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Detection of interleukin-6 and interleukin-1 production in human thyroid epithelial cells by non-radioactive *in situ* hybridization and immunohistochemical methods

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SUMMARY

Human endocrine thyroid epithelial cells have been described to produce cytokines *in vitro*. In order to determine whether they do so *in vivo* during thyroiditis, parallel studies on mRNA expression with a non-radioactive *in situ* hybridization technique and immunohistochemical detection for the protein were performed on frozen sections of thyroid samples from autoimmune thyroiditis (Graves' disease and Hashimoto's thyroiditis), non-toxic goitre and normal thyroid tissue. cDNA probes were sulphonated and their hybridization with mRNA was detected with a sulphonyl-specific monoclonal antibody. This signal was amplified and visualized with the alkaline phosphatase–anti-alkaline phosphatase (APAAP) system. The protein products were detected with immuno-purified rabbit $F(ab')_2$ antibody fragments recognizing recombinant human cytokines, visualized by the immuno-peroxidase technique. Each sample was studied at the two levels. Both interleukin-6 mRNA and protein were found in the endocrine cells. There was no obvious difference between autoimmune thyroiditis and non-toxic goitre. However, normal thyroid epithelial cells produced less interleukin-6. Interleukin-1α mRNA and its protein were found in epithelial cells from Hashimoto's thyroiditis samples, but not in the others, except one Graves' disease sample, in which only mRNA was detected. Interleukin-1β was not detected in these cells, its mRNA was only found in one of the Graves' disease samples. These cytokines were also detected in some infiltrating cells.

Keywords cytokines non-radioactive *in situ* hybridization immunohistochemistry thyroid epithelial cells

INTRODUCTION

Human endocrine thyroid epithelial cells (TEC), which normally lack HLA class II can express these molecules *in vivo* (Hanafusa *et al.*, 1983), or *in vitro* (Todd *et al.*, 1985). This expression is essential for their antigen presenting function (Londei *et al.*, 1984; Londei, Bottazzo & Feldmann, 1985). Other properties of TEC involved in antigen presentation, such as cytokine production and adhesion molecule expression have also been investigated. Hirose *et al.* (1987) reported the production of thymocyte-stimulating activity by cultured TEC after stimulation with lentinan and indomethacin, suggestive of an 'interleukin-1-like' cytokine. Recently, we extended these experiments with purified TEC from both Graves' disease and non-toxic goitre. Using cDNA probes and bioassays, these cells were found to be able to produce interleukin-1α (IL-1α) and interleukin-6 (IL-6) *in vitro* (Grubeck-Loebenstein *et al.*, 1989).

In order to understand more about the pathogenesis of the diseases, it is important to determine whether TEC actually produce these cytokines *in vivo*, and whether there is any difference in their production between different types of thyroid diseases. *In situ* hybridization to detect mRNA with sulphonated DNA probes (S-cDNA) and immunohistochemical studies were thus performed in parallel to detect IL-1 and IL-6 on frozen sections of thyroid tissue. The samples were from Graves' disease and Hashimoto's thyroiditis, diseases that are autoimmune in nature but with different clinical manifestations, and from non-autoimmune tissue such as non-toxic goitre and a normal thyroid gland for comparison.

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SUBJECTS AND METHODS

Patients

Seven cases of Graves' disease, three of Hashimoto's thyroiditis and four cases of non-toxic goitre as well as two blocks of normal thyroid tissue were used. All clinical diagnoses were confirmed by pathological examination after the operation. Each sample was studied in parallel for both cytokine mRNA and protein.

Tissue section preparation

Samples of thyroid tissue were taken surgically and frozen in liquid nitrogen with Tissue-Tek OCT compound 4583 (Miles Scientific, Naperville, IL). The sections were cut at 5-μm thickness at $-25°C$ in a Frigocut E800 (Reichert Jung) microtome, and kept frozen until used.

In situ *hybridization*

Labelling of probes. IL-6 cDNA probe (440 bp *Taq*I–*Ban*II) was kindly donated by Prof. T. Kishimoto (Hirano *et al.*, 1986); and IL-1α (460 bp *Eco*RI–*Bam*HI; March *et al.*, 1985) and IL-1β (530 bp *Nde*I–*Bam*HI; Gubler *et al.*, 1986), by Dr P. Lomedico. The labelling was performed as described by Lebacq *et al.* (1987). Probes were dissolved in water and after denaturation by boiling, sodium bisulphite (ICN Pharmaceuticals, Cleveland, OH) was added at the final concentration of 615 mM and methoxyamine (ICN), 77 mM. The solution was vortexed and left overnight. The mixture was directly used or precipitated at $-80°C$ with 2·5 volumes of ice cold 95% ethanol for 30 min and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7·4). All reagents were ANALAR reagent grade from BDH Chemicals (Poole, UK) unless specified.

Hybridization. Sections were fixed at 4°C for 20 min in a solution containing 0·5% paraformaldehyde, 0·5% glutaraldehyde, 1·6% glucose, 0·002% $CaCl_2$, 1% dimethylsulphoxide in 0·1 M sodium phosphate buffer, pH 6·5, followed by washing in 0·15 M ethanolamine (Sigma Chemical Company, Poole, UK) for 20 min at 4°C followed by washing twice in water. Adapting the method of Pringle *et al.* (1989), these sections were digested for 30 min at 37°C with proteinase K (Sigma) at 10–25 μg/ml in 50 mM Tris–HCl, pH 7·4, solution containing 5 mM EDTA and 0·5% saponin (Sigma), followed by a wash in TSG buffer (100 mM Tris–HCl, 100 mM NaCl, 2 mg/ml glycine, pH 7·4) at room temperature. Dehydration was then performed in 30%, 60%, 90% and 99% ethanol, each for 3 min, and the sections were then air-dried at room temperature.

Based on the protocol for *in situ* hybridization by Maniatis, Fritsch & Sambrook (1983), pre-hybridization was performed at 37°C for 2 h with hybridization solution. This was 50% deionized formamide, prepared with 5 g of mixed-bed resin (BioRad, 501-X8) per 50 ml of the liquid, filtered through Whatman's no. 1 filter paper, 10% dextran sulphate, 25 mM sodium phosphate, 5 × SSC taken from 20 × SSC stock solution (17·53% NaCl, 8·82% Na_3 citrate, 2 H_2O in water), and 1 × Denhardt's solution which contained 0·02% bovine serum albumin, 0·02% polyvinylpyrolidone 400, 0·02% Ficoll 400, 3 × SSC (all from Sigma). Hybridization was followed with the appropriate probe at a concentration of 0·1–0·2 ng/μl in the hybridization solution at 37°C overnight. Then the slides were washed in 50% deionized formamide with 4 × SSC for 15 min, followed by further washes in 2 × SSC and 1 × SSC, each for 15 min at room temperature.

Detection of hybridization. As recommended by Lebacq *et al.* (1987), the sections were first blocked for 50 min with the blocking solution containing skimmed milk at 0·3 g/ml in 25 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 0·3% Tween 20 (all from ICN), and heparin 500 U/ml solution, pH 7·5, followed by dipping in ST buffer (90% normal saline, 10% 50 mM Tris–HCl, pH 7·36) and overnight incubation with monoclonal antibody (ICN cat. no. 51800G) against sulphonated DNA at 1/50 dilution in TB buffer (50 mM Tris–HCl buffer, pH 7·36) at 4°C. The slides were then washed extensively in ST buffer for 1 h at room temperature. As described by Cordell *et al.* (1984), tissue sections were treated with rabbit anti-mouse immunoglobulin antibody (Dakopatts, Glostrup, Denmark) at 1/20 in TB buffer with 20% normal rabbit serum for 1 h at room temperature and washing in ST buffer for 30 min, then incubation with monoclonal anti-alkaline phosphatase antibody linked with alkaline phosphatase (APAAP complex; Dakopatts) at 1/30 in TB buffer containing 20% normal rabbit serum for 1 h followed by washes in ST buffer for 30 min. The colour development employed fast red substrate containing 0·4 mg/ml naphthol AS-MX phosphate (Sigma), 50 mM Tris-acetate, 10 mM magnesium acetate and 2 mg/ml fast red TR (Sigma), pH 9, for 15 min at room temperature. Three washes in water followed and the sections were counterstained with Harry's haematoxylin for 30 sec, washed in running water for 5 min and mounted in glycerol–gelatin (Sigma).

Controls for in situ *hybridization.* Either RNAse or DNAse was used. The sections were incubated with the enzyme at 40 μg/ml in 0·8 M NaCl, 0·01 M Tris–HCl, 0·001 M EDTA solution, pH 7·5 at 37°C for 2 h, followed by extensive washing in TSG buffer for 45 min just before dehydration.

Specificity controls were carried out by hybridizing with the labelled probe together with the unlabelled probes at the same time, the concentration of the former was as above, but the latter was in five- or 10-fold excess.

Detection of cytokine protein

Preparation of purified biotin-conjugated antibody F(ab')$_2$ fragments, ELISA and immunostaining. The entire protocol is described in detail elsewhere by Field *et al.* (1991). Briefly, rabbit antibody F(ab')$_2$ fragments were generated by pepsin digestion, followed by immunopurification with Sepharose coupled with recombinant cytokines. The biotinylation of the F(ab')$_2$ fragments was performed in bicarbonate buffer (Zeheb, Chang & Orr, 1983). The immunoreactivity of the antibody F(ab')$_2$ fragments was confirmed by ELISA, and immunoperoxidase staining was performed on tissue sections.

IL-6 peptide coupled to thyroglobulin was kindly donated by Dr J. Rothbard, recombinant IL-6 by Immunex, recombinant human IL-1α by Dainippon Pharmaceutical Co., and IL-1β by Hoffman la Roche.

Murine anti-human thyroglobulin monoclonal antibody ID4 (Chan *et al.*, 1987) was kindly donated by Dr P. Shepherd, Guy's Hospital, London, UK.

Specificity controls. To confirm the specificity of each rabbit antibody F(ab')$_2$ used on the thyroid sections, a sepharose immunoaffinity column with the relevant antigen such as IL-6 was used to remove the specific antibodies and both the drop-through, i.e. the non-antigen-absorbed part, and the 3M

guanidine-eluted antibodies, i.e. specific antibody, were collected and used to stain the tissue. An irrelevant cytokine such as IL-1α or IL-1β affinity column was used with the same antibody in parallel and both the eluate and drop through from the column were collected for staining. Pre-bleed $F(ab')_2$ fragments were also used as a negative control.

Calculations

At least 12 non-successive sections of every sample were examined for each cytokine (six for mRNA and six for protein). Under microscopy at × 160, eight areas per slide and six non-successive slides per sample were counted for the positive stained TEC. Thus more than 2000 cells per sample were scored. The number of the positive cells was expressed as the percentage (%) of positivity according to the formula

$$\% \text{ of the positive stained TEC} = \frac{\text{Number of positive TEC}}{\text{Total number of TEC in the same area}} \times 100\%$$

RESULTS

ELISA

All antibody preparations bound specifically to the relevant cytokine in a dose-dependent manner without binding to human thyroglobulin or other cytokines (Fig. 1a–d).

IL-6 mRNa localization

IL-6 mRNA was detected in the cytoplasm of TEC from all samples of thyroid diseases (Table 1), in a similar pattern to the protein (Fig. 2b). 60% of TEC from non-toxic goitre, 66% of TEC from Graves' disease and 58% of TEC from Hashimoto's thyroiditis were found to be positive. In the normal sample, weak but positive hybridization for IL-6 mRNA was observed in the cytoplasm and the nuclei, showing 35% positivity. Treatment with RNAse prior to hybridization totally abolished the positivity (Fig. 2c), while DNAse failed to do so (Fig. 2d). Specific competition with the unlabelled probe was observed (Fig. 2e). When the concentration of the unlabelled probe was 10 times higher than the labelled one, the hybridization was almost undetectable (Fig. 2f).

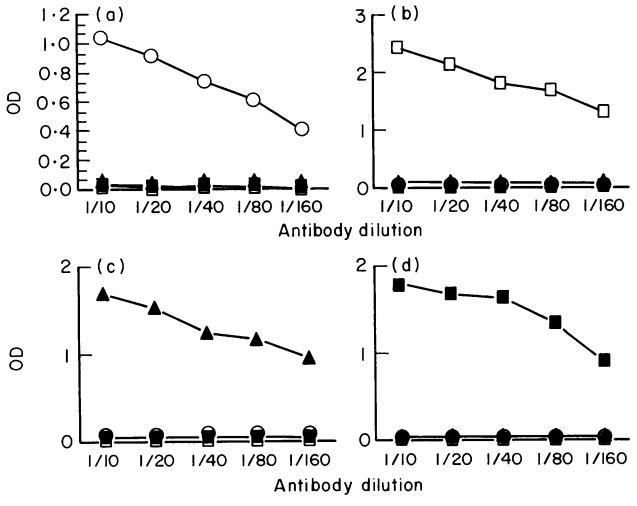

Fig. 1. ELISAs. (a) Against thyroglobulin. None of the purified anti-cytokine antibodies bound to thyroglobulin, but the anti-thyroglobulin monoclonal antibody, ID4 (○) did; (b) against IL-6. Only anti-IL-6 antibody (□) preparation, but not other antibody preparations, bound to IL-6 in a dose-dependent manner; (c) against IL-1α. Only anti-IL-1α antibody (▲) preparation, but not other antibody preparations, bound to IL-1α in a dose-dependent manner; (d) against IL-1α. Only anti-IL-1β antibody (■) preparation, but not other antibody preparations, bound to IL-1β in a dose-dependent manner.

IL-6 protein localization

In all cases, the IgG $F(ab')_2$ of the pre-bleed serum did not stain any samples (Fig. 3a), while anti IL-6 $F(ab')_2$ stained cytoplasm of TEC (Fig. 3b). The flow through of the specific antibody from recombinant IL-6-bound Sepharose column did not stain these cells (Fig. 3c), but the eluate from this column did (Fig. 3d). An IL-1α/Sepharose column failed to remove the anti IL-6 antibody activity (Fig. 3e). The eluate from this column did not stain (Fig. 3f). These controls were performed on all samples and the specificity was confirmed. The percentage of positive TEC in non-toxic goitre was 85%; in Graves' disease 90%, and in Hashimoto's thyroiditis 85%. In the normal thyroid sample, 77% of TEC were positive but the staining was less intense than others.

IL-1α mRNA localization

IL-1α mRNA was detected in 55% (range 30–74%) of TEC from all three Hashimoto's thyroiditis samples (Table 1). Cytoplasmic hybridization was observed on these cells (Fig. 2h). Only one of the Graves' disease samples hybridized with IL-1α probe on 38% of the epithelia (Table 1). Controls as mentioned above were performed, and specificity was confirmed. TEC from non-toxic goitre and normal thyroid samples did not show hybridization for this cytokine.

IL-1α protein localization

IL-1α-expressing TEC were only detected in Hashimoto's thyroiditis (Table 1), using the anti IL-1α antibody $F(ab')_2$ preparation, 73% (range 68–77%) of TEC were stained (Fig. 3g). There was no detectable IL-1α in TEC from normal, non-toxic goitre or Graves' disease. The specificity of the antibody staining was confirmed in the same manner as for IL-6 above.

IL-1β mRNA localization

In all the thyroid samples, IL-1β mRNA was not detected in TEC but was noted in some infiltrating blood cells (Fig. 2g), except for one case of Graves' disease (not the sample positive for IL-1α), in which 30% of the TEC were found to be positive. Specificity was confirmed by the controls as above.

IL-1β localization

IL-1β was not detected in TEC (Fig. 3h) in any samples, although some of infiltrating blood cells were observed to be positive. A control (synovial membrane frozen sections) was run in parallel and was stained (Covington *et al.*, 1989).

DISCUSSION

Frozen sections of operative specimens of thyroid tissues were studied to investigate whether TEC are actually producing cytokines such as IL-6 and IL-1 *in vivo* during thyroiditis. These tissues were frozen rapidly after operation, and no culture was

Table 1. Cytokine detection in thyroid epithelial cells (Percentage of positive thyroid epithelial cells)

	Grave's disease							Hashimoto's thyroiditis			Non-toxic goitre				Normal thyroid
	1	2	3	4	5	6	7	1	2	3	1	2	3	4	
IL-6															
Protein	95	77	89	92	92	92	95	84	79	91	88	82	77	89	77
mRNA	55	70	52	60	56	80	86	62	75	35	80	58	62	40	35
IL-1α															
Protein	—	—	—	—	—	—	—	75	77	68	—	—	—	—	—
mRNA	—	—	—	—	—	38	—	60	74	30	—	—	—	—	—
IL-1β															
Protein	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
mRNA	—	—	—	—	—	—	30	—	—	—	—	—	—	—	—

In all cases, some infiltrating cells were observed to be positive for both protein and mRNA of the cytokines; —, not detected.

involved. Thus, if cytokine mRNA and protein are detected, they must have been produced *in vivo*.

A new technique of non-radioactive *in situ* hybridization with cDNA probes was developed to detect the mRNA for cytokines on frozen sections. The sulphonation labelling system was first described by Budowski, Sverdlov & Monastyrskaya (1972) and improved by Verdlov *et al.* (1974). The introduced sulphone group on the cDNA probe was recognized by a specific monoclonal antibody (Povernny *et al.*, 1978). Hybridization could thus be visualized indirectly with chromatogenic-material-linked antibodies against this murine monoclonal antibody, bound to the labelled probe. An advantage of this *in situ* technique is that it can provide better localization, as there is no scatter of radioactive molecules. There is also the advantage of faster detection than with isotopic probes. Gerdes *et al.* (1989) reported an approach with this labelling system on cytospins of peripheral blood; Pringle *et al.* (1989) described biotin labelling of polydeoxythymidine; and Farquharson, Harvie & McNicol (1990) labelled oligodeoxynucleotides with digoxigenin. These methods yielded satisfactory results.

In order to enhance the accessibility for hybridization on tissue sections, treatment with HCl and proteinase K was performed. APAAP complex was employed to obtain amplification. Sufficient blocking with thorough washing yielded a very low background, as shown in Fig. 2a.

IL-6 mRNA was detected in the cytoplasm of TEC from all thyroid diseases. The normal sample showed weak positivity in the cytoplasm and some nuclei. It suggested a low production of the protein in this sample, which was confirmed by our immunohistochemical studies. Controls with DNAse free RNAse, and RNAse-free DNAse confirmed that the hybridization was on mRNA in all samples. Specificity was confirmed by the competition assay, in which excess unlabelled probe competed with the labelled one. The hybridization of the labelled probes was greatly reduced or abolished when the concentration of unlabelled probe was five- or 10-fold in excess.

Using an immunoperoxidase staining technique, we found that IL-6 was present in TEC of autoimmune thyroiditis as well as non-toxic goitre. The normal thyroid samples showed less intense staining for IL-6 on TEC than those with thyroiditis, indicating less production of IL-6, as had been suggested by our data on the mRNA detection. The purified anti-IL-6 antibody preparation showed no binding to human thyroglobulin as shown on ELISA, although the rabbit had once been immunized with IL-6 peptide coupled with bovine thyroglobulin. For the subsequent boosts and immunopurification, recombinant IL-6 was used. In order to verify the specificity of the staining, we used a cytokine-specific affinity column to remove the IL-6-binding antibody. There was no staining with the drop-through which did not bind to the affinity column, and staining with the eluate which bound to the column. Thus we could exclude any effects of antibodies against thyroglobulin. However, an irrelevant cytokine affinity column was also used in parallel to confirm that the antibody activity could not be removed by it.

It is perhaps not surprising that our results overlapped among Graves' disease, Hashimoto's thyroiditis and non-toxic goitre, as signs of inflammation such as lymphocytic infiltration and HLA class II expression (Grubeck-Loebenstein *et al.*, 1988) are present in all of three diseases. It is likely that the production of IL-6, a B and T cell activator (Hirano *et al.*, 1986; Baroja *et al.*, 1988), contributes to the stimulation of maturation of local, infiltrating lymphocytes. The presence of a low level of IL-6 in the normal thyroid sample suggests the possibility of its involvement in the physiology of these cells.

Both mRNA and the protein of IL-1α was only found in TEC of Hashimoto's thyroiditis. This production may suggest that the cytokine is related to the pathogenesis of the disease, which is destructive, in contrast to Graves' disease. In the rest of the 12 samples, IL-1α mRNA was only detected in TEC from one of Graves' disease samples, and the protein was not detected. It would suggest that there is little of this cytokine present in TEC from these samples. Our data on the protein level agree with the report by Miyazaki *et al.* (1989). As expected, some of the infiltrating blood cells produced this cytokine. This verifies that the conditions for hybridization and immunostaining were appropriate. The *in vitro* study by Grubeck-Loebenstein *et al.* (1989) reported IL-1α production by TEC purified from different Graves' disease and non-toxic goitre samples. This previous investigation revealed the potential of cultured TEC to produce cytokines *in vitro*, and our present report indicates that this potential is often expressed *in vivo* for IL-6 but, more rarely, in Hashimoto's thyroiditis for IL-

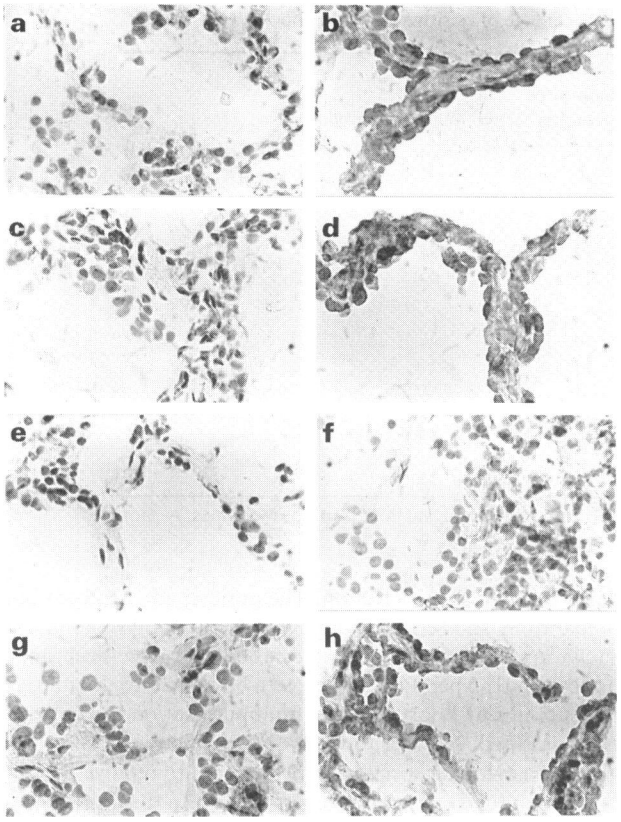

Fig. 2. *In Situ* hybridization. (a) Hashimoto's thyroiditis sample, unlabelled probe was used as a negative control; (b) hybridization with labelled IL-6 probe was observed in thyroid epithelial cells from Hashimoto's thyroiditis sample; (c) Hashimoto's thyroiditis section; after RNAse digestion, IL-6 mRNA was not detected; (d) DNAse digestion did not alter the hybridization for IL-6 mRNA, as shown here in a Hashimoto's thyroiditis sample. (Compare with b); (e) when unlabelled IL-6 probe five-fold in excess was allowed to compete with the labelled one, the hybridization of the latter was less intense, in the same sample as the previous four; (f) competition assay as in (e), but the unlabelled probe was 10 times in excess, the hybridization of the labelled probe was abolished; (g) IL-1β mRNA was not detected in epithelial cells from Hashimoto's thyroiditis samples but some of the mononuclear cells were found to be positive (lower right corner); (h) IL-1α mRNA was detected in the epithelial cells from Hashimoto's thyroiditis. Magnification × 85.

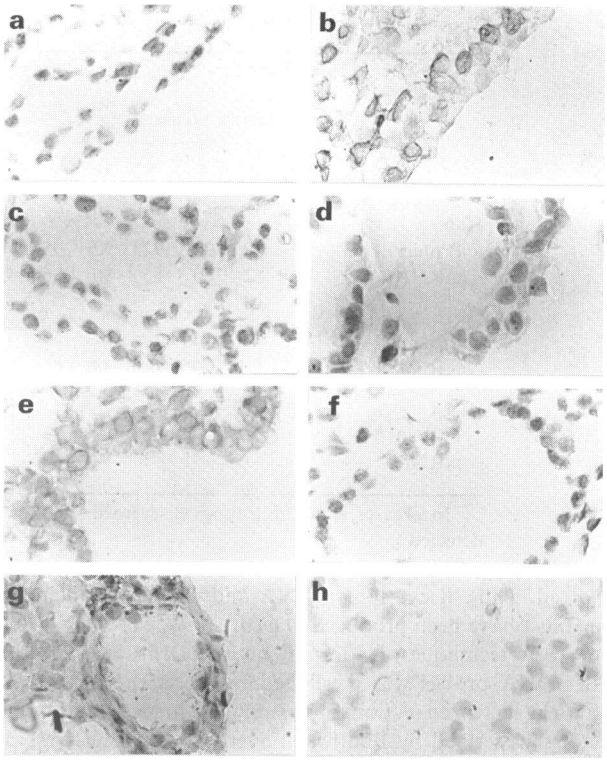

Fig. 3. Immunohistology. (a) No peroxidase staining on Graves' disease tissue with antibody $F(ab')_2$ preparation from the pre-bleed serum of the rabbit used for IL-6 immunization; (b) anti-IL-6 antibody preparation stained thyroid epithelial cells on the same Graves' disease tissue; (c) the flow-through of anti-IL-6 antibody from IL-6 column did not stain any cells. Graves' disease tissue; (d) the eluate from IL-6 column stained Graves' disease thyroid epithelial cells; (e) the flow-through of anti-IL-6 antibody preparation from IL-1α column stained thyroid epithelial cells. A graves' disease sample; (f) no antibody staining was observed with the eluate of anti-IL-6 antibody preparation from IL-1α column. Graves' disease section; (g) IL-1α was detected in thyroid epithelial cells from Hashimoto's thyroiditis; (h) IL-1β was not detected on thyroid epithelial cells. Hashimoto's thyroiditis tissue. Magnification × 170.

1α; alternatively, therapy may be more effective at diminishing IL-1α expression than IL-6 expression.

The IL-1β mRNA was not detected in TEC, but was in a few infiltrating cells (Fig. 2g), except for one of the Graves' disease patients, where TEC were found to be positive. There was no IL-1β protein in TEC. The antibody activity was confirmed by ELISA (Fig. 1), and the testing on frozen sections of rheumatoid synovial membrane prepared in the same way as the thyroid sample (Covington *et al.*, 1989). The lack of staining would suggest that there is little of this cytokine, which could not be detected by our method. Our data on the protein level agree with the report by Miyazaki *et al.* (1989) and also the *in vitro* study by Grubeck-Loebenstein *et al.* (1989).

These results establish the *in vivo* production of IL-6 and IL-1α by TEC, suggesting the involvement of this event in the pathogenesis of human thyroid diseases. It further supports the concept that TEC in an autoimmune process share all the known properties of conventional antigen-presenting cells, including cytokine production, HLA and adhesion molecule expression (Weetman *et al.*, 1990; Zheng *et al.*, 1991). The non-radioactive *in situ* hybridization with immunohistochemical techniques on frozen sections is a useful tool for investigation and localization of *in vivo* molecular events.

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