Immunology 1992 **75** 456–462

Detection of *in vivo* production of tumour necrosis factor-alpha by human thyroid epithelial cells

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Accepted for publication 23 October 1991

SUMMARY

We have established previously that human thyroid epithelial cells (TEC) from patients with autoimmune thyroiditis are able to synthesize cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6). This paper examines TEC in sections from autoimmune thyroiditis for the *in vivo* production of tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) using the combined techniques of *in situ* hybridization and immunohistochemistry. Thyroid tissue from patients with Graves' disease, Hashimoto's disease and non-toxic goitre was examined and both mRNA and the protein of TNF-α were detected in TEC on frozen sections. Representative figures of only Graves' samples are illustrated in this paper. In contrast, using the same methods, IFN-γ was detected only in the infiltrating cells and not in TEC of thyroid tissue from the patients.

INTRODUCTION

Human thyroid epithelial cells (TEC), expressing class II major histocompatibility complex (MHC) antigens, are able to support antigen-dependent T-cell proliferation and have been suggested to have the potential of presenting autoantigen(s) and thus to contribute to the development and maintenance of autoimmune thyroiditis.[1] However, it appears that expression of MHC class II antigens alone is not adequate for optimal antigen presentation and T-cell activation.[2] Both additional accessory molecules and cytokines are required. The results of recent studies have demonstrated that TEC may produce selected cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6), both of which are able to influence T-cell activation.[3-5] Analysis of TEC from patients with thyroid diseases has demonstrated the presence of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function associated antigen-3 (LFA-3) in addition to class II expression.[6,7] The expression of these proteins may be regulated by cytokines such as tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ).[7,8] The *in vivo* expression of MHC class II and adhesion molecules in autoimmune thyroiditis have been established,[6,7,9] but information concerning the *in vivo* production of regulatory cytokines such as TNF-α and IFN-γ is rather limited, although there is evidence to suggest the local release of IFN-γ by lymphocytes infiltrating the thyroid.[10]

The present study was performed in order to investigate the production of TNF-α and IFN-γ and their cellular localization. Using the combination of immunohistochemical and non-radioactive *in situ* hybridization techniques as reported before,[3] the presence of transcripts and protein were examined in tissue sections from patients with thyroiditis.

Abbreviations: IFN-γ, interferon-gamma; TEC, thyroid epithelial cells; TNF-α, tumour necrosis factor-alpha.

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MATERIALS 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 tissue from a normal thyroid gland were examined.

Tissue section preparation

Samples of thyroid tissue were taken surgically and frozen in liquid nitrogen with Tissue-Tec OCT compound (BDH Ltd, Poole, Dorset, U.K.). No tissue culture was involved. Sections were cut from these samples at 5 μm thickness, −25° in a Figocut E800 microtome (Reichert Jung Ltd, Vienna, Austria) and kept at −70° until used.

In situ *hybridization*

The non-radioactive *in situ* hybridization technique used in the study reported here has been described in detail.[3]

Probe labelling. The TNF-α probe (800 bp *Eco*RI-*Eco*RI)[11] and IFN-γ probe (980 bp *Pst*I-*Hin*cII)[12] were kindly donated by Drs M. Shepard and P. Gray (Genentech, San Francisco, CA),

respectively. These probes were dissolved in water and after denaturation by boiling, sodium bisulphite (ICN) was added at the final concentration of 615 mM and methoxyamine (ICN) 77 mM. The mixture was vortexed and left overnight, then used directly for hybridization.

Hybridization. Sections were fixed at 4° for 20 min in 0·5% paraformaldehyde and 0·5% glutaraldehyde solution, followed by stabilization in 0·15 M ethanolamine for 20 min at the same temperature. 0·2 M HCl was used to treat the sections for 30 min at room temperature before digestion with proteinase K (Sigma, Poole, Dorset, U.K.) 20 μg/ml in 50 mM Tris-HCl, 5 mM EDTA solution containing 0·5% saponin pH 7·4 at 37° for 30 min. The slides were then washed in 100 mM Tris-HCl, 100 mM NaCl solution containing 2 mg/ml glycine pH 7·4 (TSG buffer) three times at room temperature. This was followed by dehydration in 30%, 60%, 99% ethanol and air drying. Prehybridization was performed at 37° for 2 hr in hybridization solution containing 5 × SSC, 25 mM sodium phosphate, 1 × Denhardt's solution, 50% deionized formamide and 10% dextran sulphate. Hybridization was followed under the same condition with the appropriate probe at a concentration of 0·2 ng/μl in the hybridization solution overnight. The sections were washed in 50% deionized formamide with 4 × SSC for 15 min, then 2 × SSC and 1 × SSC for at least 1 hr at room temperature.

Detection of hybridization. The sections were blocked for 1 hr at room temperature with 0·3 g/ml skimmed milk (ICN) and 500 U/ml Heparin in 25 mM NaCl and 1 mM EDTA containing 0·3% Tween (ICN), pH 7·5, followed by dipping in washing buffer containing 90% normal saline, 10% 50 mM Tris-HCl, pH 7·4 (ST) and overnight incubation with monoclonal antibody against sulphonated cytidine (ICN) at 1:50 in 50 mM Tris-HCl, buffer pH 7·4 (TB) at 4°. Then the slides were washed extensively in ST buffer for at least 1 hr at room temperature, followed by incubation with goat anti-mouse immunoglobulin antibody (Sigma) at 1:20 dilution in TB containing 20% normal goat serum for 1 hr at room temperature and washing for 30 min in ST buffer. Mouse monoclonal anti-alkaline phosphatase (APAAP) complex (Dako Ltd, High Wycombe, Bucks, U.K.) was added at 1:30 in TB with 20% normal goat serum and incubated at room temperature for 1 hr followed by washes in ST buffer for 30 min. Substrate with 2 mg/ml Fast-red TR (Sigma), 0·4 mg/ml naphthol AS-MX phosphate (Sigma) in 50 mM Tris-acetate, 10 mM magnesium acetate pH 9, was added for 15 min at room temperature followed by three washes in water and counterstaining with Harris Haematoxylin. After gentle rinsing in running water for 5 min, the slides were mounted in glycerol-gelatin (Sigma).

Controls for in situ *hybridization.* Either RNase or DNase was used to confirm that the hybridization was on RNA and not DNA. The sections were incubated with the enzyme at 40 μg/ml in 800 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA solution pH 7·5 at 37° for 2 hr just before dyhydration, followed by extensive washing in TSG buffer. Specificity controls were carried out by hybridization with the labelled probe together with the unlabelled probes simultaneously. The concentration of the former was as above, and those of the latter was in five- or 10-fold excess.

Detection of cytokine protein

The entire protocol for the detection of cytokines has been described in detail elsewhere,[13-15] and is briefly outlined here.

Antibody preparation and immunostaining. Specific rabbit antibodies were generated by immunization with recombinant cytokines. Both recombinant TNF-α and IFN-γ were kindly donated by Genentech (San Francisco, CA) and IL-1α, by Dainippon Pharmaceutical Co. (U.S.A.). Pepsin digestion was performed to generate $F(ab')_2$ fragments which were immunopurified with Sepharose 4B column coupled with the appropriate cytokine. After biotinylation, the activity of the antibody preparation was confirmed by ELISA. Immunoperoxidase staining was performed with the antibody preparations.

Specificity controls. Sepharose 4B immunoaffinity column coupled with the relevant cytokine such as TNF-α for anti-TNF-α antibody preparation was used to remove the specific activity of antibodies. Both the flow through and the eluate were collected and used to stain the sections. An irrelevant cytokine affinity column (i.e. IL-1α) was used for the same antibody such as anti-TNF-α preparation and both the flow through and the eluate were collected for staining. Additionally, pre-bleed $F(ab')_2$ fragments preparations were also used in the experiments as negative controls.

RESULTS

Presence of TNF-α in TEC

Localization of TNF-α mRNA

TNF-α mRNA was detected in TEC on frozen sections from all samples of thyroid disease. The results are summarized in Table 1. Figure 1 illustrates the positive TEC from patients with Graves' disease and non-toxic goitre. In contrast, no TNF-α mRNA was detected in the normal thyroid gland (Fig. 1g). Transcripts for TNF-α were also detected in some of the mononuclear cells infiltrating the thyroid tissue. Digestion with DNase-free RNase before hybridization totally abolished the hybridization (Fig. 1c) whereas the pretreatment with RNase-free DNase did not alter the results (Fig. 1d). Furthermore, specific competition with unlabelled probe showed graded decrease of the hybridization with the labelled (probe (Fig. 1e and f) and, at a 10-fold excess concentration, hybridization was almost undetectable. (Fig. 1f).

Detection of TNF-α protein

The TNF-α protein was detected in TEC on frozen sections that were examined from all patients with thyroid diseases as summarized in Table 1. Representative pictures of thyroid sections from a patient with Graves' disease are shown in Fig. 2. In a proportion of the infiltrating cells the presence of TNF-α protein was also identified. There was no evidence of TNF-α in the normal thyroid gland. The column eluate but not the flow through of anti-TNF-α antibody preparation passed over a TNF-α–Sepharose column stained the TEC (Fig. 2c and d). In contrast, an IL-1α column did not remove the anti-TNFα antibody activity with only the flow through from the column being able to immunostain TNF-α in the TEC (Fig. 2e and f).

Localization of IFN-γ mRNA and protein

Frozen thyroid tissue sections from patients with thyroid diseases were examined for the presence of IFN-γ (summarized in Table 2) by *in situ* hybridization and immunohistochemistry. No mRNA transcripts or the protein of IFN-γ were detected in

Table 1. Detection of TNF-α in thyroid epithelial cells and the expression of surface molecules

	TNF-α		Expression of	
	mRNA	Protein	ICAM-1*	LFA-3*
Graves' disease				
Sample 1	++	+++	(+)	(+)
Sample 2	+++	+++	(+)	(+)
Sample 3	++	+++	(+)	(+)
Sample 4	++	++	(+)	(+)
Sample 5	+	+++	(+)	(+)
Sample 6	++	+++	(+)	(+)
Sample 7	+	+++	(+)	(+)
Hashimoto's disease				
Sample 1	+++	+++	(+)	(+)
Sample 2	+++	+++	(+)	(+)
Sample 3	+	++	(+)	(+)
Non-toxic goitre				
Sample 1	+	++	(+)	(−)
Sample 2	++	++	(+)	(−)
Sample 3	++	++	(+)	(−)
Sample 4	++	++	(+)	(−)
Normal thyroid tissue	−	−	(−)	(−)

At least 1000 epithelial cells were scored under microscopy and the percentage of positive cells is expressed as follows: +, less than 50% of the cells are positive; ++, more than 50% and less than 80% of the cells are positive; +++, more than 80% of the cells are positive; −, negative.

* The data in brackets have been reported elsewhere.[7] In all the pathological samples, infiltration of blood cells and the expression of HLA class II antigens on TEC have been observed.

Table 2. Detection of IFN-γ in infiltrating cells of the thyroid samples

	mRNA	Protein
Graves' disease		
Sample 1	+	+
Sample 2	++	++
Sample 3	+	+
Sample 4	++	++
Sample 5	+	+
Sample 6	+++	+++
Sample 7	++	++
Hashimoto's disease		
Sample 1	++	++
Sample 2	+++	+++
Sample 3	+	++
Non-toxic goitre		
Sample 1	+	+
Sample 2	+	+
Sample 3	++	++
Sample 4	++	++
Normal thyroid tissue	NA	NA

At least 1000 cells per sample were scored under microscopy and the percentage of positive cells is expressed as the follows: +, less than 10% of the cells are positive; ++, 10–19% of cells are positive; +++, 20–30% of cells are positive.

NA, non-applicable. No infiltrating cells were found in the normal sample. However, in all the pathological samples, infiltration of blood cells and the expression of HLA class II on TEC have been observed.

TEC. However, some inflammatory cells infiltrating the thyroid gland from each of the disease groups contained both IFN-γ mRNA and protein. Figure 2g illustrates this on a section from a patient with Graves' disease. The normal thyroid sections contained no infiltrating cells and TEC were negative for IFN-γ, both at the level of mRNA and protein.

DISCUSSION

The present study demonstrates that human epithelial cells (TEC) from thyroiditis are able to produce TNF-α *in vivo*, whereas IFN-γ identified in the sites of pathology are derived from infiltrating mononuclear cells. This contrasts with the situation in normal thyroid tissue in which neither cytokine is detectable in these areas.

The capacity of TEC to express MHC class II proteins[9,16] and adhesion molecules[6,7] has been established. It supports the hypothesis that these cells may develop the potential to function as antigen-presenting cells and to serve as the target cells in autoimmune thyroiditis *in vivo*.[1] It has been shown that TEC induced to express MHC class II molecules *in vitro* acquired the capacity to present both exogenous and auto-antigens;[1,17] and if this were to occur *in vivo*, it might trigger autoreactive T cells. However, even if potentially autoreactive T cells were to be present, the failure of normal TEC to express MHC and adhesion molecules *in vivo* would prevent the development of autoimmune disease. Therefore, it is insufficient only to demonstrate the expression of class II and adhesion molecules; their regulation by cytokines such as TNF-α and IFN-γ should also be investigated.

TNF-α displays a variety of activities essential to immunological responses; these include enhancing expression of MHC, ICAM-1 and LFA-3 molecules on many cell types, and the induction of other cytokines.[18,19] In this study, TNF-α mRNA was found in TEC from thyroiditis. In order to confirm that the hybridization detected mRNA, DNase-free RNase was used before the hybridization. This totally abolished the hybridization whereas the RNase-free DNase did not alter the hybridization. The specificity of the hybridization was confirmed by competition assays allowing high concentration of the unlabelled probe to compete with the labelled one. A graded decrease of hybridization with labelled probe was observed. TNF-α protein was detected in TEC by immunostaining. The specificity of antibody staining was confirmed by removing the TNF-α-specific antibody through a TNF-α affinity column to generate negative staining. An irrelevant affinity column (IL-(α) failed to remove the TNF-α specific antibody $F(ab')_2$ fragments, excluding the possibility of non-specific removal of the specific antibody preparations through inappropriate affinity columns.

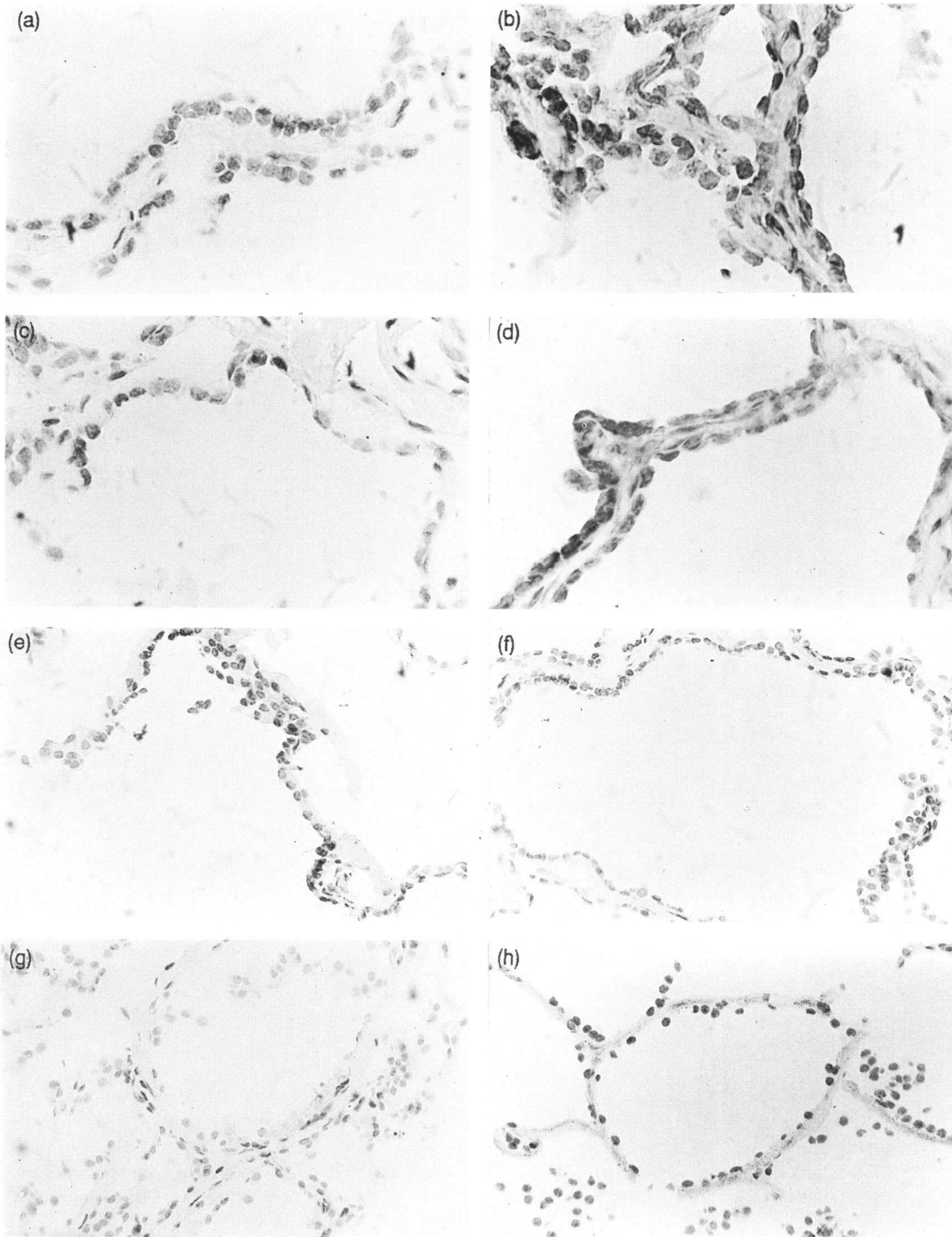

Figure 1. *In situ* hybridization on frozen sections. (a) A thyroid section from a patient with Graves' disease hybridized with unlabelled TNF-α probe as a negative control. Magnification ×160. (b) Hybridization with labelled TNF-α probe was observed in thyroid epithelial cells. ×160. (c) After RNase digestion, mRNA for TNF-α was not detected by the TNF-α probes. ×160. (d) DNase digestion did not alter the hybridization for TNF-α mRNA, as shown here in the section of Graves' disease. ×160. (e) Unlabelled TNF-α probe fivefold in excess was allowed to compete with the labelled probe, the resulting hybridization of the labelled probes was less intense. ×128. (f) Competition assays as (e) the unlabelled probe was present in 10 times excess and the hybridization of the labelled probe was abolished. ×128. (g) TNF-α mRNA was not detected in normal thyroid tissue obtained from a patient with parathyroid disease. ×128. (h) TNF-α mRNA was detected in thyroid epithelial cells on frozen sections from non-toxic goitre. ×128. (a–f) from the same sample of Graves' disease.

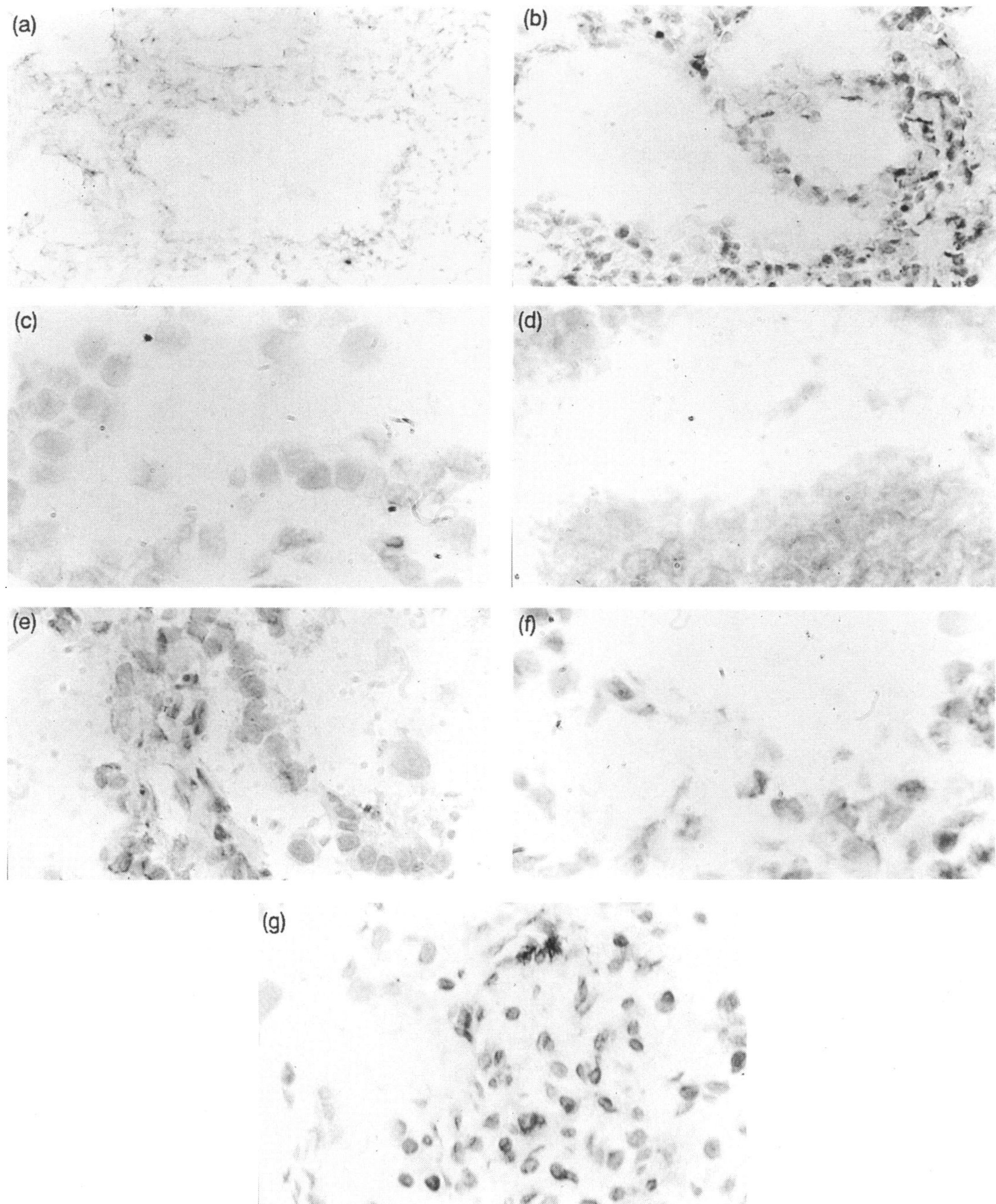

Figure 2. Immunostaining on frozen thyroid sections from a patient with Graves' disease were immunostained as indicated. (a) No peroxidase staining is detectable using an immunoglobulin $F(ab')_2$ preparation from the pre-bleed serum of the rabbit used for TNF-α immunization. Magnification × 128. (b) Anti-TNF-α antibody preparation stained thyroid epithelial cells. × 128. (c) The flow through of anti-TNF-α antibody from TNF-α column did not stain any cells. × 320. (d) The eluate from TNF-α column stained thyroid epithelial cells from Graves' disease. × 320. (e) The flow through from IL-1α column of anti-TNF-α antibody preparation stained thyroid epithelial cells. × 320. (f) No staining was observed using the eluate from IL-1α column of anti-TNF-α antibody preparation. × 320. (g) Thyroid epithelial cells were negative for immunoperoxidase staining of IFN-γ using purified antibodies to IFN-γ. × 160.

Our findings demonstrate that TEC in thyroiditis acquire the capacity to produce TNF-α *in vivo*. It is likely that this production may contribute to the disease process, in which infiltration of lymphocytes, expression of MHC class II antigens, adhesion molecules and presence of other cytokines are observed.[5]

Recently, other types of human epithelial cells have also been reported as producing TNF-α.[20] Northern blot, ELISA and bioassays were performed demonstrating that cultured human keratinocytes were able to synthesize and secrete TNF-α. Using immunohistochemical methods, Oxholm *et al.*[21] observed the presence of this cytokine in skin epithelial cells *in vivo*.

IFN-γ has been shown to regulate the MHC class II and ICAM-1 expression by TEC in culture.[6,7,16] Using the combined approaches of *in situ* hybridization and immunohistochemistry, we explored the presence of IFN-γ and its cellular origin *in vivo*. In agreement with other immunohistochemical analyses, this cytokine was detected in infiltrating cells (not TEC) on sections from patients with thyroiditis. *In vitro* studies have shown that IFN-γ may also synergize with TNF-α in the induction of HLA-DR expression by TEC.[8] It is possible that this synergy may occur also *in vivo*. Potentially, IFN-γ produced by mononuclear cells in the thyroid may synergize with TNF-α from TEC to promote the aberrant expression of MHC class II molecules. This, taken together with the abilities of TNF-α and IFN-γ to induce TEC to express adhesion molecules and to produce cytokines,[3,22] may allow TEC to become competent antigen-presenting cells *in vivo* and so facilitate the development of autoimmune thyroiditis.

Our observation that the *in vivo* production of cytokines were present in both autoimmune and non-autoimmune samples of thyroid diseases would also suggest that this cytokine production is likely to be involved in general inflammation rather than specific for autoimmunity. Other factors such as the recent demonstration of the presence of autoreactive T cells specific for thyroid peroxidase antigen and thyroglobulin may play a very important role in the pathogenesis of the diseases.[23,24] It is possible that the production of cytokines and the expression of MHC and adhesion molecules are to create an appropriate environment for the activation of T cells, and only when the autoreactive T cells are present, autoimmunity would take place. If these T cells are not available, non-autoimmune response may occur.

Our results demonstrated the *in vivo* production of TNF-α and IFN-γ. Details of the inter-relatioships between different cytokines, their cellular origins and relative importance in the immunopathology are being addressed. However, the primary events initiating autoreactivity and or non-autoimmune responses and the array of antigens involved remains ill-defined.

ACKNOWLEDGMENTS

We thank Dr Grubeck-Loebenstein of the Dept. of General and Experimental Pathology, University of Vienna for providing the samples. This work was supported by the Wellcome Trust, Medical Research Council, Sino-British Friendship Scholarship, the Arthritis and Rheumatism Council, Multiple Sclerosis Society and the Sunley Trust.

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