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Enhancement of thyrocyte HLA Class II expression by thyroid stimulating hormone

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SUMMARY

HLA Class II molecules are expressed by human thyroid epithelial cells (thyrocytes) in thyroid autoimmunity, although these cells are normally Class II$^-$. γ-Interferon (γ-IFN) is probably involved in this expression, as suggested by its ability to induce Class II in cultured normal thyrocytes. We have now found that thyroid stimulating hormone (TSH) enhances Class II expression induced in cultured thyrocytes by γ-IFN, and effects similar to those of TSH were obtained with dibutyryl cyclic AMP. A proportion of thyrocytes also expressed Class II following treatment with TSH or dibutryl cyclic AMP in the absence of γ-IFN, but the optimal activity of these mediators then appeared to be dependent upon the occurrence of some pre-existing Class II expression. These findings give insights into how a variety of mediators may influence Class II expression in thyroid autoimmunity.

Keywords thyroid autoimmunity thyroid epithelium HLA Class II γ-interferon thyroid stimulating hormone

INTRODUCTION

Major histocompatibility complex (MHC) Class II molecules (HLA-D/DR in humans) are of restricted tissue distribution, being expressed particularly by cells of the immune system, which is consistent with their role in antigen presentation. In accordance with this is the observation that thyroid follicular cells (thyrocytes) do not normally express HLA Class II. They can, however, be induced to do so by culture with plant lectins (Pujol-Borrell *et al.*, 1983) and it is particularly striking to find that thyrocytes strongly express HLA Class II in most patients with autoimmune thyroid diseases (ATD) (Hanafusa *et al.*, 1983; Jansson, Karlsson & Forsum, 1984; Aichinger, Fill & Wick, 1985). These findings suggested the hypothesis that expression of MHC Class II molecules by epithelial cells such as thyrocytes might enable these cells to present their own surface molecules as autoantigens thus by-passing the involvement of conventional antigen presenting cells like macrophages and dendritic cells. Such inappropriate Class II expression would thus play a role in the perpetuation, and possibly the initiation, of the autoreactive T cell activation which results in autoimmune disease (Bottazzo *et al.*, 1983). The wide applicability of this hypothesis is indicated by the inappropriate HLA Class II expression which has been noted in epithelial cells of other human tissues undergoing autoimmune attack (reviewed by Bottazzo *et al.*, 1986). Furthermore, direct evidence for the functional capability of these HLA-D/DR molecules was provided by the demonstration that Class II$^+$ thyrocytes can present exogenous peptide antigen (Londei *et al.*, 1984)

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and endogenous autoantigens (Londei, Bottazzo & Feldmann, 1985) to relevant cloned T cell lines in an antigen-specific, MHC Class II-restricted fashion.

Given these findings, it was important to investigate the nature of the stimulus for the thyrocyte Class II expression in ATD. In this regard, recombinant human interferon (γ-IFN) was found to induce HLA Class II in cultured normal human thyrocytes, whereas α-IFN, β-IFN and interleukin 2 (IL-2) did not (Todd *et al.*, 1985). Although these findings implicate γ-IFN in the Class II expression in ATD, this does not exclude the possible involvement of other factors. We have therefore investigated the effects of thyroid stimulating hormone (TSH) on Class II expression, since this substance is known to have important regulatory effects on thyrocytes. Our findings demonstrate that TSH enhances thyrocyte Class II expression induced by γ-IFN.

MATERIALS AND METHODS

Reagents. Recombinant human γ-IFN (kindly provided by Dr G. R. Adolf, Boehringer-Ingelheim, Vienna, Austria; produced by Genentech, Inc., California, USA) was purified to >99% homogeneity from *E. coli*: endotoxin contamination was <0·125 ng/mg protein. Bovine thyroid stimulating hormone (bTSH, 'Thytropar') obtained from Armour Pharmaceutical, Eastbourne, UK, had a specific activity of 0·4–0·5 U/mg. Human TSH (huTSH; specific activity 6·6 U/mg), dibutyryl-cyclic AMP (dbcAMP), isobutyl-methyl-xanthine (IBMX), carbamyl choline, and cyclosporin A were from Sigma Chemical Company, Poole, Dorset, UK. Three IgG1 murine monoclonal antibodies were employed in immunofluorescence: MID-3, directed against a non-polymorphic HLA Class II determinant, P11 anti-mouse thyroglobulin, and anti-SRC/21 raised against sheep erythrocytes. Hybridoma supernatants of MID-3 and P11 (kindly provided by Dr P. Lydyard), were used at a dilution of 1/2; anti-SRC/21 in ascitic fluid was employed at an immunoglobulin concentration of 10 μg/ml.

Thyroid specimens and cultures. Thyroid tissue was employed from eight individuals operated on for reasons other than thyroid autoimmunity: one diffuse goitre (HT-98), one colloid goitre (HT-104), five laryngeal carcinomas (HT-52,56,79,107,115), one kidney donor (HT-84). Fresh or cryopreserved thyrocytes obtained by collagenase digestion of the tissue were cultured for 1–2 days on glass coverslips in supplemented RPMI-1640 medium plus 10–15% fetal calf serum (FCS; Sera-Lab, Crawley, UK), as previously described (Pujol-Borrell *et al.*, 1983; Todd *et al.*, 1985). The adherent thyrocytes were then either assayed for Class II expression already present *in vivo* or washed three times with serum-free balanced salt solution (BSS) and further cultured in supplemented medium plus 1% FCS together with γ-IFN and/or other additives. In some experiments, further washings and additions of reagents were performed, as appropriate.

The importance of using low FCS concentrations during stimulation of the cultures was shown by initial experiments with 10–15% FCS throughout the culture period in which the effects of TSH on thyrocyte Class II expression were low and variable, and dbcAMP was ineffective.

Detection of HLA Class II molecules on the cultured thyroid cells was performed by indirect immunofluorescence as previously described (Pujol-Borrell *et al.*, 1983) with a test or control MoAb as the first layer followed by FITC conjugated rabbit anti-mouse immunoglobulin (FITC-RaM) (Dakopatts, Glostrup, Denmark). In all the experiments described here the staining was performed on viable (unfixed) thyrocyte monolayers so that cell surface Class II molecules were detected. The proportion of cultured thyrocytes expressing Class II was either calculated by scoring at least 100 thyrocytes by phase and fluorescence microscopy, or was estimated following extensive scanning of the monolayers. Staining with the MoAb anti-SRC/21 and P11, which served as specificity controls, was routinely negative.

Flow cytometric analysis of thyrocytes. Thyrocytes were cultured in 25 cm^2 flasks at 2–3 $\times$ 10^6 per flask, and the adherent cells washed and stimulated, as described above. About 16 h before harvesting, the cells were washed three times with Ca^{2+}/Mg^{2+}-free BSS and incubated in the same solution for the remainder of the culture period. This led to shrinking and separation of the cells in the adherent monolayers such that some detached from the plastic, and clumping of the remainder was minimized when they were harvested by digestion with 0·04% trypsin (Sigma) for 15 min at

37°C. The digestion procedure and all subsequent washing and staining steps were performed in Ca^{2+}/Mg^{2+}-free BSS plus 10 μg/ml DNase I (Sigma) (De Aizpurua, Ungar & Toh, 1983). Staining in suspension of the recovered cells was performed at 4°C with the appropriate MoAb followed by FITC-RaM. The size distribution and fluorescence intensity of the stained cells was analysed in an EPICS-C (Coulter Corporation, California).

RESULTS

TSH enhances Class II expression. The results depicted in Fig. 1 confirm our previous observation that treatment of cultured monolayers of human thyrocytes with recombinant human γ-IFN induces HLA Class II expression by these cells (Todd *et al.*, 1985). Figure 1 further shows that greatly enhanced cell-surface Class II expression resulted when bovine TSH (bTSH) was added to the cultures together with γ-IFN. This was evident in both the proportion of thyrocytes which were Class II^+ and the intensity of their expression. These observations, made by UV microscopy on thyrocyte monolayers stained by indirect immunofluorescence, were confirmed by flow cytometric analysis of similarly stained thyrocyte suspensions (Table 1). The optimal dose of bTSH was 0·1 mU/ml and its effect was most marked in conjunction with suboptimal concentrations of γ-IFN (Figs 1 & 2). Much higher doses of TSH (eg. 50 mU/ml) had little, or no, enhancing effect. Human TSH (huTSH) behaved similarly to bTSH in enhancing γ-IFN-induced Class II expression by thyrocytes *in vitro*, but was more effective at 1 mU/ml than 0·1 mU/ml (Fig. 3).

HLA Class II expression was also observed in a proportion of thyrocytes in monolayers stimulated with TSH alone (Figs 1–3, Table 1), showing the effect of TSH not to be entirely dependent on addition of γ-IFN. However, the following observations suggest the effect of TSH to be a secondary enhancing phenomenon dependent upon activation of Class II genes by some other means. Firstly, TSH alone stimulates at most 30–40% of cultured thyrocytes to express Class II (Figs 1, 2 & 3) whereas more than 90% of thyrocytes are normally induced by an optimal dose of γ-IFN (100–1000 U/ml). Secondly, preliminary investigations suggest that the degree of Class II expression stimulated by TSH alone roughly correlates with the degree of 'spontaneous' Class II expression which these specimens display at a low but variable level before stimulation *in vitro*.

The combined effects of γ-IFN and optimal concentrations of TSH were synergistic, rather than additive (eg. with 0·1 mU TSH/ml + 1U γ-IFN/ml in Fig. 1a), but γ-IFN and TSH do not have to act

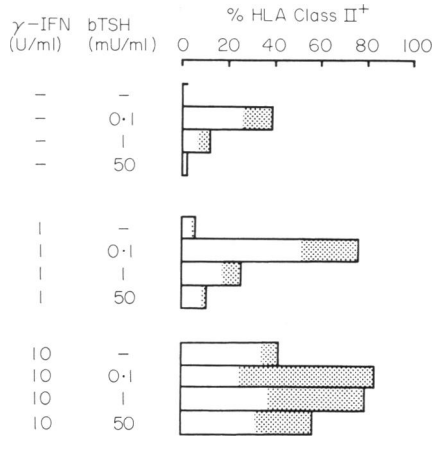

Fig. 1. Induction of HLA Class II on thyrocyte monolayers by γ-IFN and bTSH. Class II expression was detected by indirect surface immunofluorescence (MID-3 followed by FITC-RaM) under a u.v. microscope. HT-79 thyrocytes were stimulated for 5 days. Fluoresence intensity was scored as weak to moderate (□) or strong (▨).

Table 1. Flow cytometric analysis of Class II expression by thyrocytes stimulated with γ-IFN and/or bTSH

Additions to culture*			
γ-IFN (U/ml)	bTSH (mU/ml)	MoAb used in staining†	% fluorescent cells above gate‡
0	0	none	6·6
10	0	anti-SRC/21	14·4
0	0	MID-3	14·6
10	0	MID-3	25·8
0	1	MID-3	20·2
10	1	MID-3	47·7

* HT-79 thyrocytes were stimulated as indicated for 7 days before staining.

† Binding of the MoAb was detected by subsequent incubation with FITC-RaM.

‡ A large peak of autofluorescence occurred at low fluorescence intensity (presumably due to cytoplasmic lipofuchsin granules): this was excluded from the analysis by an arbitrary gate. The forward angle light scatter indicated the cells to have a wide size distribution.

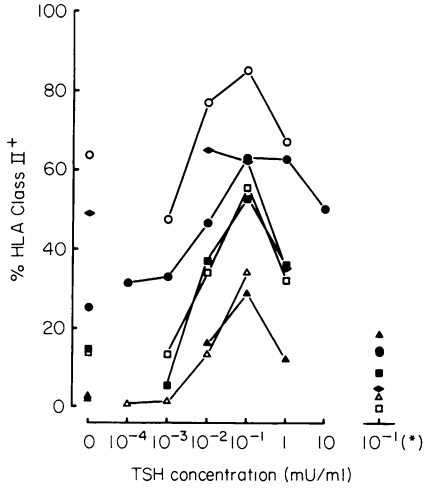

Fig. 2. Expression of HLA Class II on thyrocyte monolayers cultured with γ-IFN and bTSH for 5–6 days and then stained with MID-3 followed by FITC-RaM. The thyroid specimens and concentrations of γ-IFN used in the different experiments were, HT-52: (♦) 5 U/ml; HT-79: (△) 2·5 U/ml, (■) 10 U/ml; HT-84: (□) 10 U/ml; HT-104: (▲) 1 U/ml, (●) 7·5v U/ml, (○) 15 U/ml. (*) indicates expression following treatment with 0·1 mU bTSH/ml in the absence of γ-IFN. Monolayers cultured without γ-IFN or bTSH showed very little (< 1%) or no Class II expression.

simultaneously in order to exert their synergistic effects. Thus, TSH can potentiate the response to γ-IFN when used to treat the cells either before or after the stimulation with γ-IFN (Fig. 4).

Effect of dibutyryl-cyclic AMP. Isobutyl-methyl-xanthine (IBMX, an inhibitor of cyclic nucleotide phosphodiesterase) and dibutyryl-cyclic AMP (dbcAMP) also enhanced Class II

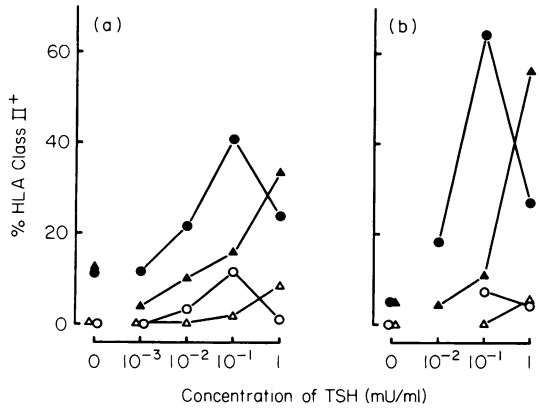

Fig. 3. Expression of HLA Class II by thyrocyte monolayers stimulated for 5–7 days with 7·5 U γ-IFN/ml and/or bTSH or huTSH. HT-104 thyroid specimen was used in both experiments (a, b). The cells were stimulated as follows: (●) γ-IFN ± bTSH; (▲) γ-IFN ± huTSH; (○) ± bTSH alone; (△) ± huTSH alone. Class II expression was detected by staining with MID-3 followed by FITC-RAM.

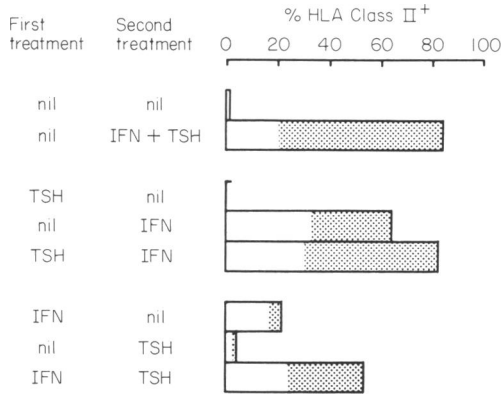

Fig. 4. Induction of HLA Class II on HT-107 thyrocyte monolayers by sequential treatments with γ-IFN (20 U/ml) and bTSH (0·1 mU/ml). The cells were first stimulated, as indicated, for 5 days then washed three times with BSS before the second treatment for 6 days. Class II expression was detected by staining with MID-3 followed by FITC-RaM, and was scored as weak (□) or moderate to strong (▨).

expression in cultured thyrocytes (Fig. 5). Although both IBMX and dbcAMP were clearly effective in the cultures at 10^{-3} M, lower concentrations ($\leqslant 10^{-4}$ M) of either had little, if any, effect. As with TSH, dbcAMP and, to a lesser extent, IBMX, caused Class II expression by thyrocytes in the absence of γ-IFN (Fig. 5). Indeed, the combined effects of dbcAMP and γ-IFN tended to be additive rather than synergistic. Furthermore, IBMX and dbcAMP caused no more enhancement than bTSH at 1 mU/ml (Fig. 5), which was generally found to be a superoptimal concentration (Figs. 1, 2 and 3).

The above findings suggest that the Class II enhancing activity of TSH is at least partly mediated via cyclic AMP. Consistent with this is the observation that thyrocyte Class II expression was not modulated by 10^{-3}–10^{-5} M carbamyl choline, which reportedly stimulates an increase of calcium levels and cyclic GMP in thyrocytes (reviewed by Carayon & Amr, in press).

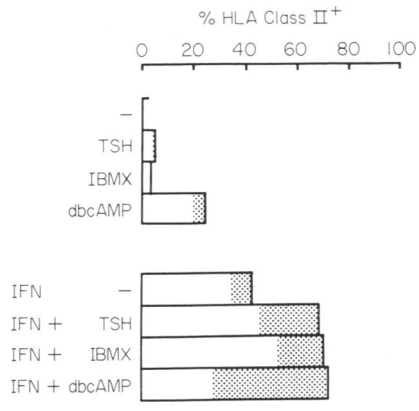

Fig. 5. Expression of HLA Class II by HT-79 thyrocytes cultured for 5 days with 10 U γ-IFN/ml and/or 1 mU bTSH/ml, 10^{-3} M IBMX, or 10^{-3} M dbcAMP. The details of the assay and scoring of fluorescence intensity were as in Fig 1.

DISCUSSION

The results presented here clearly show that TSH enhances Class II expression by thyrocytes treated with γ-IFN (Figs 1–3 and Table 1). The finding that TSH could exert its effect when used to treat the cells either before or after γ-IFN (Fig. 4) suggests that the respective activities of these two stimulators are not strictly interdependent. Thus, the enhancement of Class II by TSH could be one aspect of the more general effects of this hormone on thyroid differentiation and metabolic function, including stimulation of RNA and protein synthesis (referenced by Carayon & Amr, in press). However, the observation by Rayner *et al.* (1987) that Class II expression can be induced in the FRTL-5 rat thyroid cell line by γ-IFN, but that this induction is not enhanced by a growth-promoting dose of bTSH, suggests that the pathways through which TSH mediates its various effects are not completely overlapping.

Class II expression was also observed on thyrocytes treated with TSH alone (Figs 1–3, Table 1), as others have observed (Wenzel *et al.*, 1986). However, as outlined in the Results, the available evidence favours an enhancing, as opposed to an inducing, role for TSH. This enhancing effect appears to be facilitated by pre-existing activation of Class II genes in a variety of thyroid glands. The existence of such Class II expression by thyrocytes in glands from patients not usually considered to be suffering from thyroid autoimmunity has been reported previously (Lloyd *et al.*, 1985; Grubeck-Loebenstein *et al.*, 1985; Lucas Martin *et al.*, unpublished observation) and is consistent with the relatively high prevalence of sub-clinical focal thyroiditis and/or thyroid autoantibodies. Restriction of the Class II stimulation by TSH to 'pre-activated' thyrocytes could explain the limitation of this effect to a minority of the cultured cells. Nevertheless, the potency of TSH on those cells which are sensitive to its effects is underlined by our observation that, after 5–8 days of culture without stimulation, addition of bTSH to thyroid monolayers still results in Class II expression, even when these cells have essentially lost any 'spontaneous' surface Class II expression at the time TSH is added. The possibility that the apparent effects of TSH alone are dependent upon γ-IFN production in the cultures by contaminating lymphocytes is countered by our finding that TSH still exerts its effects in the presence of cyclosporin A (1 μg/ml) or anti-γ-IFN neutralizing antibodies (Novick *et al.*, 1983; kindly provided by Dr Z. Eshhar).

The finding that dbcAMP enhanced thyrocyte Class II expression, whereas carbamyl choline did not, suggests that at least part of the TSH effect on Class II is mediated via intracellular cyclic AMP, as has been demonstrated for several other effects of TSH on thyrocytes (reviewed by Carayon & Amr, in press). However, in contrast to TSH, the effects of dbcAMP tended to be additive rather than synergistic with those of γ-IFN. This could be due to TSH itself having additional effects, or may simply indicate the limited efficiency of exogenous dbcAMP in this *in vitro*

system. Weetman *et al.* (1985) reported that neither TSH nor dbcAMP influenced human thyrocyte Class II expression. This may be explained by their use of different concentrations of these substances and higher amounts of FCS in the cultures (see Materials and Methods).

Whether γ-IFN or another agent is the initial stimulus for thyrocyte Class II expression *in vivo* is not known. However, the production of γ-IFN by activated, autoreactive T cells infiltrating the thyroid could induce Class II in thyrocytes and thus potentiate and propagate autoimmune attack. The present findings indicate that TSH could contribute to thyrocyte Class II, with implications for the pathogenesis of those thyroid diseases characterized by hypothyroidism and raised levels of TSH. This could clearly apply to Hashimoto's thyroiditis, but the effect of TSH may also be relevant to cases of simple endemic goitre or iodine deficiency, which often show increased circulating TSH (Chopra, Hershman & Hornabrook, 1975) and autoimmune features (Grubeck-Loebenstein *et al.*, 1986). Other effects of TSH underline its potential role in facilitating autoimmune activation. For example, this hormone influences the quality as well as the quantity of thyrocyte Class II expression, particularly promoting the expression of HLA-DP and HLA-DQ products (Todd *et al.*, 1987). Also, investigations by others have indicated that TSH enhances production of thyroid microsomal antigen (Chiovato *et al.*, 1985) and thyroglobulin (Chaboud *et al.*, 1980), which are major thyroid autoantigens.

The contribution of other mediators should also be considered. The TSH-receptor stimulating antibodies of Graves' disease may enhance thyrocyte Class II expression (eg. Wenzel *et al.*, 1986), just as they mimic the action of TSH in a variety of other respects. Furthermore, we have recently found that tumour necrosis factor and lymphotoxin enhance induction of thyrocyte Class II expression by γ-IFN (unpublished observation). It is thus clear that a variety of mediators can enhance thyrocyte Class II expression. This might help to explain the different dose-response profiles of the bovine and human TSH preparations employed in the present studies (Fig. 3): the bTSH was approximately ten times less pure than the huTSH (specific activities 0·4–0·5 U/mg and 6·6 U/mg, respectively) and could therefore contain higher amounts of contaminants which might have Class II-enhancing effects additional to those mediated directly by TSH.

The synergism between γ-IFN and TSH should be viewed within the general context of immunological phenomena often requiring a two-mediator signal for optimal induction. Examples involving γ-IFN include synergism with lymphotoxin in anti-proliferative activity (Lee *et al.*, 1984), and with interleukin 2 in stimulating immunoglobulin synthesis and secretion (Bich-Thuy & Fauci, 1986). In terms of Class II expression, a dramatic example is provided by human pancreatic islet cells, which are almost insusceptible to Class II induction by γ-IFN alone (Pujol-Borrell *et al.*, 1986) whereas stimulation by γ-IFN together with tumour necrosis factor or lymphotoxin is effective in this respect (Pujol-Borrell *et al.*, 1987). With regard to the thyroid, a not dissimilar situation is seen in the synergism between TSH and a dose of γ-IFN, which, by itself, is virtually without effect (eg. △ in Fig. 2). A requirement for dual triggering will limit the circumstances in which effective stimulation of Class II expression can occur and could therefore prevent precocious and potentially harmful activation.

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