Evaluation of an enzyme-linked immunosorbent assay for the measurement of autoantibodies against eye muscle membrane antigens in Graves' ophthalmopathy

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Abstract. We have tested for antibodies against human and pig eye muscle membrane antigens in the serum of patients with Graves' ophthalmopathy using an enzyme-linked immunosorbent assay (ELISA). Several different membrane preparations were used as source of putative antigen including a 100 000 × g pellet, a pellet depleted of the 100 000 × g (microsome) fraction, and solubilized membranes. With eye muscle membrane pellets there were no significant differences for either serum or immunoglobulins between patients with ophthalmopathy, those with autoimmune thyroid disorders without eye disease, and normal subjects for either human or pig membranes, although tests were positive determined from the upper limit of normal in a few patients with or without eye disease. This was the case regardless of the enzyme-antibody conjugate used, the membrane protein concentration or serum or immunoglobulin dilution. Pre-absorption of tissue fractions, serum, or immunoglobulins, with red blood cells or liver powder, eye muscle membranes or skeletal muscle membranes did not significantly reduce background binding which was often very high, or enhance the difference between patients with ophthalmopathy and normal subjects. It was found that non-specific binding to the plastic surface of the microplates and/or tissue proteins, the presence, in human tissues, of blood-derived immunoglobulins which gave strong reactions in the ELISA, and variable fixation of membrane pellets to the plates were factors which made ELISA unsatisfactory when crude membrane pellets were used as antigen. When eye muscle membranes solubilized with standard agents including SDS, Triton X-100 and deoxycholine were tested, again no differences were demonstrated between patients with Graves' ophthalmopathy and normal subjects. However, when membranes were solubilized with the zwitterionic agent ‘CHAPSO’ approximately 20% of patients with Graves' ophthalmopathy and smaller proportions of patients with thyroid disease without apparent eye involvement had positive tests with both human and pig eye muscle. While availability of human monoclonal antibodies should soon allow the isolation and purification of soluble eye muscle membrane autoantigens for use in sensitive and specific antibody tests, the ELISA, as presently used with crude tissue fractions, appears too variable and prone to non-specific immunoglobulin-binding for routine clinical use.

Antibodies against eye muscle antigens were first reported by Kodama et al. (1982) who found antibodies against a partially purified soluble antigen derived from human eye muscle in the serum of 74% of patients with active Graves' ophthalmopathy. Kendal-Taylor and colleagues (Atkinson et al. 1984) reported antibodies against fresh pig eye muscle membranes, although they also found activity in the soluble fraction. More recently Faryna et al. (1985) detected antibodies against a human eye muscle membrane antigen using a staphylococcus protein A binding assay and eye muscle obtained as long as 24 h after death. In very recent studies Wall and colleagues (Mengistu et al. 1986; Wall & Kuroki 1985) have confirmed the existence of antibodies against human eye muscle antigens using the immunofluorescence
test. Finally, other studies from our laboratory (Kuroki et al. 1985a,b, 1986; Wall & Kuroki 1985) have shown that antithyroglobulin autoantibodies in the serum of patients with thyroid disorders, with or without associated ophthalmopathy, and monoclonal antibodies against human thyroglobulin react with an antigen in human orbital connective tissue membranes shared with human thyroglobulin.

Although antibodies against soluble (cytosol) antigens may be important as markers of the autoimmune process and as diagnostic tests, it is likely that membrane-directed antibodies are more closely associated with the underlying pathogenesis (Wall 1984, 1985). For example, while antithyroglobulin is a very reliable marker of the thyroid autoimmune reaction it is not pathogenetic, antibodies against the microsomal (cell surface) antigen almost certainly being the cause of the thyroid cell killing which is a characteristic of Hashimoto’s thyroiditis (Bogner et al. 1984). It thus seemed important to further study the nature and significance of antibodies against membrane antigens in patients with Graves’ ophthalmopathy. In this study we report our extensive attempts to demonstrate such antibodies against human and pig eye muscle membrane antigens using the enzyme-linked immunosorbent assay (ELISA). Although antibody tests were positive in a few patients with active eye disease of recent onset when CHAPSO-solubilized membranes were used as antigen, tests were mainly negative using crude membrane pellet and membranes solubilized with other agents. Moreover, major problems with the ELISA when used with crude tissue preparations were revealed.

Patients and Methods

Patients
The studies concerned 67 patients, 12 males and 55 females, aged 14–68 (mean age 43 years) with Graves’ ophthalmopathy (GO) of whom 52 had active disease and 15 inactive (‘burnt out’) disease. Of these, 53 patients had associated hyperthyroidism, 6 Hashimoto’s thyroiditis (HT) and 8 no associated thyroid disease (‘euthyroid Graves’ disease’). Twenty were hyperthyroid, 2 hypothyroid and 45 euthyroid at the time of testing. No patient was being treated with immunesuppressive drugs or steroids. The severity of the eye muscle component was quantitated 0–5, where 0 = no eye muscle involvement, 1 = mild restricted of upward gaze, 2 = moderate restriction, 3 = severe restriction, 4 = restriction of gaze in other directions and 5 = complete ophthalmoplegia. Also studied were: 1) 31 patients, 2 males and 29 females aged 20–62 (mean age 35 years), with Graves’ hyperthyroidism (GH) without evidence for eye disease of whom 8 were hyperthyroid, 21 euthyroid following treatment and 2 hypothyroid following treatment; 2) 34 patients, one male and 33 females aged 14–60 (mean age 40 years), with Hashimoto’s thyroiditis (HT) without eye disease in whom the diagnosis was confirmed from significant titres (> 1/256) of thyroid antibodies and biopsy evidence for lymphocytic infiltration and/or Hürthle cells and a firm, indurated goitre; 3) 22 patients, 5 males and 17 females aged 22–72 (mean age 44 years) with the following presumed non-immunological thyroid disorders: transient post-partum thyroiditis (2 patients), subacute thyroiditis (11 patients), and thyroid cancer (2 patients); 4) 45 normal subjects, 10 males and 35 females aged 18–60 (mean age 34 years) as controls.

Antigen preparation
Normal human eye muscle, skeletal muscle and liver were obtained from autopsy less than 4 h after death. Pig eye muscle, skeletal muscle and liver were obtained from normal animals immediately after sacrifice. Tissues were finely minced using scissors and homogenized in a mechanical blender. Washing and dilution of all tissues were carried out with phosphate buffered saline (PBS), pH 7.4. The homogenate was centrifuged at 400 × g to remove unbroken cells, nuclei and debris and the supernatant centrifuged at 100 000 × g to obtain the membranes (‘standard pellet’). The final supernatant was retained as the soluble (cytosol) fraction. In some cases the supernatant obtained after the first, low speed, centrifugation step was spun at 10 000 × g, the pellet (‘microsomes’) being discarded and the supernatant further centrifuged at 100 000 × g following the protocol used by Kendall-Taylor and colleagues (Atkinson et al. 1984; Kendall-Taylor et al. 1984). All membrane pellets were washed once in PBS.

Human and pig eye muscle membranes (the 100 000 × g pellet) were solubilized using a variety of agents including: Triton X-100 (Fischer, NJ), 0.2 M NaCl, NP-40 (BRL, Gaithersburg, MD), 3-[3-cholamidopropyl]dimethylammonio]-1-[2-hydroxy-1-propanesulfonate], 3H2O ‘CHAPSO’ (Calbiochem-Behring, La Jolla, CA), 3 M NaCNS, 2% SDS, 1% DOC, 2% SDS + 8 M Urea, 0.5% trypsin 3 M KY, 3 M KCL, 6 M G-HCL, and 2% NP-40. In all cases PMSF in acetone, at a final concentration of 1 M, was added to inhibit protease activity. The membranes were re-suspended to give a final protein concentration of 2–4 mg/ml buffer. The suspension was stirred at 4°C for 30 min. At the end of stirring the...
mixture was centrifuged at 100,000 x g at 4°C for 30 min. The supernatant, which contained solubilized proteins, was aliquoted and stored at -70°C.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

SDS-PAGE was performed on preparations of solubilized pig liver and eye muscle membranes. Fifty µg of solubilized proteins were added to 12.5% gel and run overnight at 10 mA keeping the temperature constant in a water cooling system. The protein bands were then transferred to nitrocellulose paper by electroblotting for 5 h at 40 V. The blots were incubated separately with patient and normal sera, then with anti-human IgG enzymes and finally developed using Coomassie blue dye.

Enzyme linked immunosorbent assay (ELISA)

Serum autoantibodies against pig and human eye muscle membrane antigens were measured in an ELISA. Standard methods were used, although many modifications were made as will be outlined in the results section. The concentrations of tissue fractions and antigens used were different for each tissue, and in some cases several concentrations were tested. In most cases the optimal concentration ranged from 5 to 100 µg/ml. Coating of the tissue fractions was overnight at 4°C (in most cases) or at 37°C for 2 h. For membrane pellets coating was performed both with and without 0.1% gluteraldehyde which may be necessary for adherence of particles to the plastic plates. The microtiter plates ('Immunolon') used were obtained from Dynatech (Alexandria, VA). After antigen coating the residual binding sites were blocked with 1% BSA in coating buffer. Incubation with serum or purified Ig was overnight at 4°C (in most experiments) or at 37°C for 3 h. Coating buffer used in all experiments was a bicarbonate/carbonate buffer (pH 9.6). Marker antibodies used were directed against IgG, the f(6b)2 or fc fragments, or IgG + IgM + IgA, and were labelled with alkaline phosphatase, horse radish peroxidase (HRP) or avidin-biotinylated anti-human IgG-HRP (Zymed Labs Inc, San Francisco, CA) at appropriate dilutions determined in preliminary experiments. Antibodies were raised in goat, pig and rabbit. The substrates used varied with the enzyme-conjugates. For alkaline phosphatase it was p-nitrophenol, and for avidin-biotinylated and horse-radish peroxidase conjugates o-phenylenediamine dihydrochloride. Controls, included in all tests, were 1% bovine serum albumin (BSA), coating buffer instead of antigen, tests with no second antibody and serum or IgG from age/sex matched normal subjects. Tests were read in a semi-automatic ELISA reader and results expressed as optical density (OD). A positive test was defined as an OD > upper limit of normal determined as mean +2 SD for normal subjects tested concurrently. Tests were set up in duplicate. Equal numbers of patients and normals were tested on the same ELISA plate.

Immunoglobulin preparation

(1g) the gamma-globulin fraction of patient serum was isolated by ammonium sulphate precipitation followed by chromatography on DEAE + cellulose according to standard methods. Ig was diluted in PBS, aliquoted, and stored at -70°C until use. Contaminating blood derived Ig was removed from human tissues by incubating tissue preparations with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Results

Sera from patients with Graves' ophthalmopathy, and thyroid disorders without eye involvement, were studied for antibodies against human and pig eye muscle membrane antigens. Optimal antigen concentration and serum dilution were assessed prior to each experiment. On the same 96-well plate rows of wells were coated with increasing concentrations of the antigen preparations (5 to 100 µg/ml), then decreasing dilutions of a pool of GO patients sera (1:1 to 1:1000) were applied to each well-line. Optimal reaction was taken as the highest Δ OD, calculated by subtracting the background OD from the test OD. In the first series of experiments tests were carried out using a crude 100,000 x g pellet fraction comprising all of the membranes including the microsomes, mitochondria and the endoplasmic reticulum. A membrane concentration of 50 µg/ml and serum dilution of 1/10 were found to be optimal when sera from patients with GO, GH and HT and normals were tested. There were no significant differences between any of the patient groups and normals for either human or pig tissue, and only two patients (with GO) had positive tests with pig eye muscle membranes and none with human eye muscle membranes. Next, tests were carried out using, as antigen, the pellet with which Kendall-Taylor et al. (1984) demonstrated antibodies in patients with GO. This pellet excludes a 10,000 x g fraction, but is otherwise prepared in the same way. The optimal antigen concentration and serum dilution were determined as before. Many assays were carried out using this pellet and sera from patients with GO and autoimmune thyroid disorders without eye di-
ease. Again, there were no significant differences between the groups when either human or pig eye muscle membranes were used, although a few patients in all patient groups had positive tests for both human and pig tissue. It was noted that in this and most other assays background (non-specific) binding was very high, i.e. values with normal sera were often > 0.6. Although this was less when avidin-biotinylated enzyme conjugates were used, background binding was still unacceptably high for all membrane preparations tested. In order to test the possibility that positive results in patients were being masked by non-specific binding giving false positives in normals, a series of modifications were performed in an attempt to reduce background binding.

Firstly, absorption experiments were carried out by pre-incubating test sera with pig eye muscle membranes and, as control, liver membranes and re-testing them against pig eye muscle membranes. It was found that for all patient groups, as well as normal subjects, pre-absorption of sera with eye muscle membranes and liver membranes reduced the binding to eye muscle membranes only slightly and that this was equal for patients and normals. Absorption performed with other skeletal muscle or red blood cells was equally ineffective.

Next, we tested purified Ig, prepared from patients with GO and normal subjects by DEAE + cellulose chromatography, for reactivity against the same membrane preparations. In preliminary experiments optimal concentrations of Ig and eye muscle membranes were determined. There was a marked tendency for increasing concentrations of Ig to be associated with higher readings in the ELISA, for both patients with GO and normal subjects. This was equally the case for pig eye muscle and human eye muscle membranes. Thus when Ig from patients with GO and normals were tested at concentrations of 1 mg/ml and 0.1 mg/ml, although there were no significant differences between the two groups at either concentrations, values were much higher (in both groups) at the higher concentration (Fig. 1a). When Ig prepared from patients with GO and from normal subjects

![Fig. 1.](image)

Levels of antibodies against a 100 000 × g eye muscle membrane pellet depleted of the 10 000 × g membrane fraction in the Ig fraction prepared from the serum of patients with Graves' ophthalmopathy (GO) and normal subjects (NOR), assessed using an ELISA. Results are expressed as optical density at 410 nm. Pellet protein concentration was 10 µg/ml. Horizontal lines represent mean (± sd) values. Broken lines at 1.4 (b) and 1.3 (c) represent upper limits of normal (ULN) calculated as mean ± 2 sd for normals. Ig concentration was 100 µg/ml. The enzyme conjugate was avidin-biotinylated anti-human IgG horse–radish peroxidase. a) Ig at 1 mg/ml and 0.1 mg/ml, with pig eye muscle. b) Ig at 0.1 mg/ml with human eye muscle. c) Ig at 0.1 mg/ml with pig eye muscle. □ Patients with Graves' ophthalmopathy. □ Normal subjects.
Levels of antibodies in the Ig fraction prepared from the serum of patients with Graves' ophthalmopathy and normal subjects, against human eye muscle and human skeletal muscle membranes solubilized with DOC, SDS, NP-40 and Triton X-100 assessed using an ELISA. Results are expressed as mean (± sd) optical density at 410 nm. Protein concentration was 10 μg/ml. The enzyme conjugate used was goat anti-human IgG (H + L chain specific) conjugated to β-galactosidase. □ Patients with Graves' ophthalmopathy (n = 8). □ Normal subjects (n = 8).

were tested at 0.1 mg/ml there were no significant differences between the two groups for either human (Fig. 1b) or pig (Fig. 1c) eye muscle membranes, and no patient had values above the upper limit of normal for either tissue. For both normal subjects and patients with GO, pre-absorption with neither eye muscle membranes nor liver membranes significantly reduced the subsequent reactivity of Ig with eye muscle membranes suggesting that, as for serum, reactivity in both groups comprised a large degree of non-specific binding which was not related to a specific reactivity with putative eye muscle antigens. Thus, by carrying out tests in which one component at a time was eliminated, significant optical density readings were found both in tests without serum and in those without antigens (but in the presence of serum or Ig from both patients and normals) suggesting that the enzyme-anti-human IgG conjugate bound non-specifically to the plastic surface, or tissue proteins. In addition, it was noted that fixation of unsolubilized membrane pellets to the plastic plates was variable and unpredictable, giving misleading protein concentrations. In a further attempt to reduce non-specific responses we solubilized eye muscle membranes allowing the removal of the bulk of the membrane proteins. Several standard solubilizing agents were used, their efficiency determined by carrying out electrophoresis on 8% SDS-PAGE in a preliminary experiment. Protein concentrations were measured by a modification of conventional Lowry procedure with and without TCA/DOC precipitation of the protein. The highest yield of protein bands occurred with SDS and DOC, while the new detergent CHAPSO solubilized, at 20.5% concentration, approximately 40% of the crude preparations. On the other hand trypsin and Triton X-100 were poor solubilizers of eye muscle membrane proteins. Solubilized proteins prepared from human eye muscle and human skeletal muscle by treatment with DOC, SDS, NP-40 and
Triton-X were used as antigen and tested with Ig (0.1 mg/ml) from patients with GO and normal subjects at an antigen concentration of 10 µg/ml. As can be seen in Fig. 2, responses to both human muscle tissues in both groups were high, and there were no differences between the two groups for any preparation of solubilized muscle membranes tested.

In order to further test the nature of this apparently non-specific response in the ELISA, human eye muscle tissue solubilized with Triton X-100 was depleted of blood derived Ig, which gives strong positive responses in the ELISA, using a staphylococcus protein A (SPA) chromatographic method. Despite a markedly lower background reactivity to all human tissues tested for both patient and normal Ig and serum, this did not significantly increase the difference between patients with GO and normal subjects, and no patient had positive tests (results not shown). Next, we used eye muscle membranes solubilized with a new zwitterionic agent, CHAPSO, which is an efficient, but gently, method for solubilizing membrane proteins. In preliminary experiments, the optimal conditions for CHAPSO-solubilized eye muscle membranes were established. Many assays were carried out using both human and pig eye muscle membranes and several different antibody-enzyme conjugates. At a serum dilution of 1/100 and antigen concentration of 10 µg/ml significant differences were shown between patients with GO and normal subjects for both human eye muscle (0.22 ± 0.02 SE, n = 42; 0.111 ± 0.02 SE, n = 28, t-test (P < 0.001) and pig eye muscle (0.38 ± 0.04 SE, n = 38; 0.22 ± 0.03 SE, n = 12, P < 0.05), while 11 and 9 patients, respectively, most of whom had severe eye disease of recent onset, had positive tests (Fig. 3a,b). However, tests were also positive in 4 patients with HT without clinically apparent eye disease and in 5 with presumed non-immunological thyroid disease (subacute thyroiditis 3 cases, cancer 1 case, adenoma 1 case) with human tissue, and in three patients with GH and no eye disease, 2 with HT and no eye disease, and in one patient with subacute thyroiditis with pig tissue. When a 1/10 serum dilution and 25 µg/ml membrane protein concentration were used values were much higher for both patients with GO and normal subjects (Fig. 3c,d). In this case there were no significant differences between patients with GO, those with thyroid disorders without eye disease and normals.

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**Fig. 3.**
Serum levels of antibodies against CHAPSO-solubilized eye muscle membrane antigens in patients with Graves' ophthalmopathy (GO), Graves' hyperthyroidism without eye disease (GH), Hashimoto's thyroiditis without eye disease (HT) other (non-immunological) thyroid disorders (OTH) and normal subjects (NOR) assessed using an ELISA. Results are expressed as optical density at 410 nm. The enzyme conjugate used was avidin-biotinylated anti-human IgG-horse-radish peroxidase. Horizontal line represent mean (± SD) values. Broken lines at 0.25 a), 0.46 (b), 1.34 (c) and 1.4 (d) represent the upper limits of normal (ULN) calculated as mean ± 2 SD for normals. a) Human eye muscle, serum dilution 1/100, antigen concentration 10 µg/ml. b) Same as (a), but using pig eye muscle. c) Human eye muscle, serum dilution 1/10, antigen concentration 25 µg/ml. d) Same as (c), but using pig eye muscle.
Membrane fractions of pig liver (lane 1) and pig eye muscle (lane 2) previously solubilized with CHAPSO were electrophoresed, electroblotted and immunodetected with human sera and 1) biotinylated anti-human IgG antiserum-horse radish peroxidase (blots A, B), 2) anti-human IgG (gamma chain specific)-horse radish peroxidase (blots C, D), 3) anti-human IgG (Fc fragment specific)-horse radish peroxidase (blots E, F) and 4) anti-human IgG (Fab' 2 fragment specific)-horse radish peroxidase (blots G, H). Blots A, C, E, G were incubated with serum (diluted 1:10) from a normal individual. Blots B, D, F, H were incubated with serum (diluted 1:10) from a patient with Graves' ophthalmopathy.

for either tissue, and only 3 patients with GO had positive tests with human tissue, while none were positive with pig tissue.

Reactivity of serum antibodies with a CHAPSO-solubilized pig eye muscle membrane antigen was confirmed by gel electrophoresis. As can be seen in Fig. 4, although serum from a patient with GO and that from a normal subject, both showed many bands with pig eye muscle membrane in PAGE electroblotted onto nitrocellulose paper using four different enzyme-antibody conjugates, there was an extra band in tests with the patient's serum (lanes B, D, F and H) which was not found with normal serum (lanes A, C, E and G) and not with liver membranes with either serum. There were no significant correlations between OD readings and the severity of the eye muscle involvement expressed as a clinical index for either CHAPSO-solubilized membranes ($r = 0.024, P = NS$), or the 100 000 x g pellet ($r = 0.456, P = NS$). Similarly, there were no significant correlations between OD readings and the duration of the eye muscle disease for either fraction ($r = 0.527$ and $r = 0.407, P = NS$, respectively).

**Discussion**

Antibodies against putative eye muscle antigens may be directed against eye muscle specific antigens, those present in all forms of skeletal muscle or against antigens shared between the thyroid gland and eye muscle. As discussed above several
groups have reported antibodies against human or pig eye muscle soluble (Kodama et al. 1982) or membrane (Atkinson et al. 1984; Kendall-Taylor et al. 1984; Faryna et al. 1985; Bemetz et al. 1986) antigens. As determined by immunofluorescence antibodies against eye muscle also react with other skeletal muscle, and some patients with other inflammatory muscle disorders were shown to have antibodies against eye muscle antigens (Mengistu et al. 1986). The possibility that thyroid directed autoantibodies may cross-react with eye muscle cell surface antigen(s) is currently being tested in our laboratory. Autoantibodies against soluble and membrane-derived orbital connective tissue antigens may also exist in Graves' ophthal-mopathy (Wall 1984, 1985).

In the present studies we have been unable, despite numerous modifications of the test and testing both serum and purified immunoglobulins, to convincingly show antibodies against unsolubilized human or pig eye muscle membrane pellet antigens using the ELISA. On the other hand, we showed that approximately 20% of patients with Graves' ophthalmopathy and smaller proportions of patients with autoimmune thyroid disease without apparent eye disease have antibodies against a CHAPSOSolubilized eye muscle membrane antigen. Although tests were also positive in 3 patients with subacute thyroiditis and two with thyroid tumours it is possible that such patients have subclinical eye muscle disease. Alternatively, this may represent in these situations primary thyroid antibodies which cross-react with eye muscle antigens. Detection of antibodies against membrane antigens by immunofluorescence (Mengistu & Wall, unpublished observations), of cytotoxic cell surface directed antibodies (Wang et al. 1985), and production of human monoclonal antibodies against various orbital antigens (Salvi & Wall, not yet published) confirms that such antibodies exist. The prevalence of antibodies against solubilized eye muscle membrane antigen(s) is much lower than that reported by Faryna et al. (1985) in their Protein-A binding assay. This could be attributed to the fact that a soluble antigen, when immobilized on the surface of a plastic plate, can present an altered configuration with exposition of epitopes which otherwise would not be exposed by the antigen in solution (Vaidya et al. 1985).

Because of the problems associated with the ELISA when crude fractions are used as antigen, we are attempting to purify, by affinity chromatography, soluble (cytosol) and solubilizable (membrane) eye muscle and orbital connective tissue antigens using monoclonal antibodies. Using a mouse monoclonal antibody (No. 50-24) we have been able to isolate a soluble eye muscle-derived antigen, S, against which a large proportion of patients with active Graves' ophthalmopathy were shown earlier to have serum autoantibodies (Kodama et al. 1982). This antigen is presently being further characterized and the role of the corresponding autoantibodies assessed. We are also using human monoclonal antibodies prepared from lymphocytes of patients with Graves' ophthalmopathy, reactive against soluble eye muscle antigens, to isolate the corresponding autoantigens. At that time it may be possible to use the ELISA for clinical testing. In the meantime it is the opinion of the authors that the ELISA is unsuitable for detection of antibodies against unpurified eye muscle membrane antigens.

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