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IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE

Emma J. O'Neill^{a,*}, Michael J. Day^b, and David C. Wraith^c

^aSchool of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, Belfield, Dublin 4, Ireland ^bDivision of Veterinary Pathology, Infection and Immunity, Department of Clinical Veterinary Science, University of Bristol, Langford House, Langford, BS40 5DU, UK ^cDepartment of Cellular and Molecular Medicine, University of Bristol, Medical School, University Walk, Bristol BS8 1TD, UK

Abstract

We have shown previously that intranasal administration of encephalitogenic peptides in soluble form to H-2^u and H-2^s mice affords protection from experimental autoimmune encephalomyelitis (EAE). Here we demonstrate that this method of disease protection can be induced in C57BL/6 mice by administration of the soluble peptide 35–55 from myelin oligodendrocyte glycoprotein. This protective effect was demonstrated by the evaluation of both clinical EAE scores and central nervous system histopathology; the latter showing minimal inflammatory infiltrates in treated mice. The employment of an IL-10^{-/-} congenic strain allowed an appraisal of the involvement of IL-10 in this process. The lack of disease protection in these mice clearly demonstrates the non-redundant role of IL-10 in this process.

Keywords

Experimental autoimmune encephalomyelitis; Histopathology; Myelin oligodendrocyte glycoprotein (MOG); Tolerance induction

1. Introduction

Approximately 1 in 20 individuals in Europe and North America suffer from an autoimmune disease. Current treatments for serious autoimmune diseases are based on therapies that suppress the immune system non-specifically. Such therapies are potent and can have adverse consequences for the patient including; 1. Non-specific targeting of host defenses can result in an impaired ability to fight off infection. 2. Immunosuppression is required continuously until the disease is controlled. 3. Non-specific immunosuppression can hamper the natural induction of tolerance. 4. Life-long immunosuppression is expensive. In view of this, there is clearly the need to design more specific therapies. Attempts to focus therapy more specifically have been undertaken in diseases such as multiple sclerosis (MS). Thus, IFN- β , although initially tested as an anti-viral agent, has now been shown to have anti-inflammatory effects that reduce relapse rate in relapsing–remitting MS (Zhang et al., 2002). Furthermore, glatiramer acetate, a random co-polymer of amino acids that mimics myelin basic protein, has been shown to induce a shift from Th1 to Th2 responses in vitro

(Duda et al., 2000) and is now a frontline treatment for relapsing–remitting MS (Goodin et al., 2002).

Recent studies in animals have investigated more antigen-specific therapies for autoimmune diseases (Wraith, 2004). In particular, the use of peptides based on the sequences of known disease-associated antigens has been studied in both allergy and autoimmune disease (Larche and Wraith, 2005). Many of these studies have employed experimental autoimmune encephalomyelitis (EAE) as an animal model for MS. Intranasal peptide deposition has been successfully used to prevent and treat EAE in both mouse and rat models (Anderton and Wraith, 1998; Metzler and Wraith, 1993; Xu et al., 2000). Indeed peptide treatment has been shown to promote immunological tolerance towards the antigen in question and in addition to induce bystander suppression (Anderton and Wraith, 1998). As yet, however, little is known about the mechanism of tolerance induction. The use of a T-cell receptor transgenic mouse model has shown that repeated administration of soluble peptide leads to the induction of IL-10 secreting regulatory T-cells (Burkhart et al., 1999) that depend on this cytokine for their function in vivo (Sundstedt et al., 2003). As yet, however, there is little information on the role of IL-10 in tolerance induction, especially in non-transgenic animals. Here we study the role of IL-10 in peptide therapy of a mouse model of EAE and demonstrate the non-redundant role that this cytokine plays in disease protection.

2. Materials and methods

2.1. Antigen

The peptide used was comprised of amino acids 35–55 from Myelin Oligodendrocyte Glycoprotein (MEVGWYRSPFSRVVHLYRNGK), and was synthesised using standard Fmoc chemistry on an AMS 422 multiple peptide synthesiser (Abimed, Langenfeld, Germany). The peptide was purified into endotoxin free PBS (GIBCO, Life Technologies, Paisley, UK) at a final concentration of 4 mg/ml for intranasal administration. The solution was filter sterilized and stored at -80°C prior to use.

2.2. Mice

C57BL/6 and C57BL/6 IL-10^{-/-} mice were purchased from Harlan-Olac (Bicester, UK), then bred and maintained under SPF conditions at the University of Bristol. EAE experiments were performed when the majority of mice were between eight and twelve weeks of age, with all treatment groups age- and sex-matched. Initial experiments involved male and female treatment groups, however later experiments utilized female-only groups as the disease severity was more marked in this sex. All experiments gained ethical approval and were performed under license from the UK Home Office.

2.3. Induction and evaluation of EAE

Priming mice to induce EAE involved the inoculation of 200 μg peptide antigen in a volume of 100 μl adjuvant. Antigen for injection was prepared by homogenising equal volumes of 4 mg/ml peptide solution and Complete Freund's Adjuvant (CFA, Difco Laboratories, Detroit, MI, USA). Mice were primed subcutaneously on day zero at the base of the tail with 0.1 ml of peptide-adjuvant. On days 0 and 2 post-immunization, 200 ng of Pertussis toxin (Porton Products, Salisbury, UK) was administered intraperitoneally in 0.5 ml of PBS (Sigma-Aldrich). Mice were monitored daily thereafter until day 30 for the development of neurological deficits. Mice were scored daily by a single observer and graded as shown in Table 1.

2.4. Intranasal administration of antigen

Intranasal administration was performed under a light plane of anaesthesia induced using Halothane (Fluothane, Rhone-Merieux). Peptides used for intranasal (IN) administration were made up as a 4 mg/ml solution in Dulbecco's PBS (Gibco) and were given as a total dose of 25 μ l into both nostrils. The dosage regime employed was a total of three doses given on days -7, -5 and -3 prior to disease induction on day zero.

2.5. Histopathology

Freshly isolated spinal cord and brain material were immediately placed into an excess of 10% neutral buffered formalin. Brains and spinal cords were embedded in paraffin wax, sectioned longitudinally and then stained by haematoxylin and eosin. Histopathological examination of the brains and spinal cords were carried out in a blinded fashion. The scoring method used has been previously described (Racke et al., 1994) and was as follows:

Grade of inflammation

0	no inflammatory cells
1	a few scattered inflammatory cells
2	organisation of inflammatory infiltrates around blood vessels
3	extensive perivascular cuffing with extension into adjacent parenchyma, or parenchymal infiltration without obvious cuffing

The areas examined were the entire brain (longitudinally sectioned) and the entire spinal cord (also longitudinally sectioned). Duplicates of each section (brain and cord) were present on one slide and both were evaluated. The meninges were examined for the brain sections, but the means of collection of the spinal cord (via syringe extrusion from the vertebral canal) meant that the cord meninges were not present for examination.

2.6. Statistical analysis

The Kruskal–Wallis test with the Dunn's multiple comparison post-test was used for statistical analysis of multiple-group EAE data.

3. Results

3.1. The profile of disease induced in C57BL/6 mice with MOG 35–55 peptide

Previous studies have shown the suitability of the B6/MOG 35–55 model for studying the immune response and EAE. As yet, however, there has not been a systematic analysis comparing disease score with histopathology. A large cohort ($n=52$: 21 female and 31 male) of mice were primed for EAE and during the 30-day study period, at least five mice (at least 2 female and 3 male) were culled at regular time points (days 6, 9, 12, 14, 16, 18, 20, 23, 26 and 30) in order to obtain samples for histopathology. The results shown in Fig. 1A reveal a monophasic pattern of disease with the majority of mice recovering by d27.

Histopathological examination of the mice demonstrated a mixed mononuclear cell infiltrate (macrophages, lymphocytes and plasma cells) affecting predominantly the peripheral white matter of the spinal cord, but in some individuals extending deeper into the parenchyma. Lesions within the brain predominantly affected the cerebellum and hind-brain white matter. Mild lesions generally comprised small perivascular inflammatory cuffs. More severe lesions in either the brain or spinal cord appeared as diffuse infiltrates extending from the perivascular location into surrounding parenchyma. In some mice meningitis was also identified. This tended to be regional, occurring particularly in the cerebellar region and

again comprising a predominantly mononuclear infiltrate. Fig. 1B shows some examples of the typical EAE lesions observed in the C57BL/6 wild-type mice.

Fig. 1C and D summarises the histopathological findings from the experiment shown in Fig. 1A. The overall disease profile correlated well with the EAE score, showing a very similar, monophasic time course with an almost total resolution of lesions. The onset of histopathological change appeared to lag slightly behind the clinical signs, with the peak inflammation score occurring about two days after the peak clinical score. The time courses for brain and spinal inflammation were approximately equivalent.

3.2. Intranasal peptide protects from EAE

Previous studies from our laboratory have shown that protection from EAE can be afforded by intranasal (i.n.) administration of the relevant encephalitogenic epitope (Anderton and Wraith, 1998; Metzler and Wraith, 1993). These studies, however, utilized mice on either the H-2^u or H-2^s backgrounds. Fig. 2 shows that intranasal administration of the MOG 35–55 peptide prior to induction of disease resulted in protection from EAE in the C57BL/6 (H-2^b) mouse.

3.3. Intranasal peptide fails to protect from EAE in IL-10^{-/-} mice

Having established that disease generated by MOG 35–55 could be abrogated by the prior administration of this encephalitogen in soluble form, the C57BL/6 model and the corresponding IL-10^{-/-} congenic strain were used to investigate whether IL-10 played a role in the induction of tolerance. As expected, peptide treatment of wild-type mice provided clear disease protection (Fig. 3). Equivalent peptide treatment of age- and sex-matched, IL-10^{-/-} mice failed, however, to provide any significant protection from disease ($P < 0.001$).

Previous studies of peptide therapy in EAE have focused on clinical score but had not investigated inflammatory infiltration into CNS tissue. As shown in Fig. 1C and D, wild-type mice show an early peak of inflammatory lesions in brain and spinal cord at day 15 of disease. This time point was therefore chosen as the most appropriate to allow comparison of histopathological lesions in control and peptide treated animals. Groups of wild-type and IL-10^{-/-} mice were established as before (Fig. 3) and the experiment was terminated at d15. Fig. 4A shows that peptide treatment protected wild-type mice from EAE, whereas similar treatment of IL-10^{-/-} mice was without effect. Histopathological findings mirrored this clinical assessment. Peptide treatment of wild-type mice led to a significant reduction in inflammation of CNS tissue (Fig. 4B). Identical treatment of IL-10^{-/-} mice, however, failed to confer any protection from inflammation. Representative histopathological sections are presented in Fig. 5.

4. Discussion

The C57BL/6 model has been used extensively for analysis of EAE and immunotherapy of this disease. As yet, however, there has not been a systematic analysis of the correlation between clinical disease and histopathological features. The histopathology associated with EAE has been described at specific time points but the detailed kinetics of lesion development have not been described. For example, Baker et al. (1990) studied cellular infiltration of the CNS during different phases of chronic relapsing EAE in the Biozzi mouse. Here we noted that the cellular infiltrate in the CNS correlated closely with clinical disease. Notably, recovery from disease correlated with resolution of the inflammatory infiltrate. This result confirms previous studies in the Biozzi mouse that showed loss of cellular infiltrate during remission from disease (Baker et al., 1990). The onset of histopathological change found in our study lagged slightly behind the clinical signs, with

the peak inflammation score occurring about two days after the peak clinical score. This is to be expected, as the early stages of EAE pathology are known to be mediated by soluble factors with subsequent infiltration of inflammatory cells in response to the effects of these mediators (Eng et al., 1996).

A number of recent studies have shown ways in which peptides derived from the antigens associated with allergy and autoimmune disease may be used as vaccines to treat these disorders (Larche and Wraith, 2005). Various routes of administration have been evaluated including systemic and mucosal routes (Wraith, 2004). Mucosal routes of administration are favoured since the immune system has evolved to limit inflammation at mucosal surfaces. We have previously shown that the intranasal route is more effective than the oral route for peptide therapy in the H-2^u model of EAE (Metzler and Wraith, 1993). Further studies have emphasised the importance of peptide solubility for tolerance induction (Shen et al., 2003). Intranasal peptides have been shown to be protective in several models of rodent EAE (Anderton and Wraith, 1998; Metzler and Wraith, 1993; Xu et al., 2000). This is the first description, however, of effective protection from EAE in the C57BL/6 mouse using intranasal administration of the MOG 35–55 peptide.

These studies have shown a close correlation between inhibition of cellular infiltration afforded by peptide therapy and the expression of clinical disease. This is notable since immunotherapeutic intervention in other models of autoimmune disease has not shown such a correlation. For example, in the NOD mouse model of type-1 diabetes, treatment with antibodies to cell adhesion molecules will prevent disease without impacting upon peri-islet infiltration (Kommajosyula et al., 2001). Similarly, work presented by Akkaraju et al. (1997) demonstrated that sub-clinical organ inflammation may occur in the absence of overt autoimmune disease, and that this infiltrating cell population may function in disease regulation. This group utilized T-cell receptor (TCR) transgenic mice specific for hen egg lysozyme (HEL) crossed onto various lines of mice expressing HEL systemically or on pancreatic islet beta cells or thyroid epithelium. They demonstrated that expression of HEL within the thyroid and pancreas resulted in the infiltration of these organs with inflammatory cells but no progression to disease and, in addition, showed that varying degrees of T-cell hyporesponsiveness in vitro resulted from the different autoantigen expression patterns. In view of these findings, the simultaneous evaluation of histopathology in addition to clinical disease scores, in the present study of EAE protection, provides important supplementary information. The peptide administration not only protects from disease, it prevents target organ infiltration and does not appear to mediate its effect via the local accumulation of hyporesponsive cells.

Although intranasal peptide therapy has been effectively reproduced in various models of autoimmune disease (Wraith, 2004), the mechanism involved is not clear. Intranasal peptide treatment has led to immune deviation towards the Th2 phenotype in some models (Daniel and Wegmann, 1996; Tian et al., 1996) or the induction of IL-10 secreting regulatory T-cells in others (Maron et al., 1999; Wraith, 2003). Studies from our own laboratory have shown that intranasal peptide treatment can induce bystander suppression whereby administration of a peptide from proteolipid protein inhibits disease directed towards myelin basic protein (Anderton and Wraith, 1998). Intranasal peptide delivery enhanced differentiation of IL-10 secreting cells that would actively transfer suppression (Sundstedt et al., 2003). Furthermore, protection from EAE mediated by peptide therapy resulted from secretion of IL-10 since protection was reversed by neutralising the cytokine in vivo with an anti-IL-10 antibody (Burkhart et al., 1999). Why peptides should induce Th2 cells in one model and IL-10-secreting cells in another is not clear. This may, however, relate to the length of the peptide administered and the genetic background of the recipient (Wraith, 2004).

We chose to study the MOG 35–55 model of EAE since IL-10 deficient mice were available on the H-2^b background. Bettelli et al. (1998) previously provided evidence that IL-10 contributes to peripheral immune regulation in this EAE model. They noted that IL-10 deficient mice were more susceptible to EAE induced with antigen in the absence of pertussis toxin. There was, however, little difference in the incidence or severity of disease induced by antigen and pertussis toxin in wild-type versus IL-10 deficient mice. Similarly, we found no difference in susceptibility to EAE in our wild-type and IL-10 deficient strains although it should be emphasised that our studies included pertussis toxin in the disease induction protocol. There was, however, a striking difference in the ability to induce tolerance by intranasal peptide administration. The IL-10 deficient mice were not rendered tolerant by this treatment, suffering a similar grade and incidence of clinical disease as the controls, along with equivalent levels of CNS inflammation. This demonstrates that IL-10 plays a non-redundant role in the disease protection afforded by peptide therapy.

How might IL-10 influence tolerance in this model? We believe that IL-10 may play a role in both the induction of regulatory cells and their effector function. Groux et al. (1996) first demonstrated that T-cells grown in vitro in the presence of IL-10 would develop a so-called Tr1 phenotype. These Tr1 cells produce IL-10 and some IL-5 and interferon-gamma, with or without TGF-beta but with little or no IL-2 and IL-4 production, and proliferate poorly after polyclonal TCR-mediated activation (Cottrez et al., 2000). In addition, Levings et al. (2001) showed that human naïve CD4⁺T-cells derived from peripheral blood require both IL-10 and IFN-gamma for Tr1 differentiation. The involvement of IL-10 in the in vivo generation of Tr1-like regulatory cells remains less defined although its involvement in their function has been clearly demonstrated. Thus, IL-10 transfected myelin-specific T-cells suppress EAE on transfer into susceptible mice (Mathisen et al., 1997). Furthermore, intranasal peptide treatment of Tg4 transgenic mice induces IL-10 producing CD4 cells. These cells protect against EAE in an IL-10 dependent fashion (Burkhart et al., 1999). Transfer of T-cells from peptide-treated Tg4 mice has shown that the suppression mediated by the cells is an active, IL-10 dependent process (Sundstedt et al., 2003). In view of these findings, it seems highly likely that IL-10 plays a role in both the induction and effector phases of tolerance in the C57BL/6 model of peptide therapy. However, further work is required to dissect the relative role of IL-10 in both the induction of tolerance and the modulation of disease pathogenesis.

Recently, clinical studies of peptide therapy in allergy and autoimmune disease have pointed to a role for IL-10. Administration of peptides from allergens has induced a shift from a Th2 response to one dominated by IL-10 production. This has been achieved in patients suffering from allergy to either bee venom (Akdis et al., 1998) or cat dander (Oldfield et al., 2002) and IL-10 secretion correlated with a marked reduction in allergic symptoms. Furthermore, treatment of patients suffering from rheumatoid arthritis with a heat shock protein peptide (dnaJP1) resulted in a shift from a Th1 response to the peptide to a Th2 response dominated by IL-10 production (Prakken et al., 2004). The results of these clinical investigations demonstrate that the observations from peptide therapy studies in animal models have direct relevance to the use of this approach for immunotherapy in man.

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Fig. 1. Characterisation of MOG 35–55 EAE in wild-type C57BL/6 mice. C57BL/6 mice were injected with 100 µg pMOG_{35–55} emulsified in CFA (day 0). Pertussis toxin (200 ng) was administered intraperitoneally on days 0 and 2. Mice ($n=52$: comprising 21 females and 31 males) were clinically scored daily, as described in Section 2.2. At least 2 females and 3 males were randomly selected and culled for histopathological sampling at ten time points over the 30-day monitoring period (days 6, 9, 12, 14, 16, 18, 20, 23, 26, 30). A) Clinical time course of MOG 35–55 EAE in C57BL/6 mice. The graph represents the mean disease score for mice remaining in the group and error bars depict the standard error of the mean. B) Representative photomicrographs of inflammatory lesions in the brain (i and ii) and spinal cord (iii) of a C57BL/6 wild-type female mouse, following administration of MOG 35–55 in complete Freund's adjuvant and two doses of pertussis toxin (see Section 2.2). (i) Low power view of the cerebellum with several perivascular cuffs identifiable within the white matter. Bar=1 mm. (ii) Hind brain showing focal perivascular inflammation of the meninges with extension into the underlying parenchyma. Bar=200 µm. (iii) Spinal cord with multiple perivascular inflammatory infiltrates throughout the white matter. Bar=500 µm. C) Correlation of clinical disease and inflammation scores in spinal cord. The graph shows the mean disease (open circles) and inflammation (black circles) scores over time with error bars depicting standard error of the mean. The delayed onset of CNS inflammation after clinical disease can be clearly seen in each case.

Fig. 2.

Efficacy of intranasal administration of MOG 35–55 peptide in protecting C57BL/6 mice from EAE. This graph shows mean EAE clinical score against time for soluble peptide-treated and control C57BL/6 mice. Soluble MOG_{35–55} peptide was administered on three occasions (days –7, –5, –3) intranasally (IN MOG) prior to disease induction (day 0) with MOG_{35–55} + CFA (day 0) and Pertussis toxin (days 0 and 2). Control mice (PBS) received only PBS prior to disease induction. EAE was scored daily in individual mice and this graph shows the average score for each group. There are 8 female mice in each group. This graph is one of the three experiments, with $n=8–10$ in each group, all of which demonstrate substantial disease protection in both the IN-treated groups. One of these experiments comprised equal numbers of male and female mice, the other two contained female-only groups.

Fig. 3.

Intranasal administration of MOG 35–55 peptide failed to protect IL-10^{-/-} C57BL/6 mice from EAE. This graph shows disease burden in groups of IL-10^{-/-} and wild-type C57BL/6 mice treated with soluble peptide IN and compared to control mice. A composite of three experiments is presented in this graph. Each experiment comprised two groups of age-matched mice; one group of C57BL/6 wild-type mice and one of C57BL/6 IL-10^{-/-} mice. Each of these groups was divided at random into two further groups. Soluble MOG 35–55 peptide was administered on three occasions (days -7, -5, -3) to one of each of these two sub-groups; IN (grey triangles). The second groups were control mice (black circles) and received only PBS on three occasions. Disease was induced on day 0 as outlined in Section 2.2. EAE was scored daily in individual mice and then the disease burden in EAE Days calculated for each mouse, as outlined in Section 2.2. The number of mice in each group was 17–19. The median clinical score of the IN peptide-treated wild-type group was significantly different to that of both the wild-type control group and IN peptide-treated IL-10^{-/-} group (both comparisons $P<0.001$). Two of the experiments contained only female mice, one contained equal numbers of male and female mice.

Fig. 4.

EAE clinical score against time for groups of IL-10^{-/-} and wild-type C57BL/6 mice treated with soluble peptide IN and compared to control mice. This experiment comprised two main groups of age-matched female mice (C57BL/6 wild-type mice (open circles) and C57BL/6 IL-10^{-/-} mice (solid circles)). Each group was divided at random into two further sub-groups. Soluble MOG 35–55 peptide was administered IN (grey) on three occasions (days -7, -5, -3) to one group of each mouse background. The other groups were control mice (black) and received only PBS on three occasions. Disease was induced and EAE scored daily in individual mice for 14 days and then samples for histopathology were collected, as outlined in Sections 2.2 and 2.3. The number of mice in each group was 9 in each wild-type group, 8 in the control knockout group and 7 in the IN-treated knockout group. A) shows the mean disease score for each group against time. The graph demonstrates that although clear disease protection is provided by IN peptide in the wild-type mice, this effect is lost in the IL-10 knockout mice. B) shows a graphical summary of the histopathological findings from this experiment. The columns show the average pathology score for each group calculated from brain and spinal cord results. Peptide-treated mice are shown in black whereas the PBS-treated, control mice are shown as hatched columns. The error bars indicate standard error of the mean. The medians of the groups were found to be significantly different ($P=0.0003$). The post-test analysis showed that the wild-type control and peptide-treated groups were significantly different ($P<0.05$), as were the two peptide-treated groups ($P<0.01$). The two control groups and IL-10^{-/-} groups were not significantly different ($P>0.05$).

Fig. 5.

Representative photomicrographs of brains (A, B, D and E) and spinal cords (C and F) of intranasal peptide-treated C57BL/6 wild-type (A, B and C) and IL-10^{-/-} (D, E and F) mice when primed with MOG 35–55 in complete Freund's adjuvant and given two doses of pertussis toxin (see Section 2.2). Samples were processed as outlined in Section 2.4 and stained with haematoxylin and eosin. A) Low power view of the cerebellum of a peptide-treated wild-type mouse with no inflammatory infiltrates. Bar=1 mm. B) Higher magnification of section (A) showing a blood vessel with no associated inflammatory cells. Bar=200 μ m. C) Low power section of spinal cord of a peptide-treated wild-type mouse with no inflammatory infiltration of either white or grey matter. Bar=200 μ m. D) Cerebellum of a peptide-treated IL-10^{-/-} mouse showing multiple perivascular cuffs within the white matter. Bar=500 μ m. E) Higher magnification of section (D) demonstrating two mononuclear perivascular cuffs with extension of the inflammatory infiltrate into the surrounding parenchyma. Bar=100 μ m. F) Low power view of spinal cord from a peptide-treated IL-10^{-/-} mouse. Multiple perivascular foci of inflammatory cells can be clearly identified at different levels within the white matter. Bar=500 μ m.

Table 1

Grade	Symptoms
0.5	Paretic tail
1.0	Flaccid tail
1.5	Slow righting reflex
2.0	Absent righting reflex
2.5	Absent righting reflex, pelvic limb paresis
2.75	Absent righting reflex, marked pelvic limb paresis
3.0	Pelvic limb paralysis
3.5	Pelvic limb paralysis, thoracic limb paresis
4.0	Pelvic limb paralysis with paralysis of one or both hind and thoracic limbs
5.0	Moribund or dead

EAE Disease Burden: The cumulative total EAE score for an individual mouse within the experimental period. Units: EAE Days. This score is equivalent to the area under the curve of an EAE score against time graph for an individual mouse and hence provides an overall measure of disease severity over time in each mouse.