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Characterization of monoclonal antibodies directed towards the microsomal/microvillar thyroid autoantigen recognized by Hashimoto autoantibodies

J. P. BANGA, RITA MIRAKIAN, LINDA HAMMOND, G. PRYCE, S. BIDEY,* F. BOTTAZZO, A. P. WEETMAN,† A. M. McGREGOR & I. M. ROITT

*Department of Immunology and *Department of Molecular Endocrinology, The Middlesex Hospital Medical School, London, and †University of Wales College of Medicine, Heath Park, Cardiff, UK*

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SUMMARY

Monoclonal antibodies reacting with thyroid microsomes have been described. In this report, we present evidence that some of these monoclonal antibodies (MoAb) MAH C3 and perhaps MAH C6 are directed towards the thyroid microsomal antigen towards which microsomal autoantibodies from Hashimoto patients are directed. Immunofluorescence on cryostat sections with MAH C3 and MAH C6 on thyroid gland gave cytoplasmic staining patterns that were comparable to those obtained with autoantibodies. By western blotting, reactivity of MAH C3 was shown to be directed towards a 105,000 mol. wt component, comparable to that recognized by autoantibodies by immunoprecipitation. Double immunofluorescence with MAH C3 and autoantibodies on cultured, viable thyroid monolayers revealed a large number of comparable binding sties; in contrast, binding sites for asioloagalactothyroglobulin were different of binding of MAH C3 by the autoantibodies suggesting that the epitopes recognized were different. This was confirmed with the use of a rat thyroid cell line FRTL-5 and fluorescence activated cell sorter analysis which showed binding to the rat microsomal component with autoantibodies but negligible binding with MAH C3 and MAH C6. It therefore appears that the monoclonal antibodies recognize species specific determinants while the autoantibodies recognize species cross rective determinants. These monoclonal antibodies will be useful for purification and immunochemical analysis of this autoantigen in human thyroid disease.

Keywords monoclonal antibodies thyroid microsomal antigen Hashimoto autoantibodies

INTRODUCTION

Several thyroid autoantibodies have been described against different autoantigens in thyroid autoimmune disease, including those directed towards the thyroid microsomal/microvillar antigen (Roitt, Doniach & Bottazzo, 1979). These thyroid autoantibodies of various specificities lead to destructive pathological or blocking processes, as in patients with Hashimoto's thyroiditis and primary myxoedema or to abnormal stimulation of hormone synthesis or excessive growth stimulation as in Graves' disease and in patients with recurrent goitre formation (Bottazzo Drexhage & Khoury, 1982; Weetman & McGregor, 1984).

The thyroid microsomal antigen has been characterized by immunoprecipitation with human autoantibodies as a discrete protein of 105 kD which is poorly glycosylated and probably stabilized

Correspondence: J. P. Banga, Department of Immunology, St Thomas' Hospital Medical School, The Rayne Institute, London, SE1 7EH, UK.

by intrachain disulphide bonds (Banga *et al*, 1985). Although the microvillar form of the antigen is serologically and biochemically similar, with comparable molecular weight to the microsomal counterpart, both forms of the autoantigens are distinct from soluble or membrane bound thyroglobulin.

Purification of this autoantigen by biochemical fractionation procedures or with human autoantibodies has so far proved to be difficult. The presence of large amounts of thyroglobulin in the gland inevitably lead to thyroglobulin contamination in the fractions containing microsomal reactivity, even with high resolution systems such as High Performance Liquid Chromatography (HPLC) (Banga *et al.*, 1985). In addition, approaches utilizing human sera containing antimicrosomal autoantibodies for affinity purification of this protein have not been reported.

Monoclonal antibodies (MoAb) have recently been produced to the human thyroid microsomal fraction by initially immunizing mice with partially purified microsomes (Weetman *et al.*, 1985). There specificities have been established by sensitive enzyme-linked immunosorbent assays (ELISA) (Weetman *et al.*, 1983) and it has been shown by immunolocalization studies utilizing peroxidase-conjugated antibody that they stain the cytoplasm of human thyroid sections (Weetman *et al.*, 1985). As an extension of this work, we now present further characterization of the thyroid microsomal/microvillar autoantigen recognized by the MoAb raised in the aforementioned study based on enlarged immunofluoresence studies using thyroid cryostat sections, viable thyroid cells in culture and the rat FRTL-5 thyroid cell line. Biochemical evidence on the nature of the autoantigen recognized by one of the MoAb is also presented, including definition of some epitopes recognized by monoclonal and human autoantibodies on the microsomal microvillar autoantigen. These MoAb will be useful for purification of the microsomal/microvillar autoantigen, which will be a subject of a separate report.

MATERIALS AND METHODS

Patients' sera and autoantibody tests

Autoantibody to thyroglobulin and microsomal antigen were measured using the Thymune-T and Thymune-M kits (Wellcome, Beckenham, UK) respectively. Serum from patients known to contain autoantibodies to microsomal/microvillar thyroid antigens or thyroglobulin was utilized for immunofluorescence as previously described (Khoury *et al.*, 1981). For immunoprecipitation studies with antimicrosomal/microvillar autoantibodies, serum from two patients utilized in a previous study (Banga *et al.*, 1985) was used.

Preparation of monoclonal antibodies

Monoclonal antibodies reacting specifically with thyroid microsomal membrane constitutents were prepared by immunizing mice with enriched microsomal antigen preparation from a Graves' thyroid as described (Weetman *et al.*, 1985). Seven monoclonal IgM antibodies specific for thyroid microsomal antigen were used in this study and one of these clones MAH C3 was selected for further study. In addition, anti-thyroglobulin MoAb MAHTg B4 was also used.

Tissue culture

Human thyroid cell monolayers were grown on glass coverslips essentially as described (Khoury *et al.*, 1981). A 36 h culture of $\geqslant 90\%$ thyroid cells was employed for indirect immunofluorescence, using either a patient's serum containing a high titre of microsomal antibody ($\geqslant 80$ by haemagglutination) or monoclonal MAH C3.

The FRTL-5 rat thyroid cell line was maintained and grown as previously described (Ambesi-Impiobato *et al.*, 1980; 1982; Bidey *et al.*, 1984). To obtain isolated cells from the confluent cultures for cell surface fluorescence analysis, the cells were treated with a mixture of trypsin/collagenase/chick serum (1:10 ratio) (Ambesi-Impiobato *et al.*, 1980). After careful sedimentation of cells (200 ***g***, 7 min), cells were resuspended by gentle pipetting: by this procedure isolated cells of $\geqslant 90\%$ viability were usually obtained and used for fluorescence analysis.

Immunofluorescence

Cytoplasmic staining on frozen human thyroid tissue. Snap frozen, 4 μm cryostat sections were incubated with patient's serum (1:10 dilution) or 1 mg/ml MoAb. Human blood Group O stomach, adrenal, pituitary and pancreas tissues were stained as tissue controls with the MoAb.

Cell surface staining on thyroid monolayers. This was performed by similar protocol already described (Roitt *et al.*, 1984). Thyroid cell cultures from a thyrotoxic gland grown on coverslips for 48 h were incubated for 30 min with serum from a patient with Hashimoto's thyroiditis (diluted 1/10) known to contain autoantibodies to the microsomal/micovillar thyroid antigen (titre 1:320). After washing this was followed by 25 min incubation with tetramethylrhodamine-conjugated rabbit anti-human IgG (Dako, High Wycombe, UK). The second step of this procedure was performed by applying ascitic fluid (diluted 1/50) containing the MAH C3 MoAb to microsomal/microvillar antigen for 30 min. After washing, the cultures were fixed for 10 min with 5% ethanoic acid (acetic acid) in ethanol at −20°C followed by a 25 min incubation with fluoresceinated-rabbit anti mouse Ig which did not crossreact with human immunoglobulin.

Fluorescent activated cell analysis (FACS of the FRTL-5 rat thyroid cell line). Indirect immunofluorescence was performed with saturating amounts of MoAb or patient's serum. Washed FRTL-5 cells in Hams' medium containing 5% new born calf serum (5×10^5 cells) were resuspended in 50 μl aforementioned medium containing 0·1% sodium azide to which was added 50 μl monoclonal neat superantant or 50 μl patients serum diluted 1:10 in the latter medium. For controls, the cells were stained with either fluorescein conjugated concanavalin A (positive controls) or the first step antibody was omitted and cells stained with fluorescein conjugated sheep anti-mouse (or human) Fab. The stained cells were analysed for forward angle light scattering (cell size) and scatter gated fluorescence using fluorescence activated cell sorter (FACS IV, Beckton Dickinson, Erembodegem, Belgium).

Western blotting (immunoblotting). Thyroid microsomal membrane polypeptides, separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper (Towbin, Staehelin & Gordon, 1979). Following transfer, the nitrocellulose paper was stained with monoclonal reagents, essentially as described (Banga *et al.*, 1984). Immunoprecipitation analysis on solubilized radiolabelled thyroid microsomes with patients autoantibodies was performed as described previously (Banga *et al.*, 1985).

RESULTS

Monoclonal antibodies recognizing thyroid cell constituents

Seven MoAb reacting specifically in ELISA with thyroid microsomal components and one MoAb reacting with human thyroglobulin, whose preliminary characterization has already been reported (Weetman *et al.*, 1985) were used and their characteristics are shown in Table 1. The MoAb MAH C3 showing microsomal reactivity for further study.

Immunofluorescent staining of human thyroid gland

All the seven MoAb showed cytoplasmic reactivity on unfixed and acetone fixed cryostat sections of thyroid gland (Table 1). MAH C3 in Fig. 1a shows a typical staining pattern of microsomal reactivity which is comparable to that seen with patients anti-microsomal autoantibodies (Fig. 1b). The remaining MoAb in Table 1 gave a typical microsomal staining pattern also, but the fluorescence intensity was consistently weaker and less defined than that obtained with MAH C3 and MAH C6. Only the MoAb to thyroglobulin (MAHTG B4) reacted with methanol fixed cryostat section of the gland (Table 1) and this staining pattern (Fig. 1c) was similar to that observed with patients' anti-thyroglobulin autoantibodies (not shown).

None of the above MoAb showed any reactivity by fluorescence with other organs commonly involved in organ specific autoimmune such as stomach, adrenal, pituitary and pancreas (Table 1).

Immunofluorescent staining of human thyroid cultures

Both MAHC3 and MAHC6 gave cell surface fluorescence patterns on viable thyroid monolayer

Table 1. Specificity of anti-microsomal and anti-thyroglobulin MoAb

Antibody	Class	Specificity by ELISA	Microsomal immunofluorescence: Cryostat section: Unfixed	Cryostat section: Methanol fixed	Cryostat section: Acetone fixed	Cell surface	X-reaction on other organs involved in organ specific autoimmunity: Pituitary	Pancreas	Adrenal	Stomach	Mol wt of component recognized
MAH C3	IgM	Microsomal	++++	−	++++	++	−	−	−	−	105 kD
MAH C6	IgM	Microsomal	+++	−	+++	+	−	−	−	−	(105 kD)w
MAH C5	IgM	Microsomal	++	nt	+++	w+	−	−	−	−	−
MAH B6	IgM	Microsomal	+	−	nt	nt	−	−	−	−	−
MAH A5	IgM	Microsomal	+	nt	++	nt	−	−	−	−	−
MAH A2	IgM	Microsomal	++	nt	++	nt	−	−	−	−	−
MAH C1	IgM	Microsomal	w+	nt	nt	nt	−	−	−	−	−
MAHTg B4	IgM	Thyroglobulin	nt	++	+	−	−	−	−	−	−

(w) weak.
(−) Negative.
nt Not tried.

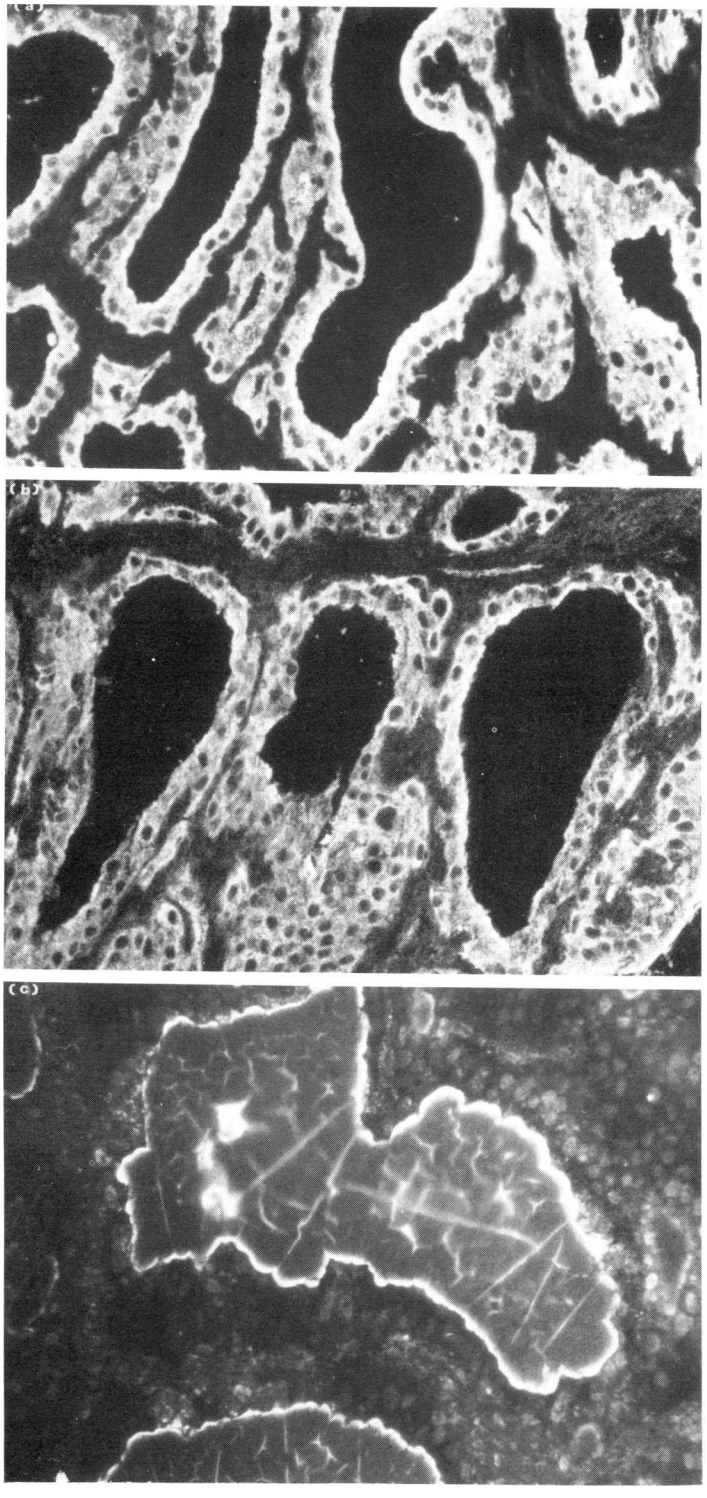

Fig. 1. Indirect immunofluorescence staining of frozen tissue section of a thyrotoxic gland showing binding of (a) monoclonal anti-microsomal antibody MAH C3, (b) Hashimoto patients anti-microsomal autoantibody. Both show comparable staining patterns consisting of linear staining on the apical border and intracellular staining of the thyrocytes, without any staining of the colloid. (c) Shows staining with monoclonal anti-thyroglobulin antibody MAH Tg B4, consisting of staining of the colloid. $\times 250$.

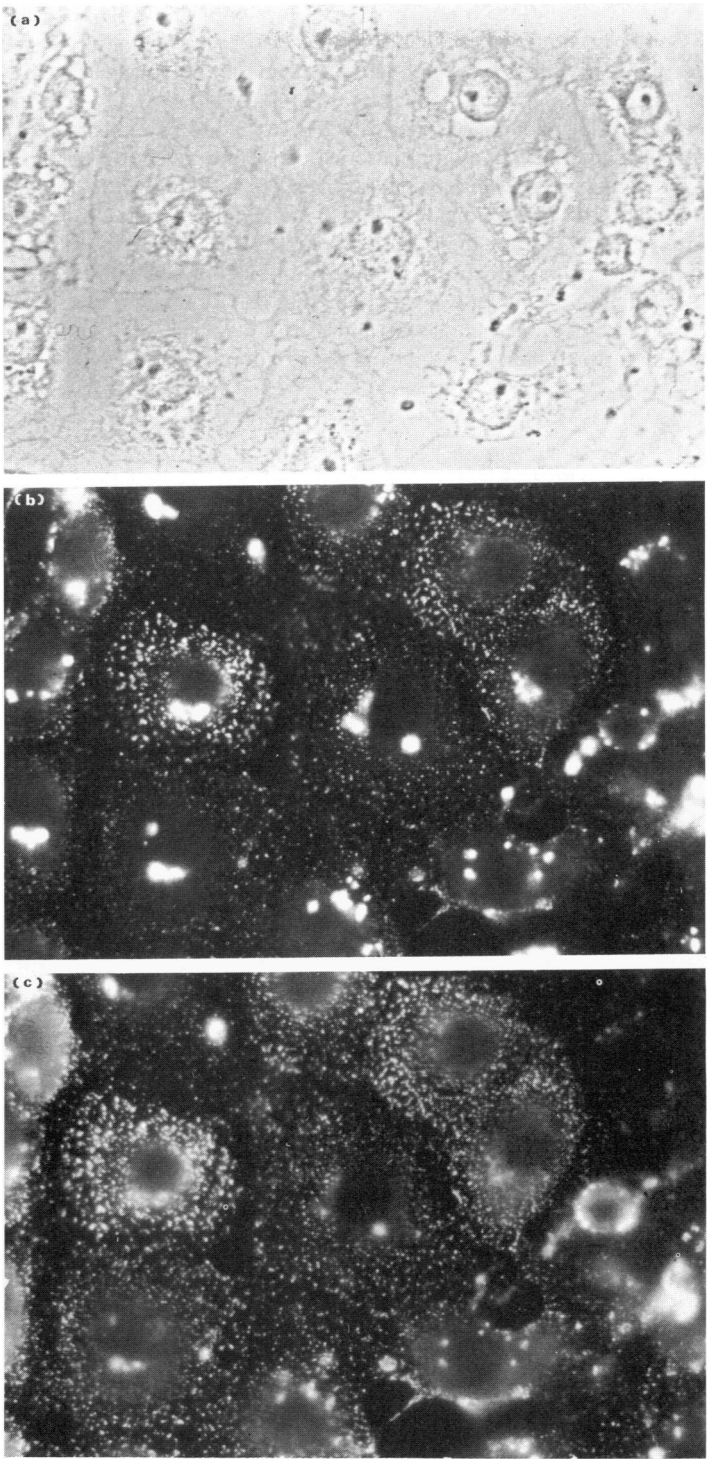

Fig. 2. Double immunofluorescence staining of viable, cultured thyroid monolayers with MAH C3 and microsomal autoantibody from a Hashimoto patient. (a) Phase contrast of the field, (b) photograph to show distribution of microsomal autoantibody. ×450. The two antibodies showed closely comparable fluoresence.

cultures while MAH C5 gave a much weaker fluorescence pattern (Table 1). Anti-thyroglobulin MoAb (MAHTG B4) did not show any cell surface fluorescence on viable thyroid monolayers confirming that thyroglobulin cannot be demonstrated on the plasma membrane of these thyrocytes by immunofluorescence techniques (Roitt *et al.*, 1984).

Simultaneous cell surface double staining with MAH C3 and patient's microsomal autoantibody gave closely comparable staining patterns consisting of dotted, discrete membrane fluorescence characteristic of the microvillar antigen (Fig 2b,c). Approximately 95% of the fluorescent spots contained both conjugates while the remainder appeared to be stained with the polyvent autoantibody only. Although in the double staining technique the monoclonal antibody was applied after the patient's autoantibody, clearly no blocking of binding of the monoclonal reagent by the human autoantibodies was apparent (Fig 2b,c). In contrast, concomitant staining with asialoagalacto-thyroglobulin (fluorescein) and either microsomal autoantibodies or MAH C3 (rhodaminated) gave distinctly separate dots and distribution patterns (not shown).

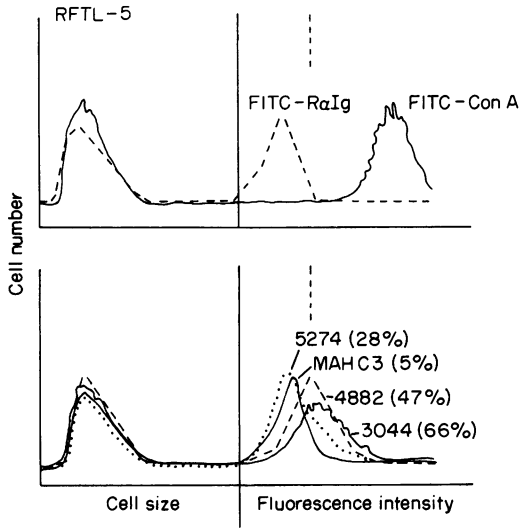

Fig. 3. Indirect immunofluorescence analysis of trypsin/collagenase treated, isolated rat thyroid cell line FRTL-5 with various antibodies and reagents. The analysis was performed on a FACS IV instrument with forward light scatter analysis. Fluoresence intensity is on a log scale.

Upper panel. Fluorescence intensity of cells labelled with FITC-Con A showing 100% positive cells compared with a negative control with FITC-rabbit anti-human Ig (FITC- RαIg) ($\leqslant 5\%$ positive). An identical frequency distribution was obtained with FITC-rabbit anti-mouse Ig (not shown). In the fluorescence intensity section, all cells on the left of the dotted, vertical line are scored negative.

Lower panel. Fluorescence analysis with MoAb MAH C3 compared with that obtained with three different patients sera containing different autoantibodies. The human autoantibodies had the following titres:

Serum no.	TGHA	MCHA
5274	1280	−ve
4882	−ve	1280
3044	5000	1280

The values in brackets show the percentage of positive cells. No specific binding with MAH C3 (or MAH C6, not shown) was seen while specific binding was apparent with autoantibodies.

Immunofluorescent staining of FRTL-5 rat thyroid cell line

The reactivity of the anti-microsomal MoAb MAH C3 (and MAH C6) with FRTL-5 cells was assessed by indirect immunofluorescence and FACS analysis and compared to binding of several human microsomal and thyroglobulin autoantibodies. Binding of fluoresceinated Concanavalin A gave cells which were very brightly positive; in contrast, under these conditions, negative controls such as fluoresceinated anti-mouse or anti-human immunoglobulin showed less than 2% weakly positive cells (Fig. 3). Cell surface expression of thyroglobulin on FRTL-5 was clearly demonstrable using anti thyroglobulin autoantibodies from Hashimoto patient's serum (serum 5274), which was free of microsomal antibodies. Comparable number of positive cells were also seen with two other sera containing anti-thyroglobulin (not shown); however, MoAb to human thyroglobulin MAHTg B4 did not bind specifically to these cells (not shown). A human serum containing microsomal antibodies reacted with 47% of the cells (serum 4882; Fig. 3); other sera with similar autoantibodies showed positive cell numbers of 43% (serum 4100), 54% (serum 3567) and 46% (serum 3278) (not shown). In contrast, MAH C3 (and MAH C6, not shown) showed negligible binding to the FRTL-5 cell lines comparable to controls labelled with the second layer, fluorescent antibody only (Fig. 3).

Immunochemical characterization of thyroid microsomal antigen(s) reacting with monoclonal MAHC3 antibodies

The microsomal membrane components reacting with MoAb were examined by immunoprecipi-

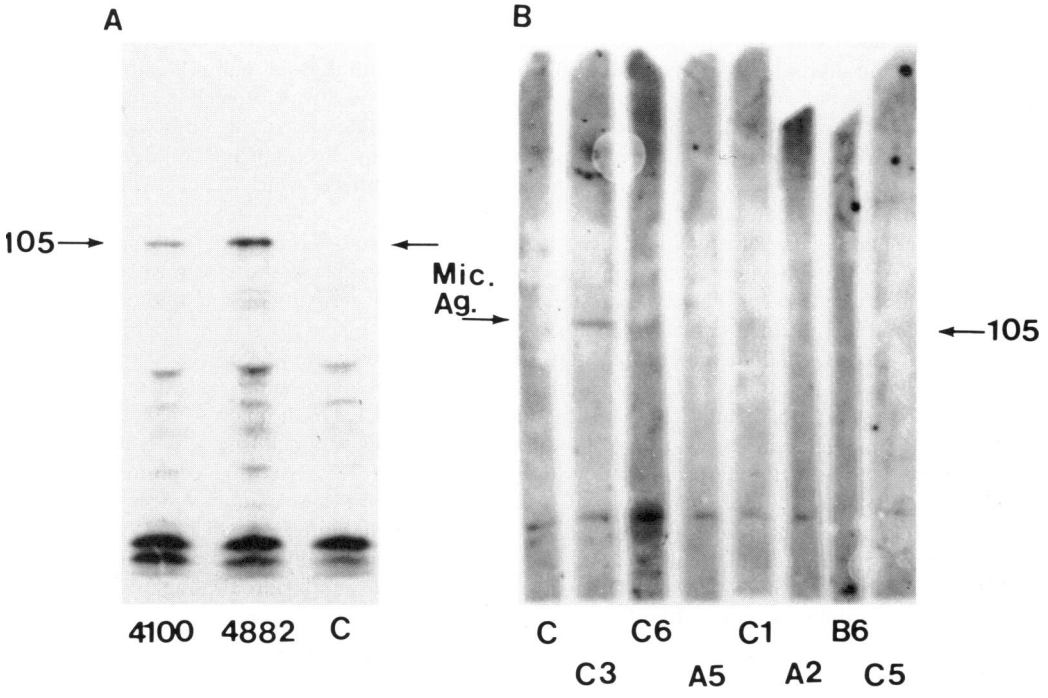

Fig. 4. Biochemical analysis of human thyroid microsomal antigen. (A) the microsomal antigen was immunoprecipitated with two sera containing antimicrosomal auto-antibodies (patients 4100 and 4882). No thyroglobulin autoantibodies were detectable in these sera. Lane C represents a control, where autoantibody was omitted. (B) western blot of human thyroid microsomes following SDS-PAGE and staining with seven anti-microsomal monoclonal antibodies. ^{125}I-sheep anti mouse FAB was used to detect the bound antibody.

The microsomal polypeptide in (A) migrates with a mol. wt of 105,000; the microsomal component reconized in (B) with MAH C3 migrates in this mol. wt region also—the remaining MoAb antibodies do not show any reactivity. The band seen with the tracking dye in panel B is non-specific. (The gels relating to (A) and (B) are different so that the mol. wt markers have not migrated to comparable positions).

tation techniques. Using solubilized radiolabelled microsomal membrane, none of the monoclonal antibodies described in Table 1 showed any specific components on immunoprecipitation, when analysed by SDS-polyacrylamide gels (not shown); modifying the conditions such as incubating antibodies and microsomal membranes for 24 h to allow maximum precipitate formation did not immunoprecipitate any specific components either. This may be due either to low affinity antibody, or SDS labile determinants or to a specificity for glycolipid determinants. Reactivity of the monoclonals was therefore examined by Western blotting. The results in Fig. 4B shows that MAH C3 reacts with a polypeptide component of approximately 105,000 mol. wt under reducing conditions. The remaining monoclonal antibodies do not show any specific reactivity under these conditions although MAH C6 may perhaps show some binding to the 105,000 mol. wt component (Fig. 4B). A prominent band migrating with the tracking dye was seen with all the MoAb and represents non-specific binding. For comparison, in Fig 4A, microsomal autoantibodies characteristically identify the 105,000 polypeptide by immunoprecipitation as the microsomal antigen. None of the patients' sera used in this study showed any reactivity by immunoblotting (not shown). The results therefore unequivocally show that MAH C3 identifies a polypeptide of 105 kD, comparable in molecular weight to the protein identified as the thyroid microsomal antigen by immunoprecipitation utilizing microsomal auto-antibodies (Banga *et al.*, 1985).

DISCUSSION

The work reported in this paper describes the characterization of MoAb reacting with thyroid microsomal/microvillar autoantigen. By an ELISA, several of these MoAb showed specificity towards human thyroid microsomes and negligible reactivity with thyroglobulin (Weetman *et al.*, 1983). As there are likely to be a large number of components derived from intracellular membranes of thyroid cells towards which these monoclonals could be possible directed, this study was initiated to determine whether some or all of the monoclonals described by Weetman and colleagues (1985) were directed exclusively to the thyroid microsomal autoantigen which reacts with human microsomal autoantibodies.

Immunofluorescence on an unfixed, cryostat sections of thyroid glands with, the MoAb MAH C3 (and MAH C6) gave fluorescence patterns that were comparable to those seen with patients' microsomal autoantibodies. There was no staining of the colloid, verifying the immunolocalization data on paraffin embedded tissue (Weetman *et al.*, 1985) and thus excluding the possibility that denaturation of the colloid moieties had occurred during the fixation steps. The similarity in immunofluorescence patterns on tissue sections and on viable thyroid monolayers with microsomal autoantibodies and MoAb indicated that the latter could be directed to the microsomal component which is the major autoantigen in thyroid autoimmunity. This was reaffirmed by concomitant staining with the patient's microsomal autoantibodies and the MoAb MAH C3 on cultured thyroid cells using two different fluorescent probes. Close inspection of the double fluorescence on thyroid monolayers showed that virtually all the dots reacted with the autoantibodies and MAH C3, although there were a small number of binding sites which stained only with the autoantibodies. This may be due to a higher titre of the antibodies in the patient's serum although one cannot exclude the possibility that the autoantibodies recognize a further minor antigenic species relative to the MoAb. The failure of the polyclonal human autoantibodies to block the binding of the MoAb MAH C3 suggests that the two epitopes on the microvillar autoantigen are spatially distinct, even though close to each other. The specificity of this double technique was confirmed with the use of asialagalactothyroglobulin which is known to bind to the surface of cultured thyroid cells at a site distinct from the microsomal antigen (Roitt *et al.*, 1984). Concomitant staining of either microsomal autoantibodies or MAH C3 together with asialoagalactothyroglobulin gave distinct dots and exhibited entirely different distribution patterns, confirming our earlier study and giving some confidence in the discriminating power of the double immunofluorescence technique.

The unique nature of the serologically distinct epitopes on the microvillar autoantigen recognized by autoantibodies and MAH C3 (and MAH C6) was further confirmed with the use of the rat thyroid cell line FRTL-5, which is known to express the rat counterpart of the microsomal/

microvillar antigen (Chiovato *et al.*, 1985). Cell surface binding studies with autoantibodies and MoAb on isolated FRTL-5 cell analysed by FACS clearly indicate that the epitope(s) recognized by autoantibodies are present on the rat counterpart of the microvillar antigen while the epitopes recognized by MAH C3 and MAH C6 are clearly absent. These findings demonstrate that autoantibodies recognise species cross-reactive determinants on the microsomal/microvillar antigen of human and rat thyroid cells while the two monoclonal antibodies are restricted in their specificity to species specific epitopes on the human molecule. We have not investigated if MAH C3 and C6 recognize separate or identical determinants.

The ability of the MoAb to stain both the cytoplasm and the plasma-membrane of the thyroid cell shows convincingly that the antigens in these two locations share closely similar if not identical specificity and confirms earlier studies with polyvalent autoantibodies which indicated immunological identity between the microsomal and microvillar antigens (Khoury *et al.*, 1981).

All the seven MoAb described in this study retain their organ specificities and none showed any reactivity with other tissues commonly involved in organ specific reactivity. Although the epitope specificities of the hybridomas studied were shown to be disparate from the epitope recognized by autoantibodies, our results favour the hypothesis that different autoantibodies react with different autoantigens in tissues involved in organ specific autoimmunity. Other arguments in favour of this proposal include our preliminary data on mol. wt characteristics of the microsomal autoantigens in different tissues such as the adrenal where the antigen recognised by the serum of patients with Addison's disease reacts with a discrete 38,000 mol. wt polypeptide (Banga *et al.*, 1985), which is very different from the thyroid microsomal component. In addition, by careful tissue absorption studies, Adams and colleagues have shown clearcut disparity in the autoantibody specificities to different tissues, with autoantibody of one tissue specificity involved in organ specific autoimmunity being unable to be absorbed by a different tissue homogenate (Knight *et al.*, 1984a,b). This is directly in contrast to reports from Notkins and colleagues who have murine and human MoAb which react with identical or common determinants in different tissues involved in organ specific immunity (Haspel *et al.*, 1983; Satoh *et al.*, 1983; Prabhakar *et al.*, 1984), which has thus given rise to the suggestion that autoantibodies exist with some specificities reacting with common antigenic determinants in different tissues.

Finally, these MoAb may prove to be useful reagents to isolate and purify the human thyroid microsomal autoantigen for immunochemical analysis. Although the hybridomas are of IgM class due to fusion following primary responses and not suitable for antibody affinity purification of the antigen, we are now endeavouring to produce IgG hybridomas solely for this purpose.

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