Eye muscle antibodies in Graves' ophthalmopathy: Pathogenic or secondary epiphenomenon?

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ABSTRACT. The extra ocular (eye) muscles are one of the principal tissues involved in the autoimmune-mediated inflammation of Graves' ophthalmopathy (GO). Several eye muscle proteins are targeted by autoantibodies or sensitized T lymphocytes, or both, and include: G2s, which is now identified as the terminal 141 amino acids of the winged-helix transcription factor FOXP1, the flavoprotein (Fp) subunit of the mitochondrial enzyme succinate dehydrogenase, the so-called "64kDa protein", a non-tissue specific membrane protein called 1D and the calcium binding protein calsequestrin. Of these, antibodies against G2s and Fp are the most sensitive markers of eye muscle damage in patients with thyroid autoimmunity even though neither antigen is specific to eye muscle and neither antibody is specific to GO. However, the recent finding that the calsequestrin gene is 4.7 times more expressed in eye muscles than other skeletal muscles suggests that we should reconsider the possible role of anti-calsequestrin autoantibodies in ophthalmopathy. GO may comprise two main subtypes with different pathogenetic mechanisms, namely ocular myopathy in which eye muscle inflammation predominates and congestive ophthalmopathy where inflammatory changes occur in the periorbital connective tissues in the absence of eye muscle dysfunction. Anti-G2s and anti-Fp antibodies are closely associated with the ocular myopathy subtype of GO while antibodies targeting type XIII collagen, the only member of the collagen family to have a transmembrane domain, are closely linked to congestive ophthalmopathy. Since both G2s and Fp are intracellular antigens it is unlikely that either antibody causes eye muscle fiber damage in GO, although a role in the later stages of the disease when the fiber has released its cellular contents has not been excluded. Eye muscle antibodies that are cytotoxic to eye muscle cells in antibody-dependent cell-mediated cytotoxicity (ADCC) are more likely to play a role in eye muscle fiber damage since they target a putative eye muscle cell membrane antigen, the identity of which is currently being investigated. While anti-G2s and anti-Fp antibodies are probably secondary to an underlying reaction, such as cytotoxic T lymphocyte targeting of an eye muscle membrane antigen that has yet to be identified, they are reliable markers of immunologically mediated eye muscle fiber damage in patients with Graves' hyperthyroidism. In conclusion, while a pathogenic role for eye muscle antibodies has not been excluded, they are most likely secondary to cytotoxic T cell reactions in GO and, as such, good markers of this autoimmune disease.

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INTRODUCTION

Graves' disease is best described as a "limited multisystem autoimmune disease" caused by a mixed B and T lymphocyte reactivity against the TSH receptor (TSHr), various "eye muscle antigens" and poorly characterized antigens in the orbital and skin connective tissue and fat and lachrymal gland (1). The most frequent feature, hyperthyroidism, is almost certainly caused by antibodies that activate the TSHr on the thyrocytes and is the best characterized component of Graves' disease. The eye changes, or ophthalmopathy, are characterized by immune-mediated inflammation of the extraocular (eye) muscles and surrounding orbital connective, adipose tissues. Since ophthalmopathy occurs in a small proportion of patients with Hashimoto's thyroiditis the combination of ophthalmopathy and Graves' hyperthyroidism or Hashimoto's thyroiditis is best termed thyroid-associated ophthalmopathy (TAO). However, for the sake of consistency, we will use the term GO in this chapter. Gritty and sandy sensation, increased lacrimation, photophobia, blurring of

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vision, and diplopia are the most frequent symptoms of ophthalmopathy (2) and reflect the diffuse inflammation in the orbit. On the basis of the clinical signs, orbital imaging features and serological findings, GO can be classified into two main subtypes namely; congestive ophthalmopathy, characterized by inflammatory changes in the orbital connective/adipose tissues manifest as chemosis, lid swelling, epiphora, and conjunctival injection, but with minimal abnormalities of eye muscles, and ocular myopathy, in which there is marked swelling of the eye muscles, double vision and eye muscle dysfunction but few inflammatory changes in the orbital connective/adipose tissues (3, 4). Most patients are expected to have a combination of the two, or "mixed" ophthalmopathy. Finally, chronic eyelid lag may be a separate subtype of GO that may occur alone or with other eye signs (5).

PATHOGENESIS OF THE THYROID-ORBIT LINK

The strong and unique association between ophthalmopathy and autoimmune thyroid disease is best explained by immunologic cross-reactivity against shared auto-antigens in the thyroid and orbital tissue (6-8) and this is still the best working hypothesis for the eye disease. The TSHr and various proteins identified in eye muscle by gel electrophoresis and Western blotting (to be discussed below) are the best candidates (9), even though all are expressed in other tissue as well. The significance of these "shared antigens" and the mechanism(s) underlying the association of the ophthalmopathy with thyroid autoimmunity remain to be clarified. It is also unclear whether the extra ocular muscles or orbital connective tissue is the main target tissue in the orbit or whether both may be targeted separately, as inferred from the GO subtypes notion. Apart from the TSHr, several connective tissue antigens have been identified and partly characterized including, laminin and collagen type II (10), and type XIII collagen (11), the only member of the collagen family to have a transmembrane domain (12). Of these, the TSHr is best characterized in the orbit (13, 14) and by many is considered the main target antigen in GO. A possible relationship between the TSHr autoantibodies and the eye changes (15) is controversial and needs to be confirmed. Interestingly, there is now good evidence that the TSHr is expressed in the eye muscle cell rather than the orbital fibroblast (16) which throws further doubt on the validity of the much promoted notion that ophthalmopathy is caused by cross reactivity against the TSHr in thyroid follicular cell and orbital fibroblast. It is our opinion that the role of antibodies targeting a TSHr-like protein in the pathogenesis of GO is far from certain.

TARGET TISSUES IN GO

One of the main controversies concerning the pathogenesis of GO is whether or not eye muscle inflammation and damage is a primary abnormality, as we propose (1), or secondary to inflammation in the orbital connective/adipose tissues, a notion which is held by many workers. Earlier, we proposed that the eye muscles were the primary targets of the autoimmune reaction, the changes of orbital connective/adipose tissues being the secondary (1, 17). A modification of this hypothesis is that there are two main subtypes of GO, as defined and described above, and that eye muscle and orbital connective tissue reactions are separate. We now propose that T-lymphocyte-mediated immunity against G2s/FOXP1, some other as yet unidentified eye muscle membrane protein or, possibly, the TSHr, may be the initial and key reaction that leads to the development of eye muscle damage, and that specific targeting of collagen XIII or a TSHr-like protein, or non-specific stimulation of orbital fibroblasts by immunoglobulins or cytokines, may lead to congestive ophthalmopathy. Others (13, 14, 18) believe that the initial reaction is in the orbital connective/adipose tissues and that damage to the eye muscle fiber is secondary to fibroblast stimulation, glycosaminoglycans overproduction and fibrosis, to be discussed in detail by others elsewhere in this volume.

Enlargement of the eye muscles and increased volume of orbital connective/adipose tissues are well identified changes found on orbital imaging and at surgery in patients with GO and the majority of patients with Graves' disease who have overt clinical ophthalmopathy have abnormalities of the eye muscles, such as diplopia and reduced eye movement in the various gazes. Even Graves' patients without clinical eye signs or symptoms can be demonstrated to have enlarged eye muscles on orbital computerized tomography or magnetic resonance imaging (MRI) (19), suggesting that all patients with Graves' disease might have some eye muscle involvement, a challenging notion. Infiltration of mononuclear cells, proliferation of fibroblasts, and deposition of glycosaminoglycans in the interstitium between the eye muscle fibers and in the orbital connective/adipose tissues are characteristic histological findings during the active stage of GO (20, 21) although it is unclear whether the eye muscle fiber or some connective tissue cell(s) is the target of the immune reaction. The eye muscle fibers themselves do appear to be spared from abnormalities as far as it can be determined from the few specimens studied. The infiltrating mononuclear cells in the orbital tissue consist predominantly of T lymphocytes (22). However, when orbital tissue from patients with severe and fairly recent eye disease are examined by electron microscopy, mitochondrial abnormalities are observed in the eye muscle fibers while the orbital fibroblasts appear normal (23). The mitochondria are of

larger size and have prominent clear-ballooned spaces between the cristae (23). Although those mitochondrial abnormalities could also reflect a nonspecific result of muscle fiber necrosis, the findings do support our working hypothesis that primary eye muscle damage is an early event in GO and separate from the connective/adipose tissue inflammation.

EYE MUSCLE ANTIGENS

The great majority of patients with active GO have one or more eye muscle antibodies in their serum although their significance is still unclear. None of the target antigens are expressed exclusively in the eye muscle and all are intracellular, suggesting that they would only be exposed to the immune system after eye muscle fiber damage.

Antibodies against eye muscle antigens in GO patients were first reported by Kodama et al. (24) and later by other investigators (25-29). Initially the antigens were identified according to their mol wt (MW) in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of porcine and human eye muscle membranes mainly and immunoblotting with serum from patients with ophthalmopathy (and Graves' disease without eye signs and normals as controls). The main antigens were proteins of 25-, 35-, 50-, 55-, 64- and 95 kDa and the corresponding serum antibodies were detected in 20-90% of patients with GO (8, 17, 24, 30).

The two eye muscle antigens most consistently targeted by antibodies in GO patients serum are eye muscle membrane proteins of 55 and 64 kDa MW, although reactivity against a 95 kDa protein, seen as a doublet, was also common and identified as part of a reaction "pattern" with bands at 55 and 64 kDa in the initial study (31). Serum antibodies against -55 and -64 kDa membrane antigens are also most closely related to the clinical features of ophthalmopathy and its activity (28, 29, 31-33). Antibodies targeting a "55-kDa protein", possibly different from that which we identified, were reported to be closely associated with eye muscle enlargement as demonstrated by orbital computed tomography (29). Recently, the 55-kDa eye muscle protein has been cloned, and called "G2s" (32), and later shown to be the terminal 141 amino acids of the winged-helix transcription factor FOXP1 (29, and see below). The identity of the 64-kDa eye muscle protein has been more difficult to ascertain and the subject of over 10 yr of work in our laboratory. At first we reported that the 64-kDa protein was common to both eye muscles and thyroid but not skeletal muscles (30, 31). However, "the 64kDa protein" was reported to be present in a wide variety of tissues (34) although it is impossible to know if these were the same proteins. Indeed, it turns out that there are at least 3 different "64 kDa proteins" namely, the flavoprotein (Fp) subunit of the mitochondrial succinate dehydrogenase, which actually has a mol wt (MW) of 67-kDa, a non-tissue-specific membrane protein called 1D, and the calcium binding protein calsequestrin which has a MW of 63 kDa. This was determined by Kubota et al. (35) using specific antibodies in Western blotting. The antigens that we will describe more in detail are G2s, Fp, calsequestrin, sarcalumenin and the 1D protein.

G2s/FOXP1

G2s is a novel protein shared in thyroid and eye muscles, which has been cloned by screening a human eye muscle cDNA library with human antibodies reactive with a "55 kDa eye muscle membrane protein" (32). G2s is now identified from the human genome program as the terminal 141 amino acids of the winged-helix transcription factor FOXP1. Whilst FOXP1 is expected to be expressed in the cytoplasm and nuclei of all cells, the G2s fragment has one putative transmembrane domain (32) which could be seen by antibodies and T-lymphocytes. G2s is strongly expressed in both thyroid and eye muscle, but not in orbital connective/adipose tissue. G2s is also expressed in systemic (abdominal) skeletal muscle and to a lesser extent in pancreas, liver, lung, and cardiac muscle (32). In immunoblotting, antibodies reactive with a G2s fusion protein were identified in 70% of patients with active GO, 50% with chronic GO, 40% with Graves' hyperthyroidism without evident ophthalmopathy, 20% with Hashimoto's thyroiditis, in 20% of patients with non-immunological thyroid disorders, and in 20% of normal control subjects (32). In enzyme-linked immunosorbent assay (ELISA), the tests were positive in 50% of patients with active GO, 30% with chronic GO, 40% of Graves' hyperthyroidism, 50% with Hashimoto's thyroiditis, and 10% of normal control subjects (32). Interestingly, the prevalence of positive anti-G2s antibodies was lower in black South African patients with overt GO (36), which suggests an ethnic/genetic factor

Flavoprotein

The flavoprotein (Fp) subunit of mitochondrial succinate dehydrogenase is the so-called "64kDa protein" (31), which was eventually shown to have a correct molecular mass of 67 kDa (37). Succinate dehydrogenase is a flavoenzyme consisting of the Fp subunit that contains the active site, the flavine adenine dehydrogenase (FAD) cofactor of the enzyme and an iron sulfur subunit containing three non-identical ion-sulfur clusters that catalyzes oxidation reactions. The enzyme is most highly expressed in skeletal muscle, cardiac muscle, and liver, where it is involved in energy production. Fp is bound to the matrix surface of the mitochondrial inner membrane where it is unlikely to be seen by immunocompetent cells or antibodies. Using Fp from highly purified bovine succinate dehydrogenase as antigen in immunoblot-

ting, anti-Fp antibodies are detected in approx. 70% of patients with active GO, 30% with chronic GO and in 30% of patients with Graves' hyperthyroidism without ophthalmopathy, but in only 10% of normal control subjects (37). In ELISA, anti-Fp antibodies are detected in sera from approx. 60% of patients with active GO, 5% with chronic GO, 15% with Graves' hyperthyroidism without ophthalmopathy, 20% with Hashimoto's thyroiditis, in 15% of patients with non-immunological thyroid disorders and 15% of normal subjects (37). When recombinant Fp was used as antigen in ELISA, anti-Fp antibodies were detected in 30% of patients with GO, 30% with Graves' hyperthyroidism without ophthalmopathy, 15% with Hashimoto's thyroiditis, in 15% of patients with non immunological thyroid disorders and in 5% of normal control subjects (38). The prevalence of anti Fp antibodies correlated with the number of extra ocular muscles involved clinically (39). The prevalence of anti-G2s antibodies was also extremely low in black South African GO patients (36). Kemp et al. (40), using a radio binding assay incorporating ^{35}[S]-labeled human recombinant Fp produced in an *in vitro* transcription-translation system and immunoprecipitation demonstrated a lower prevalence of anti-Fp antibodies in GO patients. The reason for this important discrepancy is unclear but may reflect methodological differences; the assay used appears to be less sensitive than ELISA incorporating recombinant human Fp as antigen since the antibodies were not detected in normals while we consistently find them in about 8-10% of subjects without ophthalmopathy (8), or case selection.

RECENT CLINICAL STUDIES OF EYE MUSCLE ANTIBODIES

Prospective study of Graves' patients treated with radioiodine (RAI)

In a preliminary study of the first 15 patients of a cohort of 31 Caucasian patients with newly diagnosed Graves' hyperthyroidism treated with RAI or antithyroid drugs and followed for up to 2 yr, none of the 4 patients treated with anti thyroid drugs developed anti-Fp or-G2s autoantibodies or demonstrated features of extra ocular disease (41). On the other hand, the antibodies were detected in 8 out of the 9 patients treated with radioiodine, in 5 cases after therapy. Of the 6 patients who had eye muscle involvement as defined by increased intraocular pressure on upgaze, diplopia or restricted motility, all received RAI and had detectable serum autoantibodies (41). None of the results were statistically significant, possibly due to the small numbers of patients so far analysed.

Eye muscle antibodies and GO subtypes

Recently, De Bellis et al. (unpublished data) determined the prevalences of antibodies against G2s, Fp and collagen type XIII in sera from patients with Graves' disease and correlated these with clinical characteristics of any ophthalmopathy. Eighty-five newly diagnosed patients with TAO who were euthyroid after methimazole therapy were studied. These comprised; 71 patients with prominent orbital connective adipose tissue (OCT) involvement [clinical activity score (CAS); congestive ophthalmopathy subtype] and mild involvement of the extra ocular muscle (group 1) and 14 with predominant involvement of extra ocular muscle (ocular myopathy subtype) without congestive signs (group 2). Also studied were 43 patients with Graves' hyperthyroidism without ophthalmopathy (group 3) and 43 healthy controls (group 4), as controls. Antibodies were measured in a standard ELISA. These results are summarized in Table 1. The prevalences of anti-G2s and anti-Fp antibodies were significantly increased in groups 1 and 2 (Students t test, $p<0.001$) compared to the control groups (3 and 4), but decreased in group 1; prevalences of anti-collagen XIII antibodies were significantly increased in group 1 compared to all groups but, in group 2, significantly increased only compared to group 4 patients ($p<0.001$). A positive correlation was observed between CAS and anti-collagen XIII antibody levels in both groups. These findings suggest that antibodies targeting the cell-membrane located type XIII collagen could be a useful early marker of congestive ophthalmopathy and for monitoring the effectiveness of steroid therapy in patients with active eye disease.

To summarize, Fp is the main target eye muscle antigen of "64 kDa MW" and we no longer test for antibodies against 1D or calsequestrin since the corresponding

Table 1 - *Prevalences of serum antibodies against eye muscle antigens and type XIII collagen in patients with GO subtypes, Graves' hyperthyroidism and normal subjects, measured in enzyme-linked immunosorbent assay (ELISA).*

Autoantibodies	Group 1 Congestive ophth. (no.=71)		Group 2 Ocular myopathy (no.=14)		Group 3 Graves' hyper. (no.=43)		Group 4 Normals (no.=43)	
	Positive (n)	%	Positive (n)	%	Positive (n)	%	Positive (n)	%
G2sAb[1]	43	60.5	4	100	15	35	4	9.3
FpAb[2]	8	11.3	13	92.8	5	11.6	3	7
coll XIIIAb[3]	31	43.6	2	14	4	9.3	1	2.3

[1]: G2sAb = anti-G2s antibodies; [2]: FpAb = anti-flavoprotein (Fp) antibodies; [3]: Coll XIIIAb = anti-collagen type XIII .

serum antibodies are found in only small proportions of patients with GO. The other main antigen is the FOXP1 protein, of which G2s is a small fragment. Because anti-Fp antibodies are also detected in patients with ocular myasthenia gravis (42) and non-specific orbital inflammation (43) these antibodies, and perhaps those targeting G2s as well, are not specific to GO. Moreover, it is likely that both antibodies can arise from damage to skeletal muscle cells other than those in the eye.

Calsequestrin

Calsequestrin is a 64 kDa calcium-binding protein localized in the lumen of the junctional sarcoplasmic reticulum and mitochondria of the skeletal muscle fiber and shares an epitope with heat shock protein 60. This protein was cloned by screening a human eye muscle expression library with patients serum containing antibodies against "the 64 kDa protein" and by micro sequencing of internal peptide fragments (44). Calsequestrin is expressed in eye muscle and other skeletal muscle but not in thyroid or fibroblasts. Antibodies against calsequestrin were detected in 40% of patients with active GO, 10% with chronic GO, 15% with Graves' hyperthyroidism without ophthalmopathy, in 10% of patients with Hashimoto's thyroiditis, and in 10% of normal control subjects, by immunoblotting (44). However, the differences compared to normal subjects were not significant (χ^2-test p=NS) for any group of patients. The recent finding that the calsequestrin gene is 4.6 times more expressed in eye muscle than other skeletal muscle (45) suggests that we should reconsider the possible role of anti-calsequestrin autoantibodies in ophthalmopathy.

Sarcalumenin

Sarcalumenin is a 160-kDa glycoprotein localized in the lumen of the longitudinal sarcoplasmic reticulum of the skeletal muscle fiber where it binds calcium (46). Sequencing a fragment of a 55-kDa porcine eye muscle membrane protein identified this antigen. In a preliminary study, antibodies against sarcalumenin were detected in 40% of patients with active GO of less than 1-yr duration but no normal subject, by immunoblotting (46).

1D protein

1D is another 64-kDa protein initially considered to be an autoantigen expressed in both thyroid and eye muscles (47, 48). The initial clone, called D_1, was obtained by Dong et al. (47) by screening a thyroid cDNA library with sera from patients with Hashimoto's thyroiditis. Subsequently, non-denatured 1D was reported to have a MW of 85 kDa (49). 1D shares homology with the transmembrane region of tropomodulin, a protein that modulates the binding of tropomyosin to actin in the thin filament of striated muscle tissues. The protein is expressed in slow fibers of the eye and other skeletal muscles as a component of myofibrils and is not upregulated in hyperthyroidism (50). Although 1D is expressed not only in thyroid and eye muscles but also in skeletal muscles, cardiac muscle, ovary, kidney and other tissue (51), its expression is greatest in thyroid and eye muscle (52).
In the first serological study reported from our laboratory, the preparation of 1D used as antigen was in the form of a fusion protein with β-galactosidase in ELISA. Antibodies against this protein were associated with thyroid autoimmunity rather than the ophthalmopathy per se (53). This was confirmed using, as antigen, full-length 1D expressed in Chinese hamster ovary cell membrane (54, 55), antibodies being detected in 40-50% of patients with GO and in 50-70% of patients with Graves' hyperthyroidism without GO (and 20% of normal control subjects), by immunofluorescence and immunoblotting. Since these studies of 1D did not clarify a role of the corresponding autoantibodies no further research has been performed. However, because Graves' hyperthyroidism can be considered the earliest stage of GO, a role is still possible. Indeed, it would be interesting to measure antibodies against 1D, Fp and G2s, correlating with signs of ophthalmopathy including orbital imaging findings.

95-kDa protein

Wu et al. (28) reported that "64 and 95 kDa porcine eye muscle antigens" were the main targets for serum antibodies from patients with GO by immunoblotting. The 95-kDa protein has not yet been characterized, and it is not known whether their 64-kDa protein is Fp - which seems most likely - or some other protein with a similar MW. Antibodies against 95 kDa were found in 50% of patients with GO but only in 9% of patients with Graves' disease without ophthalmopathy and 12% of healthy control subjects. The 95-kDa protein has not yet been characterized and remains a potential antigen of relevance to GO. Its relationship to the 55 and 64 kDa proteins also remains to be determined.

Immunoglobulin subclasses of eye muscle antibodies

There have been a few reports, demonstrating that various subclasses of eye muscle antibodies had some relationship with GO. Serum IgA antibodies against eye muscle were more often observed in patients with GO than IgG antibodies (56, 57). The presence of IgA and IgE in eye muscle has been shown in *in situ* studies. Of the muscle tissue obtained from 11 patients with GO all exhibited IgA1 positive staining of the endomysium and perimysium without staining of the muscle fibers themselves (58). Reactivity with anti-IgA1 was seen in 4 of 7 control muscles, but reactivity was much weaker. IgE-positive material was found in association with the majority of leukocytes and with muscle fibers (59). However, these findings have not been confirmed.

Eye muscle cell surface antigens targeted by cytotoxic antibodies

Antibody-dependent cellular cytotoxicity (ADCC) may play a role in the eye muscle damage of GO. Cytotoxic antibodies against human eye muscle cells in ADCC assay are found in 50-65% of patients with GO (60, 61) and levels of antibodies are greatest in patients with the ocular myopathy subtype of GO and in those with active disease (16). Positive reactivity against the orbital fibroblasts was a rare finding (62). The nature and identity of the eye muscle antigens, which are recognized by cytotoxic antibodies in ADCC remains unclear.

Animal models of GO

Animal models of GO generally support the role of TSHr in the pathogenesis of GO (63-65). In our own studies (66) we have investigated a role of eye muscle antibodies in a similar model produced by genetically immunizing BALB/C and outbred CD-1 mice with cDNAs encoding G2s and full-length human TSHr, separately or together and with or without IL4 or 12. Sera from the great majority of the experimental mice contained antibodies against a G2s fusion protein and Fp, with the highest levels being found at sacrifice (16 wk) (Fig. 1a). On histological examination of the orbits, edema, eye muscle fiber separation and mast cell infiltration in and around the eye muscles were found in the majority of experimental mice, but in no control mice. Splenocytes were transferred from G2s- immunized mice to normal syngeneic littermates. None of the host mice had serum antibodies against G2s or Fp, or histological changes, but their orbital tissue showed the same degree of lymphocyte and mast cell infiltration which was found in primary mice. No clear histological changes or lymphocyte infiltrates were observed in the thyroid or other skeletal muscle in either primary or host mice. We attempted to extend the model by genetically immunizing outbred CD-1 mice with G2s and TSHr, alone or in combination. As in the earlier studies, we attempted to enhance the reaction by co-immunizing with cDNAs for IL12 or IL4. Again, serum anti-G2s antibody levels were significantly different among the various experimental groups, particularly at 17 weeks, when they were significantly greater than in control animals. Anti-Fp antibodies were detected after 6 weeks, in all experimental groups, including those immunized with G2s only, and were highest in the group immunized with TSHr alone (Fig. 1b). TSHr antibody levels were highest in mice immunized with both G2s and the TSHr in the presence of IL4, but not IL12. Histological changes in the orbit, which included eye muscle fiber dissociation by edema and lymphocyte and mast cell infiltration, were detected in 80% of experimental mice at sacrifice. The greatest changes were observed in animals immunized with both G2s and TSHr. The finding of negative anti-G2s but positive anti-Fp antibodies in some mice suggests that eye muscle damage and Fp release must have been mediated by T lymphocytes targeting G2s or some other as yet unidentified cell membrane antigen. In both experiments the majority of mice immunized with G2s developed mild proptosis while all experimental mice had eyelid changes and decreased blinking reflex. We were generally unable to distinguish between mice immunized with G2s and those immunized with TSHr (65). Although this model does not exactly resemble human GO the results do support a role for anti-G2s antibodies in GO.

CONCLUSIONS

The studies to date cannot conclusively indicate whether the eye muscle antibodies play a role in the pathogenesis of the eye muscle component of GO, which we call ocular myopathy, or reflect an epiphenomenon to tissue damage. Eye muscle antibodies are proven and good markers of eye muscle damage in patients with Graves' hyperthyroidism (67). Since Fp is found within the mitochondrial membrane, it is unlikely that Fp antibodies play a role in its pathogenesis. It should however be pointed out, that in other autoimmune diseases such as Type 1 diabetes and Hashimoto's thyroiditis, identified serum autoantibodies are probably not pathogenic either but no one doubts that they are important as markers of the underlying immunologic process leading to destruction of the beta islet cells and thyroid follicular cells, respectively. Although it has not been proved, the primary reaction in the eye muscle may be T-cell autoimmunity against G2s or some other as yet unidentified membrane antigen, possibly the same antigen(s) targeted by cytotoxic antibodies in ADCC. The mechanisms for development of eye muscle antibodies in normal subjects and patients without ophthalmopathy is unclear, but may reflect non-specific skeletal muscle damage of muscle, liver or connective tissue and the tendency to multiple autoantibody production in autoimmune and connective tissue disorders. Moreover, it is often difficult to clinically exclude ophthalmopathy so that some antibody-positive patients with Graves' hyperthyroidism or Hashimoto's thyroiditis, or multi-system autoimmunity, may have early or mild ophthalmopathy.

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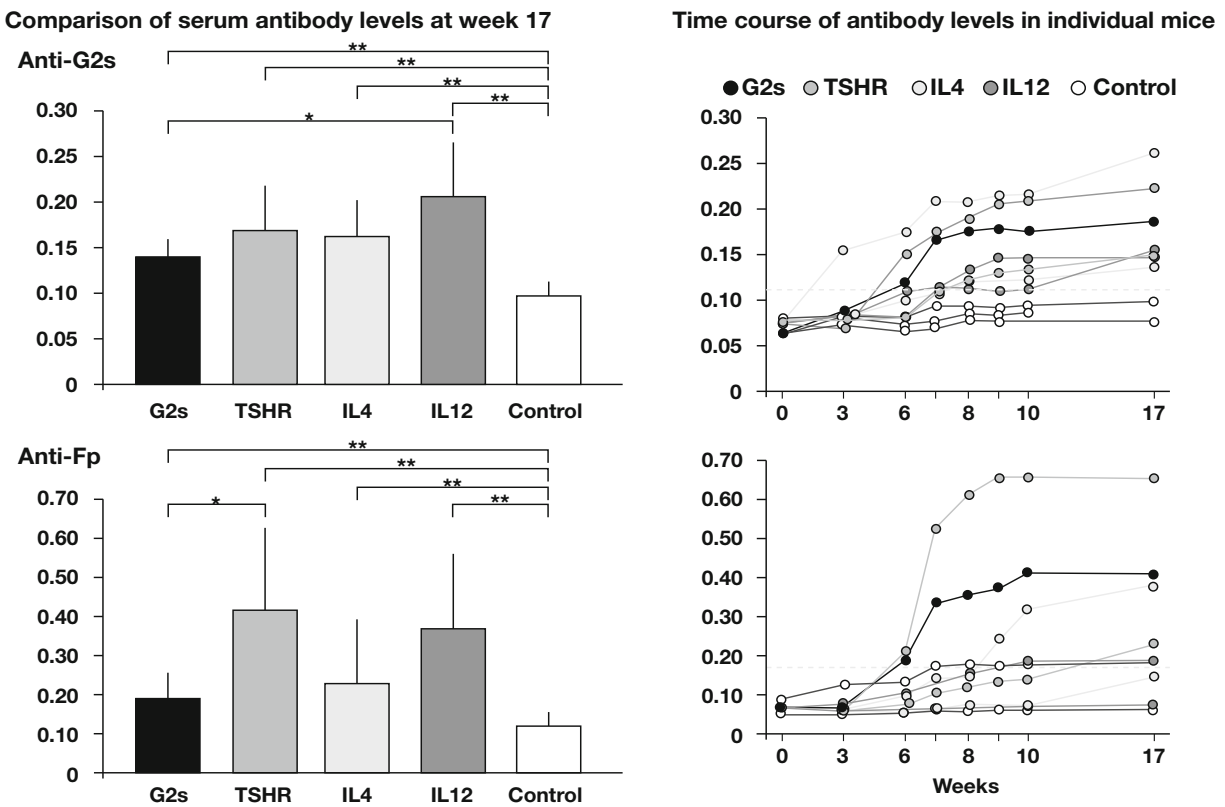

Fig. 1 - *Mean (+/- SD) serum antibodies against G2s and Fp (left panels), and levels in individual mice (right panels) in CD-1 mice genetically immunized with the G2s fragment of the FOXP1 protein (G2s), full length TSH receptor (TSHR), G2s + TSHR, G2s + TSHR + interleukin (IL)4 or G2s + TSHR + IL12. Control mice were immunized with carrier only. Results are expressed as OD at 405 nm in enzyme-linked immunosorbent assay (ELISA). The broken horizontal lines (right panels) are the upper limits of normal, calculated as mean + 2SD for the control mice. Reprinted with permission from ref. (65).*
**: Unpaired t test p<0.05; **: p<0.01*

NOTED ADDED IN PROOF

Recent data from De Bellis et al. (Clin Endo 59: 388-395, Clin Endo "in press", Clin Endo "submitted") add strong support for a role of orbital antibody testing in GO . They showed greater specificity of anti-G2s and anti-collXIII antibody tests, and less false positives, than in our earlier studies; levels of both antibodies correlated closely with disease severity and activity, especially diplopia, in patients with GO during treatment with steroids, while there was no such relationship with TSHr antibodies. Serum levels of anti-collXIII Ab were much lower, or negative, in patients with inactive ("burnt out") eye disease and both tests were usually negative in patients with no evident eye disease. Anti-collXIII Ab were closely linked to congestive features, consistent with our support for ophtalmopathy subtypes.

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