

# Canine and human myasthenia gravis autoantibodies recognize similar regions on the acetylcholine receptor

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**Article abstract**—Serum from 35 cases of naturally occurring acquired canine myasthenia gravis (MG) were assayed for patterns of autoantibody specificities against canine acetylcholine receptor (AChR) using monoclonal antibodies (mAbs) and antiserum against defined regions of the AChR as competitive inhibitors of autoantibody binding. In human MG patients and in animals immunized with AChR purified from fish electric organs or mammalian muscle, most of the antibodies are directed against the main immunogenic region (MIR), a conformationally dependent region located on the extracellular surface of the  $\alpha$  subunit away from the ACh binding site. In our studies using canine MG serum, we found that, as in human MG and in animals immunized with AChR, the antibody response is heterogeneous and predominantly IgG, with a large proportion of the autoantibodies directed against the MIR. The mAbs to the MIR blocked an average of 68% of serum antibody binding. A mAb to the  $\beta$  subunit and polyclonal antiserum to the  $\gamma$  subunit blocked an average of 34% and 39% of serum antibody binding, respectively, indicating that these subunits also contain relevant antigenic determinants, a pattern that has also been observed in human MG serum. Anti-abungarotoxin binding site antibodies made up only a small fraction of the autoantibody population in canine MG as in human MG. These and other features described here suggest that canine MG is a useful model of human MG.

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Myasthenia gravis (MG) in its acquired form is an autoimmune disorder of neuromuscular transmission in which autoantibodies have been shown to be responsible for damage to acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ).<sup>1-4</sup> As a result of autoantibody binding, there is loss of nicotinic AChRs, resulting in impaired neuromuscular transmission and marked muscle weakness. AChR loss is a result of (1) increased endocytosis as a result of cross-linking of AChRs by antibody, (2) complement activation leading to focal lysis of the postsynaptic membrane, and (3) bound antibodies directly inhibiting AChR function.

MG can be induced in animals by immunization with syngeneic AChR or AChR from the electric organ of *Torpedo californica*.<sup>4</sup> In the animal model of MG, experimental autoimmune MG (EAMG), antibodies are produced against exogenous AChRs that have <5% cross-reaction with endogenous AChR at the NMJ.<sup>4</sup> As a result of binding of this small percentage of cross-reacting antibody, there is AChR loss and profound muscular weakness. This model has provided much insight into the mechanisms of AChR damage and also into the pathologic changes occurring at the NMJ. However, it provides little information regarding the initiating factor(s) of the immune response. Also, as an

animal model for evaluating new therapeutic approaches, EAMG is limited by its lack of a self-sustaining autoimmune response.

Naturally occurring autoimmune MG has been described in dogs,<sup>5-7</sup> and rarely in cats.<sup>8-10</sup> A naturally occurring model, such as acquired canine MG (CMG), is not only valuable in investigating what initiates the autoimmune response, but also provides a system to test new modes of therapy that may ultimately be used in the treatment of human MG. CMG is very similar to human MG in clinical signs, methods of diagnosis, and modes of therapy. Generalized neuromuscular weakness, exacerbated by exercise and improved with anticholinesterase drugs, is the prominent clinical finding.<sup>11</sup> However, focal forms may occur, consisting of only ocular and pharyngeal weakness or esophageal dilatation (Shelton, unpublished observations). Esophageal dilatation occurs in most dogs with MG, a feature that is not present in human MG, due to the presence of striated muscle along the entire length of the esophagus in the dog and only in the proximal one-half in humans. As in human MG, a decrementing response to repetitive nerve stimulation can be observed.<sup>11</sup> The presence of immune complexes at the NMJ can be demonstrated *in situ* by immunocytochemical methods.<sup>12</sup> Antibodies

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**Table 1. Antibodies used to inhibit binding of autoantibodies to canine AChR**

Specificity	Anti-body	Source of immunogen	Titer for immunogen ( $\mu\text{M}$ )	Titer for canine muscle (nM)
<b>mAbs</b>				
$\alpha$ , MIR	35	<i>Electrophorus electricus</i>	350	947
$\alpha$ , MIR	203	Human muscle	44.5	196
$\alpha$ , not MIR	64	Fetal bovine muscle	5.9	250
$\beta$	73	Fetal bovine muscle	31.9	94
$\beta$	109	<i>Torpedo californica</i>	25	0
<b>Antiserum</b>				
$\gamma$	anti- $\gamma$	<i>Torpedo californica</i>	0.5	14
$\delta$	anti- $\delta$	<i>Torpedo californica</i>	0.8	13

against canine AChR have been demonstrated in CMG serum<sup>6</sup> and fixed to AChR in muscle from affected dogs.<sup>6</sup> A decrease of muscle AChR content, similar to that occurring in human MG, has also been demonstrated in dogs with MG.<sup>6</sup>

In human MG patient serum<sup>13</sup> and in serum from animals with EAMG,<sup>14,15</sup> antibodies have been shown to be directed largely to a conformationally dependent region of the AChR known as the main immunogenic region (MIR). The MIR is defined by competitive binding of antibodies and may not consist of a single epitope formed by a few amino acids. Antibodies bound to a protein surface would be expected to obscure a much larger area than the few amino acids responsible for specific binding. When binding sites for monoclonal antibodies (mAbs) on AChRs are mapped in detail by competitive binding and schematically represented as a series of partially overlapping circles, a pattern is often observed in which a single mAb may be seen to partially compete with several other mAbs to distinct epitopes.<sup>16,17</sup> The MIR is located on the extracellular surface of the  $\alpha$  subunit, distinct from the acetylcholine binding site.<sup>15,18</sup> Peptide mapping studies have localized the amino acid sequences forming the MIR in AChR from *Torpedo* electric organ to the general area of  $\alpha 46$  to  $\alpha 127$ .<sup>19,20</sup> Similar localization is expected in mammalian AChR because of the highly conserved sequence of  $\alpha$  subunits (80% identity between *Torpedo* and human).<sup>21</sup> Crystallographic studies of *Torpedo* AChR have recently shown that the MIRs are on the sides of the  $\alpha$  subunits between the synaptic surface and the cell membrane.<sup>22</sup> The proposed position of the MIR is consistent with the fact that mAbs to the MIR are very effective at cross-linking AChR molecules to each other.<sup>23</sup> Cross-linking of AChRs by antibodies to the MIR triggers antigenic modulation resulting in AChR loss.<sup>23</sup> Antibodies to the MIR are pathogenically significant, as indicated by the observations that (1) mAbs to the MIR can cause antigenic modulation,<sup>23</sup> (2) a Fab fragment of a mAb to the MIR can prevent 68% of antigenic modulation caused by MG patient serum on muscle cells in culture,<sup>14</sup> and (3) mAbs to the MIR can passively transfer EAMG.<sup>24</sup> While originally described

for *Torpedo* AChR, the MIR appears to be a well-conserved region between species, since most antibodies that cross-react between species are directed to the MIR.<sup>15,18</sup> In other studies, some but not all human MG patient serum contained antibodies directed against the abungarotoxin ( $\alpha\text{Bgt}$ ) binding site.<sup>25,26</sup> In this study, a panel of rat mAbs and polyclonal antiserum against defined regions of the AChR was used to competitively inhibit binding of canine patient serum to canine muscle AChR. By this method we investigated whether the patterns of autoantibody specificities in canine MG serum were similar to those of human MG and EAMG serum.

**Methods. Purification of canine AChR.** For use as antigen to detect the presence of autoantibodies in canine MG serum, AChR was solubilized from muscle membranes of near-term canine fetuses in 2% Triton X-100 and a crude extract prepared as previously described.<sup>27</sup> The concentration of AChR was determined<sup>27</sup> and expressed as moles of <sup>125</sup>I- $\alpha\text{Bgt}$  binding sites per liter.

**Preparation of mAbs and antiserum.** Preparation and characterization of mAbs and antiserum used in this study against AChR from the electric organs of *Torpedo californica* or *Electrophorus electricus*, or from fetal bovine muscle that cross-reacted with canine muscle AChR (table 1), have been previously described.<sup>18,28,29</sup> Titers of mAbs and antiserum against the immunogen and against canine AChR, expressed as moles of <sup>125</sup>I- $\alpha\text{Bgt}$ -labeled AChR bound per liter, were determined as previously described.<sup>27</sup>

**Canine MG patients.** A presumptive diagnosis of MG in 35 dogs (table 2) was based on the clinical findings of generalized muscular weakness that worsened with exercise and improved with rest and/or esophageal dilatation, alleviation of muscular weakness by the intravenous administration of short-acting anticholinesterase drugs, electrophysiologic evidence of a decrementing muscle action potential on repetitive nerve stimulation, or the immunocytochemical localization of immune complexes at the NMJ either in situ in a muscle biopsy sample<sup>12</sup> or following incubation of the patient's serum with normal canine muscle.<sup>30</sup> In this study, none of the CMG patients had radiographic evidence of a thymoma. The diagnosis of MG was confirmed by immunoprecipitation radioimmunoassay, as previously described,<sup>27</sup> with the following modifications. Unlabeled  $\alpha\text{Bgt}$  (1  $\mu\text{M}$ ) was added to triplicate serum controls for each serum sample to determine individual variations in background values. AChR (1 nM), labeled overnight with <sup>125</sup>I- $\alpha\text{Bgt}$  (20 nM), was used for all assays. Fixed *Staphylococcus aureus* (25  $\mu\text{l}$ , Pansorbin Cells, Calbiochem-Behring Corp, La Jolla, CA) or goat anti-dog IgG (40  $\mu\text{l}$ , Cooper Biomedical, Malvern, PA) were used to precipitate the labeled AChR-anti-AChR complexes. Titers were expressed as moles of <sup>125</sup>I- $\alpha\text{Bgt}$  binding sites per liter of serum. Normal values, as determined from the serum of 17 normal dogs of various ages, breeds, and sexes was  $0.144 \pm 0.438$  nM (mean  $\pm$  3 SD) of  $\alpha\text{Bgt}$  binding sites precipitated per liter of serum. The upper limit of normal was set at 0.6 nM. In this study, only dogs with titers  $> 3$  nM were included.

**Competition assays for determining AChR antibody specificities.** Aliquots (100  $\mu\text{l}$ ) of  $\alpha\text{Bgt}$ -labeled canine muscle extract (0.1 pmol of AChR plus 2 pmol  $\alpha\text{Bgt}$  in 0.5% Triton X-100, 10 mM Na phosphate, pH 7.5, 100 mM NaCl, 10 mM Na<sub>2</sub>SO<sub>4</sub>) were incubated overnight at 4 °C with a tenfold molar excess (1 pmol in the same buffer) of the competing mAb or antiserum, according to the general method of Tzartos et al,<sup>13</sup> using microfuge tubes instead of microtiter plates. A limiting amount of canine MG serum (0.05 pmols) was added and

Table 2. Clinical data on 35 dogs with acquired myasthenia gravis

Case no.	Breed	Sex	Age at onset (yrs)	AChR* antibody titer (nM)	$\alpha$ Bgt binding-site antibody titer (nM)
1	Australian shepherd	FS	8	35	0
2	Belgian shepherd	MC	4	7	2.6
3	Cocker spaniel	FS	5	10	0.4
4	Cocker spaniel	M	2	51	0.9
5	Collie	MC	2	6	0.05
6	Dachshund	F	3	26	0.5
7	Dachshund	FS	10	7	1.4
8	Dachshund	FS	12	7	2.2
9	Dachshund	M	3	11	2.7
10	German shepherd	F	1	6	1.8
11	German shepherd	F	2	9	0
12	German shepherd	F	4	35	0.9
13	German shepherd	FS	2	6	1.3
14	German shepherd	FS	5	8	ND
15	German shepherd	FS	7	4	1.2
16	German shepherd crossbreed	FS	9	27	1.2
17	German shepherd	M	3	22	0.8
18	German shepherd	M	3	11	0.2
19	German shepherd	M	4	4	0.4
20	German shepherd	M	4	11	4.0
21	German shepherd	M	10	10	2.2
22	German shepherd	M	12	12	2.1
23	German wirehaired pointer	FS	3	11	2.9
24	Golden retriever	F	1	8	1.1
25	Golden retriever	FS	5	12	0.9
26	Golden retriever	M	3	5	1.0
27	Golden retriever	MC	11	14	3.9
28	Gordon setter	M	10	7	0.2
29	Hound crossbreed	MC	2	4	1.5
30	Irish wolfhound	FS	7	93	1.0
31	Labrador retriever	M	9	6	0
32	Peke-a-poo	M	13	14	5.3
33	Poodle (miniature)	FS	11	11	2.6
34	Poodle (miniature)	MC	3	3	0.3
35	Springer spaniel	M	11	13	0.9

M Male.  
MC Male castrated.  
F Female.  
FS Female spayed.  
\* Normal  $\pm$  3 SD = 0.144  $\pm$  0.438 nM.  
ND Not determined.

incubated for 4 hours at 4 °C, followed by a 2-hour incubation with 40  $\mu$ l of goat anti-dog IgG that had been adsorbed with normal rat serum and centrifuged to eliminate the pellet prior to use in the assays, in order to deplete antibodies cross-reactive with rat IgG. The tubes were centrifuged and washed; then the radioactivity was measured. A species-specific mAb against *Torpedo californica* that did not bind canine AChR (mAb 109) was used as a negative control for the test mAbs and antisera. Incubation of each serum sample with excess unlabeled  $\alpha$ Bgt and  $^{125}$ I- $\alpha$ Bgt was used as control for CMG serum. Following subtraction of backgrounds, the difference in radioactivity that was precipitated by CMG serum in the presence and absence of competing antibody represented the extent of inhibition of binding of that serum by the mAb or antiserum. This procedure allowed the qualitative determination of the fraction of antibodies in CMG serum that were directed at the region to which a mAb binds. For the deter-

mination of the titer of autoantibodies directed to the  $\alpha$ Bgt binding site on the AChR, aliquots (100  $\mu$ l) of unlabeled canine muscle extract (0.1 pmol AChR) were incubated with 5  $\mu$ l of CMG patient serum or 5  $\mu$ l of normal dog serum, followed by incubation for 4 hours with  $^{125}$ I- $\alpha$ Bgt (2 pmol) and an excess of rat anti-AChR antiserum. Complexes were precipitated by a 2-hour incubation with goat anti-rat IgG. Following subtraction of radioactivity precipitated by test serum from that precipitated by incubation with normal dog serum, titers were determined and expressed as moles  $^{125}$ I binding sites per liter of serum.

**Results.** *Clinical findings of dogs with acquired MG.* Various large and small breeds of dogs were affected with acquired MG (table 2). However, German shepherds appeared especially susceptible and represented

13/35 cases. AChR antibody titers ranged from 3 to 93 nM ( $\bar{X}$  = 15 nM) and were predominantly IgG, since precipitation with either *S aureus* or goat anti-dog IgG resulted in similar titers (results not shown). Dogs with acquired MG showed a bimodal distribution in age of onset of clinical weakness (figure 1). The first group of affected dogs was younger, with a mean age of onset of about 3 years, while the second peak occurred later, at approximately 10 years of age. This bimodal type of age distribution is also described in human MG.<sup>31</sup> In hu-

mans, however, the younger onset group has a female predominance, while the older onset group is predominantly male.<sup>31</sup> In our study, the younger onset group was about evenly divided between males (intact and castrated) and females (intact and spayed). In the older group, males and females were again evenly represented; however, all of the females were spayed. While this may be consistent with a hormonal influence on later-onset MG, the incidence of intact females in the canine population was not determined, and this may reflect the fact that spaying female dogs is common practice.

*Distribution of AChR antibody specificities from dogs with acquired MG.* Properties of the mAbs and antiserum used in the competition assays are described in table 1. As shown in figure 2, most dogs with MG have autoantibodies directed to the MIR. The mAbs to the MIR (35 and 203) blocked an average of 68% of CMG serum binding to AChR (figure 3). While mAbs 35 and 203 both recognize the MIR, they probably are not recognizing identical determinants on the AChR, since there was some difference in their degrees of binding inhibition. The binding of only three sera (dogs no. 1, 18, and 19) was not significantly inhibited by mAbs to the MIR. The autoantibody response in most of the dogs was heterogeneous, as demonstrated by inhibition with mAbs or antiserum specific to other subunits. A mAb to the  $\beta$  subunit (mAb 73) and antiserum to the  $\gamma$  subunit significantly inhibited the binding of some serum to the AChR, inhibiting binding an average of 34% and 39%, respectively. A mAb to the  $\alpha$  subunit that does not bind the MIR (mAb 64) or antiserum to the  $\delta$  subunit inhibited only 14% and 13% of the binding, respectively, suggesting that these epitopes were not highly immunogenic in CMG. Similarly, when serum from dogs with

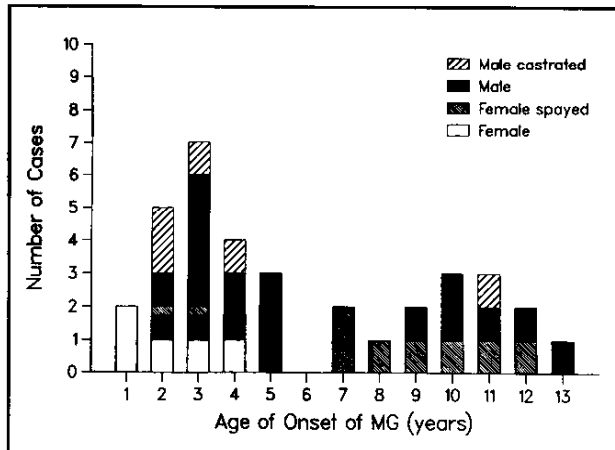


Figure 1. Dogs with acquired myasthenia gravis without evidence of thymoma have a bimodal age of onset of clinical signs. Dogs with an age of onset of <5 years are represented by both intact males and females and animals that have been neutered. In the group with an age of onset of >7 years, only males and spayed females are represented.

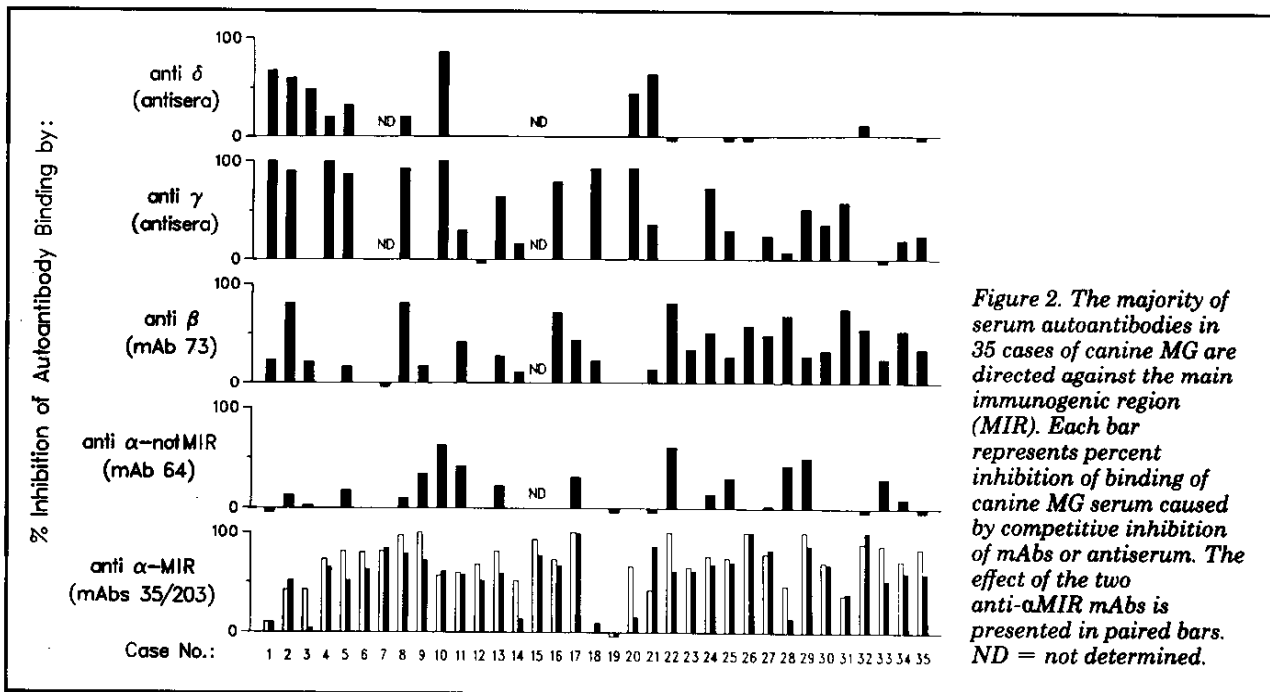


Figure 2. The majority of serum autoantibodies in 35 cases of canine MG are directed against the main immunogenic region (MIR). Each bar represents percent inhibition of binding of canine MG serum caused by competitive inhibition of mAbs or antiserum. The effect of the two anti- $\alpha$ MIR mAbs is presented in paired bars. ND = not determined.

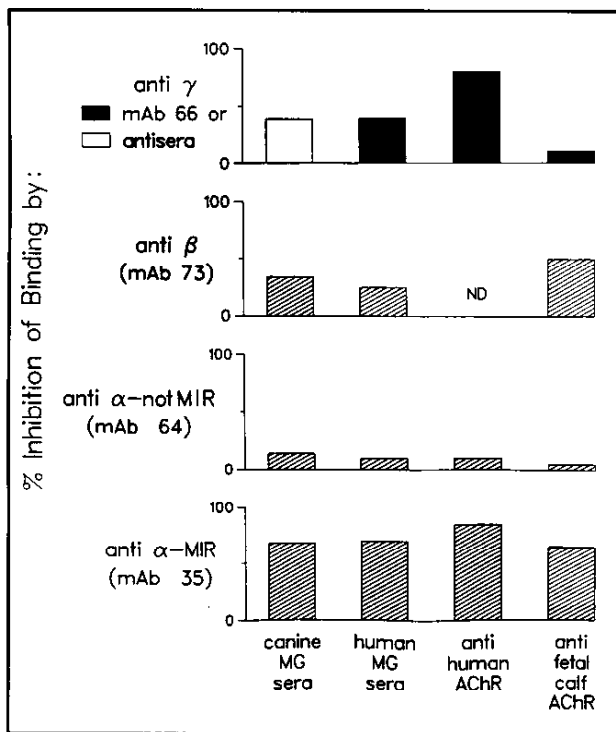


Figure 3. Serum from canine and human MG patients and from rats with EAMG have similar antibody specificities. Each bar represents the average percent inhibition of binding of canine, human, or EAMG serum by competitive inhibition of mAbs or antiserum. Values for human and EAMG serum are from Tzartos et al.<sup>13</sup>

MG were incubated with AChR prior to addition of <sup>125</sup>I- $\alpha$ Bgt, antibody titers against the  $\alpha$ Bgt binding site were <10% of anti-AChR antibody titers ( $\bar{X}$  = 1.4 nM, table 2), indicating that, in general, a large fraction of autoantibodies are not directed near the ACh binding site. While  $\alpha$ Bgt binding-site antibodies may play a pathogenic role in some CMG patients, it is not common in all cases.

Average AChR antibody specificities are similar in canine and human MG and EAMG. Antibodies in serum from dogs with MG predominantly recognized the MIR, similar to human MG patient serum and serum from rats immunized with AChR from fetal bovine or human muscle (figure 3). The proportion of antibodies to the other regions tested was also similar, with the exception that rats immunized with human muscle AChR recognized a higher proportion of the region on the  $\gamma$  subunit protected by the mAb.<sup>13</sup>

**Discussion.** CMG has been shown to have many similarities to its human counterpart,<sup>6,7</sup> and this study provides further evidence that CMG is a good model for the study of myasthenia gravis. Since acquired CMG is naturally occurring, it could provide insight into what initiates and sustains the autoimmune response to AChR in MG and a system for testing new therapeutic modalities for the treatment of human MG.

In human MG, there is clinical evidence for three

patient groups: patients <40 years of age with thymic hyperplasia and HLA-B8; patients >40 with thymic atrophy and HLA-B7; and older patients with thymoma.<sup>31</sup> It has been suggested that these three patient groups might reflect different pathways for induction of the autoimmune response to AChR.<sup>31</sup> In our study, we found a similar bimodal age distribution in dogs without thymoma, with one group of dogs having an age of onset of <5 years and the other group >7 years of age. While none of the dogs in this study had a thymoma, thymoma with associated MG has been reported in the dog.<sup>32,33</sup> In one study,<sup>32</sup> the average age of onset was 9 years (range, 5 to 13 years) with 6/7 dogs spayed females and 1/7 an intact male. Thymic changes in CMG, other than in cases of thymoma, have not been studied. While the genetics of CMG have also not been studied, there may be a breed predisposition, as indicated by the large proportion of German shepherds in this study. There clearly is evidence for three groups of dogs with MG having age distributions similar to those found in human MG.

From our study using competitive inhibitors, considerable heterogeneity of AChR autoantibodies in serum from canine MG patients was evident, with marked variability in the percentage of inhibition of autoantibody binding by different mAbs or antisera and variability in the inhibition of autoantibodies from different dogs by one mAb or antiserum (figure 2). The binding of most CMG serum was blocked by multiple mAbs or antiserum; however, in a few cases the specificity was restricted (dogs no. 6, 12, and 19). In similar studies of human MG serum, heterogeneity was reported both with mAbs produced in mice to human AChR,<sup>34</sup> and with mAbs produced in rats against AChR from other species.<sup>13</sup> In these studies, no relation was found between antibody specificity and clinical state. This cannot be evaluated in the dog until an appropriate clinical classification system is defined.

It has previously been demonstrated, using competitive inhibition assays, that when Lewis rats were immunized with *Torpedo* AChR, >50% of the antibodies produced were directed to the MIR on the extracellular surface of a subunits (figure 3).<sup>13,15</sup> However, other determinants were also immunogenic. Further, a similar pattern of inhibition was observed in serum from human MG patients using the same mAbs as competitive inhibitors (figure 3).<sup>13</sup> When the patterns of inhibition of CMG serum were compared to those of rats and humans, the patterns were very similar (figure 3). It is reasonable then to assume that since similar patterns of inhibition were obtained both in animals that were immunized with native AChR and in naturally occurring cases of canine and human MG, that the inciting immunogen is some endogenous source of AChR itself.<sup>13</sup> It is not likely that the immunogen is a single similar protein epitope on a bacterial cell membrane or a viral coat, since the autoantibody specificities are so similar to those obtained by immunization with native AChR. In a few isolated cases, however, where the autoantibody specificities are restricted to inhibition by a single mAb, molecular mimicry<sup>35</sup> may play a pathogenic role. Antibodies to contractile proteins are found in MG

patient serum<sup>36</sup> and have been reported in CMG serum.<sup>7,37,38</sup> This further suggests that the inciting immunogen is an endogenous AChR present on a muscle or muscle-like cell.

The presence of antibodies to AChR that bind at or near the ACh binding site and directly impair AChR function have been described in the serum of MG patients.<sup>25</sup> In one study however,<sup>25</sup> anti- $\alpha$ Bgt binding-site antibodies were found in only a few patients, suggesting that this determinant is not generally pathogenic. Similarly, in CMG, anti- $\alpha$ Bgt binding-site antibodies were only a small fraction of the anti-AChR antibodies.

While the EAMG model has made valuable contributions to the definition of pathologic changes occurring at the NMJ and shed much light on the role of antibodies in the pathogenesis of AChR loss and resulting muscle weakness, there has been very little progress in the area of what incites the immune response in MG and also very little progress in a cure for MG. In both these areas, the canine model could make valuable contributions. While the actual frequency of MG in the canine population is not known and the natural course of the disease not fully understood, there appears to be an adequate number of cases available for study. Since recognition of clinical signs of MG by the veterinary profession is increasing and sophisticated methods of diagnosis are available at veterinary teaching hospitals, much information may be gained with cooperation between veterinarians and medical researchers to further our knowledge of MG.

**Addendum.** Recently it has been reported that several MIR mAbs bind with very low affinity but high specificity to synthetic peptides with the  $\alpha$  subunit sequence 67-76 or 61-76 (Tzartos et al, *Proc Natl Acad Sci USA* 1988;85:2899-2903; Barkas et al, *J Biol Chem* 1988;263:5916-5920). This is consistent with our previous report that the MIR was within  $\alpha$ 46-127<sup>19</sup> and our unpublished observations with synthetic  $\alpha$ 66-83.

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