Activation of microglia in mice spinal cords following passive transfer of purified IgG from patients with amyotrophic lateral sclerosis

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ABSTRACT

Objectives: To determine whether IgG from amyotrophic lateral sclerosis (ALS) patients could cause activation of microglia, proliferation of astrocytes, and infiltration by lymphocytes within mice spinal cords.

Methods: A group of 5 mice received injections of IgG purified from sera of patients with ALS. A control group of 5 mice received IgG from healthy humans, whilst a third group of 2 mice served as non-injected controls. One mouse served as a positive control and was injected with lipopolysaccharide, a known activator of microglia. Mice were culled after one week, for immunocytochemistry of spinal cord sections to localize the complement receptor CD11b on activated microglia, glial fibrillary acidic protein on astrocytes, and CD4 and CD8 receptors on lymphocytes. Histological examination was used to determine the presence of inflammatory reaction. This work was conducted at the Institute of Neurology, Queen Square London, United Kingdom, from January to July 2004.

Results: There was no significant difference in activation of microglia between mice injected with ALS IgG and mice injected with control IgG (p = 0.631), although mice injected with ALS IgG exhibited greater microglial activation than non-injected mice (p = 0.044). Proliferation of astrocytes was not significantly different between the 3 groups. CD4 and CD8 lymphocytes were both absent in mice injected with ALS IgG, mice injected with control IgG and non-injected mice.

Conclusion: Activation of microglia following passive transfer of IgG from ALS patients to mice represents a non-specific inflammatory response, rather than a primary mechanism for motor neuron degeneration.

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting motor neurons of the cerebral cortex, brainstem and spinal cord leading to death, commonly from respiratory failure. It has an annual incidence of 0.2-2.4/100,000, and a prevalence of 0.8-7.3/100,000 population worldwide.[1] The underlying mechanisms of neurodegeneration in ALS are not precisely understood. However, a wealth of evidence favors the possible roles of free radical-mediated oxidative damage, excitotoxicity, abnormalities of axonal transport, deficiency of neurotrophic factors, and immune-mediated inflammatory injury. Immune-mediated inflammation of motor neuron regions has been described in both human and animal models of ALS. For instance, in post mortem ALS brain and spinal cords, motor neurons stained strongly for IgG compared to normal controls,[2] with significant activation of microglia described in some studies,[3] though not in others.[4] Also, in-vitro studies showed that ALS IgG was cytotoxic both to cultured motor neurons[5] and to mice motor neurons,[6] where

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significant activation of microglia and increase in intracellular calcium were observed. Recently, Pullen et al[7,8] described motor neuron degeneration in mice exposed to ALS-derived IgG, a process that involved increase in cytosolic calcium, fragmentation of Golgi complex and mitochondria, abnormalities of endoplasmic reticulum, and increased levels of activated caspase 3, suggesting possible roles both for apoptosis and inflammation in motor neuron cell death. While the cytotoxic effects of ALS IgG on mice and cultured human motor neurons has been extensively studied, the question remains unanswered as to whether motor neuron cell death in ALS occurs primarily by the process of inflammation and necrosis or by the process of apoptosis. One study reported a primary role of inflammation,[9] but the repertoire of inflammatory cells studied was limited to only microglia and lymphocytes, leaving out the role of astrocytes. The present study aimed to determine whether IgG from ALS patients could cause activation of microglia, proliferation of astrocytes, and infiltration of lymphocytes within mice spinal cord ventral horns.

Methods. This work was conducted at the Institute of Neurology, Queen Square London, United Kingdom, from January to July 2004.

Sample preparation. Blood samples were obtained from 10 adults, 5 of whom were sporadic ALS patients and 5 were healthy volunteers. All patients satisfied the El Escorial-World Federation of Neurology criteria for definite ALS, while control subjects were all healthy adults, not known to be on any medication. Controls were age and sex-matched to ALS patients, with 3 male and 2 female controls matched to 3 male and 2 female ALS patients (mean age ± SD: 54.8 ± 4.7 vs. 58.6 ± 8.4 years; $p = 0.4$).

Purification of human IgG. Sera from ALS patients and controls were purified by affinity chromatography using Protein A-4% agarose columns (Pierce Inc.), and then dialyzed overnight in 100% phosphate buffer solution (PBS) to remove serine proteases according to standard protocol. Purified IgG and serine proteases were then identified on immunodot membranes. The IgG content of sera were measured by spectrophotometry at 280 nm wavelength.

Passive transfer of IgG to mice. Animal experiments were conducted according to British Home Office regulations. Two-week old male Balb*C* mice (25g average body weight) were used for the study. One group of 5 mice received 5 daily intraperitoneal (I/p) injections of IgG from ALS patients (3.5 mg total dose), with each mouse receiving serum from a single individual; a second group of 5 mice received IgG total dose of 3.5 mg from healthy human controls; a third group of 2 mice received no injections, while one mouse was injected with a total 100μg I/p lipopolysaccharide over 3 alternate days, to serve as a positive control for the detection of activated lymphocytes.

Immunohistochemical staining. Mice were culled on day 8 with I/p injection of Lethobarb (1:10 dilution), and perfused with 4% paraformaldehyde-0.1% glutaraldehyde-PBS. Spinal cords were dissected and sectioned at 35μm thickness on a vibroslice. Sections were processed and immunostained with antibodies to GFAP (polyclonal rabbit anti-GFAP; Dako, Denmark), CD4 and CD8 (polyclonal goat anti-human CD4 [C18] and CD8-α[R-15] antibodies; Santa Cruz Biotechnology, CA) and CD11b (rat anti-mouse integrin αM monoclonal antibody; Chemicon International, CA) according to standard protocol. Streptavidin-horseradish peroxidase stain was used to detect the presence of tissue-bound antibodies.

Microscopic examination. Slides were examined with a standard light microscope at low (x10) and high (x20–x40) magnifications. The unbiased optical dissector method was used to count cells, with the examiner blinded to the identity of each slide. Two frames of each ventral horn were examined in each section, 5 sections examined per slide and 15 slides per animal, at x20 magnification. Frames were re-focused after the first counting, to include positively stained cells located at other focal depths. All CD4, CD8, and CD11b-positive cells were counted, but GFAP-positive cells were only counted when their astrocytic processes and cell bodies were both clearly visible. Images were obtained with an Olympus 35 mm camera connected to a computer.

Statistical analysis. This was carried out with SPSS for Windows version 11.0. Cases and controls were compared by means of unpaired Student's t test, with an assumption of unequal variances by Levene's test. *P* values less than 0.05 were considered significant.

Results. ***Purification of human IgG.*** The presence of human IgG from both ALS patients and controls was detected on immunodot membranes at concentrations of 1μg/μL – 10ng/μL, both before and after dialysis (**Figure 1**).

Presence of serine proteases in sera. The concentration of serine proteases detected in sera before dialysis was visibly reduced after dialysis, to levels where previous studies had shown were insufficient to elicit neuronal lesions.

GFAP-positive astrocytes. The number of GFAP positive astrocytes per spinal cord section was higher among mice injected with ALS-derived IgG compared with mice injected with control IgG or

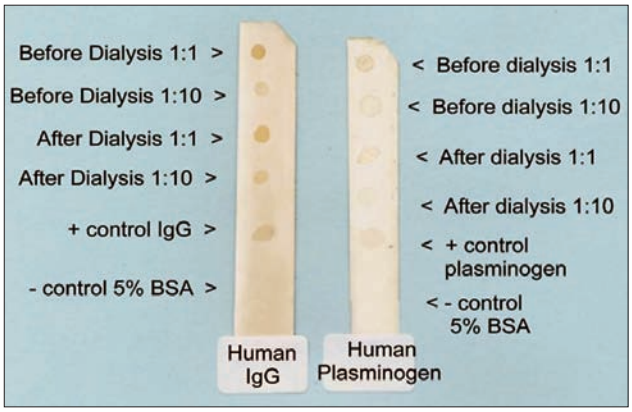

Figure 1 - Immunodot blot analysis of human IgG (H+L) and the serine protease plasminogen in an affinity-purified sample from an ALS patient before, and after dialysis. Plasminogen levels are significantly reduced after dialysis. ALS - amyotrophic lateral sclerosis. BSA - bovine serum albumin.

Table 1 - Comparison of means of GFAP-positive astrocytes per section of spinal cord in 3 groups of mice.

Group comparison	GFAP + cells, mean ± SD	*p*-value
ALS-IgG mice vs. control-IgG mice	24.28 ± 10.3 vs. 15.16 ± 5.75	0.133
ALS-IgG mice vs. non-injected mice	24.28 ± 10.3 vs. 14.80 ± 5.94	0.212
Control-IgG mice vs. non-injected mice	15.16 ± 5.75 vs. 14.80 ± 5.94	0.949

GFAP - glial fibrillary acidic protein, vs - versus, ALS - amyotrophic lateral sclerosis

Table 2 - Comparison of means of CD11b-positive microglia per section of spinal cord in 3 groups of mice.

Group comparison	CD11b + cells, mean ± SD	*p*-value
ALS-IgG mice vs. control-IgG mice	42.48 ± 21.25 vs. 37.08 ± 10.81	0.631
ALS-IgG mice vs. non-injected mice	42.48 ± 21.25 vs. 12.80 ± 7.92	0.044*
Control-IgG mice vs. non-injected mice	37.08 ± 10.81 vs. 12.80 ± 7.92	0.055

CD11b - complement receptor, vs - versus, ALS - amyotrophic lateral sclerosis, *significant value

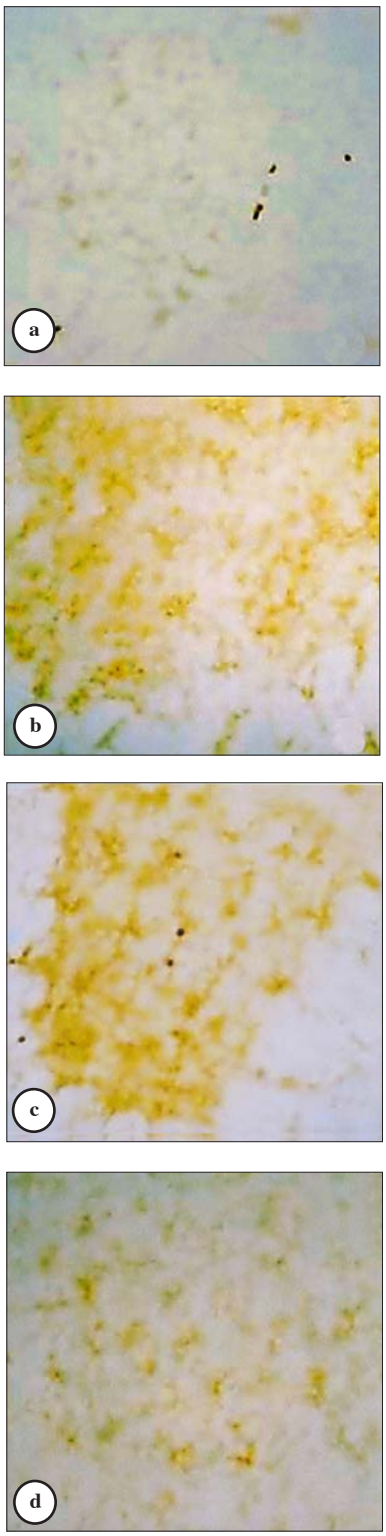

Figure 2 - Vibroslice sections of spinal cord ventral horns (35μm) immunostained for CD11b on activated microglia showing **a)** few immunostained structures in an uninjected mouse **b)** heavy staining in a mouse injected with control-derived IgG. Both **c)** marked and **d)** scanty staining is seen from 2 mice injected with ALS-derived IgG.

non-injected mice, however, these differences did not reach statistical significance (**Table 1**).

CD11b-positive microglia. The average number of microglia immunoreactive to CD11b was higher in both mice injected with ALS-derived IgG and mice injected with control-IgG, compared to non-injected mice. However, the difference between ALS IgG-injected mice and control IgG-injected mice was not significant (**Table 2**).

CD4 and CD8-positive lymphocytes. Microscopic sections of spinal cord ventral horns failed to exhibit any CD4 or CD8-positive lymphocytes in either mice injected with ALS IgG, mice injected with control IgG, or non-injected mice. However, mice injected with lipopolysaccharide showed numerous CD4 and CD8 immunoreactive cells in sections of spinal cord, spleen and thymus, thereby confirming the efficacy of CD4 and CD8 antibodies used in the study (data not shown).

Discussion. Most cases of ALS are sporadic, however, in 5-10% of patients the disease is familial, resulting from mutations of several genes, most commonly the gene for superoxide dismutase (SOD1), an enzyme involved in the dismutation of cytosolic free radicals.[10] In both familial and sporadic ALS, the pathological findings are similar and include atrophy of the primary motor cortex and spinal cord, white matter degeneration, astrocytosis, and microglial reaction. Earlier studies utilizing conventional histological stains failed to detect the presence of significant inflammatory infiltrates in ALS tissue.[4] However, the advent of immuno-histochemistry has revealed the presence of inflammatory infiltrates in areas of degenerating motor neurons, more commonly than previously recognized.[11] Modern stains that utilize monoclonal antibodies targeted specifically at receptor proteins located on the surfaces of neurons and glia have identified different inflammatory infiltrates. These include reactive astrocytes which stain for glial fibrillary acidic protein (GFAP), CD4 and CD8 lymphocytes which express the VB2-T lymphocyte receptor, and activated microglia, which stain various proteins, including the complement receptor CD11b and the immunoglobulin receptor FCγR1.[2,3,12,13]

Activated microglia have been found clustered around degenerating motor neurons in animal models of ALS, including guinea pig experimental autoimmune grey matter disease,[14] mutant SOD1 transgenic mice,[15,16] and mice injected with IgG derived from ALS patients.[9]

In the present study, activation of microglia was evident in ventral horns from mice injected with ALS-derived IgG as well as mice injected with control-derived IgG (**Figure 2**), but only in mice injected with ALS-derived IgG was this effect significant, when compared to non-injected mice ($p = 0.044$). But more importantly, the greater degree of microglial activation in the ALS-IgG group compared to the control-IgG group was not statistically significant ($p = 0.631$). This suggests that human IgG injected into mice simply caused an inflammatory response, whether it is derived from ALS patients or from healthy humans. These findings agree with earlier studies that documented the entry of IgG into the CNS,[2,16,17] an event that heralded enhanced transmitter release at nerve terminals.[18] However, our findings differed from observations made elsewhere,[9] in that there was no significant difference in microglial activation between mice injected with ALS-derived IgG and those injected with IgG from healthy controls. A possible reason for this disparity may lie with the different doses of IgG used in the 2 experiments. Whereas the present study used a total IgG dose of 3.5 mg given over 5 days with mice examined on the eighth day, the earlier study used a much higher dose of 20 mg given over 48 hours, which may have produced an acute, as opposed to a longer-term effect on motor neurons and glia. The optimal dose schedule in our laboratory was pre-determined from previous experiments in which IgG levels in ALS patients were found to be within the normal physiologic range for humans, which were then extrapolated for the weight of the smaller-sized rodent.

Although there was some degree of astrocytic proliferation in mice injected with ALS-IgG compared to control mice, this difference did not reach statistical significance. This finding supports observations made in other studies. Obal et al,[9] who utilized the same paradigm as ours, did not analyze astrocytic proliferation in their study, but other studies of mutant SOD 1 transgenic mice reported proliferation of astrocytes only at 100-120 days of age, a period corresponding to the terminal stages of motor neuron degeneration in that model of ALS.[15,19] Similarly, the paucity of CD4 and CD8 lymphocytes in spinal cord sections in this study is consistent with the known observations that although CD4 and CD8 lymphocytes were noted in post-mortem ALS brains and spinal cords,[3,11] these cells are virtually absent in SOD1 transgenic mice,[12] and in mice injected with ALS-derived IgG.[9] It would be interesting to study the effects on normal mice of purified IgG derived from SOD1 mice, but we are not presently aware of any published study that has explored this approach.

In conclusion, IgG derived from ALS patients, as well as that derived from healthy humans, both

induced activation of microglia within mice spinal cord ventral horns, an effect that did not differ significantly on the basis of IgG source. While this observation does not contradict the autoimmune theory of neurodegeneration in ALS, it supports an alternative mechanism other than inflammation as a primary cause of motor neuron cell death in this disease. Further studies are needed to investigate the possible role of apoptosis in motor neuron cell death in ALS.

References

1. Eisen A, Krieger C. Amyotrophic lateral sclerosis: A synthesis of research and clinical practice. Cambridge: Cambridge University Press; 1998.
2. Engelhardt JI, Appel SH. IgG reactivity in the spinal cord and motor cortex in amyotrophic lateral sclerosis. ***Arch Neurol*** 1990; 47: 1210-1216.
3. Kawamata T, Akiyama H, Yamada T, McGeer PL. Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. ***Am J Pathol*** 1992; 140: 691-707.
4. Hawkes CH, Cavanagh JB, Mowbray S, Paul EA. Familial motor neuron disease: report of a family with four post-mortem studies. In: Rose FC, editor. Research progress in motor neuron disease 70-98. London: Pitman Books; 1984.
5. Smith RG, Hamilton S, Hofmann F, Schneider T, Nastainczyk W, Birnbaumer L, et al. Serum antibodies to L-type calcium channels in patients with amyotrophic lateral sclerosis. ***N Engl J Med*** 1992; 327: 1721-1728.
6. Obal I, Siklos L, Engelhardt JI. Altered calcium in motor neurons by IgG from human motor neuron diseases. ***Acta Neurol Scand*** 2002; 106: 282-291.
7. Pullen AH, Demestre M, Howard RS, Orrell RW. Passive transfer of purified IgG from patients with amyotrophic lateral sclerosis to mice results in degeneration of motor neurons accompanied by calcium enhancement. ***Acta Neuropathol (Berl)*** 2004; 107: 35-46.
8. Pullen AH, Howard R, Orrell R. Necrotic and apoptotic-like degeneration in motor neurons following passive transfer of ALS/MND-derived IgG. ***Amyotroph Lateral Scler Other Motor Neuron Disord*** 2001; 2 (Suppl 2): 143-144.
9. Obal I, Soos J, Jakab K, Siklos L, Engelhardt JI. Recruitment of activated microglia cells in the spinal cords of mice by ALS IgG. ***Neuroreport*** 2001; 12: 2449-2452.
10. Andersen PM, Morita M, Brown RH. Genetics of amyotrophic lateral sclerosis; an overview. In: Brown HR, Meininger V, Swash M, editors. Amyotrophic lateral sclerosis. London: Martin Dunitz; 2000. p. 223-250
11. Engelhardt JI, Tajti J, Appel HS. Lymphocytic infiltrates in the spinal cord in amyotrophic lateral sclerosis. ***Arch Neurol*** 1993; 50: 30-36.
12. Alexianu ME, Kozovska M, Appel SH. Immune reactivity in a mouse model of familial ALS correlates with disease progression. ***Neurology*** 2001; 57: 1282-1289.
13. Mohamed HA, Mosier DR, Zou LL, Siklos L, Alexianu ME, Engelhardt JI, et al. Immunoglobulin Fcγ receptor promotes immunoglobulin uptake, immuno globulin-mediated calcium increase, and neurotransmitter release in motor neurons. ***J Neurosci Res*** 2002; 69: 110-116.
14. Engelhardt JI, Appel SH, Killian JM. Motor neuron destruction in guinea pigs immunized with bovine spinal cord ventral horn homogenate: experimental autoimmune grey matter disease. ***J Neuroimmunol*** 1990; 27: 21-31.
15. Hall ED, Oostveen JA, Gurney ME. Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. ***Glia*** 1998; 23: 249-256.
16. Fabian RH. Uptake of plasma IgG by ANS motor neurons: comparison of antineuronal and normal IgG. ***Neurology*** 1988; 38: 1775-1780.
17. Frantatoni SA, Dubrovsky AL, Uchitel OD. Uptake of immunoglobulin G from amyotrophic lateral sclerosis patients by motor nerve terminals in mice. ***J Neurol Sci*** 1996; 137: 97-102.
18. Uchitel OD, Appel AH, Crawford F, Sczupak L. Immunoglobulins from amyotrophic lateral sclerosis patients enhance spontaneous transmitter release from nerve terminals. ***Proc Natl Acad Sci USA*** 1988; 85: 7371-7374.
19. Almer G, Vukosavic S, Romero N, Przedborski S. Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. ***J Neurochem*** 1999; 72: 2415-2425.