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The expression of the microsomal/peroxidase autoantigen in human thyroid cells is thyrotrophin-dependent

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SUMMARY

In the present report the mechanisms responsible for the expression of the thyroid microsomal autoantigen (M-Ag) were studied in primary cultures of human thyroid cells prepared from Graves' or non-toxic goitres. The indirect immunofluorescence (IFL) technique using human sera positive for anti-microsomal antibody (anti-MAb) was employed to detect M-Ag. Studies were performed to ascertain whether M-Ag recognized by anti-MAb could be identified with thyroid peroxidase (TPO). Preabsorption experiments showed that, similarly to solubilized thyroid microsomes, purified human TPO abolished the binding of anti-MAb to thyrocytes, while no inhibition was obtained with control human tissues. The identity of M-Ag and TPO was also demonstrated using a double layer IFL technique which allowed a simultaneous staining of the antigen(s) recognized by anti-MAb and by a monoclonal anti-TPO antibody. After 5–15 days of TSH withdrawal from the culture medium the M/TPO-Ag disappeared from the surface and the cytoplasm of human thyroid cells. Readdition of TSH (0·1–100 mU/ml) to cells lacking M/TPO-Ag elicited its reappearance within 48–72 h. This effect of TSH was prevented by 10 μM cycloheximide but not by methimazole (0·1–2 mM). Two stimulators of the adenylate cyclase-cAMP system, cholera toxin and forskolin, and 8-bromo-cAMP mimicked TSH in inducing M/TPO-Ag. Thyroid stimulating antibody (TSAb) of Graves' disease also reproduced the effect of TSH on M/TPO-Ag reexpression in human thyroid cells. By contrast, epidermal growth factor, oestradiol or NaI were ineffective in inducing M/TPO-Ag. The present data indicate that: (i) the expression of M/TPO-Ag in human thyroid cells is dependent on TSH stimulation, through pathways which involve cAMP production and protein synthesis, (ii) TSAb reproduces this effect of TSH; (iii) oestradiol and NaI have no direct influence on the expression of M/TPO-Ag.

Keywords thyroid microsomal antigen thyroid peroxidase thyrotrophin anti-microsomal antibody

INTRODUCTION

The thyroid microsomal autoantigen (M-Ag) is a target of the immune aggression in Hashimoto's thyroiditis, idiopathic myxedema and Graves' disease (Weetman & McGregor, 1984; Pinchera *et al.*, 1985). It was previously shown in ours (Pinchera *et al.*, 1980; Fenzi *et al.*, 1982) and other (Khoury *et al.*, 1981) laboratories that M-Ag, historically considered a cytoplasmic lipoprotein, is also represented on the thyroid cell surface at the microvillar pole, and may be involved in the complement-mediated cytotoxicity of sera from patients with autoimmune thyroid disorders (Khoury *et al.*, 1984). Moreover, recent evidence indicates that in autoimmune thyroid glands follicular cells bearing HLA Class II molecules (DR) (Hanafusa *et al.*, 1983) can directly present their surface autoantigens to the immune system (Londei, Bottazzo & Feldmann, 1985). The latter mechanism could initiate and/or maintain the autoimmune process against the thyroid (Bottazzo *et al.*, 1983). In the last few years evidence was also provided that human M-Ag can be identified with thyroid peroxidase (TPO) (Czarnocka *et al.*, 1985; Kotani *et al.*, 1986; Mariotti *et al.*, 1987; Ruf *et al.*, 1987).

The mechanisms leading to the expression of M-Ag in follicular cells were unknown until we demonstrated that M-Ag is present on the surface of a peculiar strain of differentiated rat thyroid cells (FRTL-5) and that in this experimental model its expression is modulated by thyrotrophin (TSH) (Chiovato *et al.*, 1985). These results prompted us to investigate whether the same mechanism was responsible for M-Ag expression in

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human thyroid cells. Furthermore, the putative identitiy of M-Ag and TPO was investigated, both in terms of antigenic properties and TSH-dependent modulation.

MATERIALS AND METHODS

Human thyroid cells in primary culture

Eight thyroid glands (six from Graves' and two from non-toxic goitres) were obtained at surgery. Thyroid tissue was minced, digested with collagenase (Worthington type IV, 3 mg/ml) for 3 h at 37°C, and passed through a 200 μm nylon mesh to eliminate big clumps. Cell viability was checked by differential staining with acridine orange/ethidium bromide and was more than 80% in all preparations. Roughly 10^5 cells were seeded onto round coverslips plated in each well of a Costar 24-well plate (Costar, Cambridge, MA). Thyroid cells were cultured in 5% CO_2-95% air at 37°C in Coon's modified Ham F-12 medium supplemented with 5% adult calf serum, and a 6-hormone mixture (6H medium) containing: insulin (1·6 μM), cortisol (10 nM), transferrin (62·5 nM), 1-glycyl-histidyl-lysine (25nM), somatostatin (6·25 nM) and TSH (300 μU/ml). Two to three days after seeding monolayer cultures were obtained.

Sera

Sera from five patients with Hashimoto's thyroiditis were used. All had high titres (⩾1/102·400) of anti-microsomal antibody (anti-MAb) and undetectable anti-thyroglobulin antibody, as assessed by passive haemagglutination (Microsome Test Kit and Thyroid Test Kit, Fujizoki, Tokyo, Japan) and by immunoradiometric techniques (Mariotti *et al.*, 1982; Mariotti *et al.*, 1983). These sera were negative for thyroid stimulating antibody (TSAb) or TSH-blocking antibodies, as detected by methods using human thyroid membranes (Macchia *et al.*, 1981) or FRTL-5 cells (Vitti *et al.*, 1982; Chiovato *et al.*, 1987). Eight sera from normal subjects, which were negative in all thyroid antibody tests, were used as controls. IgG was prepared by DEAE Sephadex separation from serum of a patient with active Graves' disease. This IgG was strongly positive for TSAb, as measured by cAMP production assays in FRTL-5 cells and human thyrocytes.

Experimental procedure

Thyroid cells were cultured for 7–15 days in the above-described medium deprived of TSH (5H medium). At the end of this period thyrocytes were recultured for 3–7 days in 5H medium containing TSH (0·1–100 mU/ml) or other agents: cholera toxin (10 pM), forskolin (5–50 μM), 8-bromo-cAMP (0·5 mM), IgG containing TSAb (1 mg/ml), NaI (0·1–10 mM), oestradiol (3·6–73·5 nM), or epidermal growth factor (EGF, 1 nM). In other experiments thyrocytes, deprived of TSH for 7–15 days were recultured in 5H medium containing TSH (0·1–10 mU/ml) plus methimazole (MMI, 0·1–2 mM) or cycloheximide (10 μM).

Single and double indirect immunofluorescence (IFL)

Sera were appropriately diluted in Hanks' balanced salt solution (HBSS). To avoid non-organ-specific binding of human antibodies to cell membrane (Tao & Kriss, 1982), all sera were preabsorbed with acetone-treated rat liver extract (Sigma, St. Louis, MO). After incubation for 2 h at room temperature and overnight at 4°C, the supernatants were separated at 105 000 *g* for 60 min and were stored at −20°C until use. Indirect IFL was initiated by washing thyroid cells grown on coverslips with HBSS-2% bovine serum albumin (BSA). Coverslips were then incubated with preabsorbed sera for 30 min at room temperature. After washing, cells were incubated with a fluoresceinated sheep anti-human Ig conjugate (Wellcome, London, UK), preabsorbed with acetone-treated rat liver extract. After 30 min cultures were washed in HBSS-2% BSA. In the double IFL procedure, thyroid cells were incubated in the sequence with: (1) an anti-TPO monoclonal antibody (Czarnocka *et al.*, 1985) (10 ng/ml); (2) a rhodaminated goat anti-mouse Ig conjugate (Cappel, West Chester, PA) (1/30); (3) a human serum containing anti-MAb (1/16); (4) a fluoresceinated sheep anti-human Ig conjugate (1/30). At the end cells were fixed in 5% acetic acid, 95% ethanol, mounted in glycerol and examined with a fluorescence microscope. To expose intracellular antigens, cells were fixed in chilled acetone before IFL.

Preparation of human thyroglobulin (Tg) and microsomal fractions

Human Tg (19 S) was purified from saline extracts of nontoxic or Graves' goitres by ammonium sulphate fractionation followed by linear density gradient centrifugation in 10–40% sucrose (Mariotti *et al.*, 1979). Microsomes were prepared by differential centrifugation from surgical specimens of human thyroid, placenta, liver and spleen, and were solubilized with deoxycholate following the method of Mariotti *et al.* (1979).

Purification of human thyroid peroxidase (TPO)

Human TPO was purified from solubilized thyroid microsomes by affinity chromatography (Czarnocka *et al.*, 1985), using a monoclonal antibody to human TPO whose binding to thyroid membranes was specifically inhibited by anti-MAb. On the basis of previous experiments (Czarnocka *et al.*, 1985) it was estimated that TPO was purified 3 000 fold from the starting thyroid homogenate. As described in other papers (Czarnocka *et al.*, 1985; Ruf *et al.*, 1987), the affinity purified TPO shared all the physico-chemical and immunological properties attributed to M-Ag.

Absorption assay

Anti-MAb positive sera were mixed with purified TPO (10 μg) or Tg (10 μg) in 10 mM Tris-HCl buffer (pH 7·5). After overnight incubation at 4°C, samples were centrifuged at 105 000 *g* for 60 min and the supernatants were tested in the IFL procedure. Similar absorption experiments were performed using solubilized microsomes (1 mg) from human thyroid and control tissues (placenta, liver and spleen).

Chemicals

Cholera toxin was obtained from Calbiochem (La Jolla, CA), forskolin, 8-bromo-cAMP, oestradiol, NaI and cycloheximide were from Sigma (St. Louis, MO) and EGF was purchased from Flow Labs (Milan, Italy). TSH was a purified bovine preparation kindly provided by Dr L.D. Kohn, NIDDK, Bethesda, MD; its biological activity in the mouse bioassay was 25 U/mg.

RESULTS

Specificity of IFL for the recognition of M/TPO-antigen (M/TPO-Ag)

All Hashimoto's sera used in this study produced a clear staining

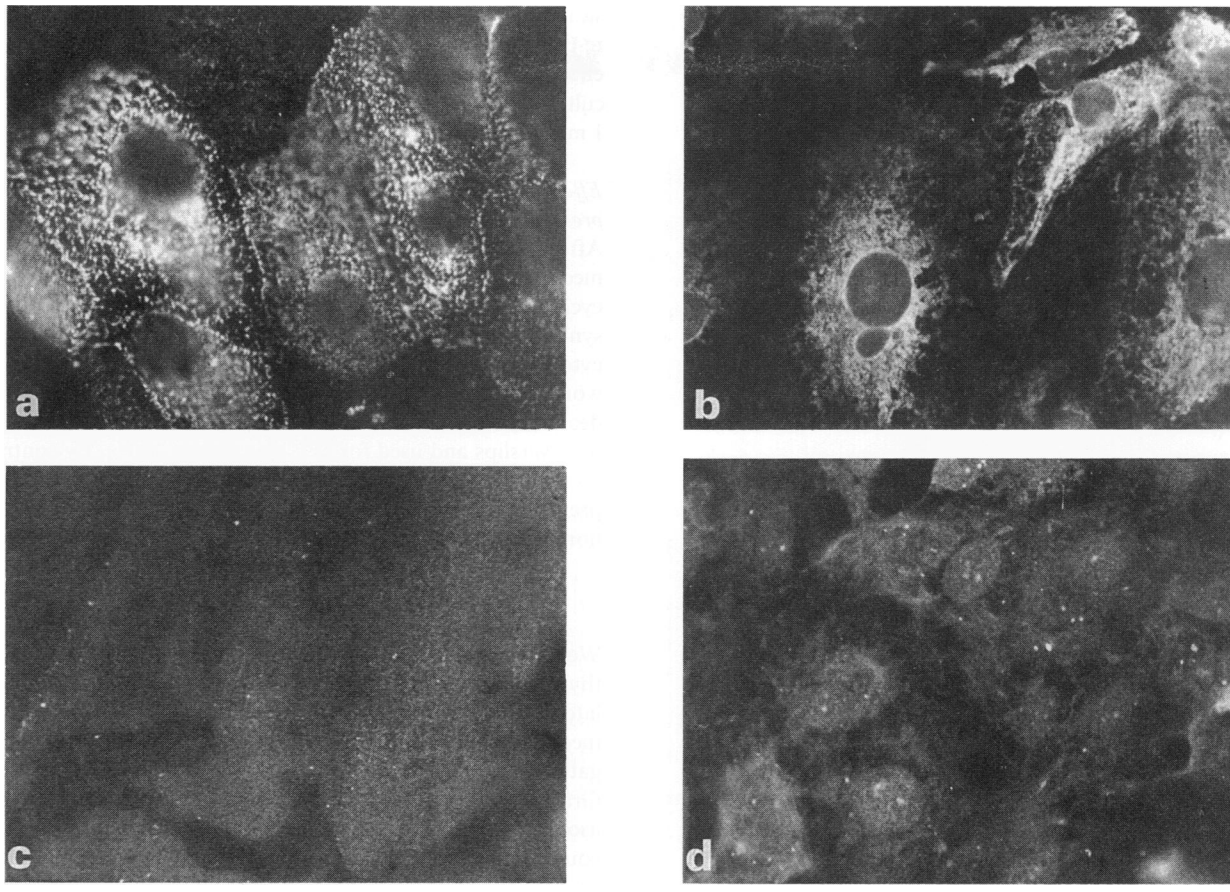

Fig. 1. Indirect IFL performed with a serum containing high levels of anti-MAb on human thyroid cells cultured in the presence of TSH for 15 days. The serum was applied before and after preabsorption with human TPO. Surface (a) and cytoplasmic (b) staining produced by the anti-MAb-positive serum before preabsorption with human TPO. Negative surface (c) and cytoplasmic (d) IFL obtained with the same serum after preabsorption with human TPO (original magnification × 1 000).

of the surface and the cytoplasm (Fig. 1, a & b) of human thyroid cells cultured in medium containing TSH. Negative results were obtained with normal sera. To demonstrate that the antigen stained by Hashimoto's sera was M-Ag and that it could be identified with TPO, preabsorption experiments and double IFL were performed. Preabsorption with solubilized thyroid microsomes and TPO abolished the staining of human thyroid cells (Fig. 1 c & d). No effect was produced by preabsorption with Tg or solubilized microsomes from control tissues. In the double IFL both human anti-MAb-positive sera and the anti-TPO monoclonal antibody produced a surface and cytoplasmic staining (Fig. 2 a & b) of thyrocytes cultured in medium containing TSH. The same cells were stained by the two antibodies and in no case was a dissociation of the green and red fluorescence observed. In the double IFL thyroid cells were incubated with the monoclonal anti-TPO antibody as a first step, since preincubation with anti-MAb-positive sera abolished the subsequent binding of the monoclonal antibody. Cross-reactivity between layers was excluded by appropriate control experiments.

Disappearance of M/TPO-Ag from human thyroid cells

When human thyroid cells were cultured in medium containing TSH the M/TPO-Ag was identified by IFL in the cytoplasm and on the surface of most thyrocytes for at least 30 days. In some experiments, thyroid cells were cultured in 6H medium for 3 days; then they were switched to medium without TSH. Single or double IFL with anti-MAb-positive sera were performed on parallel cultures after 1, 3, 5, 7, 9, 12, 15 and 30 days of culture in the absence of TSH. From day 5 to 7, depending on the different primary culture, the surface of human thyroid cells became negative for M/TPO-Ag. Disappearance of M/TPO-Ag from the cytoplasm required a longer period (7–15 days) of culture without TSH. The time required for the disappearance of surface and cytoplasmic M/TPO-Ag was longer in primary cultures prepared from Graves' goitres than in those derived from the non-toxic ones. At no time was a dissociation of the result obtained with the monoclonal anti-TPO antibody and human anti-MAb-positive sera observed. In three primary cultures (all from Graves' goitres) a limited percentage of cells (1–5%) retained their positivity for M/TPO-Ag even after 30 days of incubation in medium deprived of TSH. These positive cells were usually arranged in small clusters or in dome-like structures.

Effect of different agents on M/TPO-Ag expression in human thyroid cells

Human thyrocytes with undetectable surface (Fig. 3 a) and cytoplasmic M/TPO-Ag after 7–15 days of culture without TSH were used. Re-exposure to TSH (0·1–100 mU/ml) for 48–72 h, or

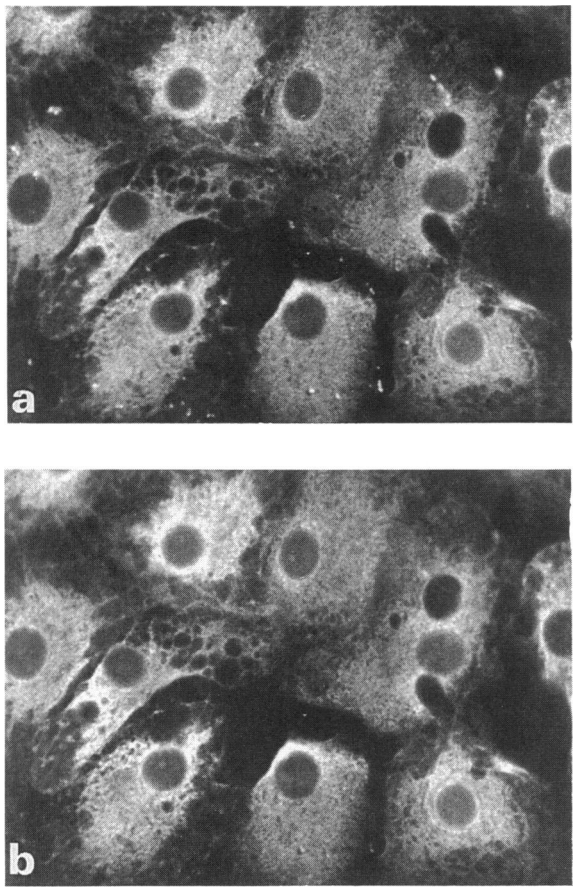

Fig. 2. Double indirect IFL staining of human thyroid cells cultured in the presence of TSH for 7 days. Cells were fixed with acetone before the IFL procedure performed as detailed in *Materials and Methods*. Cytoplasmic staining produced by the anti-TPO monoclonal antibody (a). Cytoplasmic fluorescence produced by a human serum positive for anti-MAb (b). The same cells are stained with an almost undistinguishable pattern by the monoclonal anti-TPO antibody and by the human anti-MAb serum (original magnification × 500).

longer, led to the reappearance of M/TPO-Ag on the surface (Fig. 3 b) and in the cytoplasm. The number of cells showing M/TPO-Ag increased with the dose of TSH up to 10 mU/ml, with no further change thereafter. However, the minimal concentration of TSH able to restore M/TPO-Ag varied in primary cultures obtained from different goitres and in the same thyroid cells higher doses were required after longer periods of TSH deprivation. The maximal percentage of cells recovering their positivity for M/TPO-Ag after TSH readdition ranged from 30% to 70%; it proved impossible to re-express the M/TPO-Ag in all cells.

To investigate whether this phenomenon was mediated by cAMP, TSH was substituted for by cholera toxin (10 pM), forskolin (5–50 μM) or 8-bromo-cAMP (0·5 mM). All these substances induced the surface and cytoplasmic M/TPO-Ag (Fig. 3 c). In the majority of cultures the percentage of positive cells recruited after addition of the highest dose of forskolin was greater (70–80%) than that obtained with a superoptimal concentration of TSH (Fig. 4). Reappearance of M/TPO-Ag was also produced when an IgG strongly positive for TSAb activity was added to 5H medium (Fig. 5). EGF (1 nM) stimulated the growth of human thyroid cells but was unable to re-express M/TPO-Ag at any time up to 7 days (Table 1). No effect on the reappearance of M/TPO-Ag was observed in cultures incubated with oestradiol (3·5–73·5 nM) or NaI (0·1–1 mM) without TSH.

Effect of protein synthesis inhibition on the TSH-induced expression of M/TPO-Ag in human thyroid cells

After 7–15 days of culture without TSH cells were switched to a medium supplemented with TSH (0·1–10 mU/ml) plus either cycloheximide (10 μM) or MMI (0·1–2 mM). The protein synthesis inhibitor prevented the reappearance of surface and cytoplasmic M/TPO-Ag induced by TSH at 24, 48 and 72 h. It is worth noting that, although cycloheximide produced some cell death (10–20%), the viability of thyrocytes remaining attached to coverslips and used for IFL was almost 100%. By contrast, the addition of MMI to the medium containing TSH did not prevent the reappearance of M/TPO-Ag produced by the hormone.

DISCUSSION

We have previously shown that in a differentiated strain of rat thyroid cells (FRTL-5 cells) the expression of M-Ag is modulated by TSH (Chiovato *et al.*, 1985). In the present paper the mechanisms involved in the expression of M-Ag were investigated in primary cultures of human thyroid cells prepared from Graves' and non-toxic goitres. Studies were also performed to ascertain whether M-Ag stained by IFL in human thyroid cells could be identified with TPO. Preabsorption experiments showed that, similarly to solubilized human thyroid microsomes, TPO abolished the binding of anti-MAb to human thyroid cells, while no inhibition was obtained with control human tissues or Tg. Moreover, using double IFL, the pattern of fluorescence produced on human thyrocytes by a monoclonal anti-TPO antibody was undistinguishable from that obtained with anti-MAb positive sera. These data indicate that the antigen recognized by anti-MAb in human thyroid cells is indeed TPO and that this enzyme shares most of the antigenic characteristics of M-Ag.

As previously shown in FRTL-5 cells, after some days of culture without TSH, the M/TPO-Ag disappeared from the surface and the cytoplasm of human thyroid cells. However the time of TSH deprivation required for the disappearance of M/TPO-Ag was usually longer in primary cultures obtained from Graves' goitres than in those derived from non-toxic goitres. TSAb bound *in vivo* to follicular cells could explain the delayed disappearance of M/TPO-Ag from primary cultures of Graves' goitres. At variance with FRTL-5 cells, a limited percentage (1–5%) of human thyroid cells from some Graves' goitres retained M/TPO-Ag despite prolonged starvation from TSH. This phenomenon can be explained by the presence in the original goitre of autonomous follicles already independent from TSH control *in vivo* (Studer, 1986). Following readdition of TSH, the surface and cytoplasmic M/TPO-Ag reappeared in a percentage of cells which increased with the dose of hormone up to a maximum of 30–70% in different primary cultures. Failure to recover M/TPO-Ag in all cells can be attributed to a progressive reduction of TSH receptors in cultured thyrocytes, as suggested by Bidey, Marshall & Ekins (1981). In agreement with this hypothesis, in our experiments forskolin was able to

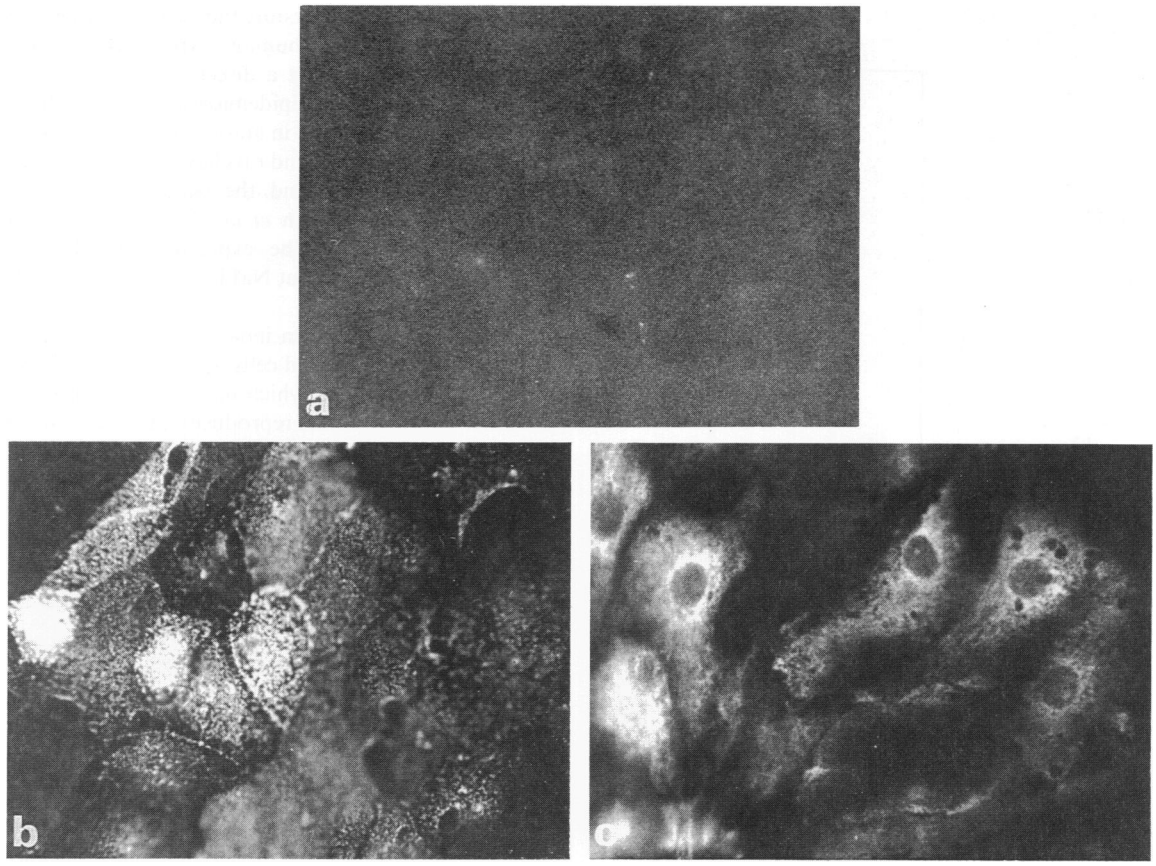

Fig. 3. Surface indirect IFL performed with a serum containing high levels of anti-MAb on human thyroid cells cultured without TSH for 12 days (a) and then challenged with the hormone (300 μU/ml) for 72 h (b). A strong dotted fluorescence is visible on the surface of cells recultured with TSH . Indirect IFL performed with a serum containing high levels of anti-MAb on human thyroid cells cultured without TSH for 12 days and then challenged with forskolin (50 μM) for 72 h (c). Cells were pretreated with acetone before the staining procedure in order to expose intracellular antigens. A strong cytoplasmic fluorescence can be observed (original magnification × 500).

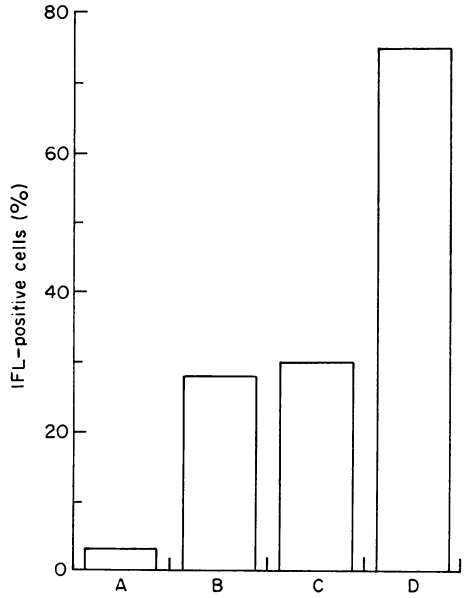

Fig. 4. Percentage of thyroid cells positive for surface M/TPO-Ag found in parallel cultures obtained from a Graves' goitre which were maintained for 12 days in medium deprived of TSH (A) and then challenged with TSH, 10 mU/ml (B) and 100 mU/ml (C), or forskolin (50 μM) (D). Surface IFL with a serum containing anti-MAb was performed after 72 h of incubation.

recruit a number of M/TPO-Ag-positive cells (up to 80%) greater than that obtained with any dose of TSH.

The lag period necessary for the re-expression of M/TPO-Ag suggested that new protein synthesis was required, and indeed cycloheximide prevented the reappearance of M/TPO-Ag induced by TSH. Methimazole, a drug blocking the enzymatic activity of TPO, was also tested as a putative inhibitor of M/TPO-Ag expression following the observation that circulating anti-MoAb decrease in thyrotoxic Graves' patients treated with MMI (McGregor *et al.*, 1980, Marcocci *et al.*, 1982). However, at least in our experimental model, MMI did not block the TSH-induced expression of M/TPO-Ag.

TSH action on thyroid cells is mediated by cAMP-dependent and cAMP-independent pathways. In the present report several agents known to increase cAMP content in human thyroid cells: cholera toxin, forskolin, 8-bromo-cAMP and TSAb mimicked TSH in the induction of M/TPO-Ag. Thus, our data suggest that cAMP is one of the intracellular signals by which TSH modulates the expression of M/TPO-Ag. To explore the possibility that cAMP-independent pathways could also be involved, the effect of EGF was studied. EGF stimulates growth, inhibits some functional activities of thyroid cells (Roger & Dumont, 1982), and reduces the accumulation of cAMP induced by TSH (Bachrach *et al.*, 1985). In our

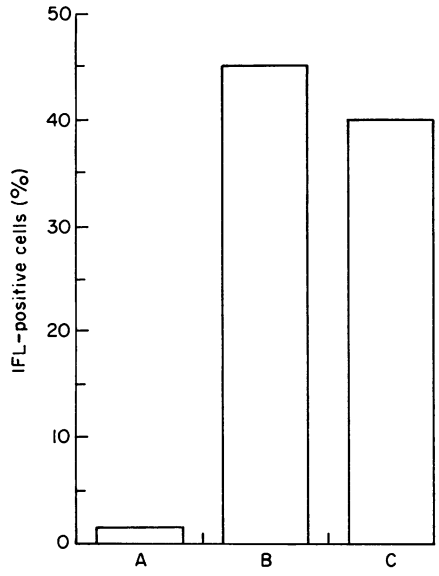

Fig. 5. Percentage of thyroid cells positive for surface M/TPO-Ag found in parallel cultures obtained from a Graves' goitre which were maintained for 12 days in medium deprived of TSH (A) and then challenged with TSH, 10 mU/ml (B) or a TSAb-positive IgG (1 mg/ml) (C). Surface IFL with a serum containing anti-MAb was performed after 72 h of incubation.

Table 1. Effect of EGF on surface M/TPO-Ag expression and cell growth in human thyroid cells previously maintained in medium deprived of TSH for 12 days. The action of TSH on parallel cultures is shown for comparison

Addition	IFL	Cell number ($\times 10^5$)*
None	Negative	$3{\cdot}5 \pm 0{\cdot}8$
EGF (1 nM)	Negative	$10{\cdot}2 \pm 2{\cdot}5$
TSH (300 μU/ml)	Positive	$4{\cdot}3 \pm 1{\cdot}2$

IFL on human thyroid cells was performed using an anti-MAb-positive serum after 48 h, 72 h and 7 days of incubation.

* Cells were counted after 7 days of culture in medium containing EGF or TSH. Results are expressed as mean $\pm$ s.d.

experiments EGF, while stimulating growth, did not produce a re-expression of M/TPO-Ag. These data support the hypothesis that the adenylate cyclase-cAMP system is the main pathway involved in the expression of M/TPO-Ag in thyroid cells, and are in agreement with the finding that in dog thyroid cells the enzymatic activity of TPO is modulated by TSH through cAMP (Magnusson & Rapoport, 1985).

All autoimmune thyroid disorders are more common in females than in males, but the reason for this remains unknown. Among different explanations, an influence of oestrogens was supposed. The question could be raised whether these steroids might influence the 'antigenicity' of follicular cells. However, oestradiol was unable to restore the expression of M/TPO-Ag in our primary cultures of human thyroid cells, indicating that these steroids do no exert a direct effect in terms of thyroid autoantigen expression. Epidemiological observations in man and experimental findings in autoimmune thyroiditis of genetically susceptible chicken and rats have suggested an association between iodide intake and the occurrence of autoimmune thyroiditis in man (Safran *et al.*, 1987). The possibility that iodide could influence the expression of M/TPO-Ag was checked in our cultures, but NaI in the absence of TSH did not induce M/TPO-Ag.

In conclusion, our data indicate that the expression of M/TPO-Ag in human thyroid cells is dependent on TSH stimulation, through pathways which involve cAMP production and protein synthesis. TSAb reproduces the effect of TSH, thus explaining why M/TPO-Ag is so abundant in Graves' goitres. The modulation of M/TPO-Ag by TSH acquires particular relevance in view of previous findings indicating that this hormone enhances the expression of HLA-DR induced by interferon-gamma in human thyroid cells (Todd *et al.*, 1987).

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