



## Titin and ryanodine receptor autoantibodies in dogs with thymoma and late-onset myasthenia gravis

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### Abstract

Similar to human autoimmune myasthenia gravis (MG), canine MG occurs spontaneously and is associated with autoantibodies against the nicotinic acetylcholine receptor (AChR). In addition to AChR, human MG patients with thymoma or late-onset MG have antibodies against titin and ryanodine receptor (RyR). The objective of this study was to establish if dogs with confirmed MG (AChR antibody titer >0.6 nmol/l) also developed titin and RyR antibodies and identify possible associations with thymoma, late age of onset, or severity of clinical signs. Sera from dogs ( $n = 430$ ) with previously diagnosed autoimmune MG ( $N = 415$ ), other immune-mediated neuromuscular disorders including polymyositis (PM) and masticatory muscle myositis ( $N = 5$ ), and control dogs ( $N = 10$ ) were evaluated for the presence of titin antibodies in ELISA using MG1-30 as antigen, a peptide representing the main immunogenic region (MIR) for human titin antibodies. Titin antibody positive sera were further examined for RyR antibodies in Western blots using a RyR fusion protein (pc2 RyR) as antigen, which covers the MIR for human MG sera. Titin antibodies were found in sera of 80/430 dogs. Thymoma was present in 11/80 and age of onset was after 4 years in 66/80 titin positive dogs. Two of the titin positive dogs had PM. RyR antibodies were found in 13/80 sera (8/13 thymoma, 12/13 age of onset after 4 years, and 1/13 PM). Neither titin nor RyR antibodies were found in sera of healthy control dogs. Acute fulminating MG was described in five dogs with both titin and RyR antibodies. From these studies we conclude that titin and RyR antibodies in canine and human MG have a similar association with thymoma, late-onset

*Abbreviations:* AChR, acetylcholine receptor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; MG, myasthenia gravis; MIR, main immunogenic region; OD, optical density; PBS, phosphate buffered saline; PM, polymyositis; RyR, ryanodine receptor; SDS, sodium dodecyl sulfate; TPBS, Tween 20 phosphate buffered saline

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MG, and possibly with more severe forms of MG. © 2001 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder of neuromuscular transmission resulting from autoantibody mediated destruction of acetylcholine receptors (AChRs) at the neuromuscular junction (Lindstrom et al., 1988). The primary mechanisms by which autoantibodies act to produce AChR loss and neuromuscular blockade are complement-mediated focal lysis (Engel et al., 1974; Engel et al., 1977a,b) and antigenic modulation (Drachman et al., 1978; Heinemann et al., 1977). The neuromuscular blockade is characterized clinically by muscle weakness and fatigability that improves with rest, but there is a poor correlation between severity of the weakness and the magnitude of the antibody titer.

The autoimmune form of canine MG was described by Lennon et al. (1978). Since that initial report numerous cases of spontaneous canine MG have been described and it has become apparent that MG is likely the most common neuromuscular disease that can be diagnosed in this species (Shelton, 1998, 1999). Canine autoimmune MG has many similarities with human MG including the spontaneous occurrence of AChR antibodies, a bimodal age distribution, a diversity of clinical presentations including focal and generalized forms, an association with other autoimmune diseases, and an association with thymoma (Shelton, 1998, 1999). The AChR autoantibodies produced in both canine and human MG have essentially the same pattern of antigenic specificities (Shelton et al., 1988). Seronegative cases are present in both canine and human MG (Shelton, 1998, 1999). Since MG in dogs is virtually the same disease as occurs in humans, further studies of canine MG may provide valuable information that may advance our knowledge of MG in general.

Autoantibodies against non-AChR skeletal muscle proteins including titin and ryanodine receptor (RyR) have been described in human MG patients with thymoma (Aarli et al., 1990; Mygland et al., 1992; Williams et al., 1992; Gautel et al., 1993) and in patients with onset of MG after 40 years of age (Skeie et al., 1995). Circulating RyR antibodies have also been associated with a severe form of thymoma-associated MG in human patients (Mygland et al., 1994). While antibodies against skeletal muscle striations have been observed in myasthenic dogs with thymoma (Garlepp et al., 1984; Shelton, unpublished) the precise antigens have not been identified. The purpose of this study was to evaluate the occurrence of titin and RyR antibodies in sera from dogs with spontaneous MG and examine the association of these autoantibodies with thymoma, severity of disease, and age of onset.

## 2. Materials and methods

Sera from 430 dogs with generalized weakness or regurgitation associated with a megaesophagus were tested by immunoprecipitation radioimmunoassay for the presence

of autoantibodies against AChR using near-term fetal canine muscle as antigen as previously described (Shelton et al., 1988). The dogs ranged in age from 1 to 14 years. These sera were submitted from veterinary clinicians within the United States and Canada to the Comparative Neuromuscular Laboratory at the University of California, San Diego, CA. Positive AChR antibody titers were previously established for this laboratory at  $>0.6$  nmol/l (Shelton et al., 1988). The serum samples were numbered and shipped on dry ice to the University of Bergen for testing of antibodies to titin and RyR. Presenting clinical signs were reviewed and classified as focal (dysphagia and megaesophagus only), mild generalized (weakness in the absence of dysphagia and megaesophagus), generalized (weakness and megaesophagus), acute fulminating (rapid loss of muscle strength resulting in severe weakness and recumbency), or thymoma-associated MG. A Student's *T*-test was used to compare the mean AChR antibody titer between groups of dogs that were titin antibody positive and negative with the assumption that the group variances were unequal.

### 2.1. Titin antibodies

Sera were tested for the presence of IgG antibodies to purified titin antigen MGT-30 in a standard ELISA (Gautel et al., 1993). MGT-30 is a 30 kDa peptide representing the main immunogenic region of the titin protein as defined by immunoscreening muscle cDNA libraries with sera from human thymoma MG patients and cloning a number of immunopositive cDNAs. All clones coded for the MGT-30 peptide (Gautel et al., 1993). MGT-30 was produced in an *E. coli* vector.

Microtiter plates (Costar flat bottom, Cambridge, MA) were coated with MGT-30 diluted to 3.5  $\mu$ g/ml in phosphate buffered saline (PBS) at pH 7.4 with 5% sucrose and incubated for 1 h at 37°C before 4°C overnight incubation. This coating concentration gave the best discrimination between positive and negative sera in preliminary antigen titration experiments. The plates were then aftercoated with bovine serum albumin (BSA) diluted to 1% in PBS with 0.05% Tween 20 (TPBS) for 1 h at 37°C. Thereafter, the plates were incubated with sera diluted in TPBS with 0.05% BSA for 1 h at 37°C. The optimal dilution was determined by testing twofold dilutions of selected sera against the best coating concentration, starting at 1:50. A dilution of 1:200 gave the best discrimination between positive and negative sera. The plates were then incubated with peroxidase-conjugated goat anti-dog IgG (Cappel, ICN-Pharmaceuticals, USA) diluted 1:3000 in TPBS with 0.05% BSA. Three washings with TPBS were performed after each incubation. The plates were developed with *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) 5 mg in 12.5 ml phosphate citrate buffer and 5  $\mu$ l H<sub>2</sub>O<sub>2</sub>, and the reaction stopped with 2.5 N H<sub>2</sub>SO<sub>4</sub>. Optimal density (OD) values were obtained at 492 nm.

All sera were tested in two parallel wells with antigen and in one control well without antigen. MG and control sera were always tested simultaneously on the same microtiter plates. The OD values for the sera were reported as the mean of the parallel wells minus the control well. Differences in OD values less than 10% between the two parallels were accepted. Sera with OD values exceeding 0.250 were considered titin antibody positive.

## 2.2. RyR fusion protein Western blot

The fusion protein pc2–RyR was used as antigen (Skeie et al., 1999). Electrophoresis was performed on sodium dodecyl sulfate (SDS) polyacrylamide gels (12%) as described by Laemmli (1970). Two hundred microlitres pc2 (50 µg/ml) were added to 120 µl of sample buffer containing 2% (w/v) SDS, 1.5% (w/v) Tris, 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue. The mixture was heated to 100°C for 1 min. Approximately, 100 µl of the protein mix was applied per gel. Proteins separated on the gel were transblotted onto nitrocellulose sheets as described (Towbin et al., 1979). Nitrocellulose sheets were soaked in 5% (w/v) low fat dry milk in PBS for 1 h to block additional protein binding sites. They were washed three times in PBS with 0.05% Tween 20 (PBS-Tween), cut into vertical strips and incubated over night at 4°C with patient and control sera diluted 1:50 in PBS containing 0.5% dry milk and 0.05% Tween 20 (PBS dry milk Tween). After separate washings for 10 min in PBS Tween, nitrocellulose strips were incubated for 1 h with peroxidase-conjugated goat anti-dog IgG diluted 1:1000 in PBS dry milk Tween. The nitrocellulose strips were then washed and developed in a peroxidase color development solution containing 30 mg 4-chloro 1 naphthol (Sigma), 17% (v/v) cold methanol, 83% (w/v) PBS, and 0.05% (v/v) H<sub>2</sub>O<sub>2</sub>. Positive and negative control sera were applied to strips from each transblotted nitrocellulose sheet.

## 3. Results

Of the dog sera tested, 415/430 had AChR antibody titers >0.6 nmol/l consistent with a diagnosis of acquired MG (Table 1). The 10 normal dogs had no clinical signs of muscular weakness and the AChR antibody titers were <0.6 nmol/l. An additional five dogs had muscular weakness or atrophy associated with histologically confirmed polymyositis (PM) or masticatory muscle myositis (AChR antibody titer <0.6 nmol/l). Focal MG was described in 130/415 dogs (31.3%); mild, generalized MG in 27/415 dogs

Table 1  
Titin and RyR antibodies in dogs with acquired MG<sup>a</sup>

Dogs ( <i>n</i> = 430)	Titin antibody ( <i>n</i> = 80)	RyR antibody ( <i>n</i> = 13)	RyR antibody+ thymoma ( <i>n</i> = 8)
AChR antibody >0.6 nmol/l			
Focal MG ( <i>n</i> = 130)	22	0	0
Mild, generalized MG ( <i>n</i> = 27)	0	0	0
Generalized MG ( <i>n</i> = 227)	41	7	3
Acute fulminating MG ( <i>n</i> = 5)	5	5	5
Information unavailable ( <i>n</i> = 26)	10	0	0
AChR antibody <0.6 nmol/l			
PM, masticatory muscle myositis ( <i>n</i> = 5)	2	1	0
Control ( <i>n</i> = 10)	0	0	0
Total = 430	80	13	8

<sup>a</sup> AChR: acetylcholine receptor; MG: myasthenia gravis; RyR: ryanodine receptor.

(6.5%); generalized MG in 225/415 dogs (54.7%); acute fulminating MG in 5/415 dogs (1.2%). History was not available for the remaining dogs (26/415). Thymoma was associated with MG in 20/415 dogs (4.8%).

### 3.1. Titin and RyR antibodies

Sera from 80/430 dogs were found to contain IgG antibodies that reacted with the titin epitope MGT-30 (Table 1). 78/80 of the dogs were diagnosed with MG by the AChR antibody titer  $>0.6$  nmol/l and 2/80 of the dogs had histologically confirmed PM. AChR antibody titers were significantly different between groups ( $p$ -value 0.0012; titin antibody positive group: mean AChR antibody titer 9.59 nmol/l, S.D. 7.71; titin antibody negative group: mean AChR antibody titer 6.53 nmol/l, S.D. 7.04 nmol/l). Sera containing titin antibody was further tested for the presence of antibodies against RyR. Antibodies against RyR were identified in 13/80 sera samples by Western blotting using the fusion protein pc2-RyR as antigen (Fig. 1). Neither titin or RyR antibodies were identified in the 10 control dogs. One dog with PM had RyR antibodies in the absence of detectable AChR antibodies or thymoma.

### 3.2. Clinical associations

Clinical presentations in the dogs with antibodies against titin included 22/80 dogs with focal MG, 41/80 with generalized MG, 5/80 dogs with acute, fulminating MG, and 2/80 dogs with PM. Thymoma was identified in 11/80 dogs. Nine dogs with thymoma and

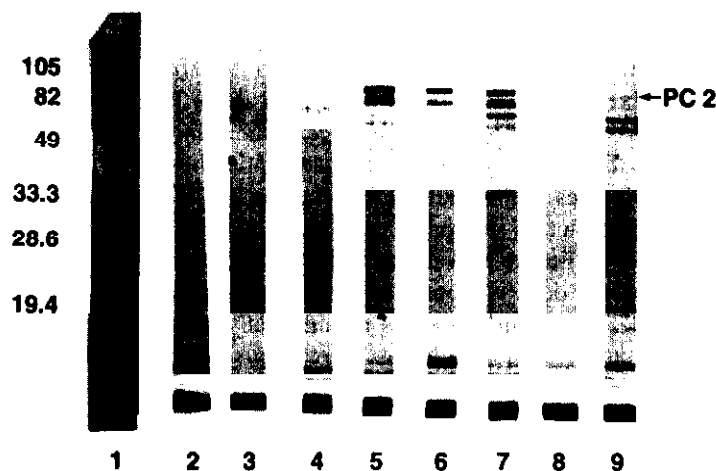


Fig. 1. Western blots of the pc2-RyR fusion protein with sera from dogs with acquired MG and control dogs. Lane 1: molecular weight markers. Lanes 2–4: canine sera positive for AChR and titin antibodies but negative for thymoma and pc2-RyR antibodies. Lanes 5–6: positive labeling of pc2-RyR fusion protein (arrow) in sera from myasthenic dogs with thymoma. Lane 7: positive control serum for antibodies against pc2-RyR obtained from a dog with thymoma and severe generalized MG. Lane 8: negative canine control serum. Lane 9: weakly positive serum from a dog with thymoma and generalized MG.

MG were titin antibody negative. Information regarding clinical presentation was not available for 10/80 dogs. 66/80 (82.5%) titin antibody positive dogs had an age of onset of 4 years or greater with 14/80 (17.5%) earlier than 4 years (this group included two dogs with PM and one dog with thymoma). In the dogs with RyR antibodies demonstrable by Western blots, 8/13 had thymoma and 12/13 had an age of onset of 4 years or greater. The acute fulminating form of MG was described in five dogs with both titin and RyR antibodies and in none of the myasthenic dogs without titin or RyR antibodies.

#### 4. Discussion

Several similarities between canine and human MG have been described including the presence of AChR antibodies (Lennon et al., 1978; Shelton, 1998, 1999), a spectrum of presenting clinical signs (Shelton et al., 1990; Dewey et al., 1997; King and Vite, 1998), and the concurrent occurrence of other autoimmune diseases (Dewey et al., 1995) and thymoma (Aronsohn et al., 1984; Klebanow, 1992). Further, there is also a good correlation between changes in the AChR antibody titer and the course of the disease within a given animal, but a poor correlation between the absolute AChR antibody titer and the severity of clinical weakness (Shelton, unpublished). In human MG, antibodies against titin and RyR have been shown to be associated with more severe disease, an older onset, or the presence of a thymoma (Mygland et al., 1992; Williams et al., 1992; Skeie et al., 1995; Aarli, 1997). This study supports similar associations in myasthenic dogs with the documentation of antibodies against titin and RyR in dogs with thymoma, an association of these antibodies with a later onset of disease, and an association of RyR antibodies with more severe disease.

Antibodies against titin were identified in 80/415 (19.2%) dogs with confirmed MG and in 2/5 dogs with PM, but in none of the control dogs. While the age range of the entire group was 1–14 years of age, the age range of the control dog sera was selected to be 4–14 years since the frequency of circulating autoantibodies, in general, increases with increasing age. Antibodies against titin were present in 11/20 dogs (55%) with thymoma and MG. Of the dogs with titin antibodies, 12/80 were reported to have acute, fulminating (King and Vite, 1998) or severe MG and died. Five of these dogs also had RyR antibody. None of the myasthenic dogs without titin antibodies were described as having severe forms of the disease. Although there was a statistically significant difference between groups, concentrations of AChR antibodies were similar in dogs with and without titin antibodies. Titin positive dogs also had an older age of onset (66/80; 82.5% with onset of 4 years or older) compared with the titin negative dogs (63% with onset >4 years of age). The age of 4 years was arbitrarily chosen to represent a later onset in dogs to approximately correlate with the age of 40 years established in a similar study in humans for later onset MG (Skeie et al., 1995).

Of the 80 myasthenic dogs with titin antibodies, 13/80 were also found to have antibodies against RyR. Of this group, 8/13 dogs (61.5%) had histologically confirmed thymoma, 5/13 (38.5%) had acute fulminating MG, and one dog had PM. The finding of 61.5% of the dogs with MG having both thymoma and antibodies against RyR is similar

to but somewhat higher than reported in human patients in which 47% (14/30) had both RyR antibodies and thymoma (Gautel et al., 1993). The dog with PM, titin, and RyR antibodies did not have detectable AChR antibody or thymoma at the time of diagnosis; however, long-term follow-up was not available. PM was also associated with titin and RyR antibodies in a human patient (Mygland et al., 1994). The association of PM, MG, and thymoma is well established in humans (Namba et al., 1974; Mygland et al., 1994) and has been described in dogs (Bellah et al., 1983; Aronsohn et al., 1984). It is possible that a thymoma may have developed at a later time in the dog with PM as detection of a neoplasm may precede or follow the onset of PM by several months (Engel et al., 1994). Thymoma has also been identified months following a diagnosis of acquired MG (Shelton, unpublished).

This study demonstrates the similarity between canine and human MG in the association of antibodies against titin and RyR with thymoma, older onset MG, and with clinically more severe disease. The functional significance of antibodies against titin, if any, is not known. The RyR, which functions as a calcium-release channel in skeletal muscle, plays an important role in the mechanism of excitation–contraction coupling and RyR antibodies could play a clinically pathogenic role in weakness associated with MG. In *in vitro* studies, it has recently been demonstrated that RyR antibodies in MG patients have high affinity for the RyR, and that the binding of antibodies probably affects calcium release from the sarcoplasmic reticulum by locking the RyR ion channel in a closed position (Skeie et al., 1998). A rat model with spontaneously occurring thymoma has also recently been described in which RyR antibodies are associated with skeletal muscle weakness in the absence of AChR antibodies (Iwasa et al., 1998). Muscle contraction studies in this model showed markedly reduced isometric twitch and tetanus of the muscle compared to normal animals. There was no difference in synaptic function between the two groups. The presence of both abnormal excitation–contraction coupling due to RyR antibodies and impaired neuromuscular transmission as a result of AChR antibodies could provide an explanation for the lack of correlation between clinical weakness and AChR antibody titers alone. Further, investigations of dogs with antibodies against AChR and RyR may provide answers to the pathogenic role of the RyR antibodies.

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