The production and characterization of monoclonal antibodies to the human thyroid microsome

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ABSTRACT

By suitable immunization of mice and fusion of their spleen cells with a non-secretor mouse myeloma line, monoclonal antibodies have been produced which react with the human thyroid microsomal (M) antigen. These monoclonal antibodies showed no reactivity by enzyme-linked immunoassay with liver microsomes or thyroglobulin and their specificity was confirmed by immunolocalization studies, in which they showed the staining characteristics of human M antibodies. All four monoclonal antibodies tested were immuno-
globulin M; three were cytotoxic to thyroid cell monolayers. The lack of cytotoxicity with the fourth monoclonal supports the concept that certain epitopes of the M antigen may be partially or completely absent at the thyroid cell surface. These monoclonal antibodies should permit further characterization of the thyroid M antigen in view of their absence of cross-reactivity with thyroglobulin.

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INTRODUCTION

Antibodies directed against the human thyroid microsome (M) antigen are frequently detected in patients with Graves’ disease and Hashimoto’s thyroiditis. These antibodies, unlike those directed against thyroglobulin (TG), fix complement and therefore are more likely to have phlogistic potential (Pulvertaft, Doniach & Roitt, 1961; Khoury, Hammond, Bottazzo & Doniach, 1981). The M antigen, present on the apical surface of thyroid cells, is nonetheless accessible to antibody in vivo (Khoury, Bottazzo & Roitt, 1984) and appears to be a lipoprotein involved in the transport of TG from the Golgi apparatus to the follicular lumen. The exact nature of the antigen is unclear; at least one study has suggested that only certain antigenic components may be expressed on the cell surface (Fenzi, Bartalena, Chiovato et al. 1982). Further purification of the M antigen has been hampered by two problems. First, preparations of M antigen are inevitably contaminated by TG (Weetman, Rennie, Hassman et al. 1984) and therefore autoimmune sera which contain antibodies to both antigens cannot be used to purify the M antigen. Secondly, production of antibodies to the M antigen in animals has proved very difficult, a fact testified to by the dearth of animal models of thyroiditis which use the M antigen as an immunogen (Weetman & McGregor, 1984). We have therefore produced murine hybridomas which secrete monoclonal antibodies to the human M antigen; this report concerns the characterization of these antibodies. A further study will describe their use in antigen purification.

MATERIALS AND METHODS

Preparation of M antigen

The M antigen was prepared from a pool of snap-frozen tissue from patients with Graves’ disease by ultracentrifugation and filtration purification on Sepharose 2B-CL (Pharmacia, Hounslow, Middlesex) as described elsewhere (Weetman et al. 1984). For specificity experiments liver microsomes were prepared in an identical fashion; crude thyroid and uterine antigens were prepared by lyophilization of 800 g tissue homogenate supernatants.

Production of monoclonal antibodies

Six-week-old female BALB/c mice were immunized with an emulsion of 100 µg M antigen (1 mg/ml) in an equal volume of complete Freund’s adjuvant, given intramuscularly and subcutaneously into the inner and


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outer aspects of the hind legs. The mice were given 30 μg M antigen (150 μg/ml saline) intraperitoneally after 7 days and then killed 3 days later. The splenic lymphocytes were mixed with cells of the non-secretor BALB/c-derived myeloma line SP1 (kindly provided by Dr J. Schalom, National Cancer Institute, Bethesda, MD, U.S.A.) in the mid-logarithmic phase of growth in the ratio of 10 : 1 spleen to myeloma cells. Fusion was performed using a 50% polyethylene glycol (mol. wt 1500; BDH Chemicals Ltd, Poole, Dorset) with 7.5% dimethylsulphoxide (Sigma Chemical Co., Poole, Dorset) and the technique of Oi & Herzenberg (1980).

After fusion, cells were washed once with RPMI 1640 (Flow Laboratories, Irvine, Strathclyde) and gently resuspended at 10^7/ml culture medium, comprising RPMI 1640, 20% fetal calf serum, 2 mm-pyruvate, 4 mm-glutamine and 40 mg gentamicin/l. Cultures were set up with 100 μl of the suspension per well of 96-well flat-bottomed plates (Nunc, Kamstrup, Denmark) to which 100 μl HAT medium (100 μM-hypoxanthine, 400 nm-aminopterin and 16 μM-thymidine in culture medium) were added, usually after 16 h. Cultures were maintained at 37°C in 5% CO2 in air and screening for antibodies was performed on day 10. Clonal growth was assessed by inspection. Positive clones were expanded with fresh BALB/c thymocytes as feeder cells and cloned three times by limiting dilution. Expanded clones were maintained in vitro in 25 cm² flasks (Nunc) or in vivo in the peritoneal cavities of tetramethylpentadecane (Pristane; Aldrich Chemical Co., Gillingham, Dorset) -treated BALB/c mice for the production of ascitic fluid. Aliquots of expanded clones were also frozen and stored in liquid nitrogen without subsequent loss of synthetic capacity. Monoclonal M antibody was precipitated from ascitic fluid using ammonium sulphate and dialysed extensively against phosphate-buffered saline before use.

**Enzyme-linked immunosorbent assay (ELISA) methods**

The ELISA for microsomal antibodies has been described elsewhere (Weetman et al. 1984). An ELISA was also developed to identify the immunoglobulin (Ig) class of the monoclonal antibodies. Wells of the ELISA plates were coated with sheep anti-mouse isotype-specific Ig (Sera Lab., Crawley Down, Sussex) using a 1:20 dilution in carbonate-bicarbonate coating buffer. After overnight coating the monoclonal antibody present in culture medium supernatant was added to the wells and the ELISA developed with rabbit anti-mouse Ig-peroxidase conjugate (Nordic, Tilburg, The Netherlands) and orthophenylene diamine as substrate. The absorbance given by the hybridoma supernatant was compared with that of culture medium on the same anti-mouse isotype-specific Ig-coated wells. Monoclonal Ig class was confirmed by adding hybridoma supernatant to M antigen-coated plates followed by a 1:20 dilution of anti-mouse isotype-specific Ig (Sera Lab.), a 1:500 dilution of donkey anti-sheep Ig-alkaline phosphatase conjugate (made in our own laboratory), and finally p-nitrophenyl phosphate (pNPP) as substrate.

**Immunolocalization method**

Fresh thyroid tissue from Graves' patients undergoing thyroidectomy was cut into small pieces, fixed in Bouin's fluid overnight and washed several times in 50% ethanol before being processed and embedded in paraaffin wax. Sections (5 μm) were cut and dewaxed using several changes of xylene without the use of an oven and brought to alcohol. Endogenous peroxidase activity was blocked using a 30-min immersion in a fresh 0.5% solution of H2O2 in methanol followed by extensive washing in Tris-buffered saline (TBS, pH 7-6).

Non-specific background staining was reduced by flooding the slides with normal rabbit serum (1 : 10 in TBS), which was replaced by the monoclonal supernatant, myeloma culture supernatant or normal mouse immunoglobulins in culture medium. After a 30-min incubation the sections were washed extensively in TBS and treated for another 30 min with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Nordic) diluted 1 : 100 in TBS. The end product was stained, after washing, by a 5-min circulation with freshly prepared 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, U.S.A.) and 0.01% hydrogen peroxidase solution in 0.05 M-Tris buffer, pH 7-6. Sections were washed well in tap water and lightly counterstained with haematoxylin, dehydrated, cleaned in xylene and mounted for viewing.

**Binding of monoclonal antibodies to thyroid cells in vitro**

The method of Feit, Bartal, Tauber et al. (1983) for detecting monoclonal antibodies which bind to viable tumour cells was adopted to investigate binding of the monoclonal M antibodies to thyroid cells. Monolayer cultures of thyroid cells from patients with Graves' disease were established using the method of Hinds, Takai, Rapoport et al. (1981) and used within 48 h of removal of the thyroid gland. Thyroid cells were removed from 75 cm² flasks with 0.05% trypsin in Earle's balanced salt solution (Ca²⁺- and Mg²⁺-free), washed, resuspended in culture medium (10%FCS) and 100 μl aliquots added to a 96-well flat-bottomed plate. After culture overnight the culture supernatants were removed, the adherent thyroid cells washed with RPMI 1640, and 100 μl monoclonal antibody to human microsomes (MAHM) C3, MAHM C6, MAHTG B4 (a mouse monoclonal antibody to human thyroid tissue).
TG produced in our laboratory) or normal mouse Ig in culture medium (0·6 mg/ml) was added to the wells. After incubation at 4 °C for 1 h, the plates were washed with RPMI 1640 and goat anti-mouse Ig-alkaline phosphatase conjugate was added for 2 h at 4 °C. The plates were then given final washes and substrate (pNPP) was added. The absorbance at 405 nm indicated binding of antibody to the cells.

Cytotoxicity assays

Cytotoxicity was assessed by three assays.

[3H]Thymidine uptake by thyroid cells
The microcytotoxicity assay which uses post-labelling of surviving cells with [3H]thymidine (Brown, Thorpe & Rosenberg, 1979) was modified as follows: thyroid cell monolayers were prepared as described above and 100 µl aliquots of a 5 x 10⁶/ml suspension allowed to adhere to the wells of a 96-well flat-bottomed plate for 4 h. Monoclonal antibody (final concentration 0·5 mg/ml) and rabbit complement (preselected serum, final dilution 1:32) were then added followed 4 h later by 30 µl [3H]thymidine (Amersham International plc, Bucks; specific activity 47 Ci/mmol) to give a total dosage of 1 μCi/well. After an 18-h incubation, the plates were washed three times with Earl's balanced salt solution (Ca²⁺, and Mg²⁺-free) and 0·05% trypsin in this medium was added to the wells for 15 min. After shaking the plate to dislodge the thyroid cells, they were harvested onto glass-fibre filter plates using an automatic cell harvester (Skatron, Lier, Norway). Thymidine incorporation was measured using liquid scintillation counting on an LKB 1215 Rack beta II counter.

Crystal violet staining
This was adapted from a method used to detect cytoxicity against tumour cells (Matthews, 1983). Thyroid monolayers were prepared and treated exactly as for the [3H]thymidine incorporation assay but without the addition of thymidine. After overnight incubation the supernatant was removed and the adherent thyroid cells were fixed to the wells by immersion of the plate in 5% formalin in water for 60 s. The plate was washed with water and then immersed in 1% crystal violet in water for 60 s. After a further wash with water the crystal violet which stained the thyroid cells was dissolved in 200 µl 33% glacial acetic acid in water. The absorbance at 540 nm was read using an automatic micro-ELISA reader (Titertek, Multiskanner; Flow Labs).

Antibody-dependent cytotoxicity
This was measured using the ⁵¹Cr release method of Calder, Penhale, McLeman et al. (1973) except for the substitution of M antigen for TG to label the chicken red cells (CRBC). Briefly, ⁵¹Cr-labelled, M-coated CRBC were incubated with monoclonal antibody (100 µg/ml) for 30 min at 37 °C and then mixed with normal peripheral blood lymphocytes (5 x 10⁵ target CRBC and 5 x 10⁵ lymphocytes), in a final volume of 150 µl. These cells were incubated overnight at 37 °C and ⁵¹Cr release into the supernatant was estimated. Background release was established by measuring release into supernatants with antibody-coated cells alone and total release by the addition of 5% Triton. Chicken red cells which were ⁵¹Cr-labelled and M-coated but which had not been incubated with monoclonal antibodies before mixing with peripheral blood mononuclear cells (PBM) served as controls to check for natural killer cell activity.

Statistics
Significance was assessed by Student's t-test.

RESULTS

Production of monoclonal antibodies
Clonal growth was found in 20–53% of wells after five separate fusions, which used three mice per experiment. Between 12 and 59% of these wells were positive for antibodies against M antigen by ELISA, and the yield was particularly good in experiment 5 in which HAT was added 4 h after fusion. Four monoclonal antibodies which gave particularly high absorbance values by ELISA on initial screening were selected for further study: these MAHM were designated C3, C4, C6 and B4.

Isotype of MAHM
The ELISA performed to determine the isotypes of C3 and C6 showed that both were IgM. Both C3 and C6 bound significantly to anti-mouse IgM (C3 mean absorbance at 492 nm was 0·272 ± 0·047 (s.d.), six triplicates, P < 0·01; C6 0·271 ± 0·023, P < 0·001) as compared with the value obtained for culture medium alone (0·194 ± 0·018). For all other isotypes (IgA, IgG, IgG₂a, IgG₂b, IgG₃) the absorbance was not above that for culture medium. The same was true for C4 and B4 in this assay and this was confirmed by adding supernatants to M antigen-coated plates. Only when sheep anti-mouse IgM was added next did significant reaction occur with anti-sheep enzyme conjugate and substrate: the mean absorbances minus background (culture medium alone) were 0·486 ± 0·094 for C4 and 0·556 ± 0·010 for B4. With other anti-mouse isotype antibodies (anti-IgA, -IgG₁, -IgG₂a, -IgG₂b and -IgG₃) the absorbances were not greater than background.
**Binding of MAHM in ELISA**

The reactivity of MAHM B4 against thyroid and liver M antigen is shown in Text-fig. 1. Similar lack of reactivity to that found against liver M antigen was also demonstrated for TG and 800 g supernatants of thyroid and uterus homogenates and the binding of the MAHM depended on the concentration of thyroid M antigen used to coat the plate (Text-fig. 2). These properties were common to the other three monoclonal antibodies examined, MAHM C3, MAHM C6 and MAHM C4 (data not shown).

![Text-figure 1](image1.png)

**TEXT-Figure 1.** Enzyme-linked immunosorbent assays (ELISA) of various concentrations of the mouse monoclonal antibody B4 to the human thyroid microsome (MAHM B4) against either thyroid microsomal antigen (○) or liver microsomal antigen (●), which were used to coat ELISA plates in separate assays at 10 µg/ml coating buffer. Each point is the mean ± s.d. of an assay in triplicate.

![Text-figure 2](image2.png)

**TEXT-Figure 2.** Enzyme-linked immunosorbent assay showing the concentration effect of various coating antigens (thyroid microsome (○), thyroglobulin (●), thyroid 800 g supernatant (■) and uterine 800 g supernatant (▲)) on the binding of the mouse monoclonal antibody B4 to the human thyroid microsome (10 µg/l). Each point is the mean ± s.d. of an assay in triplicate.

**Immunolocalization**

As shown in the Plate, MAHM C3 stained the thyroid cell cytoplasm and particularly the apical border of the cells. There was no staining of the colloid. Similar results were obtained with MAHM C6 although the apical border staining was less pronounced.

**Binding of MAHM to cultured thyroid cells**

As shown in Text-fig. 3, both MAHM C3 and C6 bound to cultured thyroid cell monolayers, and differed in their reactivity according to the thyroid source, since C3 bound equally well to preparations from both donors, whereas more C6 bound to the preparation from donor B than to that from donor A.

![Text-figure 3](image3.png)

**TEXT-Figure 3.** Binding of mouse monoclonal antibodies C3 and C6 directed against the human thyroid microsome (MAHM C3 and C6) to thyroid cell monolayer derived from two donors, A (hatched bars) and B (open bars). Each point is the mean ± s.e.m. of triplicate cultures assayed, as described by enzyme-linked immunosorbent assay. Non-specific binding was assessed using normal mouse immunoglobulin (Ig). Also shown is the absorbance given by the mouse monoclonal antibody B4, to human thyroglobulin (MAHTG B4).

By contrast, MAHTG B4, a murine IgM monoclonal antibody specific for human TG, showed no significant binding over that found with normal mouse Ig (P < 0.1, Student's t-test). The binding of MAHM C3 could only be partially inhibited by free M antigen; in one experiment the absorbance with C3 fell from 0.331 ± 0.017 to 0.235 ± 0.025 with 100 µg M antigen. A dose effect was also apparent; with a final concentration of 200 µg MAHM C3/ml in the culture medium the absorbance given by a thyroid monolayer was 0.574 ± 0.055 and with 20 µg/ml 0.363 ± 0.049.

**Cytotoxicity**

Mouse anti-human microsomal antibody C4 but not B4 was cytotoxic to thyroid cells in the presence of
complement in both $[^3]H$thymidine incorporation and crystal violet staining assays (Text-fig. 4). The mouse monoclonal antibody MAROX8 to rat T lymphocytes (Sera Lab.) served as a negative control against which the effects of MAHM C4 and B4 were assessed. By the crystal violet staining method both C3 and C6 were also found to be cytotoxic with complement. The absorbances were: normal mouse Ig 0.301 ± 0.011, Triton 0.233 ± 0.006, MAHM C3 0.271 ± 0.003 and MAHM C6 0.270 ± 0.017. These absorbances with MAHM antibodies were significantly lower than for normal mouse Ig ($P < 0.01$, Student’s $t$-test).

![Image](https://via.placeholder.com/150)

**TEXT-Figure 4**. Complement-dependent cytotoxicity assessed by (a) post-labelling with $[^3]H$thymidine and (b) crystal violet staining measured as absorbance. In both assays complement was present at the same concentration throughout. The left half of the figure shows the effect of normal (control −) and Hashimoto (control +) sera and 5% Triton. The commercially available murine monoclonal antibody (MAROX8) against a rat T lymphocyte subset served as a negative control for the effects of mouse anti-human microsomal antibodies C4 and B4 (MAHM C4 and B4). Monoclonal antibodies were present at a final concentration of 1 mg/ml with MAHM C4 and B4 each antibody was at 0.5 mg/ml. Each point is the mean ± S.D. of six replicates. **$P < 0.05$, $**P < 0.01$, $***P < 0.001** compared with MAROX8 (Student’s $t$-test).

### DISCUSSION

Murine monoclonal antibodies which bind specifically to the thyroid M antigen were produced despite contamination of the original immunizing antigenic material with TG (Weetman et al. 1984). The protocol we used produced IgM-secreting hybridomas because the period between immunization and fusion was only sufficient to generate a primary response. We also found that early addition of HAT (experiment 5) seemed to give a better yield of clones. The MAHM antibodies showed the typical distribution pattern of human M antibodies by immunolocalization and also bound to viable thyroid monolayers in vitro. It is of interest that the binding of MAHM C3 to thyroid cells was only partially inhibited by the addition of free M antigen suggesting differences in the epitope recognized by C3 in the cell surface and free forms of the antigen. Three of the four monoclonals were cytotoxic to thyroid cells in vitro in the presence of complement; the reasons for the lack of cytotoxicity with MAHM B4 are not clear. However, this monoclonal antibody

<table>
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<tr>
<th>Donor of PBM</th>
<th>$^{51}$Cr Release (c.p.m.)</th>
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<tbody>
<tr>
<td>M-CRBC + MAHM B4</td>
<td>499 ± 97</td>
</tr>
<tr>
<td>M-CRBC + PBM</td>
<td>801 ± 106</td>
</tr>
<tr>
<td>M-CRBC + MAHM B4 + PBM</td>
<td>848 ± 42</td>
</tr>
<tr>
<td>Triton</td>
<td>2318 ± 73</td>
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Background release is shown in the first line, release due to natural killer cell activity in the second line, antibody-dependent cytotoxicity in the third line and total releasable c.p.m. in the last line. MAHM, mouse anti-human microsomal antibody.

#### TABLE 1. Antibody-mediated cytotoxicity using peripheral blood mononuclear cells (PBM) from three normal donors (1, 2 and 3). $^{51}$Cr Release from microsome-coated chicken red blood cells (M-CRBC) is shown as mean (± S.D.) c.p.m. released into the supernatant.

Antibody-dependent cytotoxicity was sought using MAHM B4 (Table 1). It can be seen that untreated CRBC, that is coated with M antigen but without the addition of MAHM B4, were subject to killing (natural killer cell activity) by PBM from all three donors. However, there was a significant increase of 17 and 40% in killing when M-coated CRBC were pre-incubated with MAHM B4 using PBM from donors 2 and 3 ($P < 0.01$, Student’s $t$-test), reflecting antibody-dependent cytotoxicity. There was also a 16% increase in cytotoxicity with MAHM B4 using PBM from donor 1 but this was not statistically significant.
was capable of mediating antibody-dependent (K—) cell-mediated cytotoxicity. The differences between these two types of cytotoxicity include the form of the M antigen recognized; in the complement-dependent experiments this was the M antigen naturally expressed on the cell surface whereas for antibody-dependent cellular cytotoxicity the antigen was the ultracentrifuged, gel-filtered antigen artificially linked to CRBC. These results provide support for the incomplete expression of certain M antigen epitopes on the thyroid cell surface (Fenzi et al. 1982). Antibody B4 clearly recognizes an epitope of M antigen which is either intracellular or insufficiently concentrated on the thyroid cell surface to mediate cytotoxicity, since all IgM isotope antibodies should fix complement. The differences in immunolocalization and binding between C3 and C6 and in complement-dependent cytotoxicity between C4 and B4 suggest that these antibodies recognize different epitopes on the M antigen.

The production of stable monoclonal M antibodies overcomes the difficulties of producing specific polyclonal sera in animals for the further characterization of the M antigen. Although these monoclonal antibodies are xenogeneic, they nonetheless behave as human M antibodies do in immunolocalization and cytotoxicity studies (Khoury et al. 1981, 1984). These antibodies are therefore suitable for purification of the M antigen and for use in defining the nature of thyroid cell surface antigens by double-immunofluorescence techniques. These experiments will be the subject of a further report.

ACKNOWLEDGEMENTS

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REFERENCES


DESCRIPTION OF PLATE

Immunoperoxidase staining by the mouse monoclonal antibody C3 to the human thyroid microsome bound to thyroid follicular cells (Fig. 1) compared with control section without monoclonal antibody (Fig. 2) (× 507).