



## Possible therapeutic vaccines for canine myasthenia gravis: Implications for the human disease and associated fatigue <sup>☆</sup>

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### Abstract

Myasthenia gravis (MG) is caused by T cell-dependent antibodies reactive with acetylcholine receptors. These autoreactive antibodies cause muscle weakness by interfering with neuromuscular transmission via removal of acetylcholine receptors from the neuromuscular junction as well as changing the architecture of the junction itself. Consequently, muscle fatigue is a debilitating aspect of MG often leading to more general feelings of tiredness not directly due to muscle weakness. We have previously described two peptides that are mimetics of antigen receptors on certain autoreactive T and B cells that are involved in MG. When used as vaccines in the rat model of MG, these peptides prevented and ameliorated disease and muscle fatigue by blunting acetylcholine receptor antibody responses. Such disease protection resulted from vaccine-induced anergizing antibodies against acetylcholine receptor-specific T and B cell antigen receptors. The present study prospectively evaluated the efficacy of these two vaccines in spontaneous acquired MG in pet dogs. When compared to historical controls that were prospectively studied, the vaccines increased the proportion of remitted dogs from 17 to 75%. In comparison to retrospectively studied historical controls that spontaneously remitted from MG, the vaccines accelerated the rate of decline in acetylcholine receptor antibody titers which resulted in a 3-fold decrease in the mean time to remission. These results are suggestive of a new type of targeted therapy that can drive autoimmune responses into long-term remission and possibly afford a means of determining whether correction of a physical cause of muscle weakness also corrects the perception of chronic, generalized fatigue.

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### 1. Introduction

In general medical practice, fatigue is one of the most frequent patient complaints (Ruffin and Cohen, 1994). In contrast to healthy individuals who report experiencing fatigue after prolonged exercise (McConnell et al., 1999), many neuroimmune disease patients describe fatigue as

occurring often and being a debilitating aspect of their illness (Barroso, 1999; Krupp and Pollina, 1996; McComas et al., 1995). Fatigue is multifactorial with altered aspects of both physical and mental energy. Relationships between these two components of fatigue are not completely understood and are a challenging area of behavioral study. As a consequence of the frequent association of fatigue with diseases of neuroimmune origin, such as multiple sclerosis (MS) and myasthenia gravis (MG), these disorders represent fertile areas to study how aberrant interactions between the nervous and immune system impact behavior.

In the autoimmune disease myasthenia gravis (MG) and its model, experimental autoimmune (EA) MG, autoreactive

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(auto) antibodies (Ab) are frequently directed against residues  $\alpha 61-76$  (termed the main immunogenic region, MIR) of the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. The production of such autoAb in the Lewis rat is helped by T cells specific to residues  $\alpha 100-116$  of the AChR (for review, see Weathington and Blalock, 2003). These autoAb cause muscle weakness by interfering with neuromuscular transmission via removal of AChR from the neuromuscular junction as well as changing the architecture of the junction itself. This decreased muscle response in MG manifests itself as fatigue. However, in one study, almost nine in 10 MG patients reported “fatigue at night” (Ochs et al., 1998). Thus, it appeared that MG patients may not differentiate muscle fatigue from a general feeling of physical tiredness. Indeed, Paul et al. (2000) reported that “cognitive fatigue is an important symptom of MG and that fatigue produces pervasive impairments in important aspects of patients’ lives.” In the present study, we are evaluating therapeutic vaccines that may correct the neuromuscular deficit in MG and consequently may abrogate fatigue. If successful, this might well establish an important paradigm to determine whether restoration of normal neuroimmune interactions will lead to beneficial behavioral changes in terms of not only physical fatigue but also the perception of fatigue.

The aforementioned therapeutic vaccines were developed with a previously described algorithm for the design of peptides that apparently assume complementary contours to proteinaceous autoantigenic determinants (for review, see Villain et al., 2000; Weathington and Blalock, 2003). These peptides, termed complementary peptides, can bind the target determinant and in a sense behave as antigen (Ag) receptor mimetics (ARM). Consequently, when used as vaccines they elicit anti-idiotypic (Id) and anti-clonotypic Ab responses against the combining sites of Ag receptors (i.e., T cell receptor, TCR, and B cell receptor or Ab) on certain autoreactive T and B lymphocytes (for review, see Villain et al., 2000; Weathington and Blalock, 2003). By targeting ARM vaccines to the aforementioned AChR T or B cell epitope (termed T and B cell vaccines, respectively), Ab were induced that specifically recognized and bound Ag receptors on AChR  $\alpha 100-116$  reactive T cells and AChR  $\alpha 61-76$  reactive B cells, respectively. The resulting anti-ARM Ab/Ag receptor interactions interfered with autoreactive T cell help, anergized autoreactive B cells and consequently dramatically reduced the levels of AChR reactive autoAb. This in turn led to marked clinical improvement in terms of reduced muscle fatigue, lowered mortality and preservation of AChR levels on muscles (Araga et al., 1993, 1996, 2000; McAnally et al., 2001; Xu et al., 2000).

The purpose of the present study was to evaluate the aforementioned T and B cell vaccines for the ability to diminish AChR Ab levels as compared to historical controls in a clinical trial in spontaneous acquired autoimmune MG in pet dogs. A control group of myasthenic pet dogs immunized with carrier protein (without ARM peptides) in

adjuvant was not included at this time because of the probable lack of benefit based on rat EAMG studies (Araga et al., 1993, 1996, 2000; McAnally et al., 2001; Xu et al., 2000) and, more importantly, reports that routine vaccination can precipitate a recurrence of MG in dogs that have remitted (Garlepp et al., 1984; Shelton and Lindstrom, 2001). Thus ethical considerations precluded a placebo control in this initial study. These possible carrier protein and adjuvant effects, however, were controlled by the study design (see Section 3). In addition to potential vaccine benefits for pets, canine MG would seem to have considerable prognostic value for eventual use of these vaccines in human MG since the two conditions are very similar. The similarities include natural occurrence of the disorder, shared environments between humans and dogs, similar clinical presentations, diagnostic AChR autoAb of related epitope specificity (including the MIR), co-morbidity of canine MG with other autoimmune disease (Shelton et al., 1988). If the vaccines were eventually proven beneficial in humans, they would provide a means to test whether the correction of the physical neuromuscular problem would also correct the perception of chronic, debilitating fatigue.

## 2. Methods

### 2.1. Myasthenic dogs

Twenty-nine dogs diagnosed with autoimmune MG with positive AChR Ab titers ( $>0.6$  nM) by an established clinical radioimmunoassay were initially enrolled in the study (Shelton et al., 1988). All standardized clinical AChR Ab assays for the present study as well as historical controls were performed by the same veterinary service (UC San Diego, Comparative Neuromuscular Laboratory). Five animals (17%) died of aspiration pneumonia and one died acutely following surgery for a thymoma prior to complete vaccination. The death rate in the present study is equivalent to the 6/35 (17%) and 2/12 (17%) observed by Shelton et al. (1990) and Lennon et al. (1981), respectively. Three animals were lost to follow-up and four were dropped from the study for noncompliance. One dog was withdrawn from the study by the owner before complete vaccination because of a skin reaction at the site of immunization. Five animals spontaneously remitted after diagnosis but before the initiation of vaccination. Thus, 10 dogs (five males and five females, 1–10 years old) completed the study and none of these animals had thymoma or were on corticosteroids or other immunosuppressants. These dogs represented two outbred animals and eight different breeds, half of which overlapped with those listed in the prospective historical control group (Shelton et al., 1990). The time from onset of clinical symptoms to confirmed diagnosis with AChR Ab assay for these animals was 2–4 weeks. Prominent clinical findings include neuromuscular weakness, exacerbated by exercise and improved with anticholinesterase drugs; difficulty in swallowing, choking, and regurgitation. Remission refers to the long-term return of the AChR Ab titer to the normal range ( $<0.6$  nM) and to the dog as clinically normal in the absence of acetylcholinesterase inhibitors. Transient remission refers to one or more measurements of AChR Ab titers below 0.6 nM prior to complete resolution of MG and long-term return of AChR Ab to normal values. Pet owners were provided with informed consent and the study was approved by the Institutional Animal Care and Use Committee of the University of Florida.

### 2.2. Historical controls

To date, there have been two reports on the natural course of canine MG that included relatively large numbers of dogs and long-term follow-

up. Thus, data sets on the natural course of canine MG are available for comparison to this initial trial. In a prospective study, Shelton et al. (1990) found that six of 35 dogs (17%) spontaneously remitted with AChR Ab levels returning to the normal range (<0.6 nM, upper confidence interval for normal dogs (Shelton et al., 1988)). In a retrospective study of 47 dogs that spontaneously remitted from canine MG, Shelton and Lindstrom reported the time course of AChR Ab levels and found an average time to remission of 6.4 months post diagnosis (range 1–18 months) (Shelton and Lindstrom, 2001). Parenthetically in this later study, it was suggested that the spontaneous remission rate from canine MG was 88.7% (47 of 53 dogs). Since this was a retrospective study and it was never clearly stated whether the outcome (i.e., remission) was known in advance for any or all of the cohort, it is difficult to draw a conclusion about remission rates. Also, since only dogs with very long follow-ups were included (i.e., dogs that survived MG long-term), the selection criteria were biased toward spontaneous remission. A final confounding factor is that such a high remission rate is at odds with the earlier prospective study by Shelton et al. (1990) where the spontaneous remission rate was 17% which is consistent with the 25% herein reported (see below). In any event, as a consequence of these cohorts of historical controls, we were able to evaluate two parameters of disease. First, vaccine efficacy was assessed for an increased remission rate compared to that of the prospectively studied historical controls. Second, vaccine efficacy was evaluated for an accelerated time to remission relative to dogs that spontaneously remit.

### 2.3. AChR Ab assays

AChR Ab assays are standardized, fee-based clinical assays performed by a veterinary service (UC San Diego, Comparative Neuromuscular Laboratory). The previously described procedure is an immunoprecipitation radioimmunoassay that employs purified canine AChR and <sup>125</sup>I-labeled  $\alpha$ -bungarotoxin (Shelton et al., 1988). Titers are expressed as moles of <sup>125</sup>I- $\alpha$ -bungarotoxin binding sites per liter of serum. Normal values were previously determined from the sera of normal dogs of various ages, breeds, and sexes and were  $0.144 \pm 0.438$  nM (means  $\pm$  3SD). As a consequence, the upper limit of normal was set at 0.6 nM (for further details of assay, see Shelton et al., 1988).

### 2.4. Vaccines

The T and B cell peptide vaccine sequences are NH<sub>2</sub>-Tyr-Phe-Ser-Arg-Ile-Ile-Gln-Lys-Gln-Phe-Gly-His-Val-Asn-Asn-Gly-Lys-COOH and NH<sub>2</sub>-Phe-Asn-Ser-Thr-Ile-Ile-Gly-Trp (2,4,6-trimethoxybenzyl)-Ile-Pro-Ala-Lys-Pro-His-Ile-Asn-COOH. They were synthesized and coupled to keyhole limpet hemacyanin (KLH) as a carrier protein as previously described (Araga et al., 1993, 1996, 2000; Xu et al., 2000). The B cell peptide vaccine had a 2,4,6-trimethoxybenzyl group added to the tryptophan in position 8 (Shah et al., 1992). A single dose of vaccine consisted of 500  $\mu$ g in 0.5 ml phosphate-buffered saline of each peptide—KLH conjugate emulsified in 0.5 ml of TiterMax<sup>®</sup> adjuvant (TiterMax USA, Inc., Norcross, GA). The vaccines were administered subcutaneously at a minimum of four sites. The two vaccines were administered either sequentially beginning with the T followed by the B vaccine or simultaneously in a single dose at 2 week intervals. The experimental design was to administer five doses of the combined vaccines which would equate to 10 vaccination events if they were administered sequentially as opposed to simultaneously in a single dose. Depending on the animal, remission was achieved with as few as two vaccination events and at most 10 sequential vaccination events with the two vaccines (one dog). Vaccinations were initiated at the time of confirmed diagnosis for three dogs and 2 weeks, 2 months or 4 months post diagnosis for four, two, and one dog(s), respectively.

### 2.5. Statistical analysis

All statistical analyses were performed using InStat biostatistics software (GraphPad software, San Diego, CA). For comparison of serum Ab

values and times to remission between groups, unpaired *t*-tests were used except when the limitations of the dataset indicated the use of the unpaired *t*-test with Welch correction or the Mann–Whitney test; the null hypothesis was that vaccination did not decrease AChR Ab titers or time to remission. Paired *t*-tests were used for the analysis of serum Ab titers before and after vaccination. For endpoint evaluations such as remission statistics, contingency table analysis was performed with Fisher's exact test.

## 3. Results

### 3.1. Prospective outcomes

The prospectively studied historical control group consisted of 35 myasthenic dogs of both sexes who were followed long-term and had a focal form of MG with selective involvement of esophageal, pharyngeal and facial muscles. These animals were identified as AChR Ab positive in a cohort of 152 dogs with idiopathic megaesophagus. It has been suggested (Shelton et al., 1990) that “megaesophagus in dogs, like ocular MG in humans, may be a focal manifestation of a mild, generalized autoimmune response to AChRs which is clinically evident due to the peculiarities of local musculature.” Dogs with the focal form of MG were reported to have lower mean AChR Ab titers than those with generalized MG (3.1 nM vs. 10 nM, respectively (Shelton et al., 1990)). These animals were compared to the 20 dogs (excluding the animal that died following surgery for thymoma) from our study for whom the outcome was known. Our group consisted of some dogs with the focal form of MG as well as some with generalized MG. Thus, if anything, our cohort had fewer dogs with a milder autoimmune response. The clinical AChR Ab assays for the present animals and controls were performed by the same veterinary service (UC San Diego, Comparative Neuromuscular Laboratory).

Table 1 shows that there was not a significant difference between the number of animals that died of aspiration pneumonia or choking in our population as compared to the historical controls (25% vs. 17%, respectively). There was also no difference in the spontaneous remission rate between the two groups (25% vs. 17%). These results suggest that the two cohorts are well matched. A significant difference was in the number of euthanized animals. Twelve dogs (34%) in the historical control group were euthanized due to the poor prognosis that was given. No animals in our group were euthanized since an improved prognosis was one part of the hypothesis being tested. Most importantly, there was a highly significant difference in the overall long-term remission rates between the two groups. While the historical controls had only six of 35 dogs (17%) remit with AChR Ab levels returning to the normal range (<0.6 nM), our group had 15 of 20 dogs (75%) remit. Thus of animals that survived aspiration pneumonia or euthanasia, the historical control had a remission rate of 35% (6/17) which contrasts with the apparent 100% remission rate (15/15) ( $p < 0.0001$ ) in the present study. This increased remission rate was entirely accounted for by the 10 out of 10 dogs (100%) that remitted in association with vaccination.

Table 1  
Prospective outcomes for vaccinated myasthenic dogs and historical controls

Group	Total dogs	Remitted (%)			Not remitted (%)	Euthanasia (%)	Aspiration pneumonia death (%)
		Spontaneous	Following vaccination	Total			
Historical controls	35	6/35 (17%)	–	6/35 (17%)	11/35 (31%)	12/35 (34%)	6/35 (17%)
Vaccine trial	20	5/20 (25%)	10/10 (100%)	15/20 (75%) <sup>A</sup>	0/15 (0%) <sup>B</sup>	0/20 (0%) <sup>C</sup>	5/20 (25%) <sup>D</sup>

Of 35 historical controls, six spontaneously remitted, 12 were euthanized, six died, and 11 did not recover from MG.

Of 20 vaccinated dogs, five spontaneously remitted, 10 remitted after vaccination and five died.

A:  $p < 0.0001$ , B:  $p = 0.0044$ , C:  $p = 0.0022$ , and D:  $p = 0.504$ , all by Fisher's exact test. Each comparison considers dogs with indicated outcomes against all other dogs in the group.

The remitted animals also had clear evidence for correction of muscle weakness or fatigue since they could function normally in the absence of acetylcholinesterase inhibitors.

### 3.2. Vaccination leads to an accelerated time to remission from canine MG

The second historical control group (data set from reference (Shelton and Lindstrom, 2001) consisted of 47 myasthenic dogs that spontaneously remitted and were comparable to the 10 vaccinated animals in four important respects. First, the dogs in both groups were not treated with corticosteroids or other immunosuppressants. Second, the AChR Ab assays were performed by the same veterinary service (UC San Diego, Comparative Neuromuscular Laboratory). Third, the time elapsed from clinical signs to confirmed diagnosis for vaccinated dogs (2–4 weeks) fell within the period for historical controls (1 week to 5 months). Since disease in historical controls sometimes extended out to 5 months before diagnosis, the impact of vaccination is likely understated. Lastly, the two groups consisted of roughly comparable ratios of males and females with similar age ranges and were studied during an overlapping period of time (historical control, 1990–2000 and vaccinated animals, 1997–2003).

Interestingly, in evaluating the data set for the historical controls, the AChR Ab levels were observed to follow one of two courses. The majority of cases had a monophasic decline in AChR Ab levels that led to long-term remission (Fig. 1A). That is, they show a decrease in AChR Ab titer at each sequential assay following diagnosis, they remit earlier and once in remission never relapse. The remainder showed a fluctuating pattern of increasing and decreasing AChR Ab levels leading to transient periods of disease ( $>0.6$  nM AChR Ab) or remission ( $<0.6$  nM AChR Ab) prior to long-term remission (Fig. 1B). These animals show one or more elevations in AChR Ab titer post diagnosis, can have a temporary remission(s) and have a later long-term remission. AChR Ab levels with time in vaccinated dogs also segregated into one or the other of these same two groups (see below). Thus appropriate analysis dictated that comparisons should be made within groups.

Fig. 2A shows that at the time of confirmed diagnosis of dogs showing a monophasic decline, there was not a statistical difference in AChR Ab levels between the vaccinated group ( $n = 5$ ) and historical controls ( $n = 40$ ). However, the

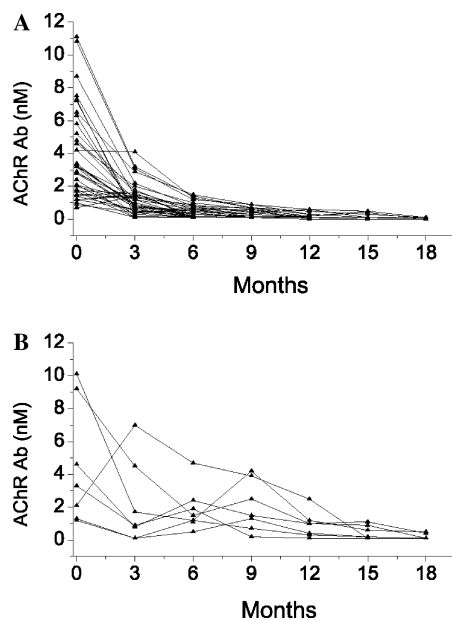


Fig. 1. Time course of AChR Ab levels in myasthenic dogs showing a monophasic decline (A,  $n = 40$ ) or fluctuating AChR Ab titers (B,  $n = 7$ ). Each line represents an individual animal and month 0 refers to the time of confirmed diagnosis.

vaccinated group showed an accelerated rate of decline in AChR Ab titers. This was mirrored by a faster rate of remission in the vaccinated group (Fig. 2B). This accelerated remission among vaccinated dogs is most evident at the 3-month time point, where the relative likelihood of remission was 3.56 for vaccinated dogs vs. controls by Fisher's exact test ( $p = 0.019$ ). Likewise, the serum Ab titers at this time point were significantly lower ( $p = 0.027$  by unpaired  $t$ -test) in vaccinated dogs (mean = 0.453 nM; lower and upper 95% CI's = 0.039 and 0.868, respectively) than for animals in the historical control group (mean = 1.21 nM, lower 95% CI = 0.913, upper 95% CI = 1.507). The mean time to remission ( $\pm$ SEM) of  $2.35 \pm 0.96$  months for vaccinated dogs was significantly shorter ( $p < 0.0014$ , Mann–Whitney test) than the  $7.05 \pm 2.35$  months for the historical controls (Fig. 2C). One hundred percent of vaccinated dogs had remitted by 6 months post diagnosis as compared with 12 months for historical controls.

Fig. 3 shows the AChR Ab levels with time after confirmed diagnosis for historical controls (3A) and vaccinated animals (3B) showing a fluctuating pattern of autoAb con-

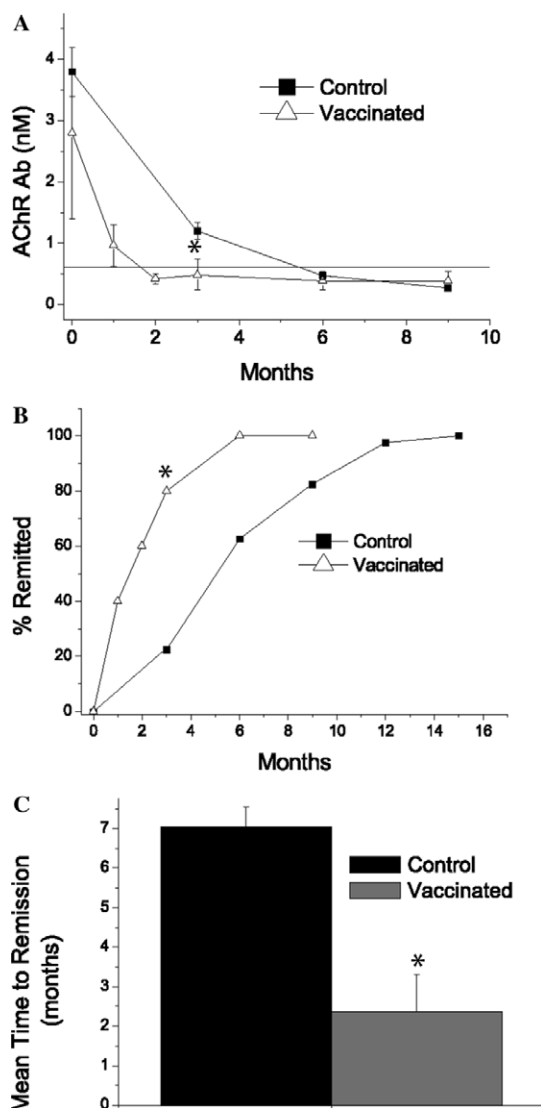


Fig. 2. (A) Effect of T and B cell ARM vaccines on AChR Ab levels (means  $\pm$  SEM) in vaccinated dogs with canine MG ( $n = 5$ ) as compared to nonvaccinated historical controls ( $n = 40$ ) showing a monophasic decline in AChR Ab titers. The horizontal line represents the upper limit ( $<0.6$  nM) of AChR Ab levels in normal dogs. Month 0 refers to the time of confirmed diagnosis.  $*p = 0.027$ . (B) Long-term remission rate for vaccinated dogs as compared to nonvaccinated historical controls from the upper panel. Percent remitted refers to the number of animals with long-term AChR Ab titers less than  $0.6$  nM as a percentage of the total starting number of myasthenic vaccinated ( $n = 5$ ) and nonvaccinated historical control animals ( $n = 40$ ). Time 0 is the time of confirmed diagnosis.  $*p = 0.019$ . (C) Mean time (months  $\pm$  SEM) to long-term remission for myasthenic vaccinated versus nonvaccinated historical control dogs from upper panel. This represents the average time following confirmed diagnosis that an animal first showed a sustained AChR Ab level below  $0.6$  nM.  $*p < 0.0014$ .

centrations. While the initial AChR Ab titers (nM  $\pm$  SEM) were not significantly different between the vaccinated dogs ( $5.98 \pm 2.33$ ) and historical controls ( $4.54 \pm 1.4$ ) there appeared to be a marked diminution in the overall amplitude of the fluctuations for the vaccinated dogs at times post diagnosis. This was reflected in a reduction in the mean time to the first observed transient remission in vac-

inated dogs ( $5.45 \pm 1.46$  months) as compared to the historical controls ( $12 \pm 2.62$  months) ( $p = 0.04$ , unpaired  $t$ -test). In contrast, the mean time to long-term remission was the same for the two groups (historical controls,  $14.5 \pm 1.38$  vs. vaccinated,  $14.3 \pm 2.03$ , Fig. 3C;  $p = 0.47$ ). Thus it seems that while the duration of the disease is the same in the two groups, vaccinated dogs may spend more time in remission during the course of the disease. To assure that the increase in animals showing a transient return to normal AChR Ab levels was not due to more frequent sampling of the vaccinated group relative to the historical controls (sampling at 3-month intervals), the AChR Ab levels were considered only at 3-month intervals post diagnosis. The results showed that all vaccinated dogs with fluctuating AChR Ab levels versus 2/7 historical controls had at least one blood sample within the normal range ( $<0.6$  nM) for AChR Ab at a 3-month interval beginning at diagnosis and before complete resolution of disease. In total, there were eight episodes of normal AChR Ab titers in the five vaccinated animals [1 dog with 3, 1 dog with 2 and 3 dogs with 1 episode(s)] compared to three episodes in the seven historical controls [1 dog with 2, 1 dog with 1 and 5 dogs with 0 episode(s)]. Thus the mean number of episodes of transient remissions per dog was significantly higher ( $p < 0.0251$ , Mann–Whitney test) in the vaccinated animals than the historical controls (Fig. 3D).

Prior to long-term resolution of MG and considering all available blood samples (not simply the 3-month intervals), four of five vaccinated dogs with oscillating levels of AChR Ab showed more than one episode of transient remission (two animals with three episodes and two animals with two episodes, total of 10) which corresponded to the administration of a vaccine dose. Fig. 4A shows a highly significant correlation ( $r = 0.955$ ,  $p < 0.0001$ ) between the time of administration of a particular vaccine dose and the time of the onset of the subsequent period of remission. Thus temporally, vaccination is very strongly associated with transient remission and is independent of when the vaccine dose was given relative to diagnosis. Importantly, the  $Y$  intercept for this relationship is 2.4 months which corresponds very closely to the mean time to remission ( $2.35 \pm 0.96$  months) of vaccinated dogs showing a monophasic decline in AChR Ab titers (Fig. 2C). Thus regardless of the pattern of AChR Ab levels, vaccination leads to a very similar time to remission suggesting a similar mechanism may be involved in both courses.

There was also a quantitative relationship between single vaccination events and a reduction of AChR Ab levels at the next sampling time (2–4 weeks) that was observed in all vaccinated dogs with fluctuating AChR Ab titers. Of 35 vaccination events, 28 (80%) resulted in a diminution in AChR Ab titer (Fig. 4B). The 7 vaccination events that did not lower AChR Ab levels were distributed among all vaccinated animals. Overall, the mean AChR Ab levels declined from  $1.63 \pm 0.21$  before to  $1.14 \pm 0.17$  nM after vaccination ( $p = 0.0015$ , two-tailed paired  $t$  test).

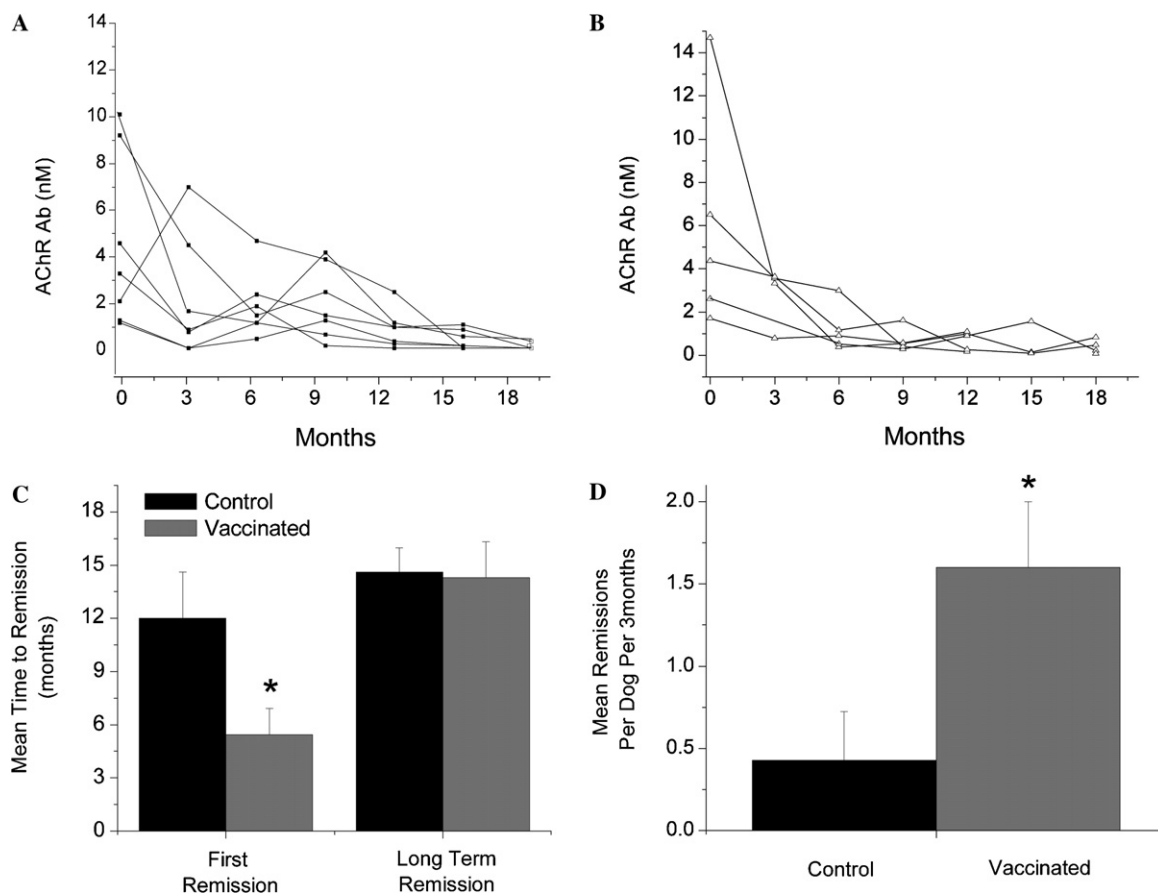


Fig. 3. Time course of AChR Ab levels in myasthenic nonvaccinated historical control dogs (A,  $n = 7$ ) as compared to vaccinated myasthenic dogs (B,  $n = 5$ ). Each line represents an individual animal and month 0 is the time of confirmed diagnosis. (C) Mean time (month  $\pm$  SEM) to first remission and long-term remission for myasthenic nonvaccinated historical control and vaccinated dogs from (A) and (B), respectively. First remission refers to the first time post confirmed diagnosis that an animal had a transient or sustained level of AChR Ab below 0.6 nM. Long-term remission refers to the time post confirmed diagnosis that an animal had a sustained level of AChR Ab below 0.6 nM. \* $p = 0.04$ . (D) The effect of T and B cell ARM vaccines on the mean number of episodes ( $\pm$ SEM) of transient remissions per dog in vaccinated myasthenic animals ( $n = 5$ ) compared to nonvaccinated historical controls ( $n = 7$ ). An episode of transient remission refers to a transient level of AChR Ab below 0.6 nM at any 3-month interval after confirmed diagnosis and before long-term remission (i.e., sustained AChR Ab levels below 0.6 nM). The mean number of episodes/dog is calculated from the total number of episodes for all dogs in a group divided by the total number of dogs/group. <sup>ast</sup> $p < 0.0251$ .

### 3.3. Combined administration of T and B cell vaccines is superior to their sequential administration

The vaccination protocol was performed in one of two ways. The T and B cell vaccines were given at the same time or a course of T cell vaccine doses at 2 week intervals was followed sequentially by a course of B cell vaccine doses at 2 week intervals. Since the mean time between vaccination and long-term remission for dogs showing a monophasic decline (2.35 months, Fig. 2) was the same as the mean time between an initiation of a vaccination course and the subsequent transient remission (2.4 months, Fig. 4) for dogs with fluctuating AChR Ab levels and in this later group was independent of the time of vaccination post diagnosis, comparisons were possible for the efficacy of the two vaccination protocols regardless of the pattern of AChR Ab levels. Fig. 5A shows the mean time to the first remission (either long-term or transient) following the initiation of vaccination. There was a trend, which approached significance ( $p = 0.064$ ), towards a shorter time to remission that was 2.7

times faster when the T and B cell vaccines were administered simultaneously rather than sequentially. On average, more vaccinations per animal were required when the T and B cell vaccines were given sequentially ( $3.86 \pm 1.06$ ) as opposed to simultaneously ( $2.67 \pm 0.333$ ) to achieve a remission. When we factor in the number of T and/or B ARM vaccine doses to achieve a remission, together with the time to remission, there is a significant decrease ( $p = 0.0167$ , two-tailed Mann–Whitney test) in the time to remission per dose when the vaccines are administered simultaneously rather than sequentially (Fig. 5B). Furthermore, when vaccines were administered sequentially only four of seven animals showed a remission following T cell vaccination alone. Thus, optimal efficacy appears to require that both vaccines be administered together. Since the same adjuvant (TiterMax) and carrier protein (KLH) were used in both vaccination protocols, it is unlikely that they were responsible for the effect. If so, one would have expected to observe similar mean times to first remission following the same amount of vaccines for the two protocols. Conse-

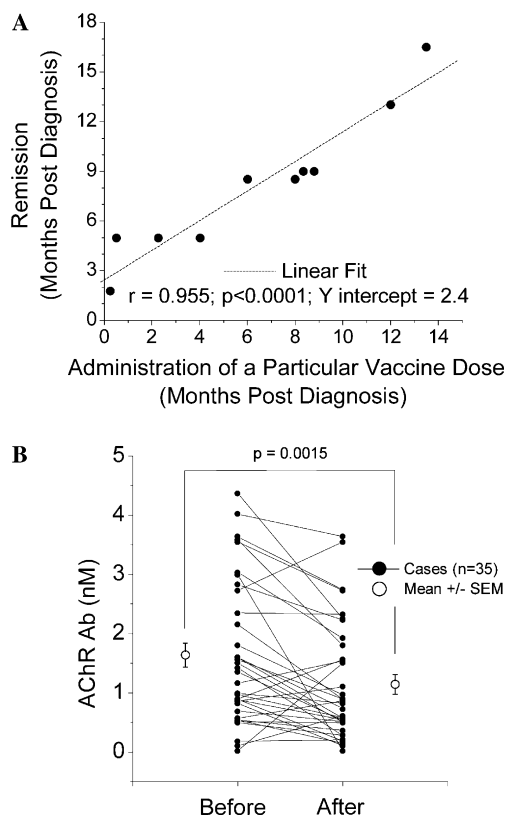


Fig. 4. (A) Correlation between the time relative to confirmed diagnosis (0 time) of the administration of a particular dose of T and/or B cell ARM vaccine and the first transient remission post diagnosis. Remission refers to the first measurement of a transient AChR Ab level below 0.6 nM. (B) Correlation between AChR Ab levels (nM  $\pm$  SEM) in blood samples drawn immediately before administration of a dose of T and/or B cell ARM vaccine with the AChR Ab level at the first sampling thereafter (2–4 weeks).

quently, the vaccine effect appears dependent on the T and B ARM peptides and independent of the adjuvant and carrier.

#### 4. Discussion

This study provides proof in principle that therapeutic vaccines can be developed for spontaneous autoimmune diseases. In particular, the results seem to demonstrate that the two vaccines described in this report are able to abrogate the AChR autoAb response in spontaneously acquired canine MG. As a consequence of the correction of this response, there was objective evidence that the muscle weakness (fatigue) experienced by afflicted dogs was alleviated. Specifically, vaccinated animals no longer required an acetylcholinesterase inhibitor, mestinon, to counter activity or exercise-mediated muscle weakness or fatigue. Thus, in a sense, correction of the underlying neuroimmune disorder resulted in a more normal behavioral response in terms of lack of excessive muscle weakness or fatigue due to normal daily activities. Obviously with dogs or other animals, it is impossible to know whether their perception of fatigue was altered as a result of treatment. However, answering this

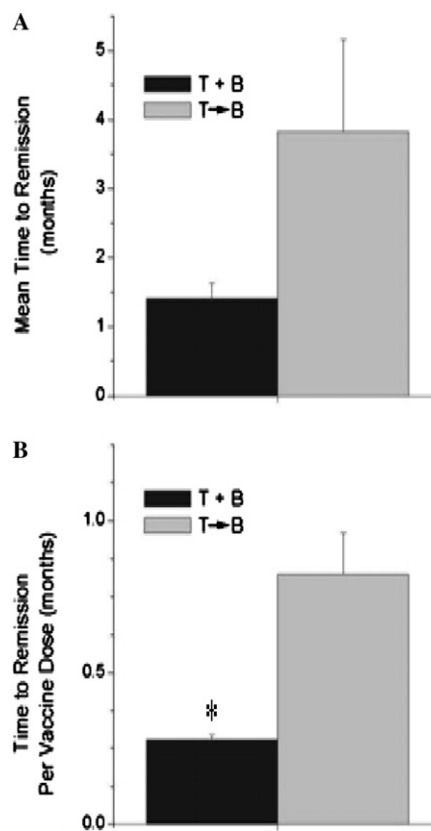


Fig. 5. (A) Comparison of the mean time (month  $\pm$  SEM) to remission post confirmed diagnosis for myasthenic dogs immunized with sequential doses of T and B cell ARM vaccines (T  $\rightarrow$  B) ( $n = 7$ ) or combined doses (T + B) of the two vaccines ( $n = 3$ ). Remission refers to the first transient or sustained AChR Ab level below 0.6 nM. (B) Comparison of the mean time (month  $\pm$  SEM) to remission per dose of vaccine administered for myasthenic dogs immunized with sequential doses of T and B ARM vaccines (T  $\rightarrow$  B) or combined doses (T + B) of the two vaccines. Remission refers to the first transient or sustained AChR Ab level below 0.6 nM. \* $p = 0.0167$ .

important behavioral question may now be possible if the vaccines are effective in human MG patients.

This study also illustrates three important points with regard to spontaneous acquired autoimmune MG in dogs. First, during the natural course of the disease in dogs that spontaneously remit, AChR Ab levels follow one of two courses. They either show a monophasic decline to normal levels or they fluctuate sometimes entering and emerging from the normal range. While both courses ultimately lead to disease resolution with normal AChR Ab titers, such long-term remissions occur on average two times faster in animals showing a monophasic ( $7.05 \pm 2.35$  months) as compared with a fluctuating pattern ( $14.5 \pm 1.38$  months). Interestingly, some human MG patients also show a fluctuating pattern of AChR Ab levels with time (Dwyer et al., 1984). Thus this may represent yet another similarity between human and canine MG, and it would be interesting to determine whether the pattern of AChR Ab levels in humans is prognostic for disease duration or remission.

The second major point is that compared to historical controls that spontaneously remit the previously described T and B cell vaccines can accelerate the rate of AChR Ab decline in the monophasic group and cause dogs with fluctuating AChR Ab levels to more frequently have AChR Ab titers in the normal range. Collectively, these results suggest that these vaccines can benefit spontaneous canine MG by shortening the duration of the disease even in dogs that spontaneously remit. This is borne out in the third major point: when compared to historical control myasthenic dogs that were followed prospectively as in the present study, vaccination is associated with a profound increase in the remission rate. Specifically, every dog (10/10) that received the T and B cell vaccines and survived aspiration pneumonia remitted, while one would have expected a 35% remission rate at most based on the historical controls. Furthermore, the vaccines alleviated muscle fatigue as evidenced by normal function in the absence of acetylcholinesterase inhibitors and appeared to be safe as no overt side effects, other than a self-resolving skin reaction at the injection site in one dog who dropped from the study and one who completed the study, were noted. Two reports have suggested that routine vaccination against infectious agents can precipitate a recurrence of MG in dogs that have previously remitted (Garlepp et al., 1984; Shelton and Lindstrom, 2001). If this is a general occurrence, then the present vaccines are unique in that they never caused a single re-occurrence when given after remission had been achieved.

Canine and human MG, as well as EAMG in rats, are characterized as having an AChR Ab response that is predominantly (68%) against the MIR (Shelton et al., 1988). Furthermore, MIR-specific-Ab can passively transfer a myasthenic phenotype. Considering this together with the ability of the B cell vaccine to induce an anti-Id Ab response to MIR-specific-Ab and B cells in rat EAMG, lowering AChR Ab levels and ameliorating disease, it does not seem particularly surprising that this vaccine appeared to diminish AChR Ab levels in dogs. The efficacy in rat EAMG and the suggestion of an effect in spontaneously acquired canine MG, suggests a potential utility of the B cell vaccine in the human disease. Indeed, naturally occurring anti-Id Ab against AChR Ab has been reported in 40% of human MG patients, and its presence is associated with lower anti-AChR Ab titers and clinical improvement (Dwyer et al., 1983, 1984). Furthermore, in other Ab-mediated autoimmune disorders, including systemic lupus erythematosus, Sjogren's syndrome and autoimmune necrotizing systemic vasculitis, anti-Id Ab that binds autoreactive Ab can be detected with the appropriate complementary/ARM peptides (Pendergraft et al., 2004; Routsias et al., 2002, 2003). Thus, B cell vaccines of the type herein described may be useful in inducing or bolstering a natural and perhaps regulatory anti-Id Ab response in not only MG but other adult autoimmune diseases. One might wonder why an anti-Id response would suppress rather than stimulate an Id Ab response. A possible explanation could be due to

the well known observation that anti-Id Ab given during the fetal or neonatal period often enhances production of the corresponding Id Ab while injection of anti-Id Ab during adulthood almost always inhibits Id Ab production (Holmberg et al., 1989). Consequently as these vaccines are further developed, special attention should be given to a recipient's age.

In contrast to the B cell vaccine, it is puzzling how a vaccine designed against the dominant T cell epitope of the AChR for the Lewis rat is apparently effective in dogs. One possible scenario is that anti-clonotypic Ab against the T cell vaccine may induce or expand the newly described and naturally occurring CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells via an interaction with their TCR (for review, see Maloy and Powrie, 2001). If this were the case, these cells would specifically react with AChR  $\alpha$ 100–116 but would nonspecifically suppress other autoreactive T cells for more dominant epitopes of the AChR at the neuromuscular junction. Such a mechanism, if operational, would suggest that the vaccines may be effective against human as well as canine MG. In preliminary studies, Ab to certain T cell ARM peptides that cross-react with the appropriate TCR can induce and/or expand the number of CD25<sup>+</sup> CD4<sup>+</sup> T cells (unpublished observation). This seems particularly interesting considering that naturally occurring regulatory anti-TCR Ab have been recently found in MG patients where they predicted clinical improvement (Jambou et al., 2003) and that immunization with V-region peptides (of which ARM peptides are analogs) from TCR or Ab upregulate CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells (Sharabi et al., 2006; Vandembark, 2005). This trial versus historical controls suggests that, as in rats, these two MG vaccines may be effective in dogs. The results indicate that their development as a pet medication warrants further study in a randomized trial. The apparent positive findings in a second species raise cautious optimism for the eventual application of these vaccines in humans with ad hoc carrier/adjuvant (McAnally et al., 2001). If these vaccines prove successful in humans, they would afford an opportunity to assess whether correction of the physical cause of muscle fatigue in MG would also correct the perception of more general chronic fatigue.

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