T and B cell reactivity to extraocular and skeletal muscle in Graves' ophthalmopathy

A P WEETMAN,¹ P FELLOWS,² AND B SHINE²

From the ¹Department of Medicine, University of Cambridge Clinical School, Addenbrooke's Hospital, Level 5, Cambridge CB2 2QQ, and the ²Institute of Ophthalmology and Moorfields Eye Hospital, London EC1V 9AT

SUMMARY We have sought human eye muscle membrane binding antibodies in patients with Graves' ophthalmopathy using an enzyme-linked immunoassay. Antibodies were found in patients with thyroid autoimmunity irrespective of eye signs, and binding correlated closely ($r=0.94$) with binding to skeletal muscle, showing that these antibodies are not site-specific. T cells from patients with thyroid autoimmunity proliferated in response to eye muscle, but again this was not specific for eye muscle or the presence of ophthalmopathy. No single antigen was responsible for inducing proliferation. These results fail to confirm a recent report of eye muscle membrane binding antibodies in a high proportion of patients with ophthalmopathy, and suggest instead that T and B cell autoreactivity to striated muscle antigens is a frequent feature of autoimmune thyroid disease, unlikely to be directly related to eye disease.

Graves' ophthalmopathy appears to be an autoimmune disease, but despite extensive studies, reviewed recently,¹ the immunological mechanisms responsible for the condition have not yet been elucidated. Studies of both retrobulbar pathology and the evolution of disease as revealed by CT scanning have shown that the extraocular muscles are the primary site of involvement.² The eye muscles become infiltrated by lymphocytes early in the course of disease, with subsequent oedema; this leads to fibrosis and muscle fibre atrophy later.

Further understanding of Graves' ophthalmopathy should come from elucidation of the antigens recognised by these autoreactive cells, since this would aid identification of the pathogenic components of disease – for example, whether autoantibodies or cytotoxic cells are involved. The possibility that antibodies against eye muscle antigens are a feature of ophthalmopathy was suggested by recent studies using porcine eye muscle as an antigen in an enzyme linked immunosorbent assay (ELISA) in which 64% of patients with Graves' ophthalmopathy were positive, compared with only one of 22 Graves' disease patients with no clinical evidence of eye disease.³ However, others have failed to obtain such clear results,^{4,5} and the possibility exists that

species differences may preclude reactivity of positive human sera in such an assay. T cell responses to retrobulbar antigens have also been assessed by a variety of assays, but again with conflicting results.⁶⁻⁸ The T cell proliferative response to retrobulbar antigens has not to our knowledge been assessed previously, though this is a simple test of T cell sensitisation; lacrimal gland extracts do stimulate T cell division in about 40% of Graves' ophthalmopathy patients.⁹

In the present study we have tested T and B cell reactivity to human skeletal and eye muscle antigens, using proliferative responses and an ELISA respectively, with the aims of identifying any autoantigens which are unique to the eye and which elicit responses only in patients with Graves' ophthalmopathy.

Materials and methods

PATIENTS

Samples were obtained from the following group of subjects (1) Graves' ophthalmopathy ($n=23$, three men), mean age (SD) 58(15) (range 14-84), all with grade III or greater eye changes,¹⁰ which were active or had been treated within the preceding six months. Nineteen patients also had Graves' disease; the

Correspondence to Dr A P Weetman.

remaining four were ophthalmic Graves' disease, with hypothyroidism in three. (2) Graves' disease ($n=18$, seven men), mean age 40(14) (range 19-65), all with minimal (grade 0-II) eye changes; 11 were untreated and seven were receiving carbimazole, (3) Hashimoto's thyroiditis ($n=14$, two men), mean age 50(17) (range 19-75). (4) Normal controls ($n=16$, six men), mean age 36(12) (range 18-52) taken from hospital staff with no history of thyroid disease.

ANTIGEN PREPARATION

Snap-frozen extraocular muscle (EM) from surgery for an intraocular malignancy, and skeletal (arm) muscle (SM) from an amputation, were minced and homogenised at 4°C in phosphate-buffered saline (PBS), pH 7.4, in a Polytron homogeniser (Kinematika, Lucerne, Switzerland). The homogenates were spun at 2000 g for 20 minutes at 4°C and the pellets discarded. The supernatants were further centrifuged at 10 000 g for 40 minutes at 4°C and the resulting membrane fractions, in the pellets, used as antigens. The protein concentration in each antigen was determined spectrophotometrically with Coomassie blue G250¹¹ and the antigens stored in aliquots at -70°C.

ELISA

Preliminary experiments revealed that both antigens contained IgG in concentrations sufficient to cause high background readings in the ELISA. Prior to coating assay plates, therefore, the antigen preparations were sonicated (4×15 s at 15 kHz) on ice and then incubated with Protein-A-Sepharose beads from Pharmacia, Uppsala, Sweden (1 mg antigen in 1 ml PBS with 0.5 ml packed beads) for 15 minutes on a rotator; antigen was recovered by centrifugation and used to coat ELISA plates overnight at 4°C with 10 µg antigen/ml coating buffer (carbonate buffer; pH 9.6). The next morning the plates were washed and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour. Serum was tested at 1:100 dilution in PBS-0.05% Tween 20. After two hours at room temperature the plates were washed and incubated with 1:1000 dilution of antihuman IgG alkaline phosphatase conjugate (Sigma) for a further two hours. After the washing a substrate (*p*-nitrophenyl phosphate 1 mg/ml in carbonate buffer plus MgCl₂ 100 µg/ml, pH 9.6 was added and the absorbance at 405 nm read on a Titertek Multiskanner (Flow Labs, Irvine, Scotland). Results are the mean of duplicate estimates. Variation between duplicates never exceeded 15% and was usually less than 10%.

BLASTOGENESIS

Peripheral blood mononuclear cells (PBM) were

isolated on Ficoll-Hypaque gradients and incubated at 10⁵ cells/well of a 96-well flat-bottomed plate in 200 µl final volume of culture medium (RPMI 1640 with 10% fetal calf serum). Sonicated muscle antigens (sterilised by irradiation) were added in varying concentrations at the beginning of culture. After four days [³H]-thymidine, 1 µCi/well, was added and the cells harvested on to filters 16 hours later; [³H]-thymidine incorporation was measured by liquid scintillation counting and results expressed as counts per minute (cpm). All assays were performed in triplicate, and control as well as patient lymphocytes were included in each set of cultures.

To delineate putative antigens further we used the fractionation method described by Abou-Zeid *et al.*¹² In brief, muscle antigens were separated on a 5-15% sodium dodecyl sulphate (SDS)-polyacrylamide gradient gel and transferred electrophoretically to nitrocellulose; conditions were as defined previously for porcine eye muscle.⁵ The transferred proteins were then divided into 4 mm band fractions by cutting the nitrocellulose into 20 pieces, 4 mm deep and 10 mm wide. These pieces were dissolved in dimethylsulphoxide and resuspended, in particulate form, in 1 ml carbonate buffer, pH 9.6. The fractions were then washed, resuspended in culture medium, and used at 20 µl/well for stimulation of blastogenesis.

Results

ELISA

The results of antibody binding to EM by ELISA are shown in Fig 1. With the mean absorbance of the control sera plus 2 SD taken as the upper limit of normal, 2(11%) of the ophthalmopathy sera gave

Fig. 1 ELISA using eye muscle as coating antigen. The dotted line represents 2SD above the control mean. GO=Graves' ophthalmopathy, GD=Graves' disease, HT=Hashimoto's thyroiditis.

Fig. 2 Correlation between ELISA for reactivity against eye and skeletal muscle; $r=0.94$, $p<0.0001$. GO=Graves' ophthalmopathy, GD=Graves' disease, HT=Hashimoto's thyroiditis.

positive binding, compared with 4(22%) of the Graves' disease sera and 9(64%) of the Hashimoto's disease sera. The absorbance values did not differ significantly between controls and Graves' disease patients with or without ophthalmopathy, but the Hashimoto sera absorbances were significantly higher than those of controls ($p<0.01$, Wilcoxon rank test). When the absorbances produced by binding to EM were compared with those against SM, a significant correlation was found, as shown in Fig 2 ($r=0.94$, $p<0.001$).

T CELL BLASTOGENESIS

Using 100 μg of EM/ml culture medium, we found the PBM from thyroid disease patients ($n=13$) showed significant stimulation of proliferation over background ($p<0.01$, paired t test), but this had no obvious relationship to the presence of eye signs. These results are shown in Fig. 3 as the difference in cpm between cultures in the presence and in the absence of antigen. By comparison, PBM from control subjects ($n=7$) showed no stimulation with EM antigen. The response was dose dependent, being maximal at 100 μg antigen/ml (Table 1). Again there was a significant correlation between the blastogenic response to EM and SM, both at 100 μg /ml, as shown in Fig. 4 ($r=0.98$, $p<0.001$).

To identify further the antigens responsible for stimulating T cell proliferation the EM was

Fig. 3 Blastogenic response of peripheral blood T cells to eye muscle membranes (100 $\mu\text{g}/\text{ml}$). Results are shown as the difference in cpm between cultures with and without antigen. To the left of each column is the group mean ± 1 SD. Probability values are given for a paired t test between cultures with and without antigen. GO=Graves' ophthalmopathy, GD=Graves' disease, HT=Hashimoto's thyroiditis.

fractionated by polyacrylamide gel electrophoresis; 28 bands were identifiable by Coomassie blue staining. These were all transferred to nitrocellulose as verified by amido black staining of the nitro-

Table 1 Dose response for various concentrations of eye muscle antigen in T cell blastogenesis

	Patient	Antigen concentration ($\mu\text{g}/\text{ml}$)			
		Nil	100	10	1
Graves' ophthalmopathy	1	538*	932	701	517
	2	1105	1863	1487	1298
	3	193	2866	352	NT
	4	939	1202	1572	1302
Graves' disease	1	5549	10800	5588	NT
	2	4213	6767	4165	NT
Hashimoto's thyroiditis	1	1089	5013	3164	NT
	2	1695	4537	2314	NT

*Values are mean (of triplicate) cpm. Replicates were always within 15% of the mean. NT=not tested.

Fig. 4 Correlation between the blastogenic responses to eye and skeletal muscle both at 100 µg/ml; $r=0.98$, $p<0.001$. Results are expressed as in Fig. 3. The open circles are patients with Graves' ophthalmopathy, the open triangle Graves' disease, and the solid triangles Hashimoto's thyroiditis.

cellulose. The dissolved fractions corresponding to the separated antigens were added to cultures of PBM from seven patients with ophthalmopathy and two patients with Hashimoto's thyroiditis; control cultures were also set up with nitrocellulose free of antigen. In five sets of cultures there was no striking stimulation of T cell proliferation with any single fraction, though in all cases some fractions stimulated proliferation 2-3 fold above the control values. In the remaining four cultures stimulation was seen with certain fractions that was 4-13 fold above control values (Table 2). These fractions were numbers 2 and 3 (approximate MW 72-78 kd) for 2 patients, 5 and 6 (approximate MW 52-56 kd) for one patient, and fraction 20 (approximate MW 12 kd) for the other patient. This latter patient had Hashimoto's

thyroiditis. It can be seen in Table 2 that there was often considerable variability between the triplicates, reflected by a high SD; this probably represents a low precursor frequency of cells responsive to certain antigens, as discussed by others.¹²

Discussion

These results show that B and T cells are sensitised to eye muscle antigens in patients with autoimmune thyroid disease, irrespective of clinically apparent ophthalmopathy, and these antigens are common to striated skeletal muscle. We have used a human eye muscle antigen preparation, in contrast to previous studies with porcine material^{3,5}; this gave a low background once IgG had been removed. Previous studies with human eye muscle membranes have given conflicting results in an assay using protein A to detect immunoglobulin binding, but IgG was not depleted prior to using the antigen.^{13,14} Our results confirm recent studies showing that porcine muscle membrane-binding antibodies are not tissue specific.^{4,5} In the present series only the group of patients with Hashimoto's thyroiditis showed significantly higher binding than controls. Patients with thyroid autoimmunity also have antibodies against tubulin¹⁵; possibly this and other cytoskeletal antigens are being detected in the ELISA. The greater reactivity of the Hashimoto's group could reflect heightened and broader autoreactivity in these patients.

We also examined T cell sensitisation to muscle antigens by measuring proliferation. Patients with thyroid autoimmunity, again irrespective of eyes signs, gave a positive response, and this also was not specific for extraocular muscle. With the migration inhibition factor assay and a retrobulbar connective tissue antigen, 30-60% of Graves' disease patients without ophthalmopathy gave a positive response, in addition to the positive responses in 90-100% of ophthalmopathy subjects.^{6,16} To our knowledge T cell proliferation with extraocular muscle has not been assessed previously, though 41% of ophthalmopathy patients did give a blastogenic response to a lacrimal gland extract.⁹ The present findings show in addition that T cells may be sensitised to several different antigens within these crude membrane preparations, as revealed by the fractionation studies. No single molecular weight determinant was found which reliably produced proliferation, but fractionated antigens with molecular weights of about 75, 55, and 12 kd warrant further study.

In summary, our results do not confirm the existence of ophthalmopathic immunoglobulins in Graves' ophthalmopathy,³ but they show instead that

Table 2 Blastogenic responses to fractions of eye muscle

Patient	Nil	Plus antigen (fraction number)*
1	2319 (465)†	23175 ± 15883 (2) 27688 ± 2721 (3)
2	1089 (402)	10743 ± 1742 (2) 9193 ± 2488 (3)
3	842 (464)	8645 ± 2665 (5) 11281 ± 708 (6)
4	2343 (565)	10253 ± 9231 (20)

*Numbers in parentheses are the fraction number used to stimulate blastogenesis; other fractions gave stimulation of less than four times control values.

†Results are mean cpm(SD).

thyroid autoimmunity is associated with B and T cell reactivity to striated muscle antigens of either extraocular or skeletal muscle origin. This makes the strong association of Graves' disease, but not Hashimoto's thyroiditis, with ophthalmopathy difficult to understand, unless the unknown sensitising antigen (which could be thyroid or retrobulbar in origin) induces a response only in Graves' disease patients:

APW is a Wellcome senior research fellow. We are grateful to Miss C Panagiotidi for technical help and to Miss C Jones for typing the manuscript.

References

- Jacobson DH, Gorman CA. Endocrine ophthalmopathy: current ideas concerning etiology, pathogenesis and treatment. *Endocrine Rev* 1984; **5**: 200-20.
- Trokel SL, Jakobiec FA. Correlation of CT scanning and pathologic features of ophthalmic Graves' disease. *Ophthalmology* 1981; **88**: 553-64.
- Atkinson S, Holcombe M, Kendall-Taylor P. Ophthalmopathic immunoglobulin in patients with Graves' ophthalmopathy. *Lancet* 1984; **ii**: 374-6.
- Kadlubowski M, Irvine WJ, Rowland AC. The lack of specificity of ophthalmic immunoglobulins in Graves' disease. *J Clin Endocrinol Metab* 1986; **63**: 990-5.
- Ahmann A, Baker JR, Weetman AP, Wartofsky L, Nutman TB, Burman KD. Antibodies to porcine eye muscle in patients with Graves' ophthalmopathy: identification of serum immunoglobulins directed against unique determinants by immunoblotting and enzyme-linked immunosorbent assay. *J Clin Endocrinol Metab* 1987; **64**: 454-60.
- Munro RE, Lamki L, Row VV, Volpé R. Cell mediated immunity in the exophthalmos of Graves' disease as demonstrated by the migration inhibition factor (MIF) test. *J Clin Endocrinol Metab* 1973; **37**: 286-92.
- Wall JR, Walters BAJ, Grant C. Leukocyte adherence inhibition in response to human orbital and lacrimal extracts in patients with Graves' ophthalmopathy. *J Endocrinol Invest* 1979; **2**: 375-8.
- Mullin BR, Levinson RE, Friedman A, Henson DE, Winand RJ, Kohn LD. Delayed hypersensitivity in Graves' disease and exophthalmos: identification of thyroglobulin in normal human orbital muscle. *Endocrinology* 1977; **100**: 351-66.
- Wall JR, Trewin A, Fang SL, Ingbar SH, Braverman LE. Studies of immunoreactivity to human lacrimal gland fractions in patients with ophthalmic Graves' disease. *J Endocrinol Invest* 1978; **11**: 253-8.
- Van Dyk HJL. Orbital Graves' disease: a modification of the 'No specs' classification. *Ophthalmology* 1981; **88**: 479-83.
- Rylatt DB, Parish CR. Protein determination on an automatic spectrophotometer. *Anal Biochem* 1982; **121**: 213-4.
- Abou-Zeid C, Filley E, Steele J, Rook GAW. A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro by converting lines cut from Western blots into antigen bearing particles. *J Immunol Methods* 1987; **98**: 5-10.
- Faryna M, Nauman J, Gardas A. Measurement of auto-antibodies against human eye muscle plasma membranes in Graves' ophthalmopathy. *Br Med J* 1985; **290**: 191-2.
- Sikorska H, Wall JR. Failure to detect eye muscle membrane specific autoantibodies in Graves' ophthalmopathy. *Br Med J* 1985; **291**: 604.
- Rousset B, Bernier-Valentin F, Poncet C, *et al.* Anti-tubulin antibodies in autoimmune thyroid disorders. *Clin Exp Immunol* 1983; **52**: 325-32.
- Mahieu P, Winand R. Demonstration of delayed hypersensitivity to retrobulbar and thyroid tissues in human exophthalmos. *J Clin Endocrinol Metab* 1972; **34**: 1090-2.

Accepted for publication 23 June 1988.