Unsuccessful reproduction of Experimental Autoimmune Neuritis (EAN) in C57BL/6J mice using published protocol and variations

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Abstract:
This study aimed to establish an experimental model of Guillain-Barré Syndrome (GBS), a peripheral inflammatory neuropathy in humans, using the Experimental Autoimmune Neuritis (EAN) model in C57BL/6 mice. This approach was informed by a comprehensive bibliographic analysis. While this experimental model has been developed successfully in rabbits, rats, and the SJL mouse strain, the literature on its application in C57BL/6 mice is limited. Our protocol analysis encompassed twenty-one articles utilizing the P0180-199 neurogenic peptide to induce EAN in this genetic background. Based on this literature, we selected one protocol for replication and made three adaptations.
Despite using high-quality $P0_{180-199}$ peptide, we were unable to reproduce the selected EAN protocol or induce any pathological signs, even under optimized conditions.
As a control to validate our reagents and methodology, we replicated a different model of inflammatory neuropathy targeting the central nervous system: the well-established Experimental Autoimmune Encephalitis (EAE) protocol. This replication was successful using the same C57BL/6 mouse genetic background and reagents, with the sole exception of the neurogenic peptide employed (MOG_{35-55}).

Categories:

- **Nature:** negative result, repetition/replication
- **Model:** in vivo
- **Specie:** mouse
- **Data type:** methods

Keywords: neuroinflammation, autoimmunity, demyelination

Introduction:

Animal models replicating human symptoms are pivotal for the study of autoimmune neuroinflammatory disorders like Multiple Sclerosis (MS) and Guillain-Barré Syndrome (GBS) (1,2). These models have been developed using species-specific antigens (protein extracts or peptides) analogous to human auto-antigens. Disease is induced through immunization with these antigens, combined with Complete Freund's Adjuvant (CFA) to boost the immune response. Additionally, pertussis toxin (PTx) is administered to facilitate immune access to endogenous antigens by disrupting blood-brain or blood-nerve barriers.

MS is characterized by chronic inflammation, in which the immune system attacks the central nervous system's oligodendrocytes, particularly targeting Myelin Oligodendrocyte Glycoprotein (MOG). This leads to demyelination, inflammation, gliosis, and neuronal damage, with a relapsing-remitting or progressive course. MS-related mortality often results from severe disability complications rather than direct muscle control loss. The Experimental Autoimmune Encephalomyelitis (EAE) model, widely used in MS research, mirrors these MS features. Employing MOG peptides and PTx, it induces central nervous system demyelination and is supported by extensive literature in C57BL/6 mice.

GBS manifests as acute inflammation, where the immune system targets Schwann cells in the peripheral nervous system, particularly focusing myelin proteins such as Myelin Protein Zero (P0). This results in rapid demyelination, inflammation, and axonal damage, with a typically acute monophasic course. This lead to loss of muscular control, paralysis, respiratory dysfunction, and in severe cases to death. Post-acute phase, remission occurs without recurrence. Experimental Autoimmune Neuritis (EAN) is a well-documented model for GBS in rabbits, rats, and the SJL mouse strain. In these models, Myelin Protein Zero (P0), is injected for inducing autoimmune peripheral demyelination. However, published protocols of EAN in the C57BL/6J mouse strain are scarce and heterogeneous despite its high relevance: the availability of numerous genetically modified mice in this genetic background make it a tool of choice to investigate GBS disease and its pathogenic mechanisms *in vivo*.

In this study, we aimed to set up a reproducible EAN model in C57BL/6 strain. The EAE protocol, which is in practice in our laboratory (3), was employed as an experimental control since all reagents are identical, with the exception of the specific peptides used. This ensured the adequacy of the components used for immunization and of the administration protocols. We assessed clinical symptoms of neurological impairment and muscular function disruptions, including the "flaccid tail" a robust and easily-observable marker of disease onset. Herein, we detail our unsuccessful efforts to implement the EAN model in the C57BL/6J genetic background by replicating a published protocol despite our optimization approaches.

Results and conclusion:

We conducted a review of articles detailing the EAN protocol and found only twenty-one studies (see supplemental Table S1) that have enabled the induction of EAN in the resistant C57BL/6 mouse strain (4-14,16-24) using myelin $P0_{180\text{-}199}$ peptide (4-24). One laboratory accounted for more than half publications. They made several changes over time in the immunization protocol, PTx administration, or C57BL/10 genetic background used (4-16).

The protocol outlined by Meyer zu Hörste G. and colleagues (22) beared similarities to standard EAE protocol with no immunization boost at D8. However, it utilized a doubled PTx dose (500ng) while maintaining the timing (D0 and D2) and similar intraperitoneal (IP) route of administration. Furthermore, the immunization employed $P0_{180\text{-}199}$ peptide doses (200µg) comparable to the MOG peptide dose that we used in the EAE protocol (3). A second protocol (15) was also similar to EAE protocol but with a boost at D8 and PTx at D-1 (400ng) and D1 (300ng) IV. We decided to use these two protocols as references to set up our EAN trials (15,22).

Across studies (4-24), P0 peptide doses ranged from 50 µg to 200 µg per injection at D0 up to D8, and it had been noted that, like in EAE, higher doses of pertussis toxin led to a faster onset and increased severity of EAN clinical symptoms (17). The PTx timing also varied across different publications, including D-1/0/1/3 (4), D-1/0/3 (5,6,20,24), D-1/0/2 (7,8,9,10), D-1/1/3 (11,12,13,14,16,17,18,19,21), D-1/1 (15), D0/2 (22), and D-1/1/2 (23).

We implemented what appeared to be the most promising formulation, consisting of the highest dose of peptide (200 µg), the highest dose of HKMtb adjuvant in CFA (500 µg in a 50 µl immunization mixture), and the highest dose of PTx (500 ng/IP injection). We assumed that this approach would consistently elicit a robust, rapid, and efficient EAN response and that the high PTx dose would allow a prolonged action and superior Blood-Nerve disruption, rendering the choice of the day of administration less important.

We set up the original EAN protocol as described by Meyer zu Hörste G. and colleagues (22, EAN1) and three additional variations in PTx administration timings (EAN2, EAN3, EAN4) which we compared to the efficient and robust EAE protocol used routinely in our laboratory (depicted in Figure 1A). The groups were as follows:

- EAN1 and EAN2 groups: PTx on D-1 and D2, with immunization boost at D8 for the EAN2 group.
- EAN3 and EAN4 groups: PTx on D0 and D2, with immunization boost at D8 for the EAN4 group.
- EAE group: PTx on D0 and D2, with immunization at D0 (and no boost).

We used the EAE group as a 'positive control' for evaluating the EAN protocol. To minimize animal use, we did not include a sham-immunized (no peptide) group in the parallel EAN and EAE experiment (as shown in Figure 1C and 1D). However, this sham control was included in an independant, separate EAE experiment using the same batch of reagents (Figure 1E and 1F). In line with what was already reported, weight loss in an EAE group began at day 11 and was statistically significant at day 12 as compared to the sham control (Figure 1E), while general clinical scores were observed at day 11 and became significant at day 12 (Figure 1F). The general clinical score is the better current quantitative "tool" utilized by researchers in the field to determine disease progression (4-24). It is determined by assessing the progressive appearance of several phenotypes, such as partial tail paralysis, paralysis of one limb, etc. (Figure 1B).

In our experiment, none of the four EAN groups exhibited weight loss (Figure 1C) or any neurological symptoms (Figure 1D). There were no statistical differences in weight variations and clinical scores between the different EAN groups (Figure 1C and 1D). Within the same experiment, as expected, the EAE group began to lose weight at day 10, and this became statistically significant at day 12 (Figure 1C) and to develop clinical signs at day 11(Figure 1D).

In conclusion, we were unable to replicate the selected EAN protocol (22) and to induce any neurological symptom, despite using highly potent induction conditions. As a result, we discontinued further attempts to establish this model in our laboratory.

Currently, we cannot pinpoint the reasons that might explain this lack of success in our attempt to reproduce EAN protocol. The use of high adjuvant dosage and/or PTx dose and the administration timing should have triggered neurological symptoms - even temporary or mild - during the time frame of the experiment. We also verified the quality of the neurogenic peptide, which must be extremely high (>98% purity). A mass spectrometry expert reviewed the company's QC report of the peptide we used, ensuring that its quality met the required standards (see materials & methods; Supplemental data S2-S5).

It is possible that subtle differences in reagent quality, protocol, environmental conditions, or stress levels during injections could affect the EAN induction in C57BL/6 mice, a strain described as EAN-resistant by several authors (2,17-19). The use of anesthesia during injections, wich can reduce stress in animals, might also play a role but is only reported in four articles (17-19,23). A notable difference between the twenty-one analyzed protocols was the route of administration of PTx. In fifteen articles (4-16,21), PTx was administered intravenously (IV), while in our protocols and in six other articles, it was administered intraperitoneally (IP), as in the standard EAE protocol and Jove publication (18). We based our choice of IP administration on a study of Gonzalves D. and colleagues (17) that indicated no difference in induction between these two routes of administration. In addition, maybe IP administration of PTx only twice was insufficient to produce a sufficient long Blood Nerve Barrier disruption, as eight-

teen out of the twenty-one studies used 3 PTx injections.
Another potential reason for the EAN protocol's failure was the source of the animals. Genetic variations between suppliers for the same genetic background could explain the resistance to EAN. A significant difference between suppliers is the specific composition of the gut microbiota. Could this microbiota influence the animal's response to the EAN protocol and account for the resistance? This possibility should not be dismissed, as Guillain-Barré Syndrome (GBS) in humans is often associated with dysbiosis that frequently involves intestinal pathogens such as Campylobacter jejuni (25).

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- Writing – original draft: Marc Le Bert
- Writing – review & editing: Marc Le Bert, Nicolas Riteau

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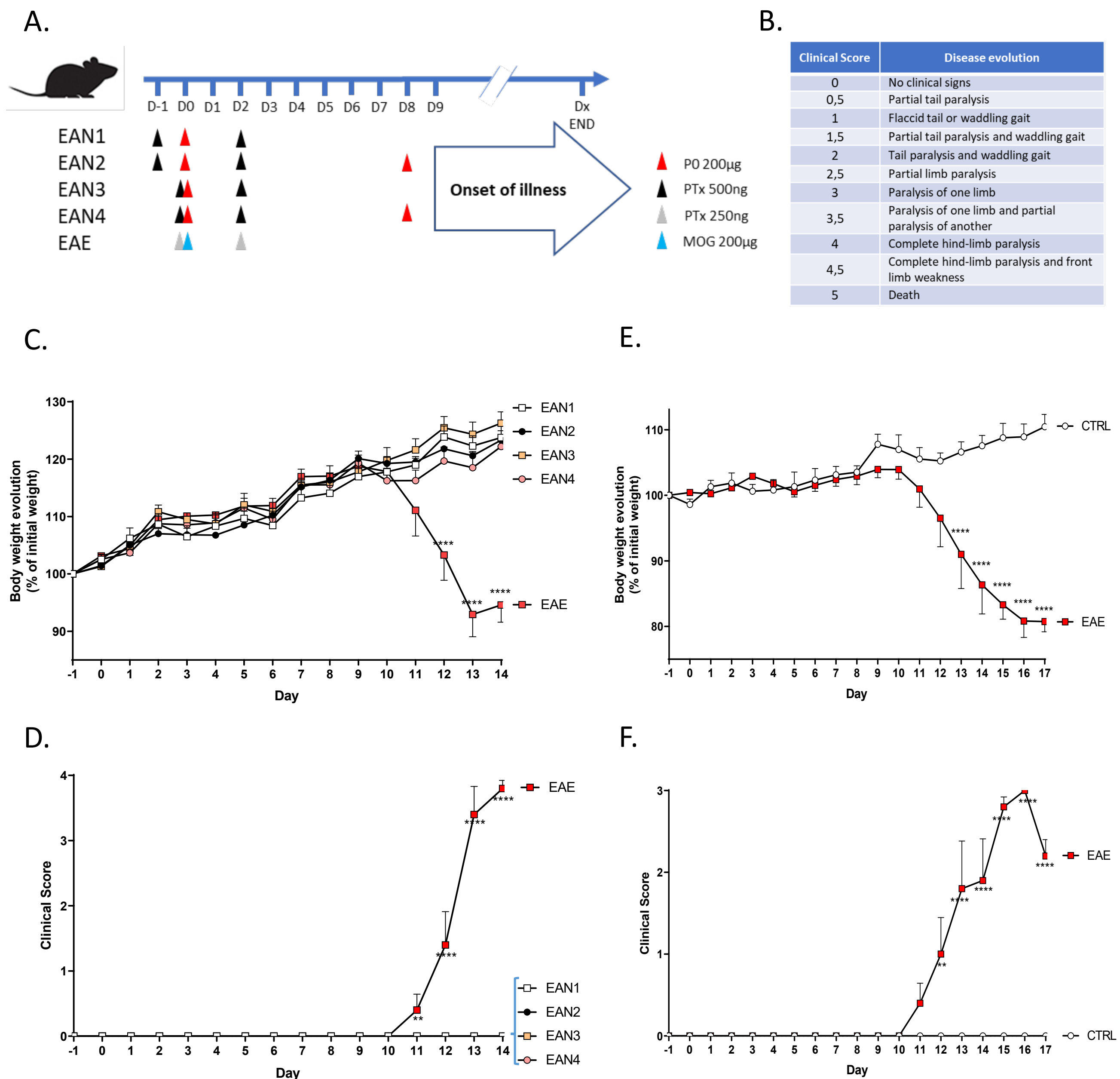

Clinical Score	Disease evolution
0	No clinical signs
0,5	Partial tail paralysis
1	Flaccid tail or waddling gait
1,5	Partial tail paralysis and waddling gait
2	Tail paralysis and waddling gait
2,5	Partial limb paralysis
3	Paralysis of one limb
3,5	Paralysis of one limb and partial paralysis of another
4	Complete hind-limb paralysis
4,5	Complete hind-limb paralysis and front limb weakness
5	Death

Figure 1

(A) Experimental onset: Male C57BL/6J mice were used for various immunization protocols to induce EAE or EAN. Day0 refer to the initial immunization injection with peptide P0180-199 (EAN), peptide MOG35-55 (EAE), or no peptide in the sham-immunization mixture (CTRL). EAN1-4 refer to different EAN protocol setups tested. Administration timing and doses variations of Pertussis toxin (PTx) are indicated. Variations in the immunization mixture are also shown. Weights and clinical scores are daily recorded.
(B) EAE/EAN Clinical score table used for disease onset scoring.
(C) Body Weight changes of animal groups during the time of the experiment.
(D) Clinical scores of the experiment.
(E) Typical EAE results: Body Weight changes of animal groups during a typical EAE experiment using 8-week-old C57BL/6J males.
(F) Typical EAE results: Clinical scores of the EAE group versus the sham-immunized (no peptide) control group.
(C-D) Experiment group composition: five groups of 5-week-old males were used ; four different EAN groups (EAN1, EAN2, EAN3, EAN4) and one EAE group as a "positive" control. In panel D, all four EAN curves (EAN1-EAN4) overlap perfectly and are represented by the first EAN1 visible curve. A brace groups these symbols to show their superposition.
(E-F) Typical EAE experiment: 8-week-old C57BL/6J males are used. 5 mice in the EAE group versus a 4-mice no peptide (sham-immunized) control group.
(C-F) Data are presented as group mean. Standard error of the mean (SEM) for each data point is plotted. For the experiment (C-D), n=5 animals were used in each group (EAE, EAN1-4). For the typical experiment (E-F), n=4 for the CTRL group and n=5 for EAE group. **$p < 0.01$ compared to all other groups; **** $p < 0.0001$ compared to all other groups. Statistical analysis was done using GraphPad Prism: data was analyzed with non parametric two-way analysis of variance (ANOVA) test adapted for small effectifs. The mice were euthanized on day14 (C-D) due to the lack of symptoms in the EAN groups or killed at day17 (E-F) for further analysis.

Abbreviations: EAE, experimental autoimmune encephalomyelitis; EAN experimental autoimmune neuritis; MOG, myelin oligodendrocyte glycoprotein; P0, myelin protein zero; CTRL, control; PTx, pertussis toxin.

Materials & methods:

Animals :

C57BL/6J mice were purchased from Janvier Labs (France) and maintained at the Joint Animal Facilities, TAAM UPS44 under French Home Office guidelines. All experiments were performed under the guidelines of the University of Orleans Research Ethics Committee. The EAN and EAE experimental models in mice were approved and followed the French government's ethical and animal experiment regulations (APAFiS 2020 #28852).

Groups of five (n=5) sex-matched male mice at the age of 6-week-old were used in the reported experiment and 8-week-old male mice for the typical EAE experiment (EAE versus sham-immunized mice).

Parts of the "Demande d'Autorisation de Projet » (DAP) :

In France, authorization to experiment with animals for scientific purposes is centralized and delivered by the MESR (Ministere de l'enseignement superieur et de la recherche) to users such as researchers, laboratories, and companies. This is done via online submission of the form using the APAFiS platform. The process includes peer-reviewed approval by an ethical committee that evaluates the protocol, the number of animals used, statistical analyses, and the severity of the procedures. Our study received authorization under APAFiS 2020 #28852, while the protocols themselves were classified as severe due to the experimental procedures and interventions outlined. The criteria for assessing animal welfare and delimiting the humane endpoint included a 20% weight loss from the initial weight and an evaluation of animal suffering and condition based on day-to-day observations.

Immunogenic peptides:

EAN Peptide immunogen: we requested a custom bulk synthesis of the EAN peptide $P0_{180-199}$, (sequence: SSKRGRQTPVLYAMLDHSRS) from ProteoGenix (France) with a purity greater than 98%, HPLC purified and MS QC verified, and certificate of analysis was delivered by the company (supplemental data S2). After the first EAN protocols failure, we submitted the QC control of the provider to an LC-MS expert of the MO2VING-MS facility. Complementary analysis were conducted at the MO2VING-MS facility to analyze the P0 peptide (supplemental data S3a,b).

A second commercial P0 peptide was obtained from MCE UK, reference: HY-P2476-10mg, >95% purity. Its purity was checked by the MO2VING-MS facility (supplemental data S4).

The translated versions of S2, S3a and S3b are available in S5 supplemental data file.

EAE Peptide immunogen: MOG_{35-55}, (sequence: MEVGWYRSPFSRVVHLYRNGK) produced by ProteoGenix through custom synthesis with >98% purity, HPLC purified and MS QC verified and analysis certificate provided. The report was checked at MO2VING-MS facility expert and it was considered as conform.

P0 Peptides Quality controls at MS Facility:

The quality control report provided by the company (supdata2) indicated that the HPLC profile under standardized conditions displayed a single peak, suggesting a purity of >98% with no contaminant. However, the provided mass spectrometry revealed the presence of a contaminating peptide with mass-to-charge ratios of m/z 1081.5 (2+), 721.35 (3+), and 541.3 (4+). It is important to note that the MS is not quantitative, and peak size is not always representative of quantity.

After a previous failure of EAN induction, the peptide was suspected, and further analysis was conducted at the MO2VING-MS facility (supdata3). It was also found by MS that there is indeed a secondary contaminating peptide. MS sequencing permitted the determination of the sequences of the two peptides. The expected peptide (sequence SSKRGRQTPVLYAMLDHSRS) was present at the correct size (m/z 2289). The analysis of the fragmentation spectrum of the second peptide (at m/z 2161), whose mass suggested the loss of a lysine (K182) or a glutamine (Q186), allowed the determination of its mass, which corresponded to the peptide missing the glutamine at position 186 (SSKRGR_TPVLYAMLDHSRS). The LC-UV-MS analysis (supdata3b) showed that the sample was contaminated with 30% of the second peptide lacking glutamine 186 (SSKRGR_TPVLYAMLDHSRS),

which may explain the failure of the immunization and EAN induction in our first attempts.

Bordetella Pertussis toxin:
Pertussis toxin (PTx) glycerol stocks (ref PT-TNG-200, Native Antigen, UK) were purchased directly from the producer and stored at -20°C until use. The exact quantities were calculated, and the volume required diluted in NaCl 0,9% just before use.

Immunizations:
To prevent skin ulceration at the injection sites, C57BL/6 mice were immunized subcutaneously on the back hind with 50µl of emulsion mixture as follow:
EAE Groups (Fig1A, C-F EAE): Groups of 5 mice were immunized only once with 200 µg of $MOG_{35\text{-}55}$ peptide (>98% purity, ProteoGenix, France; QC report checked at MS facility) in 25 µL of PBS, which was emulsified with an equal volume of CFA containing a total of 250 µg of heat killed Mycobacterium tuberculosis (strain H37RA, Difco, USA).
The sham-immunized control (Fig1E-F CTRL): Group of 4 mice was submitted to the same protocol but peptide is omitted in the immunization mixture.
In addition, each mouse received an intraperitoneal injection of 250 ng of freshly diluted PTx in 200 µL of 0.9% NaCl solution at two time points: on day 0, two hours post-immunization, and on day 2, 48 hours after the first PTx injection.

EAN Groups (Fig1A,C-D EAN1-4): Groups of 5 mice were immunized once (day 0, EAN1,3) or twice (days 0 and boost at day 8, EAN2,4) by subcutaneous injection of 200µg of $P0_{180\text{-}199}$ peptide (>98% purity, MCE, UK; QC report checked and supplemental analysis assessed by MS facility) in 25 µL saline, which was emulsified with an equal volume of CFA containing a total of 500 µg of heat killed Mycobacterium tuberculosis (strain H37RA, Difco, USA).
In addition, each mouse received an intraperitoneal injection of 500 ng freshly diluted PTx in 200 µL saline at two time points: on day 0, two hours post-immunization, and on day 2, 48 hours after the first immunization injection (EAN3,4) or on day -1, 24h before immunization and on day 2 48h post immunization (EAN1,2).

Animal wellness, weight recording and clinical scoring:
The animals' well-being was assessed and documented daily. Their weights and clinical scores were recorded each morning. Animals were scored according to the criteria outlined table in Fig1B, using a 0–5 scale as follows: 0, no clinical sign; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb involvement; 5, moribund/humane end point euthanasia.
The raw data for weight and clinical scoring can be found in Supplementary data S6.

Statistical analysis
Experiment used 5 mice in each group and data were expressed as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to compare the clinical score and weight of the groups. For all statistical analyses, the level of significance was set at $p < 0.05$.

Supporting information and files:
S1 supplemental table: Bibliographic analysis review table of EAN protocols articles 4-24
S2 supplemental data: P0 180-199 EAN neurogenic peptide - ProteoGenix QC analysis report
S3a supplemental data: P0 180-199 EAN neurogenic peptide - MO2VING-MS facility - French MS report
S3b supplemental data: P0 180-199 EAN neurogenic peptide - MO2VING-MS facility – French LC-UV-MS QC analysis report
S4 supplemental data: P0 MCE MS LC-UV-MS QC analysis report - MO2VING-MS facility French
S5 supplemental data: English versions of S2a, S2b and S3
S6 supplemental data: Raw data for weights and clinical scores