1 in a variety of ALS-related SOD1 mutations but not in its native WT conformation. Immunization with SEDI peptide applied to mice overexpressing SOD1G37R resulted in a delay of the disease onset and a prolongation of survival up to 40 days; this is greater than which was obtained by the usage of mutant SOD1 as an immunogen (Urushitani et al., 2007). SEDI peptide vaccination also produced a reduction in the accumulation of misfolded SOD1 species but, in contrast with the results reported by Urushitani’s, also damped down the neuroinflammatory glial reaction. In a transgenic SOD1 mouse model, the attenuation of Mac2-positive microgliosis after immunotherapy has been considered indicative for a beneficial anti-inflammatory effect (Liu et al., 2012). Thus, the increase in Mac-2 immunoreactivity detected in microglia after AJ10 vaccination might be interpreted as detrimental. However, it should also be considered that, in some circumstances, the increase in Mac2-positive microglia has not been associated with an adverse outcome because these cells might be involved in Fc-mediated phagocytosis and the clearance of extracellular misfolded proteins such as α-syn and tau (Bae et al., 2012; Luo et al., 2015).

A vaccination protocol involving recombinant WT non-metaled WT SOD1 was applied in the only report in which low-copy SOD1G93A transgenic mice were used as a model for an immunotherapeutic assay, (Takeuchi et al., 2010). An increase of life span and potentiation of protective lymphocyte Th2 responses were found, while a less beneficial effect was observed after immunization with SOD1G93A. Passive immunization by infusion of an appropriate anti-SOD1 monoclonal antibody has therapeutic effects on the SOD1G93A mouse model (Gros-Louis et al., 2010).

Our assay with AJ10 peptide suggested that the effects of different SOD1 vaccines on therapeutic outcomes may differ substantially. Neuroinflammation is a major contribution to MN injury during ALS (Philips and Robberecht, 2011) and chronic stimulation of microglial-dependent innate immunity exacerbates ALS caused by SOD1 mutation (Nguyen et al., 2004). However, lymphocyte-mediated immune responses may also influence the microglial phenotype and be neuroprotective (Appel et al., 2010). It has been shown that T cells play a central role in the regulation of the microglial function in ALS (Beers et al., 2008; Zhao et al., 2012). The complex immunomodulatory responses triggered during ALS progression may produce either beneficial or detrimental responses. How stimulation of the immune system after vaccination affects this subtle regulatory balance is currently unknown. It is possible to enhance the protective T cell-mediated response by therapeutic administration of the unspecific antigen copolymer-1 (Angelov et al., 2003), but other therapeutic assays with this compound did not produce positive results or accelerated the progression of the disease in both fast and slow ALS SOD1 models (Haenggeli et al., 2007). Similarly, therapeutic immunization with glatiramer did not show positive results in humans (Meininger et al., 2009).

At the present, the effects of different types of acquired immunity induced by diverse vaccines on therapeutic outcomes remain poorly understood. It is therefore possible that a host immune response may have counteracted the beneficial effects of vaccination in our experiments. In our study, we noted increased deposits of IgG in MNs and microglial cells after vaccination. The presence of IgG in these cells in ALS human and animal tissues has already been described and was probably a consequence of blood–brain barrier leakage (Engelhardt and Appel, 1990; Zhong et al., 2008). However, the larger amounts of IgGs induced by AJ10 peptide immunization may also reflect SOD1-IgGs immunocomplexes that could help to potentiate inflammatory cascades that aggravate the ALS pathological and clinical course. Further research is required to establish whether AJ10 vaccine-induced endogenous IgGs reacts with SOD1 as a self-antigen similarly to autoimmune diseases. Under this condition, IgG Fc receptor-dependent activation of the complement system would play an important role in the determination of the dangerous inflammatory/autoimmune response. If this would be the case, passive immunization with engineered immunoglobulins lacking the Fc fragment might suppress these adverse effects while maintaining the beneficial effect on misfolded SOD1 binding. New and emerging concepts in ALS research, such as the transcellular spread of SOD1 aggregates in a prion-like manner (Munch et al., 2011; Grad et al., 2014) and its targeting by antibodies, they should be taken into account as they add further interest for development and improvement of immunotherapeutic strategies for ALS.
CONCLUSIONS

In this study, we performed an active immunotherapeutic assay in SOD1G93A mouse slow model of ALS. SOD1 peptide was used as an antigen for vaccination. It had previously been demonstrated that this peptide was able to raise conformational-specific antibodies against ALS-related misfolded SOD1. Although vaccination was initiated before the onset of symptomatology, treated animals did not display any signs of clinical or pathological improvement. On the opposite, after vaccination, animals showed an increased neuroinflammatory response, which was evidenced by (i) higher levels of Iba-1 and Mac2 immunoreactive microglia, (ii) large amounts of IgG complexes in the tissue, and (iii) increased intraneuronal misfolded SOD1 deposition. Treated animals showed worse motor behavioral performance and scores. Further analysis is required to determine whether AJ10 vaccine would induce endogenous IgGs reactivity with SOD1 as a self-antigen, similarly as in autoimmune diseases.

AUTHOR CONTRIBUTIONS

J.S., A.C., and J.E. designed the research; J.S. and A.C performed the experiments and analyzed the data; GA and HR contributed to behavioral testing and J.S. and J.E. analyzed the data and wrote the paper.

DISCLOSURES

None of the authors have any conflicts to disclose. All of the authors have approved the final manuscript.

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REFERENCES


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