Re-evaluation of eye muscle autoantibody determination in Graves' ophthalmopathy: failure to detect a specific antigen by use of enzyme-linked immunosorbent assay, indirect immunofluorescence, and immunoblotting techniques

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Abstract. Sera from 41 patients with Graves' ophthalmopathy were investigated for presence of autoantibodies directed against eye muscle preparations using different methods: 1. ELISA with pork eye muscle membrane preparations; 2. Immunoblotting with glycoprotein preparations from human eye muscle; 3. Indirect immunofluorescence with human eye muscle sections. The ELISA was not suitable for detection of specific immunoglobulin binding with sera from patients suffering from endocrine ophthalmopathy. Immunoblotting exhibited only nonspecific binding to some muscle proteins; it could be prevented by pre-adsorption procedures and was not different from the pattern observed with skeletal muscle as antigen. The indirect immunofluorescence technique revealed no binding of Graves' sera to human muscle sections, whereas sera containing antibodies against skeletal muscle bound to eye muscle as well. Thus far, an antigenic structure of eye muscle specific for Graves' ophthalmopathy is not detectable with the methodology used here. The possibility that retroorbital connective tissue may be the main target of the autoimmune process must be considered.

Graves' ophthalmopathy (GO) is characterized by its common association with Graves' thyrotoxicosis (1), which is now considered as an autoimmune disease (2). However, the loose temporal relationship of the two diseases (3) as well as the detection of peripheral blood lymphocytes specifically sensitized to eye muscle or muscle membrane preparations (4–6) indicate that ophthalmopathy probably comprises a separate autoimmune disease. The mononuclear cell infiltrate is mainly present in the extraorbital eye muscles and at the site of the muscle membranes suggesting a reaction to eye muscle specific antigens (7–10). The purification of these target antigens is crucial to the study of autoimmunity in this disease and to the development of specific diagnostic tests.

Histological studies and computed tomography of the orbits (11) now suggest that extraocular muscles are abnormal in Graves' ophthalmopathy. With partially purified soluble eye muscle antigen, using monoclonal 'orbital antibodies' as probes, Kodoma et al. (12) detected circulating eye muscle autoantibodies in 17 out of 23 patients with Graves' ophthalmopathy. However, the antibodies were also positive in patients with Hashimoto's thyroiditis and subacute thyroiditis. Using a 100 000 X g pellet fraction of porcine eye muscle as well as Guinea pig Harderian gland and an ELISA system, Kendall-Taylor et al. (13) described an eye-muscle-specific binding of GO sera. With the same antigen preparation and detection method, Ahmann et al. (14) were unable to reproduce these results. Furthermore, the high specificity of the ELISA with the 100 000 X g fraction as well as with an 80 000 X g fraction of bovine extraorbital eye muscles was
not confirmed by Bemetz et al. (15). Recent results by Miller et al. (16) using an immunoblot analysis and of Mengistu et al. (17) using indirect immunofluorescence suggested a high specificity of the latter methods. However, these reports have not been confirmed so far. The aim of our study was to re-evaluate the above mentioned antibody tests and to apply further methods to the purification and characterisation of antigens which are targets for circulating autoantibodies in patients with Graves' ophthalmopathy.

Material and Methods

Sera
Sera from 41 patients with Graves' disease with overt endocrine ophthalmopathy were investigated; 23 patients presented with grade IV ophthalmopathy according to the classification of Werner (18), 16 with grade III, and 2 with grade II. The eye disease was still active with clinical signs of inflammation when the sera were drawn in 22 patients. In 19 patients, exophthalmos and/or diplopia persisted without inflammatory signs at the time of investigation. No patient received steroids at the time of blood sampling. Eight patients had received steroids or undergone orbital radiation before. Nine patients had to be treated with steroids or radiation shortly after blood sampling i.e. the eye disease was highly active when sera were won.

Out of the 41 patients, 26 (63.4%) were positive for TSH receptor autoantibodies at the time of investigation.

Eighteen healthy subjects without autoimmune endocrine disorders served as controls. They showed neither antibodies against islet cells, adrenal gland, parietal cells and thyroid gland nor antimicrobial antigens.

In ELISA experiments, 14 patients suffering from diffuse toxic goitre without endocrine exophthalmos served as a second control group.

TSH receptor autoantibodies
Autoantibodies against the thyroidal TSH receptor were determined using a commercial receptor assay (TRAK®, Henning, Berlin).

Tissues
Tissues from pork external eye muscles were obtained from the local slaughterhouse immediately after the animals had been killed. The tissues were at once put on ice. After shock-freezing in liquid nitrogen, particles were mechanically minced and homogenized. The tissue suspension was filtered through three layers of cheese-cloth and centrifuged at 800 × g. After separation of the pellet with nuclei, the supernatant was further centrifuged at 100,000 × g. The pellet was resuspended in TRIS-HCl buffer (protein concentration adjusted to 1.5 or 3.0 g/l) and stored at −80°C until further use in ELISA experiments.

Tissue from human external eye muscles were obtained from ophthalmological surgery for strabism. After resection, tissue particles were immediately put into ice-cold DMEM medium and stored in liquid nitrogen until use in the immunoblot experiments.

Human eye muscle membrane preparation
Tissues were pottered in a hypotonic lysis buffer (10 nmol/l TRIS-HCl, 1 mmol/l phenyl-methyl-sulfonyl-fluoride (PMSF), 4 mmol/l EDTA, pH 8.6) using an Elvehjem potter device at 4°C. Nuclei were separated by centrifugation at 800 × g and supernatants were centrifuged at 100,000 × g for separation of membranes. The pellet containing membranes and microsomes was resuspended in a lysis buffer consisting of 50 mmol/l HEPES, 150 mmol/l NaCl, 10,000 IU/l aprotinin, 1 mmol/l PMSF and 0.4% Triton X-100. Protein concentration was adjusted to 400 mg/l, the suspension gently mixed on ice for 90 min and then either used in immunoblot experiments immediately or again stored in liquid nitrogen.

Purification of glycoproteins
For purification of glycoproteins as antigens out of porcine eye muscle preparations, one tissue sample was subjected to affinity chromatography on a lentil-lectin-sepharose 4B column (Pharmacia-LKB, Freiburg). Lentil lectin binds α-D-glucose and α-D-mannose residues and therefore retains solubilized glycoproteins. Cytosol and membrane preparations were pre-adsorbed with 2 ml lentil-lectin-sepharose at 4°C overnight to obtain efficient binding of the slow-binding glycoproteins. After loading the column with this preparation, extensive washing was performed with PBS, and glycoproteins were eluted with PBS containing 0.5 mmol/l methyl-2-D-mannopyranosid.

Enzyme-linked immunosorbent assay
Nunclon microtitre plates (NuncIntermed, Roskilde, Denmark) were coated over 12 h at 4°C with porcine eye muscle membrane preparations as antigens in concentrations varying from 10 to 400 mg/l. Plates were incubated with sera in dilutions of 1:5 to 1:100 for 4 h at room temperature or for 2 h at 36°C. After washing three times with PBS, anti-human IgG conjugated to horseradish-peroxidase (Dianova, Hamburg) was added in dilutions of 1:10 to 1:1000. O-phenyldiamin or 3,3’-diaminobenzidin were used as substrate. Extinction was measured with a ‘Titertec Multiskan’ ELISA reader (Dynatech) at 492 nm.

In another series of ELISA testing, Nunc Immunoplates (Maxisorp F 96, NuncIntermed, Roskilde, Denmark) were coated with the glycoprotein preparation of porcine eye muscle membranes (20 mg/l coating buffer) and air-dried under a sterile hood overnight at room temperature to facilitate especially the binding of glyco-
proteins to the polystyril surface. Plates were then incubated with sera in dilutions of 1:1 and 1:10 to 1:100 for 4 h at room temperature or for 1 h at 37°C. After three washes with PBS, anti-human IgG conjugated to a biotin-streptavidin-β-galactosidase-complex was added. P-nitrophenyl-β-D-galactopyranosid served as substrate: extinction was measured at 405 nm. Washing buffers did not include any detergent in order to prevent antigen alteration or desorption of glycoproteins or glycolipids and gangliosides from the plates.

**Polyacrylamide gel electrophoresis**

Proteins were separated according to molecular weight by sodium dodecyl sulphate (SDS--) polyacrylamide gel electrophoresis (PAGE) (7.5%, 18 mA for 20 h or 30 mA for 7 h) as described by Laemmli (19), using a Bio-Rad Protean II Slab Cell (Bio-Rad, Munich, FRG). The gels were stained with Coomassie Brilliant Blue and then with a silver staining system (Bio-Rad). The relative molecular mass (mol wt) of bands was assessed using the following mol wt standards (Bio-Rad): myosin (200 000), β-galactosidase (116 000), phosphorylase b (93 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (20 000), lysozyme (14 000).

**Western blot**

Electrophoresed antigens were transferred onto nitrocellulose sheets (0.25 μm; Schleicher & Schuell, Dassel, FRG) by an electroblot according to Towbin et al. (20) at 37°C with 70 V for 4 h (transfer buffer: 20 mmol/l TRIS, 192 mmol/l glycine, 20 vol% methanol, pH 8.3). Unspecific binding to the filters was blocked with PBS containing 10% non-fatty dry milk powder (Merck, Darmstadt, FRG), 100 IU/ml aprotinin and 2000 IU/l insulin.

Patients' sera were used in a dilution of 1:25 in blocking buffer. A goat-antihuman-IgG-peroxidase-complex (Dianova, Hamburg, FRG) was chosen for detection of antibody binding (dilution 1:250, in PBS); 4-chloro-1-naphthol served as substrate.

**Pre-adsorption with platelets**

In female patients, anti-HLA-antibodies, even in high titres, are frequently present and give false positive results with HLA class I and related molecules. Therefore, sera were pre-adsorbed with an excess of platelets (pools from 10 blood donors) for 4 h at room temperature to eliminate or reduce anti-HLA class I immunoreactivity.

**Indirect immunofluorescence for eye muscle antibodies**

Four micron cryostat sections were cut from human adult and human fetal eye muscles, respectively. Tissue sections were mounted on glass slides, air-dried, and subjected to antibody testing using the indirect immunofluorescence method as described previously (21).

Patients' and control sera were incubated at room temperature for 30 min in dilutions of 1:5, 1:10, 1:20, 1:40 and 1:80. Washing steps were done in PBS, pH 7.4, with gentle agitation for 20 min at 4°C. Fluorescinated goat-anti-human IgG (Dianova, Hamburg, FRG) was used as a conjugate in a dilution of 1:40. The avidin-biotin method was also employed with all sera. The reaction was read with a Zeiss fluorescence microscope at a magnification of 1:400 by one observer (W.A.S.) without knowledge of whether the investigated sera originated from patients or controls.

**Results**

**ELISA with porcine eye muscle membrane preparations**

Using the ELISA technique described, we failed to detect in the sera from patients with endocrine exophthalmos antibodies specifically directed against cell membrane structures of porcine eye muscles. No significant differences in binding ac-
tivity were seen between patients' and control sera. Under varying experimental conditions, immunoglobulin binding was weak, and no marked differences in binding were seen between patients with active or treated and longer persistent ophthalmopathy or patients with thyrotoxicosis without endocrine ophthalmopathy.

Fig. 1 shows data from the experiment exhibiting the strongest binding of sera to the membrane preparation. Dilution of membrane fraction as well as of sera was 1:100. One female patient with endocrine exophthalmos grade IV, who had been treated with steroids before serum was collected, gave an OD far above the other values. Three other patients with Graves' ophthalmopathy and two with thyrotoxicosis without Graves' ophthalmopathy exhibited an OD above the upper limit of normal which could also indicate 'specific' binding activity.

**Immunoblotting with human eye muscle preparations**

Immunoblots of human eye muscle cytosol fraction with the sera of patients with active endocrine exophthalmos reveal a common band with a molecular weight of about 55 kD, which is also seen with a control serum (Table 1). Only sera No. 3, 5 and 9 bind to other proteins, especially sera No. 3 and 5 exhibit a high reactivity against multiple bands. However, after pre-adsorption of nonspecific antibodies directed to HLA class I molecules and others by pre-incubation with nonspecific antibodies, these reactive sera also bind only to the common 55 kD band.

In comparison, the same sera were incubated with a cytosol preparation of striated skeletal muscle generated by differential centrifugation, and revealed specific binding to one to three pro-

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**Fig. 2.**

Indirect immunofluorescence test on human eye muscle preparations with serum from a patient with Graves' ophthalmopathy (a) and positive control sera containing anti-fibrillatory antibodies to skeletal muscle (b: longitudinal section, c: cross section).
tein bands of serum No. 3, 6, 7, 9, 12 and 17. All these six sera bound to a protein of about 40 kD, the other binding sites were unique in the individual blots.

Pre-adsorption to thrombocytes decreased the number of binding sites again. Binding to the 40 kD protein was preserved in 4 sera, one further site at 66 kD was observed in serum No. 12.

Using membrane fractions of human eye muscle and skeletal muscle also prepared by differential centrifugation, comparable observations were made, but fewer bands were detected. Five patients showed a 55 kD band again with eye muscle membranes, still after pre-adsorption with thrombocytes. A 66 kD band (this is within the molecular weight range of a HLA class I molecule) stained with another patient’s serum disappeared after pre-adsorption.

Indirect immunofluorescence test for eye muscle antibodies

All the sera from patients with endocrine exophthalmos were negative in this assay (Fig. 2a).

The sera from patients with acquired myasthenia gravis containing anti-sarcolemmal and anti-fibrillar antibodies to skeletal muscle were taken as positive controls and tested on human adult and human fetal eye muscle (22). The immunofluorescence pattern was no different, irrespective of the source of striated muscle used (Fig. 2b and c).

Discussion

Characterization of the relevant autoantigens and development of a disease-specific autoantibody test in Graves’ eye disease would be beneficial for the diagnosis. Especially in cases where thyrotoxicosis is not associated with clinically overt Graves’ ophthalmopathy in the initial phase (3), specific autoantibodies could serve as a marker of an already present retrobulbar inflammation. It would also allow insight into the immunobiology of the disease as well as new approaches for early intervention in severe ophthalmopathy (23–25).

Conflicting data have been reported on the detection of reactivities regarded as candidates for autoantibodies in Graves’ ophthalmopathy, employing a great variety of cytosolic and membrane preparations of extraorbital eye muscles (13, 15, 16, 26). We have re-evaluated some of the previous ELISA and immunofluorescence tests and introduced immunoblotting of electrophoretically separated proteins from cytosolic and membrane preparations of human eye muscles. It appears from our studies that there exists no reliable method so far to detect GO-related or even specific autoantibodies.

A paper published by Mengistu et al. (17) suggests that autoantibodies specifically directed to the cytoplasm of extraorbital eye muscles can be detected by indirect immunofluorescence testing and that the presence in the serum of these antibodies is closely correlated with Graves’ ophthalmopathy. However, the figures given in that report show the pattern of anti-fibrillary antibodies, which are typical of autoimmune myasthenia gravis (27; see also Fig. 2b and c of this paper), a disease occurring in patients with Graves’ disease with a 30-fold higher frequency than in the general population (28). The antibodies were reported as positive in more than 70% of GO sera, whereas none of 39 sera from normal subjects contained these specificities. With the same, well-established immunofluorescence method (21) we were unable to detect eye muscle antibodies, either in the sera studied here, or in an additional lot of sera from 100 patients with exophthalmos (data not shown). As suggested by all previous investigators, our primary serum dilution for the detection of muscle-specific autoantibodies was 1:15 or above, because many normal human sera give the fibrillary pattern when they are applied undiluted.

Using various preparations of eye muscles in an ELISA, Miller et al. (16) failed to reproduce the results of Kendall-Taylor (13) suggesting a high specificity of eye muscle antibodies in GO sera.

Our studies are in accordance with the results of the former group (16). We too used a number of methods to prepare cytosolic and membrane antigens from eye muscles for ELISA assays, including various methods of coating such as PBS, carbonate buffer and air-drying.

Immunoblotting of electrophoretically separated antigens offers one of the most specific methods to detect antibodies directed to a mixture of non-purified protein antigens. In their above-mentioned paper, Miller et al. (16) suggested that specific bands are discriminated by Graves’ sera after electrophoresis and electrophoretic solubilization porcine eye muscles.
Ahmann et al. (14) used SDS-PAGE of the 10 000 and 100 000 × g sediment fractions of porcine eye muscle and of peripheral skeletal muscle and immunoblotting of sera for antibody detection. Eight unique eye muscle determinants of < 50 kD were detected in the serum from 6 out of 13 patients with Graves’ ophthalmopathy. Serum from 3 patients with Graves’ disease recognized a 50 000 antigen, but no single band was common to serum from all the 6 patients with Graves’ disease. The 50 kD band may be identical with our 55 kD band and represent a cytoskeleton protein, possibly the one immunoprecipitated from membrane preparations of various tissues by sera of patients suffering from autoimmune thyroid disease and identified as the intermediate filament protein desmin with a mol wt of 54 kD by Furmaniak et al. (29).

Using the 100 000 × g pellet of human eye muscle and a muscle membrane preparation with a TRIS-HCl and EDTA lysis buffer, SDS-PAGE, and Western blot technique, we were able to detect two sera recognizing multiple bands which were not stained by control sera. However, these reactivities could be removed by pre-incubation with thrombocytes and are thus to be considered nonspecific, interacting with HLA or Ia-like molecules, which are highly prevalent, for example, in female patients after pregnancy.

We share the cautious suggestion of Ahmann et al. (14), that there is a potential antigenic difference between eye muscle and skeletal muscle which accounts for the immunological specificity of eye muscle as a target in Graves’ ophthalmopathy.

However, neither our own results nor the work of other researchers were able so far to reveal the common antigen in Graves’ ophthalmopathy.

The possibility that retroorbital connective tissue may be the main target of the autoimmune process must be considered (30). We therefore propose that antigen preparation of retroorbital tissue from patients with Graves’ ophthalmopathy may provide further insight into the pathogenesis of endocrine exophthalms.

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