Clin Exp Immunol 1997; **109**:98–106

Hyperexpression of transporter in antigen processing-1 (TAP-1) in thyroid glands affected by autoimmunity: a contributory factor to the breach of tolerance to thyroid antigens?

M. SOSPEDRA*§, E. TOLOSA*§, P. ARMENGOL*§, Y. ASHHAB*§, S. URLINGER†, A. LUCAS-MARTIN†¶, M. FOZ-SALA‡, D. JARAQUEMADA*§ & R. PUJOL-BORRELL*§ **Immunology, †Endocrinology and ‡Internal Medicine Divisions, University Hospital 'Germans Trias i Pujol' and §Department of Cell Biology and Physiology, Autonomous University of Barcelona, Bellaterra, Badalona, Spain, and ¶Max-Planck-Institute for Biochemistry, Department of Molecular Structural Biology, Martinsried, Germany*

(Accepted for publication 6 February 1997)

SUMMARY

According to the 'aberrant HLA expression' hypothesis, endocrine autoimmunity is driven by presentation of self antigens by target cells over-expressing HLA molecules. In autoimmune thyroid diseases (AITD), thyroid follicular cells (thyrocytes) over-express HLA class I and HLA class II molecules. Since efficient presentation of endogenous peptides via class I requires transporters that translocate endogenous peptides from the cytoplasm to the endoplasmic reticulum, i.e. transporters associated with antigen processing (TAP) -1 and -2, the capability of thyrocytes to express TAP and whether TAP is hyperexpressed in AITD glands are issues relevant to the above hypothesis. Results from immunofluorescence and Northern blotting studies on primary thyrocyte cultures and on a thyroid cell line demonstrate that thyrocytes express constitutively TAP-1 at a low level, and that this expression is readily induced by interferon-gamma (IFN-γ) and to a lesser extent by IFN-α. In AITD, but not in non-autoimmune glands, thyrocytes hyperexpress TAP-1, as demonstrated by both immunohistopathology and flow cytometry. The cytokine pattern does not bear, as assessed by reverse transcriptase-polymerase chain reaction (RT-PCR), a clear relationship with TAP-1 expression. These results have broad implications and suggest that the core concept of the 'aberrant HLA expression' hypothesis of endocrine autoimmunity could be incorporated in the currently prevailing view of 'autoimmunity by breach of peripheral tolerance'.

Keywords transporter associated with antigen processing-1 antigen presentation HLA thyroid autoimmunity cytokines

INTRODUCTION

Human autoimmune thyroid diseases (AITD), i.e. Hashimoto's thyroiditis (HT), Graves' disease (GD) and primary myxoedema, are characterized by an intense immune response to thyroid autoantigens, of which thyroglobulin (Tg), thyroid peroxidase (TPO) and thyrotropin receptor (TSH-R) have been fully identified and characterized. Since this immune response is maintained and includes high-affinity antibodies, it must depend on help from T lymphocytes. Recognition of thyroid follicular cells (thyrocytes) and antigens by T lymphocytes is therefore central to AITD pathogenesis [1].

TAP-1 and TAP-2 are two MHC-encoded proteins that translocate peptides generated in the cytoplasm into the endoplasmic reticulum (ER), where they are loaded into the presenting cavity of MHC class I proteins and transported to the cell surface, where they can be recognized by $CD8^+$ T lymphocytes [2]. TAP expression is therefore required for cell recognition by $CD8^+$ T cells.

The possible contribution of TAP to the pathogenesis of thyroid autoimmune disease can be approached from the widely accepted view which considers that endocrine autoimmunity is a failure of peripheral tolerance [3]. According to this view, autoimmunity arises when peripheral tissues express enough of the molecules capable of efficient antigen presentation and of providing second signals [4–9]. It is therefore important to determine which of these molecules are actually expressed and at what levels in the organs affected by autoimmune diseases. It has been known for some time that 'classical' MHC products, HLA class I and class II in humans, are markedly over-expressed by thyrocytes of glands affected by AITD [10]. There are no data, however, on the expression of the other MHC-encoded products required for efficient presentation, i.e. TAP and low molecular mass polypeptides (LMP) for the class I pathway [11], and invariant chain and HLA-DM for the class II pathway [12].

Correspondence: Professor R. Pujol-Borrell, Immunology Division, Hospital Universitario 'Germans Trias i Pujol', Ctra. del Canyet s/n, 08916 Badalona, Spain.

In this context the questions of whether thyrocytes (i) have the capability to express TAP co-ordinately with class I and (ii) express elevated levels of TAP-1 in the AITD glands, are important to determine how the above concepts on tolerance can be applied to the pathogenesis of these autoimmune diseases.

PATIENTS AND METHODS

Patients

Thyroid tissue and serum samples were obtained from patients with GD (n = 16), HT (n = 1) and multinodular goitre (MNG; n = 10) who underwent thyroidectomy. Normal glands (n = 3) were obtained at ENT surgery (one gland) or from multiorganic cadaveric donors. Clinical diagnosis was made on the basis of usual routine thyroid function tests: free T4 (FT4), T3 (T3), thyroid-stimulating hormone (TSH) measured by a two-site immunoradiometric assay and thyroid autoantibodies.

Thyrotoxic patients were treated with carbimazole. Diagnoses were confirmed by histopathology. A summary of the clinical data is given in Table 1. The protocol was approved by the ethical committee of the Hospital 'Germans Trias I Pujol'.

Table 1. Summary of patients' data and results

		Thyroid antibodies			Fts ($\times 10^3$)		
Ref. no.	Age	TPO	Tg	TSH-R	TAP-1	Class I	Class II
GD ($n = 16$)							
193	67	720	1500	87	117	2218	726
204	24	Neg	Neg	49	138	1108	357
212	22	Neg	Neg	68	126	1411	749
213	22	Neg	Neg	Neg	206	3197	2320
220	26	Neg	Neg	64	132	921	334
228	52	Neg	Neg	80	288	730	436
244	21	Neg	Neg	Neg	198	242	320
252	42	ND	ND	79	154	566	146
255	30	166	225	20	145	3706	4299
257	21	598	1155	67	357	1752	1597
258	16	Neg	Neg	118	334	1258	1219
260	32	Neg	105	206	144	1338	747
269	30	Neg	Neg	33	95	433	250
270	32	Neg	Neg	96	84	291	239
278	39	Neg	Neg	16	98	468	299
286	28	Neg	Neg	ND	90	459	469
Mean					169	1250	903
s.e.m.					21	257	269
MNG ($n = 10$)							
189	56	Neg	Neg	Neg	22	873	420
198	39	Neg	Neg	Neg	138	915	246
218	38	Neg	Neg	Neg	129	922	103
226	64	Neg	ND	Neg	25	296	200
227	36	Neg	Neg	Neg	86	1450	712
238	61	Neg	Neg	ND	99	148	158
259	52	Neg	Neg	ND	46	1561	300
273	62	Neg	Neg	ND	70	790	1135
280	53	Neg	Neg	Neg	167	453	234
285	69	Neg	Neg	Neg	45	472	26
Mean					83	788	353
s.e.m.					15	146	105
HT ($n = 1$)							
290	25	584	766	Neg	431	ND	ND
Normal ($n = 3$)							
209	50	ND	ND	ND	ND	ND	ND
D1	15	Neg	Neg	Neg	ND	ND	ND
D2	15	Neg	Neg	Neg	ND	ND	ND

TPO antibodies, thyroid peroxidase antibodies, normal values <130 U/ml; Tg antibodies, anti-thyroglobulin antibodies, normal values <90 U/ml. Serum samples for TPO and Tg antibodies obtained the day of the surgical intervention. TSH-R, antibodies to thyrotropin receptor detected by their ability to inhibit the binding of TSH to a standard preparation of TSH-R obtained from porcine thyroid membranes. Results expressed as U/ml. Normal values <10 mU/ml. TSH-R antibodies are characteristic of Graves' disease (GD), and they cause thyroid hyperfunction, the main clinical manifestation of this disease. Serum samples for TSH-R antibodies were obtained at different moments during the early course of the disease. Fts, Total specific fluorescence; ND, not done.

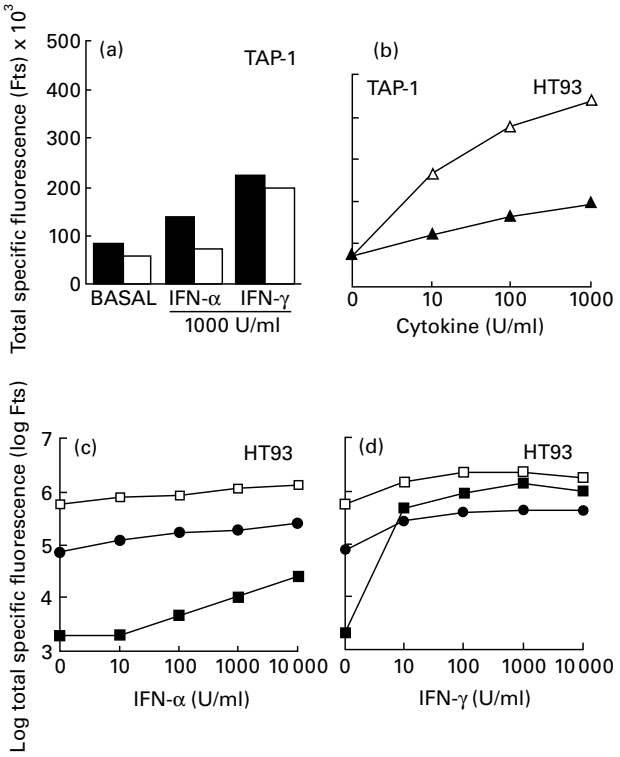

Fig. 1. Induction of TAP-1, HLA class I and class II: effect of cytokines. Comparison of TAP-1 expression induction assessed by flow cytometry and expressed as total specific fluorescence (Fts) (see text for definitions). Effect of IFN-γ and IFN-α on thyrocytes from Graves' disease (GD; ■) and multinodular goitre (MNG; □) glands (a) and on the thyroid cell line HT93 (b). Δ, IFN-γ; ▲, IFN-α. (c,d) Comparison of the dose response of the TAP-1 (●), HLA class I (□) and HLA class II (■) expression to IFN-α and IFN-γ on the thyroid cell line HT93.

Methods

Thyroid autoantibodies. Anti-TPO and anti-Tg antibodies were assayed by ELISA (Advanced Biological Products Inc., Mississauga, Ontario, Canada) and thyrotropin-binding inhibitory immunoglobulin (TBII) using the RRA TRAK kit (Behring, Marburg, Germany).

Tissue preparation and culture. Thyroid glands were processed as previously described and cells cryopreserved [13]. Blocks (0·5 cm) from each gland were snap frozen. After thawing, thyrocytes were cultured for 18 h, detached by incubation with 2·5 mg/ml trypsin (Sigma) and 0·3 mg/ml EDTA (Sigma) Ca^{2++}-Mg^{2+}-free balanced salt solution and stained as below. The HT93 human thyroid line, generated by SV40 transfection, was used to complete the study [14].

IFL staining. Series of 5-μm cryostat sections from 17 glands were stained by double indirect immunofluorescence (IFL) [15] combining the r.Ring4c rabbit anti-TAP-1 antiserum (P. Cresswell, New Haven, CN) [16] with MoAbs to HLA class I monomorphic (W6/32; Seralab, Crawley, UK), HLA class II monomorphic (RFDR2; G. Janossy, London, UK) and thyroid peroxidase (Microsome-18; P. Carayon, Marseille, France). TRITC-labelled GAM IgG and FITC GAR IgG (Southern Biotechnology, Birmingham, AL) were used as secondary antibodies. Other preparations were also stained using the MoAb 148.3 anti-TAP-1 (S. Urlinger, Martinsried, Germany) and patient's serum with high titre of TPO antibodies and the corresponding FITC GAM and TRITC goat anti-human IgG. Preparations were examined by two independent observers under a UV microscope. Between incubations (30 min for each layer), preparations were washed in PBS.

For TAP-1 flow cytometry, dispersed cell preparations were permeabilized before staining [17], and for HLA class I and class II viable cells were stained to detect only the surface molecules. Samples of 5000 cells were analysed on a FACScan using the Lysis II software (both from Becton Dickinson, San Jose, CA). For a better estimation of expression we applied an algorithm previously described and validated in our laboratory ([18] and Sospedra *et al.*, in preparation). The results of this algorithm, designated 'total specific fluorescence' (Fts), reflect the total number of fluorescence molecules expressed by the relevant cell population. The results of TAP-1 flow cytometry were clearly reproducible (CV < 10%). To assess better the distribution of TAP-1 in the cytoplasm of thyrocytes, monolayer cultures were also stained as described [19].

Culture supplements and time course experiments. To study the regulation of TAP, HLA class I and class II expression in thyrocyte and HT93 cells, cultures were exposed to different concentrations of interferon-gamma (IFN-γ) or IFN-α. After 48 h, cells were stained and analysed by flow cytometry. The cytokine sources were: rhIFN-γ (SA 2×10^7 U/mg; G. R. Adolf (Boehringer Institute,

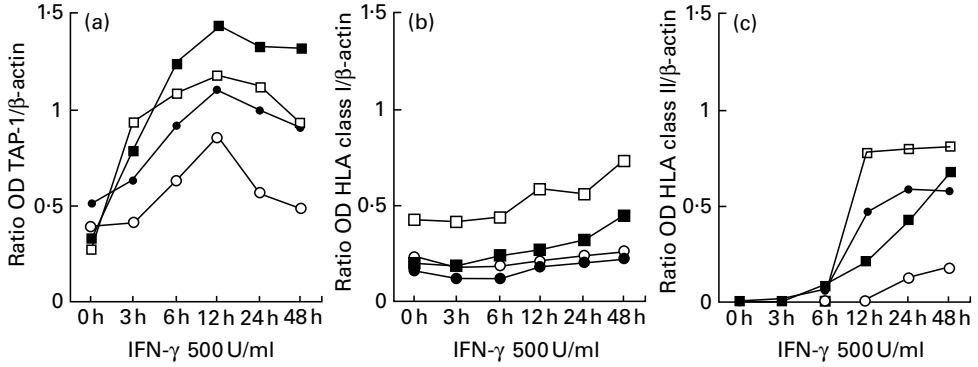

Fig. 2. Comparison of the time course of TAP-1, HLA class I and HLA class II mRNA induction. Thyrocytes from Graves' disease (GD) and multinodular goitre (MNG) glands and the thyroid cell line HT93, stimulated with 500 U/ml IFN-γ. Densitometric values from Northern blots, normalized for loading with the corresponding value of β-actin and then corrected with the corresponding positive control in each plot. (a) TAP-1, (b) HLA class I, and (c) HLA class II. ●, GD TB193; ■, GD TB228; ○, MNG TB238; □, HT93.

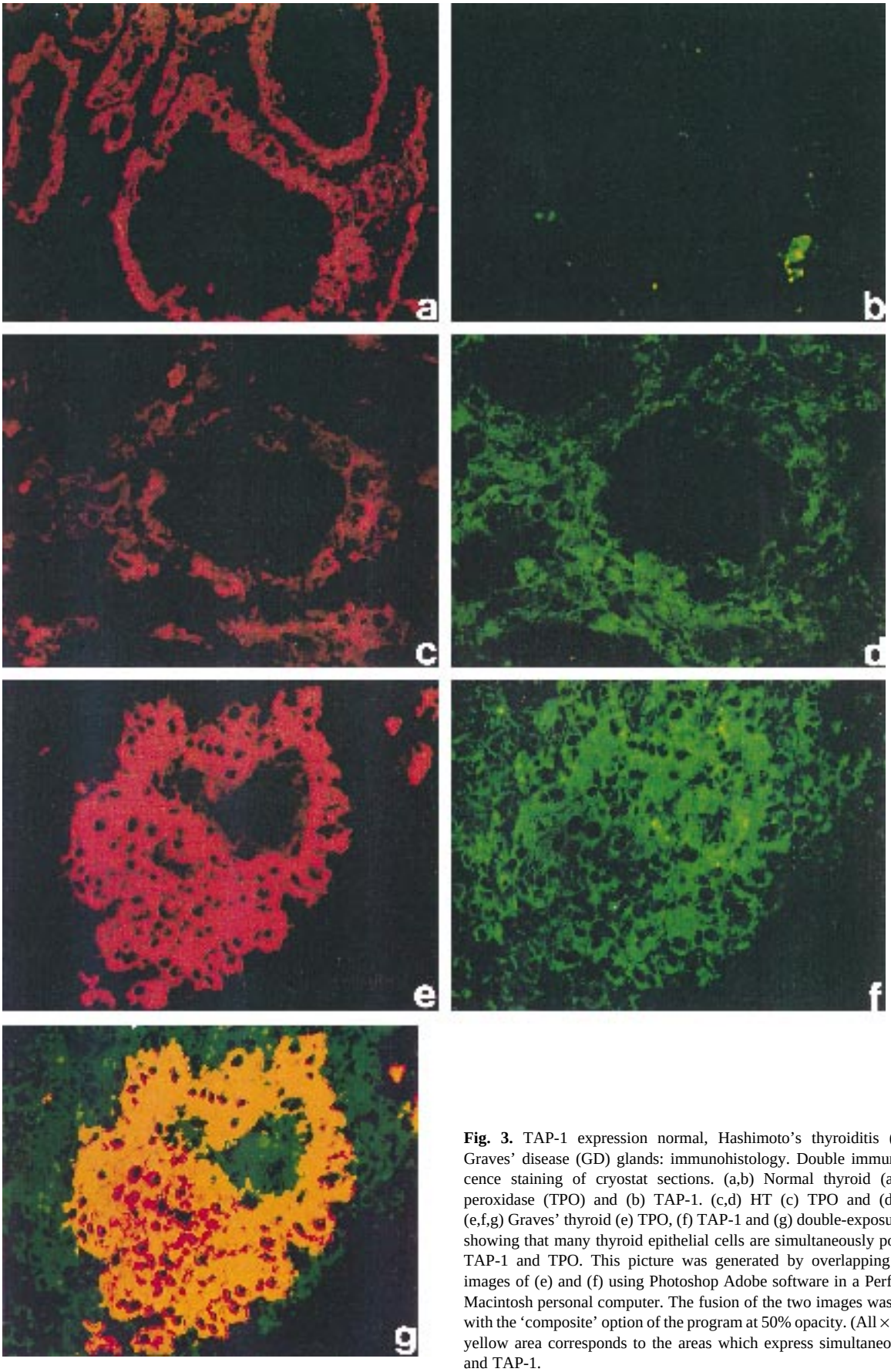

Fig. 3. TAP-1 expression normal, Hashimoto's thyroiditis (HT) and Graves' disease (GD) glands: immunohistology. Double immunofluorescence staining of cryostat sections. (a,b) Normal thyroid (a) thyroid peroxidase (TPO) and (b) TAP-1. (c,d) HT (c) TPO and (d) TAP-1. (e,f,g) Graves' thyroid (e) TPO, (f) TAP-1 and (g) double-exposure picture showing that many thyroid epithelial cells are simultaneously positive for TAP-1 and TPO. This picture was generated by overlapping digitized images of (e) and (f) using Photoshop Adobe software in a Performa 630 Macintosh personal computer. The fusion of the two images was achieved with the 'composite' option of the program at 50% opacity. (All × 400.) The yellow area corresponds to the areas which express simultaneously TPO and TAP-1.

Vienna, Austria)), and rhIFN-α (SA 2×10^8 U/mg; Schering-Plough (Madrid, Spain)).

Northern analysis. Total RNA was extracted by Chomczinsky's technique [20] from 10^7 cells of GD or MNG glands or HT93 cultures. A lymphoblastoid cell line served as positive control. Samples (20 mg) of RNA were electrophoresed, transferred, hybridized with a 2·8-kb TAP-1 (J. Trowsdale, ICRF, London), a 632-bp class I (polymerase chain reaction (PCR) product), a 1·3-kb HLA-DRa (E. Long, NIAID, NIH, Rockville, MD) and a 3·5-kb actin probe, autoradiographed and analysed using a Scanjet IIc scanner (Hewlett Packard) connected to LCIII Macintosh and the Scan Analysis program (Biosoft, Cambridge, UK).

Detection of cytokines using reverse transcription and PCR. RNA was extracted from several snap frozen blocks from a selection of thyroid glands. Positive controls were peripheral blood mononuclear cells (PBMC; 2×10^6) stimulated with 1% phytohaemagglutinin (PHA). cDNA was prepared by standard methods. All reagents were obtained from Promega (Madison, WI). PCR was performed according to the manufacturer's general instructions, using the following primers: IFN-γ (sense ATGAAATATA CAAGTTATAT CTTGGCTTT, antisense GATGCTCTTC GACCTCGAAA CAGCAT); tumour necrosis factor-alpha (TNF-α; sense GACGTGGAGC TGGCCGAG, antisense CACCAGCTGG TTATCTCTCA GCTC); IL-2 (sense ATGTACAGGA TGCAACTCCT GTCTT, antisense GTCAGTGTTG AGATGATGCT TTGAC); IL-4 (sense ATGGGTCTCA CCTCCCAACT GCT, antisense CGAACACTTT GAATATTTCT CTCTCAT); IL-10 (sense AAGCTGAGAA CCAAGACCCA GACATCAAGG CG, antisense AGCTATCCCA GAGCCCCAGA TCCGATTTTG G) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense TCTTCTTTT GCGTCGCCAG, antisense GGGGGCAGAG ATGATGACC). Primers were designed in our laboratory to span a whole intron and supplied by Oligo Etc. (Wilsonville, OR). For Southern blotting the following oligoprobes were used: IFN-γ 5′GCTCTGCATC GTTTTGGGTT3′, TNF-α 5′AGCCTCTTCT CCTTCCTGAT CGTG3′, IL-2 5′TTCTTCTAGA CACTGAAGAT GTTTCAGTTC3′, IL-4 5′TCATGGTGGC TGTAGAACTGC3′ and IL-10 5′CATTCTTCAC CTGCTCCACG G3′.

To reduce the sources of error and improve reproducibility, RNA was extracted from several blocks from each gland, equal amounts were retrotranscribed, and membranes were hybridized under standard conditions. Optical density (OD) was normalized for the amount of loaded cDNA using GAPDH as standard and expressed as the ratio to the corresponding positive control.

Statistical analysis. For comparison of the Fts values from the different groups we applied the Mann–Whitney *U*-test (two-tailed) and to determine correlations we used simple regression analysis using the SPSS program.

RESULTS

Thyrocytes have the capability to express TAP co-ordinately with class I HLA molecules

Thyroid cell preparations from one case of GD (no. 260) and one case of MNG (no. 238) were stained for TAP-1, HLA class I and HLA class II and analysed by flow cytometry. The results (Fig. 1a) demonstrate that freshly isolated thyrocytes express TAP-1 and respond to IFN-γ and IFN-α (1000 U/ml) with a rapid superinduction. Northern analysis showed that TAP-1 and HLA class I transcripts were already present in untreated thyrocytes. Kinetics of message induction were different: TAP-1 message rose sharply and started to decline after 12 h, while class II rose steadily from 12 h on. Both types of transcripts reached higher levels in thyrocytes from GD glands than in those from MNG. Class I mRNA increase was less dramatic and slower than that of TAP-1 or class II. These studies were repeated and extended using the HT93 thyroid cell line to rule out a possible contribution of mRNA from endothelial and haematopoietic cells in the thyrocyte preparations (Fig. 2). The results from FACS analysis show that IFN-γ, and to a lesser extend IFN-α, each induced a clear increase of TAP-1 expression (Fig. 1b,c,d). These differences in time course are much less evident at the protein level (data not shown).

In AITD thyrocytes hyperexpress TAP-1

A first assessment of the level of expression of TAP-1 in AITD was obtained by staining cryostat sections from normal (n = 3), GD (n = 7), HT (n = 1) and MNG (n = 4) thyroid glands. To identify clearly the thyrocytes, staining was always carried by double IFL combining a thyrocyte specific marker (TPO) with the TAP-1 antiserum.

In thyrocytes of normal glands the staining for TAP-1 was extremely weak, just above the limit of detection (Fig. 3a,b). Some TAP-1-positive interstitial cells provided an internal positive control. As expected, the stainings for HLA class I and HLA class II were very weak and negative, respectively [15]. By contrast, all the tissue samples from GD patients showed clear TAP-1 staining in thyrocytes. The distribution of TAP-1 was patchy, often including the epithelium of whole follicles (Fig. 3e,f,g). The cellular distribution of the TAP-1 was cytoplasmic, predominantly perinuclear and finely granular. On monolayer cultures supplemented with IFN-γ thyrocytes showed a diffuse cytoplasmic distribution of TAP-1 (data not shown). Of the other cell types present in this tissue, macrophages in the colloid space were often strongly positive, as were

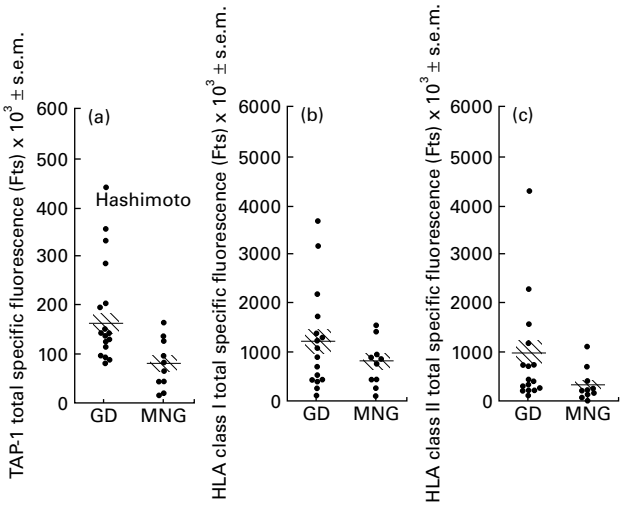

Fig. 4. Summary of flow cytometry results. (a) TAP-1 expression on thyrocytes from Graves' disease (GD) and multinodular goitre (MNG) glands expressed as total specific fluorescence (Fts) (P = 0·0084). (b) HLA class I expression on thyrocytes from GD and MNG glands (P = NS, but distribution of values is different; see Results). (c) HLA class II expression on thyrocytes from GD and MNG glands (P = 0·02). The black line indicates the mean and the hatched area represents ± s.e.m.

a few cells of dendritic morphology visible in the interstitium. The correlation between the expression of TAP-1 and HLA class I was analysed in the same glands by double staining TAP-1/HLA class I. In general the areas of hyperexpression of each molecule coincided, but the TAP-1-positive areas were smaller. There was also an overlap between TAP-1 and HLA class II-positive areas, but TAP-1 was less prominent (data not shown). The hierarchy of the expression of the three molecules in this series of glands was: HLA class I > HLA class II > TAP-1.

In the HT gland, TAP-1 hyperexpression was also strong,

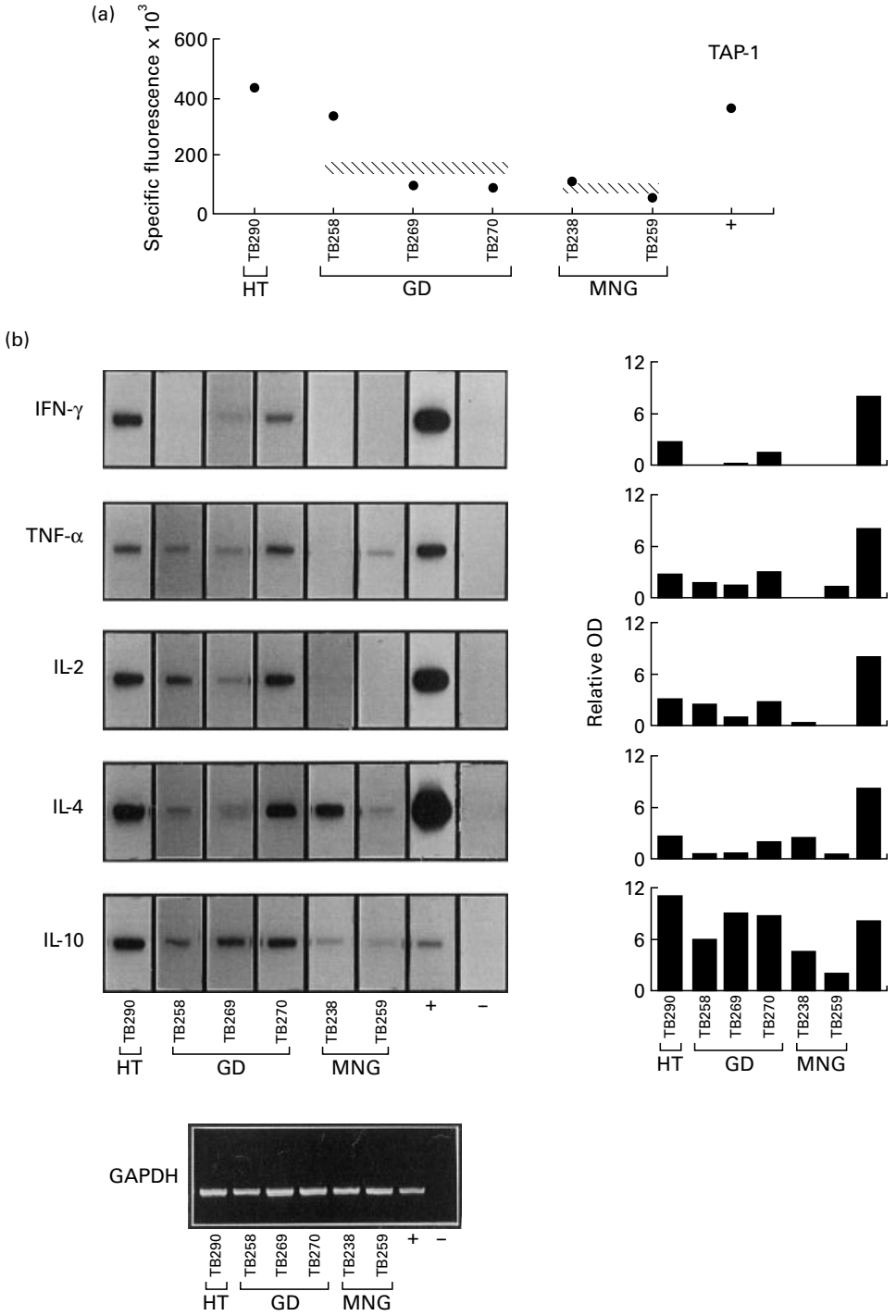

Fig. 5. Relationship between tissue cytokines as detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and TAP-1 expression as assessed by quantitative fluorescence in a selection of thyroid glands. (a) Total specific fluorescence (Fts). HT, Hashimoto's thyroiditis (TB290), GD, Graves' disease (TB258, TB269 and TB270); MNG, multinodular goitre (TB238 and TB259), +, positive control (a lymphoblastoid cell line). The hatched area represents ± s.e.m. (b) Left panel: Southern blots of the amplicons of each of the cytokines. +, peripheral blood mononuclear cells (PBMC) stimulated with 1% phytohaemagglutinin (PHA) for 18 h, –, reagents without cDNA. Right panel: densitometry histograms corresponding to the left panel showing corrected and normalized values.

but less bright than in GD tissue, probably due to its flat epithelium (a consequence of therapy) (Fig. 3c,d). The study of the MNG glands revealed some TAP-1 expression in the thyroid epithelial cells, but weak and confined to very small areas (data not shown).

To obtain a quantitative measurement of TAP-1 expression, we analysed by FACS disperse thyrocyte preparations from one HT, 16 GD and 10 MNG glands, including those studied on cryostat sections. TAP-1 expression was significantly higher in GD than in MNG glands ($P = 0{\cdot}0084$, see Table 1 and Fig. 4a). As expected, the intensity of HLA class II surface fluorescence was higher in thyrocytes from GD than in thyrocytes from MNG glands ($P = 0{\cdot}02$; see Table 1 and Fig. 4c). For HLA class I expression, the difference between the average Fts values of the two pathologies did not reach significance, but distribution of values was different in GD and in MNG, i.e. the Fts of four GD glands was above mean + 2 s.d. of the MNG mean, while the Fts of no MNG gland was above this value (see Table 1 and Fig. 4b).

The Fts values were higher for HLA class I and HLA class II than for TAP-1. This does not imply that the number of TAP-1 molecules was smaller than that of HLA class I or HLA class II: cytoplasmic and membrane stainings cannot be directly compared due to the different factors influencing the background.

Thyrocyte hyperexpression of TAP is not directly related to tissue cytokines

To investigate whether the hyperexpression of TAP-1 in thyrocytes from autoimmune glands was due to the paracrine effect of cytokines such as IFN-γ and TNF-α presumably present in the tissue, we measured the mRNA by reverse transcriptase (RT)-PCR in tissue blocks from a selection of glands with different levels of TAP-1 (Fig. 5a). We also assessed the presence of IL-2, IL-4 and IL-10 and of GAPDH message (as control) in the same samples. Results are depicted in Fig. 5b, which shows abundant transcripts of all cytokines investigated in HT tissue. Unexpectedly, in the three GD glands the levels of TAP-1 expression correlated inversely with the amounts of IFN-γ transcripts. Levels of TNF-α, IL-2, IL-4 and IL-10 tended to be higher in autoimmune glands than in MNG glands, but we could not assign Th1 or Th2 patterns to given glands or pathologies.

DISCUSSION

The data reported in this study constitute a first demonstration that: (i) thyrocytes have the capability to express TAP-1 molecules; and (ii) thyrocytes from AITD glands express significantly higher levels of TAP-1 than those from normal and to a lesser extent from MNG glands.

The concordance of the results obtained using two different antibodies in two types of preparations supports these conclusions. The cytoplasmic pattern of TAP-1 distribution in normal tissue sections and in the cytoplasm of cells in monolayer cultures is in accordance with previous descriptions [21]. We confined our study to TAP-1 because we had no access to TAP-2 antibodies. On the other hand, since the expression of the two genes is strictly co-ordinated and TAP-1 is physically bound to TAP-2 molecules to form the functional transporter complex, it is unlikely that the study of TAP-2 would add useful information.

The studies of induction of TAP-1 by IFN-α and IFN-γ using both primary thyrocyte cultures and the thyroid cell line HT93 gave similar results and demonstrate that TAP-1 expression is induced by these cytokines, IFN-γ being clearly more effective. The induction of HLA class I was parallel to TAP-1, while HLA class II was much more strongly induced by IFN-γ. At mRNA level the induction of TAP-1 was faster and shorter than that of HLA. This regulation of TAP-1 gene expression is unusual (structurally but not functionally): in spite of being located within the HLA class II region, TAP-1 genes are expressed co-ordinately with HLA class I, and yet their expression is not strictly parallel. These results are in agreement with initial reports describing TAP, in which it is mentioned that these genes are constitutively expressed in epithelial cells at low level but that they can be induced by IFN-γ [22], and are concordant with results recently obtained by ourselves in human islets [23] and by Epperson *et al.* in endothelial cells [24a].

Thyrocytes in glands from HT and GD patients express very high levels of TAP-1. The intensity occasionally reached the level of a lymphoblastoid cell line used as positive control. In tissue sections it was noticed that TAP-1 expression keeps a spatial correlation with the staining for HLA class I and class II, suggesting that they may be induced by the same factors. Flow cytometry demonstrated statistically higher expression of TAP-1 in GD than in MNG. A parallel analysis demonstrated that HLA class II expression was significantly higher in GD than in MNG glands. We believe this is the first quantitative validation of the 'aberrant' expression of HLA class II in AITD [10].

Interestingly, thyrocytes from GD glands were more easily induced to express TAP-1, protein and mRNA, than those from MNG glands. This observation is reminiscent of our recent demonstration, regarding class II induction, that thyrocytes from GD are intrinsically more responsive to IFN-γ than thyrocytes from MNG [18].

In trying to identify the factors causing TAP-1 and HLA expression changes in autoimmune thyroid glands we investigated the levels of cytokine message by RT-PCR following a semiquantitative protocol. No clear pattern (Th0, Th1 or Th2) emerged in relation to diagnosis, except for the finding that the HT gland had the higher level of all cytokines, i.e. IFN-γ, TNF-α, IL-2, IL-4 and IL-10. The level of TAP-1 expression did not bear a clear relation to the level of IFN-γ transcripts. The same lack of correlation is appreciated between HLA class I and class II expression and the pattern of cytokine message. This difficulty in establishing links between cytokine transcripts and thyrocyte phenotype may be due to the inevitable need to study only a portion of the thyroid gland, the different half-lives of cytokine message and HLA/TAP molecules, and the known shortcomings of the RT-PCR. However, since we have controlled most of the error sources we believe the difficulty in assigning to a cytokine the induction of HLA and TAP-1 expression is more probably due to the induction results from the interaction of complex mixtures of cytokines.

These results, together with preliminary data that indicate that LMP2 and LMP7 are also hyperexpressed in AITD ([24b] submitted), indicate that autoimmune thyrocytes over-express the whole set of proteins required for the presentation of endogenous peptides through the class I pathway. This finding is relevant to the current hypothesis on the origin of autoimmune endocrine diseases, which postulates that autoimmunity occurs when peripheral tissues express appropriate levels of the molecules required for efficient antigen presentation (signal 1) and of the costimulatory molecules (signal 2), so as to overcome the low affinity and number of circulating autoreactive T cells. This concept derives

from Bretscher & Cohn's two signals paradigm [25] applied to peripheral T cell tolerance [26], and its present formulation is based on observations made in a variety of transgenic mice engineered to express combinations of MHC proteins with viral antigens, cytokines or CD80 in islet β cells or other tissues typically 'peripheral' [4–9,27–29]. Our data suggest that there is a high concentration of endogenous peptide associated with HLA class I on the surface of thyrocytes, and it may be argued that this may be a crucial factor which by lowering second signal requirements facilitates recognition by autoreactive T cells [30]. To determine whether this argument can be applied to presentation via HLA class II, crucial for $CD4^+$ T cell activation, DM expression by AITD thyrocytes is now under investigation.

The observation that thyrocytes have the capability to express HLA molecules and that in glands affected by AITD these cells express high levels of HLA molecules prompted, in 1983, the hypothesis that 'aberrant' or 'inappropriate' expression of HLA-DR—emphasis was then on class II—would be driving the autoimmune response [13,31]. In the following years transgenic mice generated to test this hypothesis by expressing high levels of HLA class I or class II in the pancreatic β cells failed to show signs of autoimmunity—or even alloresponse—and these results were interpreted as a refutation of this hypothesis [32]. This is arguable, because there are many differences between the characteristics of the expression of HLA achieved by transgenic technology and that observed in spontaneous human autoimmune disease [26], not the least that transgenic mice lack the co-ordinated expression TAP which is indispensable for presentation via class I. On the other hand, the inappropriate HLA expression hypothesis of autoimmunity is not necessarily in contradiction with the current view that autoimmunity results from the breach of peripheral tolerance. In fact, its central concept, facilitation of recognition by hyperexpression of HLA in the target cells, could be easily incorporated into it, as one of the mechanisms possibly contributing to shift the peripheral tolerance towards autoimmunity.

One pending question that becomes more interesting in the light of our results is that of the characteristics of the peptides that occupy the cavity of the HLA class I molecules expressed by thyrocytes in AITD glands. Obvious difficulties have until now precluded eluting and sequencing these peptides, but from our results one would predict that they are likely to reflect the proteins synthesized by thyrocytes. This is interesting, because since thyrocytes mainly produce Tg and iodothyronine-synthesizing enzymes, a few peptides from these antigens must be dominant and very abundant in surface class I molecules. A high density of a given HLA class I–peptide combination on the surface of thyrocytes may certainly facilitate recognition by T cells.

Finally, it should be mentioned that the existence of a link between the alleles of TAP and endocrine autoimmunity has already been examined from the genetic point of view. However, in humans the contribution of TAP-1 and TAP-2 alleles to autoimmune disease has been difficult to prove, and remains controversial [33,34].

In conclusion, recent developments in the understanding of the proteins required for antigen processing and presentation led us to investigate the levels of expression and regulation of TAP-1 in normal and autoimmune thyrocytes. The marked overexpression of TAP-1 in AITD suggests that the core concept of the 'inappropriate HLA expression' hypothesis of endocrine autoimmunity should be incorporated into the currently prevailing model of 'autoimmunity by breach of peripheral tolerance'.

ACKNOWLEDGMENTS

We are grateful to Departments of Pathology (Dr M. Fernández) and Clinical Chemistry (Dr E. Abad) for thyroid tissue and for thyroid antibody tests. Dr G. Obiols is thanked for the procurement of thyroid tissue from well characterized patients. We thank all the authors quoted in the text for their presents of reagents, and Dr O. Domínguez for advice on molecular biology techniques. This work was supported by grants no. 90/945 and 92/765 of the FIS (Fondo de Investigaciones Sanitarias) to R.P.-B. and A.L.-M., by IEC (Institut d'Estudis Catalans) and by grant no. 1995SGR00510 of the Grups de Recerca de Catalunya. E.T. and M.S. were recipients of fellowships from the Spanish Ministry for Science and Education. M.S. is recipient of fellowship from IEC (Institut d'Estudis Catalans).

REFERENCES

1 Weetman AP, McGregor AM. Autoimmune thyroid disease: further developments in our understanding. Endocrine Rev 1994; **15**:788–830.
2 York IA, Roek KL. Antigen processing and presentation by the Class-I major histocompatibility complex. Annu Rev Immunol 1996; **14**:369–98.
3 Miller JFAP, Flavell RA. T-cell tolerance and autoimmunity in transgenic models of central and peripheral tolerance. Curr Opin Immunol 1994; **6**:892–9.
4 Ohashi PS, Oehen S, Buerki K *et al.* Ablation of 'tolerance' and induction of diabetes by virus infection in viral antigen transgenic mice. Cell 1991; **65**:305–17.
5 Oldstone MBA, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 1991; **65**:319–31.
6 Ohashi PS, Oehen S, Aichele P *et al.* Induction of diabetes is influenced by the infectious virus and local expression of MHC class I and tumor necrosis factor-α. J Immunol 1993; **150**:5185–94.
7 Harlan DM, Hengartner H, Huang ML *et al.* Mice expressing both B7–1 and viral glycoprotein on pancreatic beta cells along with glycoprotein-specific transgenic T cells develop diabetes due to a breakdown of T-lymphocyte unresponsiveness. Proc Natl Acad Sci USA 1994; **91**:3137–41.
8 Guerder S, Picarella DE, Linsley PS, Flavell RA. Costimulator B7-1 confers antigen-presenting-cell function to parenchymal tissue and in conjunction with tumor necrosis factor α leads to autoimmunity in transgenic mice. Proc Natl Acad Sci USA 1994; **91**:5138–42.
9 Von Herrath MG, Guerder S, Lewicki H, Flavell RA, Oldstone MBA. Coexpression of B7-1 and viral ('self') transgenes in pancreatic β cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. Immunity 1995; **3**:727–38.
10 Hanafusa T, Pujol-Borrell R, Chiovato L, Russell RGG, Doniach D, Bottazzo GF. Aberrant expression of HLA-DR antigen on thyrocytes in Grave's disease: relevance for autoimmunity. Lancet 1983; **2**:1111.
11 Lehner PJ, Cresswell P. Processing and delivery of peptides presented by MHC class I molecules. Curr Opin Immunol 1996; **8**:59–67.
12 Cresswell P. Invariant chain structure and MHC class II function. Cell 1996; **84**:505–7.
13 Pujol-Borrell R, Hanafusa T, Chiovato L, Bottazzo GF. Lectin-induced expression of DR antigen on human cultured follicular thyroid cells. Nature 1983; **303**:71–73.
14 Belfiore A, Mauerhoff T, Pujol-Borrell R, Baudenhoop K, Mirakian R, Bottazzo GF. Spontaneous HLA Class II and enhanced Class I molecule expression in SV40 transfected human thyroid epithelial cells. J Autoimmun 1991; **4**:397–414.
15 Lucas-Martín A, Foz-Sala M, Todd I, Bottazzo GF, Pujol-Borrell R. Occurrence of thyrocyte HLA class II expression in a wide variety of thyroid diseases: relationship with lymphocytic infiltration and thyroid autoantibodies. J Clin Endocrinol Metabol 1988; **66**:367–75.
16 Spies T, Cerundolo V, Colonna M, Cresswell P, Towsend A, DeMars R.

Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. Nature 1992; **355**:644–6.
17 Dodi AL, Brett S, Nordeng T, Sidhu S, Batchelor RJ, Lombardi G, Bakke O, Lechler RI. The invariant chain inhibits presentation of endogenous antigens by a human fibroblast cell line. Eur J Immunol 1994; **24**:1632–9.
18 Sospedra M, Obiols G, Santamaria Babi LF *et al*. Hyperinducibility of HLA Class II expression of thyroid follicular cells from Graves' disease. A primary defect?. J Immunol 1995; **154**:4213–22.
19 Vives M, Soldevila G, Alcalde L, Lorenzo C, Somoza N, Pujol-Borrell R. Adhesion molecules in human islet β-cells. *De novo* induction of ICAM-1 but not LFA-3. Diabetes 1991; **40**:1382–90.
20 Chomczynsky P, Sacchi N. Single step of RNA isolation by acid guanidium thiocianate-phenol-chloroform extraction. Anal Biochem 1987; **162**:156–9.
21 Kleijmeer MJ, Kelly A, Geuze HJ, Slot JW, Townsend A, Trowsdale J. Location of MHC-encoded transporters in the endoplasmic reticulum and cis-Golgi. Nature 1992; **357**:342–4.
22 Trowsdale J, Hanson I, Mockridge I, Beck S, Towsend A, Kelly A. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. Nature 1990; **348**:741–3.
23 Vives-Pi M, Armengol MP, Alcalde L, Somoza N, Vargas F, Jaraquemada D, Pujol-Borrell R. Expression of Transporter Associated with Antigen Processing-1 (TAP-1) in the endocrine cells of human pancreatic islets: effect of cytokines and evidence of hyperexpression in IDDM. Diabetes 1996; **45**:779–88.
24a Epperson DE, Arnold D, Spies T, Cresswell P, Pober JS, Johnson DR. Cytokines increase transporter in antigen processing-1 expression more rapidly than HLA class I expression in endothelial cells. J Immunol 1992; **149**:3297–301.
24b Vives-Pi M, Vargas F, James RFL *et al*. Proteasome subunits, low molecular mass polypeptides-2 and -7 (LMP2 and LMP7) are hyperexpressed by target cells in autoimmune thyroid disease (AITD) but not in insulin-dependent diabetes mellitus (IDDM): implications for autoimmunity. Tissue Antigens 1997; in press.
25 Bretscher P, Cohn, M. A theory of self-nonself discrimination. Science 1970; **169**:1042–9.
26 Jenkins MK. The role of cell division in the induction of clonal anergy. Immunol Today 1992; **13**:69–73.
27 Pujol-Borrell R, Bottazzo GF. Puzzling diabetic transgenic mice: a lesson for human type 1 diabetes? Immunol Today 1988; **9**:41–52.
28 Miller JFAP, Heath WR. Self-Ignorance in the peripheral T cell pool. Immunol Rev 1993; **133**:131–50.
29 Vives-Pi M, Somoza N, Vargas F, Pujol-Borrell R. Modulation of MHC antigen expression and disease. In: Blair GE, Pringle CR, Maudsley DJ, eds. Modulation of MHC antigen expression and disease. Cambridge: Cambridge University Press, 1995:361–89.
30 Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. Science 1996; **273**:104–6.
31 Bottazzo GF, Pujol-Borrell R, Hanafusa T, Feldmann M. Hypothesis: role of aberrant HLA-DR expression and antigen presentation in the induction of endocrine autoimmunity. Lancet 1983; **2**:1115–9.
32 Hämmerling GJ, Schönrich G, Ferber I, Arnold B. Peripheral tolerance as a multi-step mechanism. Immunol Rev 1993; **133**:93–104.
33 Martínez-Laso J, Martin-Villa JM, Alvarez M, Martin-Quiles N, Lledo G, Arnaiz-Villena A. Susceptibility to insulin-dependent diabetes mellitus and short cytoplasmic ATP-binding domain TAP2′01 allelles. Tissue Antigens 1994; **44**:184–8.
34 Ploski R, Undlien DE, Vinje O, Forre O, Thorsby E, Ronningen KS. Polymorphism of human major histocompatibility complex-encoded transporter associated with antigen processing (TAP) genes and susceptibility to juvenile rheumatoid arthritis. Human Immunol 1994; **39**:54–60.