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HLA-D subregion expression by thyroid epithelium in autoimmune thyroid diseases and induced *in vitro*

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SUMMARY

Human thyroid epithelial cells (thyrocytes) express HLA Class II molecules in autoimmune thyroid diseases (ATD). Normal thyrocytes do not express Class II, but can be induced to do so by culture with interferon-gamma (γ-IFN). We have examined HLA-D subregion expression in sections and monolayers of thyroid by indirect immunofluorescence using appropriate monoclonal antibodies. The results indicate that, in ATD, the incidence and intensity of Class II subregion expression by thyrocytes varies between patients, and follows the pattern DR > DP > DQ. The same hierarchy is observed in cultured normal thyrocytes treated with γ-IFN: strong induction of Class II, and of DP and DQ in particular, requires relatively high concentrations of γ-IFN or additional factors such as thyroid stimulating hormone. These findings suggest that HLA-D subregion expression by thyrocytes in on-going ATD is determined by the levels of disease related factors in the affected tissue.

Keywords autoimmune thyroid diseases thyroid epithelium HLA-DR HLA-DQ HLA-DP γ-interferon

INTRODUCTION

Tissues subject to autoimmune attack may play an active role in stimulating their own destruction. This is suggested by the observation that, in various autoimmune diseases, expression of HLA Class II molecules is found in epithelial cells which are normally Class II negative (Hanafusa *et al.*, 1983; Ballardini *et al.*, 1984; Bottazzo *et al.*, 1985; reviewed by Bottazzo *et al.*, 1986). Since antigen presentation depends on Class II molecules, this inappropriate expression may enable these cells to present their own surface molecules to autoreactive T cells (Bottazzo *et al.*, 1983). Indeed, such an interaction has been demonstrated *in vitro* between Class II$^+$ thyrocytes and cloned T cells derived from the mononuclear infiltrate of thyroids from Graves' disease patients (Londei, Bottazzo & Feldmann, 1985), and similar interactions have been observed in a murine system of *in vitro* stimulation of T cells by thyrocytes (Salamero & Charreire, 1983).

But what is the nature of this inappropriate Class II expression, and how does it relate to the disease process? This question arises since the HLA-D region encodes at least three types of Class II molecules, currently designated HLA-DR, HLA-DQ, and HLA-DP. Whether these molecules subserve different functions is presently unknown. It is, however, apparent that DR and DQ are differentially expressed, with DR appearing earlier in development and having a wider tissue

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distribution (Natali *et al.*, 1984). We have accordingly examined the expression of DR, DQ and DP by thyrocytes in autoimmune thyroid diseases, and have investigated the *in vitro* induction of this expression in normal human thyrocytes. The results presented here indicate a hierarchy of HLA-D subregion expression by thyrocytes with heterogeneity of expression between patients, and suggest that this may reflect the nature and levels of Class II modulating factors related to the disease process.

MATERIALS AND METHODS

Reagents. The mouse monoclonal antibodies (MoAb) used for the detection of HLA Class II molecules are listed in Table 1, which also indicates their subregion specificities. MoAb specific for sheep red blood cells (clone anti-SRC/21) or mouse thyroglobulin (clone P11) served as controls.

Human interferon-gamma (γ-IFN) (kindly provided by Dr G. R. Adolf, Boehringer-Ingelheim Vienna, Austria, produced by Genentech Inc. California, USA) was purified to high specific activity from an *E. coli* recombinant DNA source (> 99% homogeneous; endotoxin contamination < 0·125 ng/mg protein). Bovine thyroid stimulating hormone (bTSH), obtained from Armour Pharmaceutical ('Thytropar'), had a specific activity of about 0·4–0·5 units/mg.

Patients. Diseased thyroid tissue was obtained following surgery on patients with autoimmune thyroid diseases. Based on clinical and histological data, Patients 1 and 2 were diagnosed as having Hashimoto's thyroiditis (although the records of Patient 2 were not available to us). Patients 3 to 14 had Graves' thyrotoxicosis; Patient 14 additionally had a benign follicular adenoma.

All of the Graves' disease patients were treated with carbimazole (or propylthiouracil in Patient 12) before surgery, except for Patient 3. The sera of all patients contained autoantibodies to thyroid microsomal antigen or thyroglobulin or both, except for Patient 4 (and no results were available from Patient 2).

Patients undergoing surgery for carcinoma of the larynx served as a source of thyroid from individuals without autoimmune thyroid disease.

Treatment of thyroid specimens. In most cases, each thyroid specimen was divided for two types of processing: (i) small blocks were snap-frozen and sections 4 μm thick cut and stained for Class II expression; (ii) fresh tissue was digested with collagenase type IV (Cooper Biomedical) for 3 h at 37°C. The digest was filtered through a 200 μm mesh to yield thyrocytes which were either cultured immediately or cryopreserved for later use. The cells were cultured for 1–2 days on glass coverslips

Table 1. Murine MoAb employed for indirect IFL

Clone	Specificity	Isotype	Preparation	Working concentration	Source
MID-3	Class II*	IgG1	Supernatant	1:2	P. Lydyard
DA6.164	HLA-DR†	IgG	Ascites	10 μg/ml	K. Guy & V. van Heyningen
B7/21	HLA-DP*	IgG	Purified Ig	10 μg/ml	J. & W. Bodmer
TU.22	HLA-DQ*	IgG	Ascites	1:1000	A. Ziegler
			Supernatant	1:2	
SDR4.1	HLA-DQw1	IgG	Purified Ig	10 μg/ml	J. & W. Bodmer
Anti-SRC/21	Sheep red blood cells	IgG1	Ascites	10 μg/ml	I. Todd
P_{11}	Mouse thyroglobulin	IgG1	Supernatant	Neat	P. Lydyard

* Non-polymorphic.

† All DR types except for DR7.

in supplemented RPMI-1640 medium plus 10–15% fetal calf serum (FCS), as previously described (Pujol-Borrell *et al.*, 1983; Todd *et al.*, 1985), to allow the thyrocytes to adhere to the glass. The thyrocytes were then assayed for Class II expression already present *in vivo*. It was important to perform this assay after a relatively short period of culture since Class II expression is gradually lost by thyrocytes cultured for a number of days without stimulation.

In other experiments to investigate Class II induction *in vitro*, thyroid tissue from patients operated for carcinoma of the larynx was digested and cultured for 1–2 days as described above. Thyrocyte monolayers were then washed three times with serum-free balanced salt solution and further cultured in supplemented medium plus 1% FCS. At this point γ-IFN or bTSH or both were added to the cultures, as appropriate. The monolayers were assayed after several days of stimulation.

Detection of HLA Class II molecules in the frozen tissue sections and cultured monolayers 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). The stained sections and monolayers were viewed under a Zeiss UV microscope.

RESULTS

Class II subregion expression in thyroid sections. Autoimmune thyroid specimens were initially screened for Class II expression by thyrocytes detected by IFL with MID-3. Twelve Class II$^+$ specimens (two Hashimoto disease and 10 Graves' thyrotoxicosis) were thus selected and analysed for follicular expression of DR, and DP and/or DQ molecules in consecutive frozen sections. The observed intensities of expression are summarized in Fig. 1 and grouped according to the patterns found. Each point represents an average score of staining intensity for those thyrocytes which were Class II$^+$. Both Hashimoto's glands and two of the Graves' disease thyroids showed strong expression of all three subregions by thyrocytes (Fig. 1a, b). In the other cases of Graves' disease a hierarchy of subregion expression was observed in which DR was most strongly expressed, with weaker expression of DP followed by DQ (Fig. 1c, d). Indeed, in some cases DQ expression by

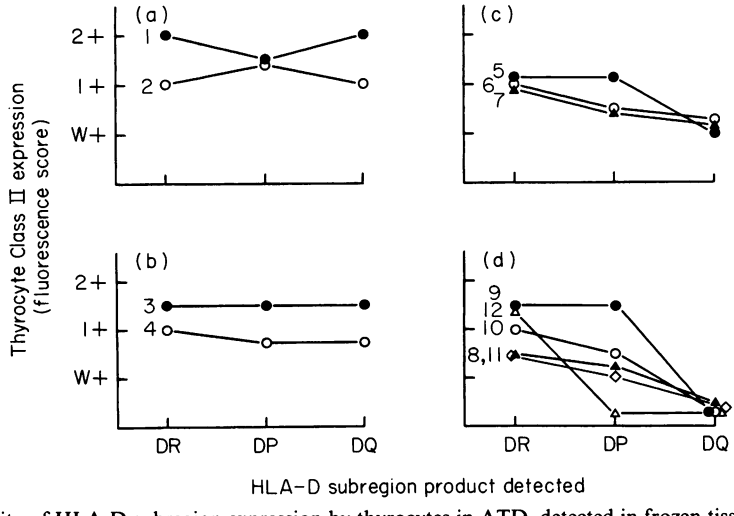


Fig. 1. Intensity of HLA-D subregion expression by thyrocytes in ATD, detected in frozen tissue sections by indirect IFL with MoAb specific for DR (DA6.164), DP (B7/21) and DQ (TU.22). Class II expression was scored in terms of strong (2+), moderate (1+) and weak (W+) fluorescence intensity. Patients 1 and 2 had Hashimoto's disease, whereas 3–12 were diagnosed as having Graves' thyrotoxicosis.

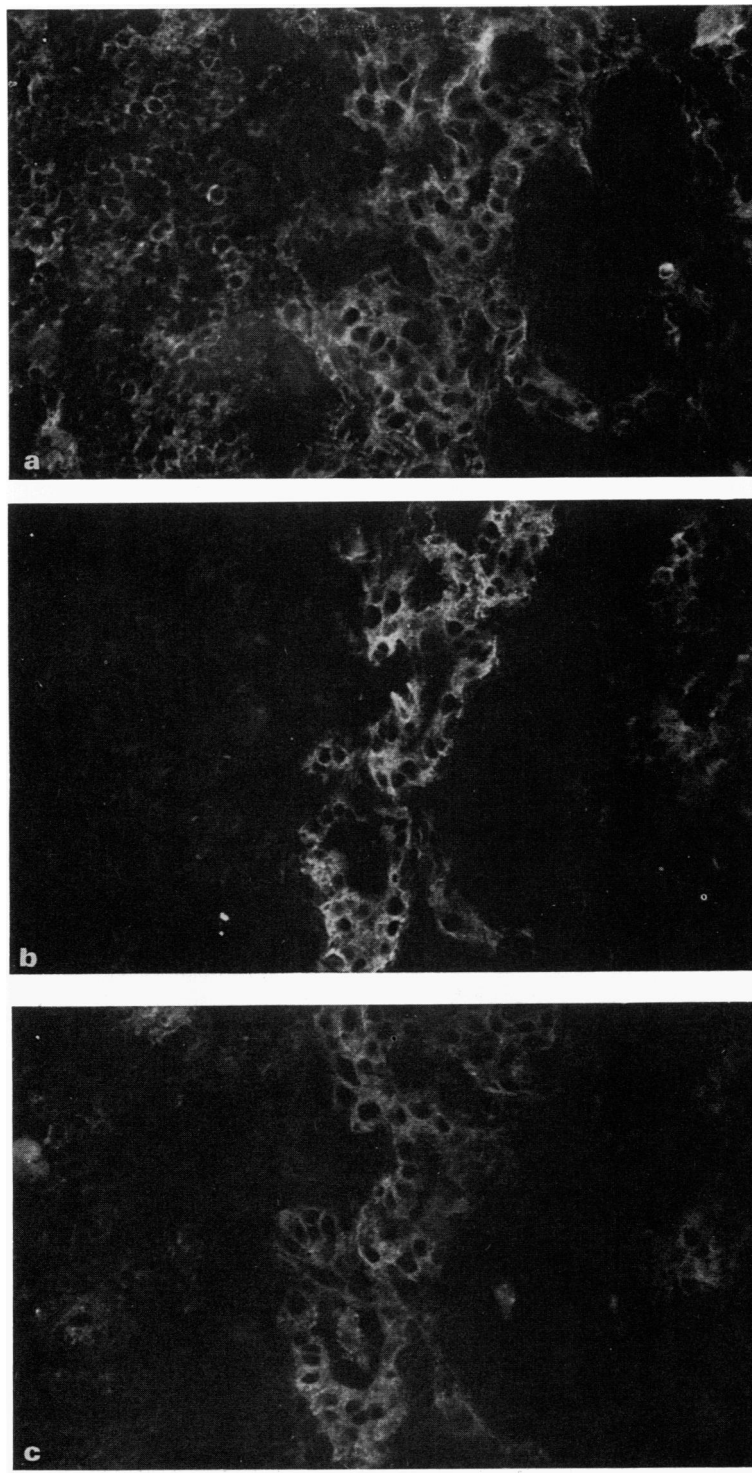
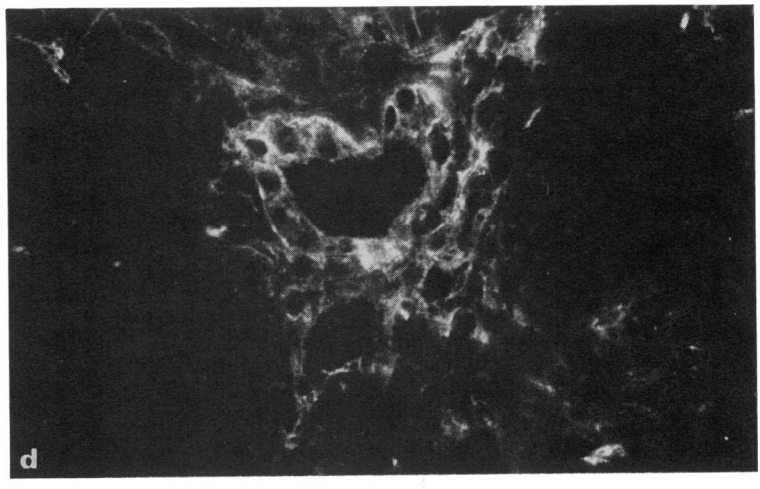


Fig. 2. Indirect IFL on consecutive sections of thyroid for detection of HLA-D subregion products: (a–c) thyroid from patient 7 × 250.; (d–f) thyroid from patient 10 × 500. The MoAb used were: (a, d) DA6.164 (anti-DR); (b, e) B7/21 (anti-DP); (c, f) TU.22 (anti-DQ).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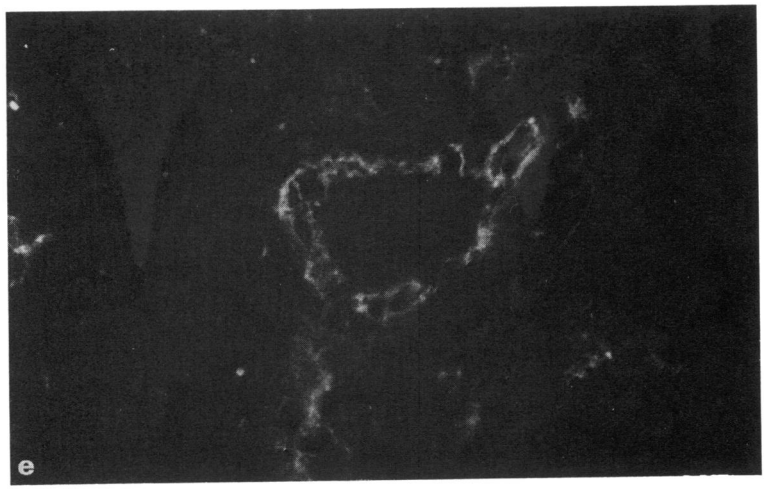
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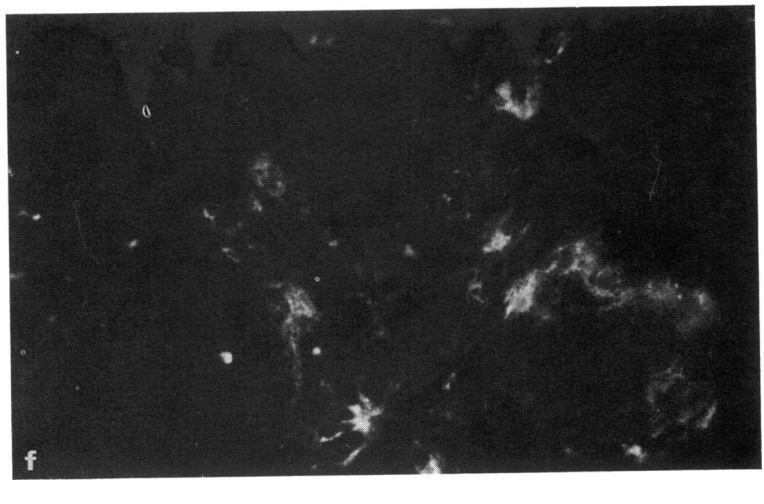
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Table 2. Extent of Class II expression by thyrocytes and infiltrate*

Patient	Extent of Class II$^+$ thyrocytes†	Extent of Class II$^+$ infiltrate†	Predominant nature of infiltrate‡
1	+++	+++	L
2	++	+++	L
3	++	++	L
4	+	+	L/M
5	++	++	L/M
6	++	+	L
7	++	++	L/M
8	+	+	M
9	++	++	L
10	+	+	L/M
11	+	+	L/M
12	+	+	L/M

* These results and those in Fig. 1 are derived from analysis of the same sections.

† Estimation of the extent of Class II expression was based on indirect IFL staining with MoAb specific for DR (DA6.164), DQ (TU.22) and DP (B7/21), but particularly with DA6.164 since DR was always the most expressed subregion. The extent of Class II expression was scored as: +++, extensive large areas; ++, limited areas; + a few areas and/or sparse.

‡ These assignments were based on morphological criteria: L, lymphocytic (i.e. relatively small, rounded cells with a low ratio of cytoplasm to nucleus); M, macrophage-like (i.e. relatively large, irregular-shaped cells with more cytoplasm).

thyrocytes was negative or barely detectable (Fig. 1d). The different patterns observed are illustrated in Fig. 2 with examples of the subregion staining in patient 7 (DQ$^+$; Fig. 2a-c) and patient 10 (DQ$^-$; Fig. 2d–f). The DR > DP > DQ pattern observed on thyrocytes appears not to be due merely to intrinsic differences in reactivity of the various HLA-D subregion-specific MoAb since, as described below, in some cases the hierarchy was different for the staining of infiltrating leucocytes and/or endothelial cells compared with thyrocytes within the same section.

The different intensities of subregion staining described above roughly correlated with the proportion of follicles expressing Class II. Thus, sections in which thyrocytes were clearly DQ$^+$ tended to have the more extensive distribution of Class II expression by follicles (Table 2), and this distribution also followed the pattern DR > DP > DQ.

A large proportion of the infiltrating leucocytes seen in the thyroid sections were Class II$^+$. In different glands they appeared to be mainly lymphocytic or macrophage-like, or a mixture of both (Table 2), with the latter type of cells often being located in the lumen of follicles. Both lymphocytes and macrophages expressed products of all three HLA-D subregions. DR, DQ and DP were either expressed at similar levels, or DR was more prominent than DQ and DP. In the two Hashimoto's glands and one of the Graves' (Patient 3) both patterns were observed, with the former occurring in foci of lymphocytic infiltration and the latter in lymphocytes dispersed around the follicles. These patterns may coincide with the predominant areas of B cells and T cells, respectively, within the infiltrate (Aichinger, Fill & Wick, 1985; Warford *et al.*, 1984). In some instances, DQ was expressed more strongly than DP by infiltrating cells: this is the opposite of the trend in thyrocytes, which also

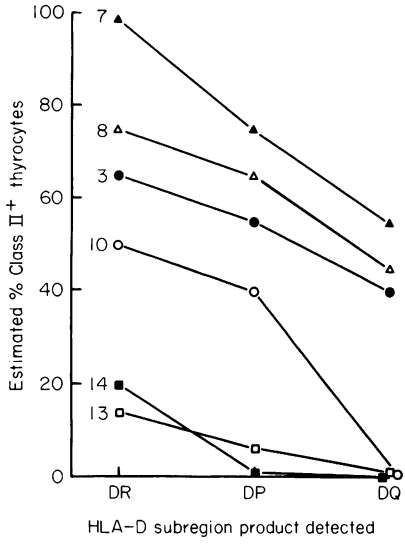

Fig. 3. Surface expression of HLA-D subregion products by thyrocytes in ATD detected on cultured thyroid monolayers by indirect IFL using the MoAb indicated in the legend to Fig. 1. The proportion of cells expressing Class II was estimated following extensive scanning of the monolayers, except for patient 13 where quantification was obtained by scoring at least 100 cells under phase and fluorescence illumination. All specimens were from Graves' disease patients: the numbering of the patients corresponds to that used in Fig. 1, except for Patients 13 and 14 whose specimens were not analysed in tissue sections.

showed stronger staining for DP and DQ than some of the infiltrate (Fig. 2a–c). The Class II expression by thyrocytes was generally in areas of infiltration, and the extent of infiltration by Class II$^+$ cells roughly correlated with the proportion of thyrocytes expressing Class II (Table 2) and with thyrocyte expression of DQ as well as DR and DP.

Expression of the three subregions was noted in capillary endothelium where examined, regardless of the thyrocyte staining.

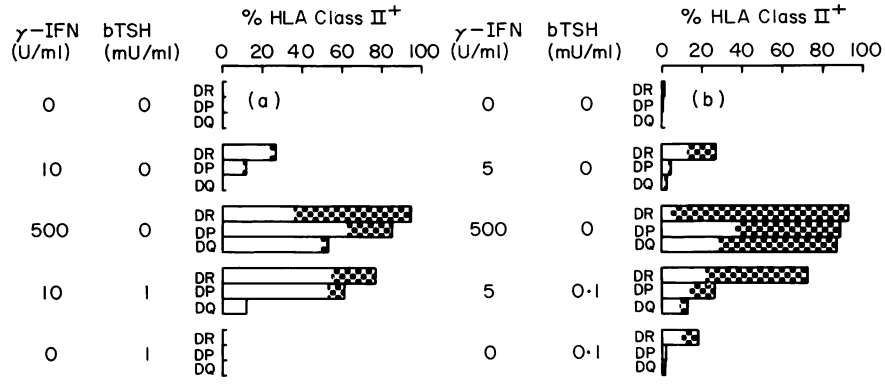

Fig. 4. Induction of HLA-D subregion expression on the surface of thyroid monolayers cultured with γ-IFN with or without bTSH. Expression was detected by indirect IFL using the MoAb indicated in the legend to Fig. 1, and quantified on duplicate cultures by scoring at least 100 cells per coverslip by phase and fluorescence microscopy. The intensity of fluorescence on each Class II$^+$ cell was scored as weak to moderate (□), or strong (▦). Different thyroid specimens were used in Figs 4a and 4b: both patients were operated for laryngeal carcinoma.

Class II subregion expression on thyroid monolayers. Class II subregion expression was also examined on thyrocytes isolated by digesting thyroid tissue from Graves' disease patients. The cells were cultured on glass coverslips for 16–44 h yielding adherent monolayers of which >95% express thyroid microsomal antigen. Class II expression present in the original specimen is largely maintained in such short term cultures. Figure 3 summarizes the subregion expression estimated on monolayers of thyrocytes from six patients, four of whom had also been examined in sections. The order of expression by the monolayers is again DR > DP > DQ.

Induction of Class II subregion expression in cultured thyrocytes. We have previously reported that normal human thyrocytes are induced to express Class II molecules by culture with plant lectins (Pujol-Borrell *et al.*, 1983) or with the physiological mediator γ-IFN (Todd *et al.*, 1985). Furthermore, Class II induction by γ-IFN is enhanced by TSH (Todd *et al.*, 1987). These stimuli were used to investigate induction of Class II subregion expression in cultures of thyrocytes from non-autoimmune patients operated for carcinoma of the larynx.

Little or no Class II expression was detected in the unstimulated thyroid monolayers. Culture with relatively low doses of recombinant human γ-IFN (5–10 U/ml) induced moderate cell surface expression of DR, much lower DP expression and little or no DQ (Fig. 4). Employing a 50-fold or 100-fold higher concentration of γ-IFN resulted in much greater induction of DR in terms of both the proportion of cells which were positively stained and the intensity of their fluorescence (indicated by the shading in the histogram). Moreover, under these conditions the induction of DP expression was similar to that of DR, and good induction of DQ was also observed (Fig. 4). Employing a low dose of γ-IFN plus bTSH also improved the induction of DP and DQ, as well as enhancing expression of DR (Fig. 4). In the experiment depicted in Fig. 4b, treatment with 0·1 mU bTSH/ml alone resulted in some expression of DR, but almost no DP or DQ.

The MoAb used to detect DQ in the above experiments was TU.22: similar results were obtained employing the anti-DQw1 MoAb SDR4.1. These *in vitro* findings indicate that the ease of induction of HLA Class II in thyrocytes is DR > DP > DQ, which accords with the levels of Class II subregion expression by thyrocytes in autoimmune glands (Figs 1 and 3).

DISCUSSION

The complexity of HLA Class II is apparent at the level of both the D region genes and the products they encode (reviewed by Feldmann & McMichael, 1986) and this must be taken into account when considering HLA Class II expression in particular situations. In autoimmunity the qualitative nature of the Class II expression by epithelial cells of the affected organs is important given the possible role of such inappropriate expression in stimulating the pathogenesis. The results presented here show expression of all three HLA-D subregions by thyrocytes of patients with ATD, but with a hierarchy of subregion expression which follows the pattern DR > DP > DQ (Fig. 1–3). At the same time, however, the analysis of thyrocyte monolayers indicates that all three subregions are modulated in parallel, with those specimens having very little or no DQ expression also having the least expression of DR and DP (Fig. 3).

Examination of Class II subregion expression in thyrocyte monolayers has several advantages over the analysis of tissue sections, not the least being that isolated thyrocytes are a mixture of the digested tissue, and hence are probably more representative of the whole specimen than are individual sections. It is thus important that in the studies described here, although the frozen tissue was not sampled repeatedly, the analysis of thyrocyte Class II subregion expression in sections led to essentially the same conclusions as those drawn from the monolayer studies (despite some differences in detail between the results from individual patients analysed by both methods: compare Figs 1 and 3). Furthermore, the scoring of sections was performed by a different investigator and at a different time than the scoring of monolayers.

Since most of the Graves' disease patients received carbimazole before surgery (see Materials and Methods), such treatment is unlikely to be a major factor in determining the differences in HLA-D subregion expression observed between patients. By contrast, the correlation generally noted between the degree and location of infiltration and Class II subregion expression by

thyrocytes (Fig. 1 and Table 2) is consistent with lymphokines derived from activated T cells, particularly γ-IFN, propagating this inappropriate Class II expression in the ongoing autoimmune situation (Todd *et al.*, 1985). Indeed, induction of Class II in thyrocytes cultured with γ-IFN and TSH reproduced the DR > DP > DQ hierarchy of expression observed in ATD (Fig. 4). These findings suggest that the nature of thyrocyte Class II expression in the ongoing autoimmune disease may be governed by the local concentrations of stimulating factors like γ-IFN and TSH. Heterogeneity of subregion expression between patients would then reflect differences in the levels of these factors which are themselves dependent upon the nature and severity of the pathogenesis. For example, the fact that γ-IFN is product of T cells and large granular lymphocytes might help to explain the high expression of all three subregions in Hashimoto's thyroiditis reported here (see also Möst, Knapp & Wick, 1986), together with a contribution from the raised levels of TSH which often accompany this disease due to the destructive attack generating hypothyroidism (for further discussion of this point, see Todd *et al.*, 1987). It should be emphasized that these considerations are pertinent to the on-going disease, but do not necessarily apply to the epithelial Class II expression postulated to play a role in initiating the autoimmune activation (Bottazzo *et al.*, 1983). This could be induced by γ-IFN produced in response to local infection, or could be triggered by other agents, as yet undefined. Indeed, one cannot assume that the mechanisms which propagate Class II expression in the infiltrated thyroid are identical to those responsible for its initial induction.

Our findings in ATD are consistent with the general observation that the expression of DQ is less than that of DR (DP expression has not been studied as extensively). Thus, DQ is expressed less widely, and arises later in ontogeny than DR in normal human tissues (Natali *et al.*, 1984). Furthermore, others have found, similar to ourselves, that DQ is induced to lower levels than DR in thyrocytes cultured with γ-IFN (Weetman *et al.*, 1985) or leucoagglutinin (Davies, 1985), and similar observations have been made on other cell types treated with γ-IFN including endothelial cells (Collins *et al.*, 1984), thymic epithelium (Berrih *et al.*, 1984) and melanoma cell lines (Carrel, Schmidt-Kessen & Giuffre, 1985). We have also observed preferential induction of DR in human islet beta cells treated with γ-IFN plus tumour necrosis factor although, interestingly, the hierarchy of induction in these cells is DR > DQ > DP (Pujol-Borrell *et al.*, 1987). In rat thyroid and islet beta cell lines I-A and I-E products are induced to similar levels by γ-IFN (Rayner *et al.*, 1987; A-M. Varey *et al.*, pers. comm.).

The discussion so far has centred on the nature of thyrocyte Class II subregion expression being a consequence of the autoimmune process. However, it should be remembered that this inappropriate Class II expression may itself contribute to the propagation, and possibly the initiation, of the pathogenesis by enabling the thyrocytes to present their surface autoantigens (Bottazzo *et al.*, 1983). An important issue is therefore how the extent to which DR, DP and DQ are expressed by thyrocytes might influence the course of the disease. Although little is presently known about the precise functional roles of the different HLA-D region products, DR, DP and DQ all seem to function in the presentation of exogenous antigens, with responses to different antigens showing different HLA-D subregion restrictions (eg. Gonwa *et al.*, 1983; Fischer *et al.*, 1985; Sone *et al.*, 1985). Since different antigens are presented in the context of different Class II molecules, one feature of ATD which might be determined by thyrocyte subregion expression is the nature of the autoantibodies produced. In preliminary investigations we have found a significant relationship between thyrocyte HLA Class II expression and the occurrence of circulating thyroid autoantibodies (Todd *et al.*, 1986; Pujol-Borrell *et al.*, 1986). Furthermore, the most significant associations were found between thyroid microsomal autoantibodies and thyrocyte expression of HLA-DR, and between thyroglobulin autoantibodies and HLA-DQ (Todd *et al.*, 1986 and in preparation). Other comparisons of this type could provide insights into the ways in which inappropriate Class II expression by thyrocytes may help to determine the course of ATD.

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