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Research Article

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Endogenous Antigen Presentation by Autoantigen-transfected Epstein-Barr Virus–Lymphoblastoid Cells

I. Generation of Human Thyroid Peroxidase–reactive T Cells and their T cell Receptor Repertoire

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Abstract

To develop a model for endogenous thyroid autoantigen presentation, we transfected EBV-transformed B lymphoblastoid cell lines (EBV-LCL), established from patients with autoimmune thyroid disease and normal controls, with cDNA for the human thyroid autoantigen thyroid peroxidase (hTPO). hTPO-antigen presentation to patient peripheral blood T cells was demonstrated after stimulation in vitro for 7 d with irradiated hTPO-transfected or untransfected autologous EBV-LCL. Anti-hTPO–reactive T cells were subsequently cloned in the presence of irradiated, autologous hTPO-transfected EBV-LCL and IL-2. 10 T cell–cloned lines exhibited specific hTPO-induced proliferation (stimulation indices of 2.1–7.9) towards autologous hTPO-transfected EBV-LCL, and were subjected to human T cell receptor (hTCR) V gene analysis, using the PCR for the detection of V α and V β hTcR gene families. The results indicated a preferential use of hTCR V α 1 and/or V α 3 in 9 of the 10 lines. In contrast, hTCR V β gene family use was more variable.

These data demonstrate a model for the endogenous presentation of human thyroid peroxidase in the absence of other thyroid specific antigens. The high frequency of antigen-specific T cells obtained from PBMC using this technique will facilitate further studies at both the functional and hTCR V gene level. (*J. Clin. Invest.* 1993. 91:1567–1574.) Key words: autoimmune thyroid disease • human thyroid peroxidase • human T cell receptor • polymerase chain reaction • Epstein-Barr virus B cells

Introduction

Both T and B lymphocytes have been implicated in the etiology of human autoimmune thyroid disease (AITD)[1] (1). It has been suggested that inappropriate antigen presentation may occur in the context of MHC class II molecules newly expressed on thyroid epithelial cells in patients with autoimmune thyroid disease (2–4), and we have demonstrated a thyroid antigen-specific T cell response to autologous thyroid cells in the absence of classical antigen presenting cells (5). The clonal investigation of thyroid antigen–autoreactive T cell populations has demonstrated MHC restriction and both phenotypic and functional heterogeneity of T cell clones infiltrating the thyroid of patients with both Graves' and Hashimoto's diseases (6–8). More recently, we have observed a restricted use of human T cell receptor (hTCR) genes by intrathyroidal T cells implicating a primary role for T cells in human thyroid autoimmune disease (9, 10).

At the clonal level, extensive analysis of human T cell clones reactive with thyroid autoantigens has encountered several practical limitations. Firstly, human T cell clones, especially those reactive to autoantigens such as human thyroglobulin and human thyroid peroxidase (hTPO), have been difficult to raise and propagate in vitro (6, 7, 11). Secondly, autologous thyroid epithelial cells, the putative target cells, are always in short supply. If available from surgical specimens, their number is limited and their survival in culture is shortlived; attempts to obtain their immortalization have been only transiently successful (12) or have been accomplished by somatic cell fusion, thereby introducing nonautologous cellular components (13, 14). Similarly, feeder cells in the form of autologous PBMC may be available only in limited quantities. To overcome these practical limitations, we have investigated autologous B lymphoblastoid cells, obtained through transformation with EBV and transfected with the cDNA for hTPO, as potential antigen-presenting cells (APCs) for T cells derived from patients with AITD. Such an approach offers several advantages. Stably transfected EBV lines provide a potentially unlimited supply of autoantigen presenting cells. Furthermore, EBV cells frequently express high levels of MHC class II antigens at their surface and are efficient APCs for exogenous antigens (15). Studies have clearly demonstrated enhanced stimulation of antigen-specific T cells when antigen-specific B cells were used as APCs, thus underlining the importance of antigen processing for efficient antigen presentation and recognition (16). Perhaps most importantly, a model in which the antigen is generated by the cell itself and handled as an endogenous protein in a potentially processed form, may closely mimick autoantigen presentation by the thyroid epithelial cell itself. Thus, the system would differ qualitatively from conventional antigen presentation systems using exogenous antigen. By the same token, such autoantigen can be expected to be glycosylated in a human form as opposed to recombinant preparations obtained from nonhuman expression systems (17), and EBV lymphoblastoid cells may be more apt to provide the second signal than epithelial cells (18). Here we describe expression of the

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1. *Abbreviations used in this paper:* AITD, autoimmune thyroid disease; APCs, antigen-presenting cells; CHO-hTPO, human thyroid peroxidase–expressing Chinese hamster ovary cells; EBV-LCL, EBV-transformed B lymphoblastoid cell lines; hTCR, human T cell receptor; hTPO, human thyroid peroxidase; phTPO-ECE, hTPO expression plasmid; RAJI-hTPO, hTPO-transfected RAJI cells.

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human thyroid autoantigen hTPO in EBV-transformed B lymphoblastoid cell lines (EBV-LCL) and their stimulation of autologous T cells subsequently characterized for their T cell receptor.

Methods

Construction of pHEBo-hTPO. The human hTPO expression plasmid (phTPO-ECE) was prepared as previously described (17) in a way similar to the original report (19). The plasmid was digested with ScaI and then subjected to incomplete digestion with BamHI. A 4.2-kb partial digestion product consisting of the region from ScaI through the BamHI site 3′ to the hTPO cDNA contained the 5′ portion of the β-lactamase gene, the SV-40 origin of replication and early promoter, the human thyroid peroxidase cDNA, and the SV-40 polyadenylation site. This 4.2-kb fragment was isolated and then ligated to a 6.2-kb restriction fragment from pHEBo (20) that had been obtained by digestion of the plasmid with BamHI along with partial digestion with ScaI. Ligated DNA was transfected into competent *Escherichia coli* (W3110), which were then screened for the presence of recombinant plasmids containing an intact β-lactamase (amp^r) gene. Plasmid DNA isolated from ampicillin resistant colonies was subjected to restriction digestion with HindIII, SmaI, EcoRI, BamHI, and XbaI, or combinations of these enzymes to confirm the proper size and orientation of the hTPO cDNA. Plasmid DNA was prepared and purified two times by cesium chloride density gradient centrifugation (Fig. 1).

Source of PBMC and their EBV transformation. PBMC were obtained by Ficoll-Hypaque density gradient centrifugation according to established methods (21). Donors (n = 2) included a patient with Graves' disease, as defined by clinical and biochemical hyperthyroidism and the presence of thyroid-stimulating hormone (TSH) receptor autoantibodies (80% inhibition, normal < 15%) (22) and high serum hTPO antibody levels (ELISA index 1.47, normal < 0.2), and a patient with autoimmune thyroiditis as defined by clinical hypothyroidism and detectable levels of serum autoantibody to human thyroid peroxidase (ELISA index 0.8). A normal donor was a laboratory employee with no history of AITD. EBV-LCL were obtained by transformation of PBMC using B95-8 supernatants (generously provided by Dr. P. Casali, New York University) in combination with T cell suppression using cyclosporin (2.9 μg/ml) (Sandoz Pharmaceuticals Inc., East Hanover, NJ) (23).

Transfection of hTPO cDNA into B cells. Either Burkitt lymphoma derived-RAJI cells (American Type Culture Collection, Rockville, MD) or EBV-LCL from normals or patients were grown in 15% FBS/85% RPMI 1640 (with 25 mM Hepes buffer and L-glutamine) (Gibco, Grand Island, NY) supplemented with 1% penicillin/streptomycin (Gibco). Cells (10×10^6) in log phase of growth were transfected via electroporation using 20 μg of pHEBo-hTPO (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) at 200 V. Transfected RAJI cells (RAJI-hTPO) and control RAJI cells were selected using hygromycin B (400 μg/ml) (Calbiochem, La Jolla, CA). Resistant cells were cultured continuously in this medium. Patient and normal PBMC-derived EBV-LCL were transfected using a similar technique but selected with increasing hygromycin B concentrations from 100 to 200 μg/ml and maintained at 200 μg/ml of hygromycin B. Control pHEBo-only transfected cells were obtained using the same procedure. Control cultures of untransfected cells all died within 4 wk after addition of hygromycin.

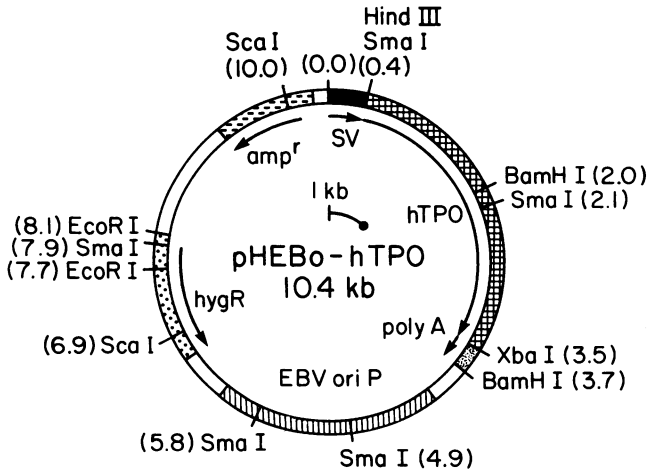

Figure 1. Schematic diagram of construct pHEBo-hTPO conferring hygromycin resistance, and a full length hTPO-cDNA sequence under control of an SV40 promoter (see Methods).

Evaluation of hTPO mRNA expression. Total cellular RNA was extracted from cell cultures using guanidinium thiocyanate and phenol (RNAzol B; Cinna/Bioteck Labs International Inc., Friendswood, TX) and stored in alcohol/saline. Northern blots (10 or 20 μg/lane) were probed with a ^{32}P-labeled 2.25-kb XhoI-SstI restriction fragment of hTPO cDNA (from clone phTPO-ECE). Thyroid tissue from a patient with Graves' disease and hTPO expressing Chinese hamster ovary (CHO-hTPO) cells (17) served as positive controls.

Evaluation of hTPO-antigen expression. Western blotting was performed using reduced and denatured samples of transfected and control cells on a mini-gel apparatus (Miniprotean Apparatus; Bio Rad, Richmond, CA) using standard techniques as previously described at concentrations of 5–20 μg protein/lane (24). The filters were blocked with 5% nonfat milk and incubated with 1/100 dilution of a standardized and pooled patient serum preparation (MS 12/89: anti-hTPO standard) (17) or normal serum control. After washing, the filters were incubated with ^{125}I protein A and subsequently exposed. For surface analysis of hTPO antigen expression, cells were stained via indirect immunofluorescence using high titer human polyclonal anti-hTPO serum (1:100 dilution) or murine mAb to natural hTPO (no. 18AS2, 1:100 dilution; kind gift of Dr. B. Rees Smith, University of Wales, Cardiff, UK) and FITC-labeled second antibody to human IgG (Sigma Immunochemicals, St. Louis, MO) or mouse Ig (Cappel/Organon Teknika, Durham, NC), respectively. A minimum of 2,000 fluorescent cells were analyzed on a three-decade logarithmic scale using laser flow cytometry (Epics C; Coulter Electronics, Hialeah, FL).

Evaluation of HLA-class II expression in transfected EBV-LCL. hTPO-transfected EBV-LCL and controls were stained using phycoerythrin-conjugated I2 (anti-HLA-D/DR) mAb (Coulter Immunology, Hialeah, FL) and fluorescent cells were analyzed by laser flow cytometry (Coulter Epics C).

Assessment of PBMC reactivity to hTPO. Peripheral blood mononuclear cells (1×10^5/well) were incubated with 1×10^4 gamma irradiated (100 Gy), autologous, EBV lymphoblastoid cells (either untransfected or transfected with the construct pHEBo-hTPO) in quadruplicates for 7 d. The culture medium consisted of 10% human heparinized plasma (derived from male donors) and RPMI 1640 (supplemented as above). Tritiated thymidine (0.5 μCi = 18.5 kBq/well) (New England Nuclear, Boston, MA) was added for the last 18 h of the cultures. Cells were then harvested (PHD cell harvester, model 200A; Cambridge Technology Inc., Cambridge, MA) and subjected to liquid scintillation counting (model LS 3801, Beckman Instruments Inc., Fullerton, CA). For expansion after this initial 7-d period, additional wells were set up in parallel at the same time and used for cloning.

Generation of hTPO-specific T cell lines. After the initial 7-d sensitization period, cells were seeded at 100/well in microwells of 96-well tissue culture plates (Linbro; Flow Laboratories, McLean, VA) together with 1×10^4 gamma irradiated (100 Gy) autologous EBV-LCL transfected with the construct pHEBo-hTPO. The medium was identical to the one used during the sensitization period but contained 10% crude IL-2 (Lymphocult T; Biotest, Frankfurt, Germany). After 12 d, the contents of wells demonstrating cell growth were transferred to 24-well plates (Costar, Cambridge, MA) with 1.25×10^4 100 Gy gamma-irradiated pHEBo-hTPO feeder cells and expanded for another

week, after which they were tested as single or pooled cultures for antigen-specific proliferation. These cultures are referred to as lines, since they were derived from cultures of ≥ 100 cells/well. We then performed a cloning procedure at 0.5 cells per well in Terasaki plates (Robbins Scientific, Mountain View, CA) in a 20-μl volume with 1–2 × 10^3 autologous EBV-hTPO feeder cells. Cells were grown in these wells for 12–14 d, after which they were transferred to 96-well plates with 1 × 10^4 autologous, irradiated EBV-hTPO feeder cells. After 6 d, the surviving cells were transferred to 24-well plates, expanded, and tested as described below.

Testing of anti-hTPO reactivity. For the testing of sensitized bulk cultures, as well as IL-2 expanded lines, T cells were examined for their reactions to 100 Gy irradiated autologous pHEBo-hTPO transfected EBV-LCL (0.4–1 × 10^4 cells/well), as well as untransfected EBV-LCL, or against the pHEBo-(wildtype)-transfected EBV lymphoblastoid cell line as control. An average of 1.2 × 10^4 T cells per well were stimulated over 2 d in triplicates or quadruplicates followed by the addition of 0.5 μCi/well of tritiated thymidine for 18 h, after which cells were processed for β liquid scintillation counting (see above).

T cell phenotyping. T cell lines with stimulation indices > 1.5 were stained for cell surface markers CD4 and CD8 using monoclonal antibodies T4 (phycoerythrin-coupled) and T8 (fluorescein isothiocyanate-coupled) (Coulter Immunology) and dual fluorescence analyzed by laser flow cytometry as described above.

T cell receptor analysis. Complementary DNA (cDNA) transcripts were prepared using oligo-dT priming and avian reverse transcriptase (Life Sciences, Inc.) as described elsewhere (9, 25) and the cDNA, synthesized from the equivalent of 1–5 μg total cellular RNA, was stored in 200 μl sterile water. For the amplification of the hTCR, mRNA transcripts, we used 18 different V α (9) and 21 V β (26) oligonucleotide 5′ amplimers prepared using a DNA synthesizer (Applied Biosystems) and paired them with 3′ primers matched to C α or C β 1 constant region genes. The predicted size of the amplified PCR fragments based on the individual V gene families was subsequently used in the recognition of V gene family specific bands. For the PCR reactions, 5 μl of denatured cDNA was amplified in a 25-μl final volume with 1 U *Taq* DNA polymerase, 0.3 μg of both primers, *Taq* polymerase buffer, and with 1.5 mM of each dNTP. We carried out 35 cycles of amplification by using a step program (95°C, 1 min, 56°C, 2 min, and 72°C, 3 min) followed by a 10-min extension at 72°C (model PTC100 programmable thermal controller; M. J. Research Inc., Cambridge, MA). Negative controls included tubes without cDNA. The amplified products were subjected to electrophoresis on 1.5% agarose gels with ethidium bromide and visualized under ultraviolet light.

hTCR V gene hybridization. To improve the sensitivity of fragment detection and to examine the specificity of the PCR products the agarose gels were transblotted onto nitrocellulose membranes (Hybond-C; Amersham Corp., Arlington Heights, IL), baked, and prehybridized as described (9, 25). All blots were subsequently hybridized with a ^{32}P-gamma ATP-labeled oligonucleotide probe specific to the C α or C β-1 regions and internal to the predicted hTCR products. Approximately 5 × 10^5 cpm/ml of probe was hybridized with each filter for 18 h at 42°C in 6 × SSC, 1 × Denhart's solution, 0.05% sodium pyrophosphate, and transfer RNA. The filters were washed in 2 × SSC with 0.05% sodium pyrophosphate at increasing temperatures (50°C, 60°C, and 70°C). After each washing, the blots were exposed to x-ray film with an intensifying screen at −80°C for 1–24 h.

Results

Human TPO gene expression in pHEBo-hTPO transfected RAJI cells. We first transfected the Burkitt lymphoma cell line RAJI using the construct pHEBo-hTPO, since high copy numbers of pHEBo had been reported in these cells after transfection (20). Total cellular mRNA, prepared from hygromycin resistant RAJI cells, contained an abundant 3.7-kb hTPO mRNA, as shown in Fig. 2. The hTPO mRNA was of similar size and equally abundant to control CHO-hTPO cells and absent from nontransfected RAJI cells. Natural hTPO mRNA prepared from Graves' disease thyroid tissue contained a smaller 3.2-kb mRNA as described previously (19) because of the lack of the additional 0.5-kb polyadenylation sequence derived from plasmid pECE (not illustrated) (27). Western blotting of RAJI whole cell lysates was complicated by the presence of a variety of (likely EBV-associated) immunoreactive bands using pooled anti-hTPO positive sera (major bands at 68, 72, and 96 kD) (Fig. 3). Nevertheless, a band at ~ 107 kD characteristic of hTPO was seen with RAJI-hTPO cells but not with control RAJI cells, and was similar to that seen in the control CHO-hTPO cells that lacked the EBV-associated bands (Fig. 3). The surface expression of the hTPO antigen was confirmed by laser flow cytometry which demonstrated hTPO on > 40% of hygromycin resistant RAJI cells when detected with high titer human polyclonal anti-hTPO (Fig. 4). However, no reactivity with murine monoclonal anti-hTPO no. 18AS2 was seen on the same RAJI-hTPO cells (Table I) suggesting that the expressed protein differed from thyroidal hTPO.

Expression of hTPO in pHEBo-hTPO transfected normal and patient EBV-LCL. Results of hTPO transfection studies using normal and patient EBV immortalized B cells are sum-

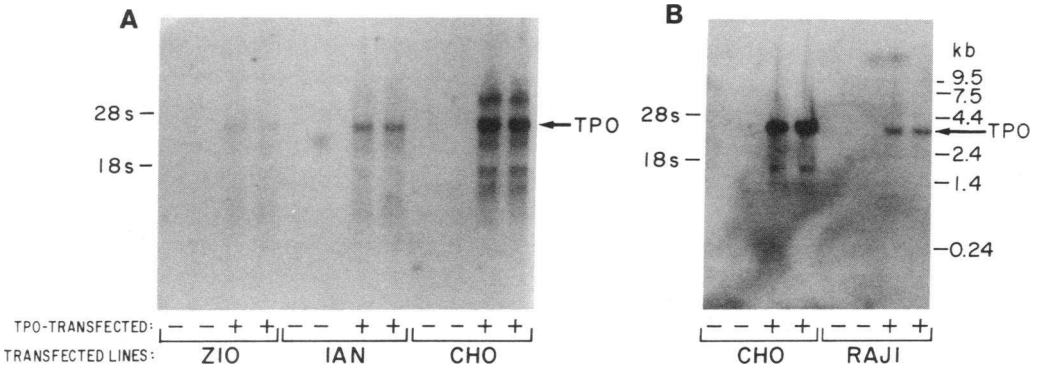

Figure 2. (*A*) hTPO mRNA expression in transfected cell lines from patients with AITD (lines ZIO-hTPO and IAN-hTPO) as assessed by Northern blot analysis. Untransfected lines were included as controls. Transfected cells are indicated by +, and untransfected controls are indicated by −. CHO-hTPO (from reference 17) and untransfected CHO cells were included as controls. The hTPO mRNA was 3.7 kb in both transfected patient lines and CHO-hTPO. (*B*) hTPO-mRNA expression in transfected RAJI cells as assessed by Northern blot analysis. Untransfected and transfected CHO cells (from reference 17), as well as untransfected RAJI cells served as controls. The hTPO mRNA was 3.7 kb in both CHO-hTPO and RAJI-hTPO.

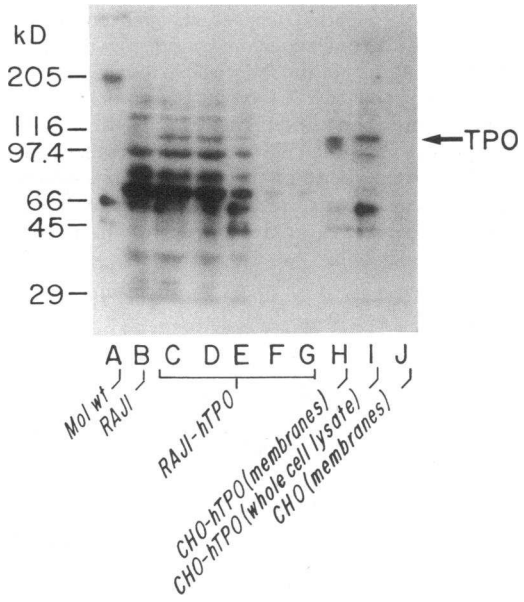

Figure 3. This figure illustrates the detection of hTPO protein by Western blot of RAJI-hTPO (whole cell lysate) using pooled human anti-hTPO serum. Both RAJI-hTPO and CHO-hTPO reveal characteristic protein bands at 100–110 kD. Additional bands in the RAJI and RAJI-hTPO preparations likely represent reactivity with EBV antigens. (Lane *A*) Molecular mass markers, (lane *B*) RAJI (8.4 μg/lane), (lane *C*) RAJI-hTPO (8.4 μg/lane), (lane *D*) RAJI-hTPO (5 μg/lane), (lane *E*) RAJI-hTPO (1.7 μg/lane), (lane *F*) RAJI-hTPO (0.34 μg/lane), (lane *G*) RAJI-hTPO (0.17 μg/lane), (lane *H*) CHO-hTPO membranes (10 μg/lane), (lane *I*) CHO-hTPO (whole cell lysate) (2.8 μg/lane), and (lane *J*) control CHO membranes (10 μg/lane).

marized in Table I. Three EBV-LCL were generated from PBMC and successfully transfected with pHEBo-hTPO (one patient with Graves' disease (IAN), one patient with Hashimoto's disease (ZIO), and one normal (KIM). One of the patient lines was also transfected with the original plasmid pHEBo as a plasmid transfection control. Northern blot analysis confirmed the presence of hTPO-specific mRNA in each of the three lines transfected (Fig. 2). There again appeared to be a difference in molecular size of both mRNAs (3.7 kb) compared to natural hTPO (3.2 kb). Analysis of mRNA expression of these transfected EBV-LCL over time revealed considerable variability in the degree of gene expression by each individual line. For example, line IAN-hTPO expressed higher steady-state levels of hTPO mRNA than line ZIO-hTPO (Fig. 2). In the normal control cell line, KIM-hTPO, there was a time dependent decline in hTPO mRNA (data not shown).

Antigen expression in the EBV-LCL was analyzed by Western blotting technique using transfected and nontransfected EBV-LCL. However, the presence of EBV antigens, detected by the human antisera, made it difficult to illustrate the hTPO bands. Although 100-kD proteins were discernable it was not possible to see hTPO protein fragments of differing sizes representative of degraded hTPO (data not illustrated). However, cell surface staining of normal and patient EBV-LCL transfected with pHEBo-hTPO using both monoclonal and polyclonal anti-hTPO revealed a heterogeneous picture of low level hTPO-antigen expression (Table I). While the polyclonal anti-hTPO did not detect surface hTPO expression in these lines, mAb 18AS2 detected hTPO molecule expression on 16% of the IAN-hTPO cells (Fig. 4).

HLA-class II expression in hTPO-transfected EBV-LCL. One potential effect of the transfected hTPO-containing con-

Table I. hTPO gene expression in transfected EBV-LCL

Human cell	mRNA (kb)	Western* (kD)	Surface hTPO		
			Poly-Ab	M-Ab	HLA-DR‖
Burkitt lymphoma cells:					
RAJI-LCL	—	—	0%	—	—
RAJI-hTPO	3.7	107	47%	0%	—
Patient EBV-lymphoblastoid cells:					
IAN-LCL	—	—	0%	0%	99%
IAN-hTPO	3.7	—	2%	16%	95%
IAN-pHEBo	—	—	—	—	87%
ZIO-LCL	—	—	0%	0%	—
ZIO-hTPO	3.7	—	1%	0%	—
Normal EBV-lymphoblastoid cells:					
KIM-LCL	—	—	0%	0%	—
KIM-hTPO	3.7	—	3%	0%	—
Control cells:					
CHO-hTPO‡	3.7	107	>90%	>70%	—
Thyroid	3.2	107	—	—	—

— Not done or undetectable.
* Using MS 12/89 anti-hTPO polyclonal serum.
‡ Enriched by flow cytometric cell sorting (17).
§ Corrected for background. Percentages ≤ 3% are within margin of tolerance of flow cytometer.
‖ Peak channels for IAN-LCL, IAN-hTPO, and IAN-pHEBo were 232, 173, and 150, respectively.

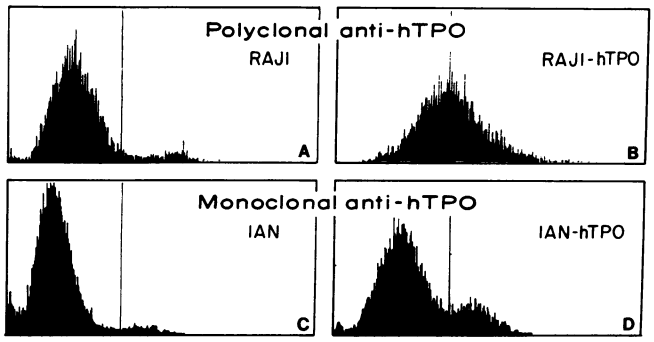

Figure 4. Flow histogram demonstrating cell surface expression of hTPO on transfected RAJI-hTPO cells and patient line IAN-hTPO. (*A*) Background fluorescence with polyclonal anti-hTPO positive human serum on untransfected RAJI cells, (*B*) Fluorescence measured after labeling with polyclonal anti-hTPO positive human serum. More than 50% of RAJI-hTPO cells expressed surface hTPO. On the patient line IAN-hTPO, hTPO was detected on 16% of cells by indirect immunofluorescence using monoclonal anti-hTPO (#18AS2) (*D*), but not on the untransfected line IAN (*C*).

struct on the EBV-LCL target cells was an enhancement of HLA class II antigen expression. Such a consequence would cause considerable difficulty in the interpretation of mixed lymphocyte responsiveness and antigen presentation. In each of the transfected EBV-LCL lines examined for HLA class II expression using mAb I2, we found > 85% of the cells to be positive. However, the intensity of HLA-DR expression on both pHEBO-hTPO–transfected and pHEBO-construct-only–transfected EBV-LCL cells was lower than on untransfected EBV-LCL (Table I).

Anti-hTPO reactivity detected in PBMC. Primary cultures of PBMC in the presence of irradiated autologous hTPO-transfected and untransfected EBV-LCL were performed to measure both anti-hTPO reactivity and to enrich for cells with such reactivity. Stimulation of T cell proliferation was induced by exposure to transfected cells known to be expressing hTPO antigen in primary cultures of patient IAN PBMC when compared to EBV-LCL controls (Table II). No antigen-specific proliferative response could be generated against the second hTPO-transfected patient EBV-LCL which expressed only low levels of hTPO mRNA (line ZIO-hTPO, Table II).

Generation of hTPO-antigen–specific T cell lines. 21 lines derived from pHEBo-hTPO stimulated PBMC (from Graves' patient IAN) were tested for reactivity against autologous untransfected and pHEBo-hTPO transfected EBV-LCL (IAN-LCL). Specific reactivity (SI > 1.5) towards the hTPO-transfected line was observed in 15 out of the 21 (71%) lines tested (SI range 1.6–8.7, mean±SEM = 2.7±0.5) (Table II). Phenotyping of these hTPO-reactive T cell lines showed a mixture of CD4+ and CD8+ T cells (mean±SEM CD4/CD8 ratio = 1.0±0.2, range 0.05–2.44), although one line showed a predominantly CD8+ phenotype (95% CD8+).

The T cell line with the highest stimulation index was tested against both the untransfected and pHEBo-(wildtype)-transfected autologous EBV-LCL (IAN-LCL and IAN-pHEBo-only) as negative controls and against IAN-hTPO. Both controls elicited only insignificant responses compared to the hTPO-transfected EBV-LCL (IAN-hTPO) at a stimulator cell concentration of 1×10^4 cells/well (Fig. 5). There was a small, nonspecific increase in thymidine incorporation towards both untransfected and pHEBo-(wildtype)-transfected EBV-LCL at higher stimulator cell concentrations, indicating a minor autologous mixed lymphocyte reaction component under these experimental conditions (Fig. 5).

T cells were subsequently cloned (0.5 cells/well, two independent limiting dilution experiments) after an initial 7-d culture of hTPO-antigen stimulated PBMC and 67 cloned lines were derived. 22 lines developed sufficiently by day 26 for antigen testing against autologous nontransfected and hTPO-transfected EBV-LCL. 10 of the 22 cloned lines reacted significantly to the hTPO antigen with SIs ranging from 2.1 to 7.9 (mean±SEM = 3.8±0.7) (Fig. 6). Thus, in two independent experiments (numbers 2 and 3 in Table II), where there was no specific reactivity in the primary cultures towards IAN-hTPO, we found it possible to derive hTPO-specific T cells. 8 of the 10 cloned lines that we were able to analyze phenotypically displayed homogeneous phenotypes: seven were CD4+ phenotype and one expressed CD8 (Table III).

Table II. Stimulation Indexes (SIs) in EBV-LCL-hTPO–Stimulated Cultures

Primary cultures				
Experiment	Patient	PBMC + EBV-LCL	PBMC + EBV-LCL-hTPO	SI
1	IAN	1,531±646	11,004±1,434	7.2
2	IAN	14,742±2,083	20,071±2,585	1.4
3	IAN	16,438±1,381	7,843±1,865	0.5
4	ZIO	33,318±3,118	24,923±2,848	0.7

Secondary Cultures				
Experiment	Patient	Responders	Number	SI (range)
1	IAN	Lines	15	1.6–8.7
2	IAN	Cloned lines	4	2.8–7.1
3	IAN	Cloned lines	6	2.1–3.3

Only secondary cultures with SI > 1.5 included. Culture time for IAN-hTPO ranged from 9–16 wk after transfection.

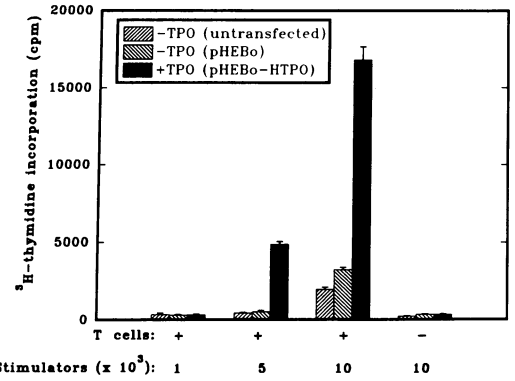

Figure 5. Reactivity of T cell line (derived from patient IAN) against autologous EBV-LCL cells that were either untransfected, transfected with pHEBo (wild type construct) (*hatched bars*) or transfected with pHEBo-hTPO (*filled bars*). Stimulator cell numbers per well are indicated on the x axis. Mean±SEM thymidine incorporation by T cells only was 320±28 cpm.

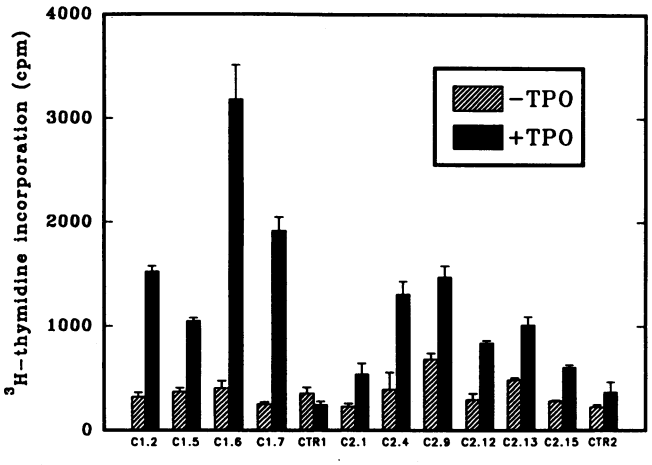

Figure 6. Stimulation of 10 hTPO-reactive T cell–cloned lines (C) (Table III) by untransfected autologous EBV transformed B cells (*hatched bars*) and the same EBV-LCL cells transfected with the pHEBo-hTPO construct (*closed bars*). Only responses with SI > 1.5 are plotted. CTR1 and CTR2 indicate thymidine incorporation by stimulator cells only.

T cell receptor V gene use by hTPO-antigen-specific T cell cloned lines. Analysis of 18 hTCR V α and 21 V β gene families used by the 10 hTPO-reactive cloned T cell lines showed between one and four different hTCR V α and V β mRNA transcripts (Table III). Such data indicated that the cultures were likely to be lines not clones. Since only PCR fragments of the correct predicted size were included, the multiple V genes were not representative of incompletely spliced transcripts or transcriptionally unproductive rearrangements. While 9 of the 10 lines used V α 1 and/or V α 3, there was no consistency in the utilization of hTCR V β genes.

Discussion

We have described a novel approach to the study of autoantigen-specific human T cells that used autologous EBV-LCL transfected with cDNA for a human autoantigen. This approach differs elementarily from conventional antigen-presenting systems in which autoantigen or synthetic peptides are added as external antigens to classical antigen-presenting cells. In these latter systems, the presumptive pathway of antigen processing is thought to involve the route of all exogenous antigens with predominant involvement of MHC class II antigens (28). In contrast, in the system we have described here, the autoantigen was encoded by its transfected cDNA and generated endogenously. The fact that most T cell clones were CD4+ suggested that MHC class II was antigen presenting and there now is ample evidence that such molecules can indeed facilitate such endogenous antigen presentation (29–32). Furthermore, this route of presentation may be the most likely way for antigen to be presented to autoreactive T cells in autoimmune disease (33). Future experiments will have to determine in a more direct manner whether the endogenously produced hTPO is presented after intracellular processing via the endogenous pathway, or whether hTPO, once at the cell surface is treated as an exogenous antigen by the EBV-LCL. The handling of thyroid antigen by human thyrocytes for antigen processing is thought to include such an endogenous route since cloned T cells can interact directly with cloned thyrocytes in the absence of other antigen presenting cells (5). The model described here may allow further investigation of these processes. It is also unknown at present whether there is a direct contribution to antigen presentation by the T cells themselves in the model described here (34) or whether these different mechanisms for antigen presentation may be operative in combination. In subsequent studies it should be possible to specifically block various processing pathways (35, 36) and determine their influence on T cell reactivity in the EBV-LCL model.

Irrespective of the antigen-processing pathway(s) involved, the endogenously generated hTPO has a number of additional advantages. Being glycosylated by a human cell, although not a thyrocyte, sets the recombinant hTPO used apart from other recombinant preparations derived from CHO cells or other expression systems (mammalian or nonmammalian) (17, 37). This approach also avoids significant contamination with other antigens (thyroidal or nonthyroidal) commonly found in antigenic preparations from whole tissue, cells, or various expression systems (38, 39). Furthermore, the limited supply of autologous (patient-derived) thyroid cells as a source of antigen-presenting cells has been an important constraint on the investigation of T cell/target cell interaction. However, with the use of autologous, hTPO-presenting EBV-transformed lym-

Table III. Characteristics of T Cell–Cloned Lines Reactive to IAN-hTPO

Cloned lines	Thymidine incorporation*		SI	Phenotype		hTCR Vα	hTCR Vβ
	Background	TPO stimulated		CD	Percentage		
1.2	322±43	1,525±50	4.7	—	—	3, 12	8, 18
1.5	370±38	1,052±27	2.8	8	47	3, 7	14, 17
1.6	405±69	3,185±129	7.9	4	98	2, 3, 8, 14	many
1.7	252±19	1,918±32	7.6	—	—	6, 8	12
2.1	227±32	541±100	2.4	4	92	1	6
2.4	392±163	1,310±120	3.3	4	95	1, 2, 3	6, 7
2.9	680±58	1,479±101	2.17	4	90	3, 10, 15, 17	3, 13, 14, 17
2.12	296±59	843±19	2.8	4	83	1, 10	5, 6, 18
2.13	484±21	1,015±78	2.1	4	84	1, 6	5, 8
2.15	281±10	610±17	2.2	4	84	1, 3, 15, 17	2

* Data are given as cpm±SEM.

phoblasts, we have now available immortal and autologous thyroid antigen presenting cells. Unfortunately, the system is not as stable or as constant as hoped and further efforts in this direction are required.

The use of "professional" antigen-presenting B cells, in the form of EBV-LCL, offers several unique immunological potential benefits: Firstly, EBV-transformed B cells frequently express high constitutive levels of HLA class II (and class I) antigens in contrast to the need for thyrocyte HLA class II antigen induction (7). This is important for antigen presentation itself and should also further facilitate the identification of HLA gene elements involved in actual hTPO presentation, as well as identification of the self-peptides responsible. Secondly, the high degree of constitutive expression of intercellular adhesion molecule-1 (CD54) on EBV-LCL (40, 41) is likely to be an integral part of antigen presentation in this system (42). The recent finding of thyrocyte intracellular adhesion molecule-1 expression and T cell binding emphasizes the need for an adequate APC model (41, 43–45). Thirdly, EBV B cells may be better providers of the controversial second signal necessary to enable a proliferative T cell response than epithelial cells (18).

Our previous experience, and that of other workers, in the generation of thyroid-antigen–specific human T cell clones from patients with AITD has always involved the utilization of intrathyroidal T cells rescued from thyroid surgical specimens (6–8). This was forced by necessity since PBMC were consistently negative in cloning studies and this greatly limited the patients who could be studied. Furthermore, clonal SIs were relatively low compared to external antigen-reactive T cell clones. Of particular concern was our inability to generate clones to hTPO while we were able to obtain cells reactive to thyroglobulin or intact autologous thyroid cells (7). This may have been due to the nature of the membrane-bound hTPO antigen which required detergent solubilization. Furthermore, natural hTPO preparations are always contaminated by thyroglobulin unless elaborate immunopurification steps are taken (46, 47). Although human hTPO-reactive T cell lines with low SIs have been reported, without excluding thyroglobulin reactivity (38), the recent use of recombinant hTPO microsome preparations has allowed the production of intrathyroidal human T cell clones that were highly reactive to hTPO (48) when presented by conventional antigen presenting cells. Using hTPO-expressing EBV-LCL, we were able to generate hTPO-specific T cell lines directly from peripheral blood via what we believe is most likely to be an endogenous processing route. The lines showed a heterogeneous pattern of reactivity, and allowed generation of a variety of cell characterization data, including analysis of the T cell receptor V gene families used. These initial studies demonstrated oligoclonality of the hTPO-reactive cells with nine of the lines using and/or hTCR V α 1 and/or V α 3 gene families. The restriction to V α rather than V β was further evidence of the importance of the V α chain in the recognition of thyroid protein, as first suggested by our previous demonstration of restricted hTCR V α gene usage in intrathyroidal T cells (9) and our subsequent demonstration that this restriction may preferentially apply to V α compared to V β (10). The hTPO-reactive T cell lines expressed multiple V gene family mRNA transcripts although they were all "clones" by traditional immunological criteria. The data also demonstrate the usefulness of V gene analysis in the definition of "clonality."

In conclusion, we have demonstrated the successful use of autoantigen-transfected lymphoblastoid cell lines for the presentation of human thyroid peroxidase to autologous T cells and the potential use of this system for the generation of hTPO-reactive T cell lines and analysis of their T cell receptor V gene use. This system should be applicable to the investigation of other human autoimmune diseases where specific autoantigens have been identified. The comparison between endogenous versus exogenous processing of peptides in relation to the T cell receptor structural elements will enhance our understanding of the molecular basis of autoimmune disease.

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