Clin Exp Immunol 1994; 98:470–477

Perforin expression by thyroid-infiltrating T cells in autoimmune thyroid disease

Z. WU, E. R. PODACK*, J. M. McKENZIE, K. J. OLSEN* & M. ZAKARIJA *Departments of Medicine and *Microbiology/Immunology, University of Miami School of Medicine, Miami, FL, USA*

(Accepted for publication 10 August 1994)

SUMMARY

Infiltration of the thyroid gland by lymphocytes is a hall-mark of autoimmune thyroid disease; it is particularly evident in Hashimoto's thyroiditis but is also seen in most patients with Graves' disease. Infiltrating cells are comprised primarily of T lymphocytes, of which only a minority appears to be activated. Their precise pathogenic role is largely unknown. Since perforin has been a marker for functionally activated cytotoxic T cells *in situ* we elected to assess the presence of perforin-containing cells in thyroid-infiltrating lymphocytes and establish their phenotype. Cells were isolated from seven subtotal thyroidectomy specimens, five from patients with Graves' disease and two with Hashimoto's thyroiditis. The novel findings were as follows: $CD4^+$ perforin-containing T cells occurred only in Hashimoto's glands, suggesting a class II-restricted component of cytotoxicity; in Graves' disease, and to a lesser extent in Hashimoto's, perforin-expressing cells were primarily T cell receptor $\alpha\beta^+$ $CD4^-CD8^-$ (double negative); double negative perforin-containing cells in peripheral blood of normal individuals were largely $\gamma\delta^+$ T cells. In Hashimoto's samples, the predominant population of T cells expressing perforin was $CD8^+$. By comparison, in studies of the synovial fluid of knee joints from patients with rheumatoid arthritis only a minor population of the perforin-containing cells was double-negative. The data suggest significant differences in cytotoxic autoimmune mechanisms between the two autoimmune thyroid diseases. Functional characterization of double-negative T cells is necessary to define their role in autoimmunity.

Keywords perforin $\alpha\beta$ T cell receptor double-negative T cells $CD4^+$ cytotoxic T cells thyroid autoimmunity

INTRODUCTION

Autoimmune thyroid disease (AITD) includes the two extremes of a clinical spectrum, Hashimoto's thyroiditis (HT) and Graves' disease (GD), characterized respectively by hypo- and hyperthyroidism. The pathogenesis of the former is primarily immune destruction of the thyroid and the typical histology includes lymphocytic infiltration of the gland, frequently with lymphoid follicle formation [1]. These features are found to a highly variable degree also in thyroids of patients with GD in which the hyperthyroidism is due to a thyroid-stimulating antibody [2,3]. Because of the physical availability of the gland, AITD is an excellent human model for study of organ-specific autoimmunity.

Although an autoimmune pathogenesis of AITD is supported by the production of autoantibodies to thyroid antigens, the presence of lymphocytic infiltration in the thyroid and a genetic association with loci of the MHC, the precise cellular immunological mechanisms mediating the disease are not clear [1,4]. Phenotypic studies of intrathyroidal lymphocytes in AITD have shown a predominance of T lymphocytes and a variety of their subsets, including suppressor/cytotoxic ($CD8^+$) and T cell receptor (TCR) $\alpha\beta^+$ $CD4^-CD8^-$ cells [5–9]. However, the relative contributions of T cell subsets to the disease are not easily determined since activated T cells are a minority within the thyroid [7,10,11].

Perforin, a cytotoxic molecule able to form pores in the membranes of target cells, is inducible in activated TCR$\alpha\beta^+$ cytotoxic T lymphocytes (CTL). It is constitutively expressed in natural killer (NK) cells [12–14] and in TCR$\gamma\delta^+$ T cells [15]. The expression of perforin in T cells has been a marker for functionally activated cytotoxic cells *in situ* [16,17]. More recently, TCR$\alpha\beta^+$ $CD4^-CD8^-$ cells, a major T cell population in autoimmune *lpr/lpr* mice [18], were shown to express perforin and to be cytotoxic [18]. To evaluate the possible

Z.W. present address: Department of Immunology, The London Hospital Medical College, 57/76 Ashfield Street, London E1 2AJ, UK.

Correspondence: Margita Zakarija, Department of Medicine (R-93), University of Miami School of Medicine, PO Box 016760, Miami, FL 33101, USA.

involvement of *in vivo* activated cytotoxic cells, we have assessed thyroid-infiltrating lymphocytes (TIL) from patients with AITD for the presence of perforin, and established the phenotype of perforin-containing cells.

Here we report that perforin is expressed by several subpopulations of TIL, including $CD4^+$ and $TCR\alpha\beta^+$ double-negative T cells. Therefore our results suggest a possible role for perforin in the pathogenesis of AITD. The significance of the phenotype of perforin-containing cells we found in the disease is discussed.

PATIENTS AND METHODS

Patients

Thyroidectomy specimens from five patients with GD and two patients with HT were studied. All patients with GD had the diagnosis of hyperthyroidism confirmed by elevated serum thyroxine levels and suppressed thyrotropin values, while patients with HT were defined by the presence of clinical and biochemical hypothyroidism and positive titres of thyroid microsomal antibody. All Graves' patients were treated with an anti-thyroid drug (propylthiouracil or methimazole) and iodide before surgery, while the two Hashimoto's patients were receiving thyroxine.

Of the seven thyroids, one from a GD patient and one from a subject with HT were simply weighed and processed for harvesting of TIL. The remaining thyroids were also analysed histologically. The four GD glands were described in a previous study [3], 1, 3, 4 and 5 of the current experiment being, respectively, 12, 11, 8 and 5 of that earlier report. As shown there, the degree of lymphocytic infiltration in 23 glands varied widely, with a derived index ranging from 0 to 50 000 [3]; the indices from glands 1, 3, 4 and 5 were 90, 155, 356 and 831. We assessed one of the Hashimoto's thyroids (H 2, Table 1) similarly and calculated an index of 50 000, i.e. 50% of the tissue was comprised of lymphocytes. The available portions of the five Graves' glands ranged in weight from 55 g to 103 g and the number of lymphocytes obtained were from $1{\cdot}4 \times 10^7$ to $2{\cdot}6 \times 10^8$. The tissue of the two Hashimoto's thyroids, weighing 44 g and 200 g, yielded $4{\cdot}7 \times 10^8$ and $4{\cdot}6 \times 10^8$ TIL, respectively. It was the 44-g fragment that was 50% infiltrated.

For comparison, lymphocytes from the synovial fluid from knee joints of six patients with rheumatoid arthritis (RA) and peripheral blood mononuclear cells (PBMC) from three normal subjects were also obtained. Tapping of synovial fluid was part of the routine clinical treatment of RA.

Preparation of lymphocytes for analyses

Following the procedure of McLachlan *et al.* [19], surgically obtained thyroid tissue was macerated, digested with collagenase (class 2, Worthington Biochemical Co., Freehold, NJ) and resulting cells were seeded into culture flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS). Unattached cells, comprising primarily lymphocytes, were removed 20 h later and separated by density centrifugation on a Ficoll–Hypaque gradient (Sigma, St Louis, MO) to yield TIL. Density centrifugation was also used for purification of lymphocytes from synovial fluid and peripheral blood. Cells, in RPMI 1640 medium supplemented with 50% fetal bovine serum (FBS) and 10% DMSO, were stored in liquid nitrogen. For analyses, they were rapidly thawed, washed in PBS, fixed in 1·5% paraformaldehyde/1% sucrose in PBS and washed again. Then they were cytocentrifuged onto poly-(L-lysine)-coated slides (Sigma) and air-dried.

Monoclonal antibodies

Mouse anti-human perforin MoAb was generated as described [20] and was biotinylated using biotinamidocaproate N-hydroxysuccinamide ester (Sigma) according to a standard procedure [21]. Anti-CD4 (T4) and CD56 (NKH-1) MoAbs were purchased from Coulter Electronics (Hialeah, FL); anti-CD8 was from Boehringer Mannheim (Mannheim, Germany); anti-$TCR\alpha\beta$ was obtained from T Cell Sciences, Inc. (Cambridge, MA); anti-CD3 was from American Type Culture Collection (Rockville, MD); and anti-$TCR\gamma\delta$ was a kind gift from Dr M. G. Lichtenheld (Department of Microbiology/Immunology of this School of Medicine).

Immunofluorescence

Two-colour fluorescence staining was performed at room temperature as follows. The slides were rinsed in PBS–0.1%

Table 1. Phenotypes of thyroid-infiltrating lymphocytes (TIL) in autoimmune thyroid disease identified by two-colour immunofluorescence

	Per cent positive cells						
TIL	$CD3^+$	$CD4^+$	$CD8^+$	$CD56^+$	$TCR\alpha\beta^+$	$TCR\gamma\delta^+$	$Perforin^+$
G 1	83	40	31	6	71	7	4·5
2	86	24	43	6	82	9	8·0
3	90	49	27	3	64	2	0·5
4	86	44	31	7	80	5	0·5
5	79	50	31	4	83	5	1·0
H 1	84	38	43	4	68	3	5·5
2	67	36	33	7	66	2	1·5

G 1–5 and H 1, 2, TIL from five Graves' (G) and two Hashimoto's (H) disease glands. In each instance at least 200 TIL were assessed to obtain independently the frequency of a surface marker-positive (red fluorescence) or perforin-positive (green fluorescence) cells.

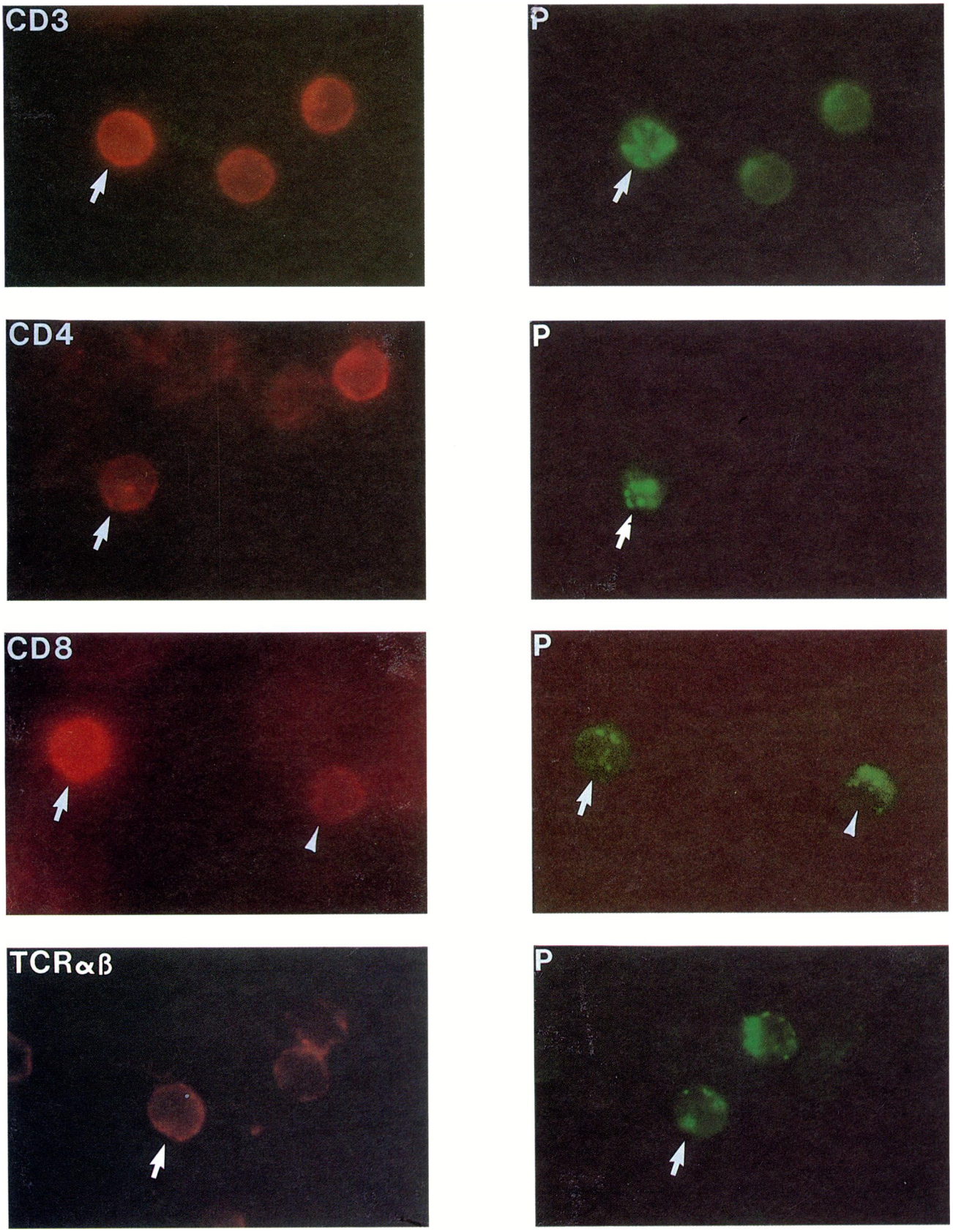

Fig. 1. Phenotypic characterization of thyroid-infiltrating lymphocytes (TIL) by two-colour immunofluorescence. The series of photomicrographs on the left show staining for surface markers as indicated (CD3, CD4, CD8, TCR$\alpha\beta$). Photomicrographs on the right show paired staining with anti-perforin (P). Arrows denote cells positive for a specified marker and perforin. In panel CD8 one cell is $CD8^+$ bright and one $CD8^+$ dim (smaller arrow). (Original magnification ×1000.)

Table 2. Surface antigens expressed by perforin-containing lymphocytes from Graves' and Hashimoto's disease glands and from synovial fluid from patients with rheumatoid arthritis as determined by two-colour immunofluorescence analysis

	Per cent positive cells						
Ly	$CD3^+$	$TCR\alpha\beta^+$	$TCR\gamma\delta^+$	$CD4^+$	$CD8^{+\,bright}$	$CD8^{+\,dim}$	$CD56^+$
G 1	86	60	10	0	11	8	22
2	88	75	5	0	14	9	9
3	86	79	8	0	14	6	11
4	88	82	8	0	12	12	13
5	80	82	1	0	20	5	7
H 1	88	72	6	9	42	8	8
2	78	74	5	12	40	21	14
RA 1	29	27	0	0	4	18	75
2	16	16	1	0	10	24	85
3	16	13	2	0	10	19	85
4	12	12	1	0	6	24	87

Ly, infiltrating lymphocytes; G1–5 and H1, 2, thyroid-infiltrating lymphocytes from Graves' (G) and Hashimoto's (H) disease glands; RA 1–4, lymphocytes from synovial fluid from patients with rheumatoid arthritis (RA). In each instance, values are from the analyses of at least 100 lymphocytes that contained perforin.

bovine serum albumin (BSA), quenched by 0·75% glycine (Sigma), and incubated for 20 min with 1% normal goat serum in PBS–BSA, that was then decanted. Slides were next incubated with control mouse IgG, or the antibodies indicated, for 2 h, washed, and then incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Southern Biotech Associates, Inc., Birmingham, AL). After rinsing, the slides were further incubated overnight at 4°C with biotinylated anti-perforin MoAb, or biotinylated mouse IgG as a control, followed by streptavidin–FITC (Sigma) staining. In each experiment perforin-containing YT cells [20] served as a positive control.

Using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) and magnification ×1000, analyses were carried out by counting at least 200 TIL for phenotype determination and 100 perforin-positive cells for their phenotype. The final calculation of the distribution of perforin-positive cells among different phenotypes was based on the following assumptions: (i) all $\gamma\delta^+$ cells are part of the double-negative T cell population; and (ii) all $CD8^{+\,bright}$, as well as $CD4^+$ cells, are also $CD3^+$.

Cell sorting and analysis of $CD4^-CD8^-$ double-negative T cells

PBMC from three normal individuals were incubated with anti-CD4 and anti-CD8 MoAbs for 30 min at 4°C. The cells were washed and subsequently stained with FITC-conjugated goat anti-mouse IgG (Southern Biotech Associates). After three washes with PBS–BSA at 4°C, the cells were incubated with biotinylated anti-CD3 (Becton Dickinson, Mountain View, CA), followed by PE-conjugated streptavidin (Southern Biotech Associates). Cells were sorted using a FACStar Plus (Becton Dickinson) and the purity of separated $CD3^+CD4^-CD8^-$ cells was checked by repeat flow cytometry. These cells were further fixed, cytospun, and incubated with anti-perforin, anti-$TCR\alpha\beta$ or anti-$\gamma\delta$ MoAbs, then stained with FITC-conjugated goat anti-mouse IgG and assessed by microscopy for their phenotype.

Statistical analysis

For assessment of statistical significance of differences the data were analysed by Student's *t*-test.

RESULTS

Phenotypes of thyroid-infiltrating lymphocytes

MoAbs to several surface antigens were used to assess the phenotype of the infiltrating lymphocytes. As shown in Table 1, the TIL were predominantly $CD3^+$, with a distribution of 24–50% $CD4^+$ and 27–43% $CD8^+$ cells. The individual CD4/CD8 ratios were higher for Graves' TIL with the exception of G2. (To exclude possible inaccuracy of the method employed, the TIL from G2 were subjected to analysis by flow cytometry and the results obtained by microscopy, as shown in Table 1, were confirmed; the percentages for $CD3^+$, $CD4^+$ and $CD8^+$ cells by the two methods were 85/86, 25/24 and 40/43.) On average, for all seven TIL, the population of NK cells ($CD56^+$), $TCR\alpha\beta^+$ and $\gamma\delta^+$ T cells accounted for 5·3%, 73·4% and 4·7%. As per cent of total $CD3^+$ cells, values (mean ± s.e.m.) for $TCR\alpha\beta^+$ and $\gamma\delta^+$ cells were 89·9 ± 4·4% and 5·7 ± 1·1%, while the ratio of $TCR\gamma\delta^+$/$TCR\alpha\beta^+$ cells was 0·061 ± 0·12; these results are comparable to those reported by McIntosh *et al.* for Graves' TIL using flow cytometry [22]. Staining with anti-perforin MoAb showed that 0·5–8% of all infiltrating cells expressed perforin in Graves' and 1·5–5·5% in Hashimoto's glands. In short, there were no predominant phenotypes among TIL that could be taken as characteristic for either disease.

Table 3. Distribution of perforin-positive cells among different phenotypes

Phenotype	Per cent perforin-positive cells (mean ± s.e.m.)† Graves' ($n = 5$)	Hashimoto's ($n = 2$)	RA ($n = 4$)
$CD3\alpha\beta^+$ $CD4^-CD8^-$	61·4 ± 3·5	21, 22	9·8 ± 4·6*
$CD3\gamma\delta^+$ $CD4^-CD8^-$	6·4 ± 1·6	6, 5	1·0 ± 0·4
$CD3\alpha\beta^+$ $CD8^{+bright}$	14·2 ± 1·6	42, 40	7·5 ± 1·5**
$CD3\alpha\beta^+$ $CD4^+$	0	9, 12	0
$CD56^+$ $CD8^{+dim}$	12·4 ± 2·6	8, 21	83·0 ± 2·7*

P values for RA *versus* Graves': * <0·001; **<0·02.

† The data were compiled under the assumption that all $\gamma\delta^+$ cells are double-negative and that all $CD8^+$ bright and $CD4^+$ are $CD3^+$.

RA, rheumatoid arthritis.

Phenotypes of perforin-containing thyroid-infiltrating lymphocytes

Characterization of the phenotype of perforin-containing cells was by two-colour fluorescence staining and representative data (TIL from H 1, Table 1) are shown in Fig. 1. Table 2 summarizes the surface antigens expressed by perforin-containing cells from five patients with GD. $CD3^+$ T cells contributed 80–86% to the perforin-positive cells. Only a small fraction of these T cells (1–10%) was $TCR\gamma\delta^+$, the majority expressing the $\alpha\beta$ receptor. Significantly, the large majority of $CD3^+$ T cells containing perforin was double-negative, $TCR\alpha\beta^+$. This can be concluded from the complete absence of the CD4 marker and the presence of only 11–20% of $CD8^{+bright}$ cells. ($CD8^{+dim}$ cells are likely to belong to the NK cell population [23]; they were included, therefore, in the total of the $CD56^+$ NK cells, shown in Table 3.) Hence $CD3^+CD4^-CD8^-TCR\alpha\beta^+$ cells contributed 61·4% of all perforin-positive cells in the five GD TIL samples analysed (Table 3). The mean ± s.e.m. of the various perforin-positive cells and their assignment by a series of two-colour fluorescence analyses are shown in Table 3.

In the TIL from two patients with HT (Table 2), 40% and 42% of the perforin-containing cells were $CD8^+$ high intensity, indicative of a greater activation of cytotoxic T cells. Of the perforin-positive cells, 9% and 12% stained for CD4, suggesting involvement of a $CD4^+$ cytotoxic component in this disease. Twenty-one per cent and 22% of the cells were double-negative $TCR\alpha\beta^+$ cells (Table 3).

Phenotypes of perforin-containing cells in synovial fluid from knee joints of patients with RA

Our finding of $CD4^-CD8^-$ T cells and $CD4^+$ perforin-containing cells raised the possibility that these phenotypes might also exist in other autoimmune diseases. To evaluate this, we analysed the phenotype of perforin-containing cells in synovial fluids from patients with RA. From the six available samples 10^6–6×10^7 mononuclear cells were obtained, but two of the six had no perforin-containing cells. These two were from patients receiving high doses of immunosuppressive drugs, i.e. methotrexate 20 mg/week and a combination of methotrexate 10 mg/week + prednisone 10 mg/day. In the four samples analysed, between 6% and 17% (12·3 ± 2·3%, mean ± s.e.m.) of the cells were perforin-positive, a value significantly higher ($P < 0·01$) than the combined result obtained with TIL from all seven thyroids (3·1 ± 1·1%, Table 1). As shown in Table 2, most perforin-positive cells were $CD56^+$ NK and 4–10% were $CD8^{+bright}$ CTL. No $CD4^+$ cytotoxic T cells were found. As summarized in Table 3, synovial fluid, compared with Graves' TIL, contained fewer perforin-positive $CD4^-CD8^-TCR\alpha\beta^+$ and $CD8^{+bright}$ cells, and approx. seven-fold more $CD56^+$ cells.

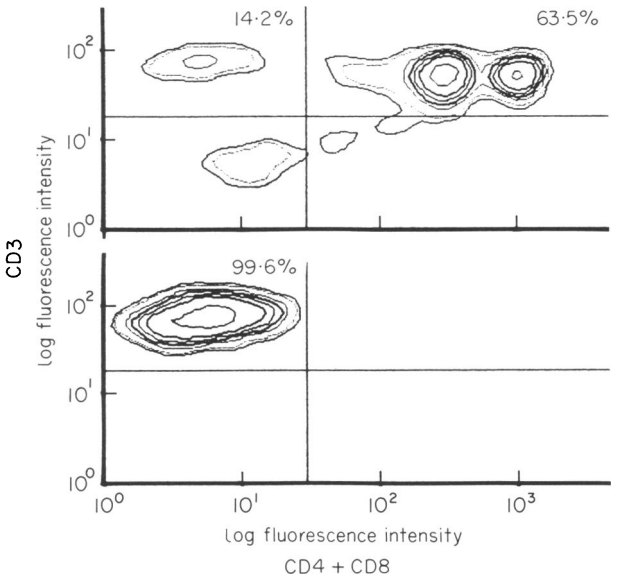

Fig. 2. Separation of $CD4^-$ $CD8^-$ T cells from peripheral blood of a normal subject. In the top panel FACS profiles during sorting show double negative T cells to comprise 14·2% of the total peripheral blood mononuclear cells (PBMC) (11% of all $CD3^+$ cells). In the post-sorting analysis, bottom panel, this population is 99·6% pure.

Expression of perforin in $CD4^-CD8^-$ T cells from peripheral blood of normal subjects

Using PBMC from three normal subjects, $CD3^+$ double-negative T cells were isolated by sorting to determine whether they expressed perforin. Of the $CD3^+$ cells 12·8 ± 6·4% (mean ± s.d.) were double-negative (9·1 ± 5·4% of all PBMC). In the post-sort analyses the double negative T cell population was 99·1 ± 0·8% pure. Representative data are shown in Fig. 2. The

Table 4. Perforin expression and T cell receptor (TCR) phenotypes of $CD4^-CD8^-$ T cells in peripheral blood from normal subjects

No.	Per cent positive cells: $Perforin^+$	$TCR\alpha\beta^+$	$TCR\gamma\delta^+$
1	56	ND	ND
2	51	ND	88
3	71	16	82

$CD4^-CD8^-$ T cells were obtained by sorting (see Fig. 2). ND, Not done.

double-negative T cells expressed perforin at a frequency of 51–71% and they were 82–88% TCR$\gamma\delta^+$ (Table 4). Peripheral blood of normal subjects therefore does not seem to contain a significant subpopulation of perforin-positive $CD4^-CD8^-$ TCR$\alpha\beta^+$ T cells that are characteristic of TIL in AITD.

DISCUSSION

We have analysed perforin expression among TIL that have an ill-defined pathogenic role in autoimmune thyroid disease [7,24]. The limited number of samples studied emphasizes the variability in the yield of TIL from Graves' thyroid [3] and the rarity of thyroidectomy in patients with Hashimoto's disease. Using a MoAb to perforin, which was previously shown not to react with human thyroid tissue [20], we found expression of perforin in lymphocytes isolated from AITD glands. The central role of perforin for cytotoxicity is supported by its delicate transcriptional regulation and its restriction to professional killer cells [25]. Perforin expression in lymphocytes infiltrating tissue *in vivo* correlates with the manifestation of T cell-mediated cytotoxicity [26–30]. Therefore, perforin expression in thyroid infiltrates may indicate that perforin-secreting cytotoxic lymphocytes play a role in the pathogenesis of AITD.

Our analysis of the phenotype of perforin-containing cells in autoimmune thyroid disease revealed two important and novel findings. First, $CD4^+$ perforin-containing cells were found only in thyroid infiltrates from HT, suggesting a class II-restricted component of cytotoxicity. Second, perforin-positive T cells of the TCR$\alpha\beta^+CD4^-CD8^-$ phenotype predominate in GD and to a lesser extent in HT. These cells are a minor population in either synovial fluid from patients with RA or in peripheral blood of normal subjects.

$CD4^+$ T cells are generally regarded as helper cells which recognize antigenic peptides presented by MHC class II molecules. On the other hand, they may also be cytotoxic, killing target cells bearing class II molecules and appropriate antigen; for instance, most human $CD4^+$ CTL specific for HIV are perforin-positive [31]. Thyroid epithelial cells *in vivo* express MHC class II molecules on their surface when the gland is infiltrated with lymphocytes [3,32], the intensity of expression paralleling the degree of infiltration [3,33]. In addition, although not found *in vivo* [34], thyrocytes can be induced to express intercellular adhesion molecule-1 [34,35] and are shown to secrete certain cytokines, e.g. IL-1α and IL-6 [36]. Thus, thyroid epithelial cells have the potential to present autoantigen directly to thyroid antigen-specific $CD4^+$ T cells without the requirement for other antigen-presenting cells. The presence of perforin-containing $CD4^+$ lymphocytes may result in the destruction of epithelial cells presenting MHC class II-specific antigen.

Our finding of a high proportion of TCR$\alpha\beta^+CD4^-CD8^-$ T cells expressing perforin in AITD represents the first demonstration that this T cell subpopulation may contribute to the disease. Artefacts are unlikely, since treatment of lymphocytes with collagenase as used for TIL isolation does not decrease the expression of cell surface antigens CD3, CD4 and CD8 [8]. A role for double-negative T cells in AITD has to be considered based on their relationship to autoimmunity [18,37–39], immunodeficiency [40], as well as tumour immunity [41,42]. TCR$\gamma\delta^+$ double-negative T cells are known to express perforin and to mediate cytotoxicity [15], presumably against microbial antigens. The role of TCR$\alpha\beta^+$ double-negative T cells is not clear, and only recently they have been shown to express perforin [18]. Another report [43] indicated that such cells obtained from peripheral blood of normal individuals express a restricted Vβ repertoire and an invariant TCRα chain. This finding is consistent with a unique role of this T cell subpopulation, whose restricting element may be a non-polymorphic class I molecule. The origin of the TCR$\alpha\beta^+$ double-negative T cells in general and those reported here is not known. However, their presence in athymic nude mice and certain properties suggest that they have not been selected in the thymus [44,45]. TCR$\alpha\beta^+$ double-negative T cells have been found in epithelial tissue such as human nasal mucosa [46] and normal skin [47], and in the epidermis of athymic mice [48]; these may be the sites providing extrathymic education to resident double-negative T cells [46–48].

Similar to infiltrating lymphocytes in the pancreas of diabetic mice [26] and during heart allograft rejection [20], only a minor fraction of TIL expressed perforin. Even in acute viral infection in mice, such as hepatitis caused by the hepatotropic LCMV strain, only about 15% of the infiltrating cells express perforin [27]. These cells, however, are critical for removal of the virus and animal survival; perforin-deficient mice die due to their inability to eliminate the virus [49].

For comparative purposes, we also analysed synovial infiltrates from patients with RA. These data offer three points of interest: (i) perforin-containing TCR$\alpha\beta^+$ double-negative T cells were present in this autoimmune disorder, though their percentage was much lower than in AITD; (ii) $CD4^+$ perforin-containing cells were not detected in the synovial fluid, which contrasts with published, but indirect, findings [30]; (iii) most of perforin-containing cells were NK cells, indicating non-specific cytotoxicity at this disease site, while there is antigen-specific cytotoxicity in AITD.

In conclusion, this study provides evidence for perforin expression in thyroid-infiltrating lymphocytes, thus implying a role for T cell-mediated cytotoxicity in AITD *in vivo*. In addition to $CD4^+$ and $CD8^+$ perforin-containing lymphocytes, a large population of TCR$\alpha\beta^+CD4^-CD8^-$ perforin-containing T cells was found *in situ*. Further characterization of these cells in the thyroid gland in AITD and in target organs in other autoimmune diseases may help in understanding their function and contribution to autoimmunity.

ACKNOWLEDGMENTS

We wish to thank Drs R. D. Altman and W. Sunshine for providing the synovial fluid from patients with RA and Mr J. Phillips for his expert assistance in cell sorting. We are grateful to Mrs M. Sanchez for typing the manuscript. This work was supported by NIH grants CA39201 (E.R.P.) and DK31391 (M.Z.). J. M. McK. is the Kathleen and Stanley Glaser Professor of Medicine.

REFERENCES

1 Weetman AP. Autoimmune thyroiditis: predisposition and pathogenesis. Clin Endocrinol 1992; **36**:307–23.

2 McGregor A. Autoimmunity in the thyroid—can the molecular revolution contribute to our understanding? Quart J Med, New Series 1992; **82**:1–13.

3 Levis S, Ghandur-Mnaymneh L, McKenzie JM, Zakarija M. Evaluation of the biological significance of leukocyte infiltration

of the thyroid in Graves' disease. Int Arch Allergy Immunol 1992; **99**:37–43.
4 DeGroot LJ, Quintans J. The cause of autoimmune thyroid disease. Endocr Rev 1989; **10**:537–62.
5 Bene MJ, Derennes V, Faure G, Thomas JL, Duheille J, Leclerc J. Graves' disease: *in situ* localization of lymphoid T cell subpopulations. Clin Exp Immunol 1983; **52**:311–6.
6 Margolick JB, Hsu S-M, Volkman DJ, Burman KD, Fauci AS. Immunohistochemical characterization of intrathyroid lymphocytes in Graves' disease. Am J Med 1984; **76**:815–21.
7 Martin A, Goldsmith NK, Friedman EW, Schwartz AE, Davies TF, Roman SH. Intrathyroidal accumulation of T cell phenotypes in autoimmune thyroid disease. Autoimmunity 1990; **6**:269–81.
8 Kawakami A, Eguchi K, Matsunaga M *et al.* $CD4^+$ $CD45RA^+$ cells (suppressor-inducer T cells) in thyroid tissue from patients with Graves' disease. Acta Endocrinol (Copenh) 1991; **125**:687–93.
9 Iwatani Y, Hidaka Y, Matsuzuka F, Kuma K, Amino N. Intrathyroidal lymphocyte subsets, including unusual $CD4^+CD8^+$ cells and $CD3^{lo}TCR\alpha\beta^{lo/-}CD4^-CD8^-$ cells, in autoimmune thyroid disease. Clin Exp Immunol 1993; **93**:430–6.
10 Ishikawa N, Eguchi K, Otsubo T *et al.* Reduction in the suppressor-inducer T cell subset and increase in the helper T cell subset in thyroid tissue from patients with Graves' disease. J Clin Endocrinol Metab 1987; **65**:17–23.
11 Mackenzie WA, Schwartz AE, Friedman EW, Davies TF. Intrathyroidal T cell clones from patients with autoimmune thyroid disease. J Clin Endocrinol Metab 1987; **64**:818–24.
12 Lichtenheld MG, Olsen KJ, Lu P *et al.* Structure and function of human perforin. Nature 1988; **335**:448–51.
13 Podack ER, Hengartner H, Lichtenheld MG. A central role of perforin in cytolysis. Ann Rev Immunol 1991; **9**:129–57.
14 Lu P, Garcia-Sanz JA, Lichtenheld MG, Podack ER. Perforin expression in human peripheral blood mononuclear cells. Definition of an IL-2-independent pathway of perforin induction in $CD8^+$ T cells. J Immunol 1992; **148**:3354–60.
15 Nakata M, Smyth MJ, Norihisa Y *et al.* Constitutive expression of pore-forming protein in peripheral blood γ/δ T cells: implication for their cytotoxic role *in vivo*. J Exp Med 1990; **172**:1877–80.
16 Podack ER. T-cell effector functions: mechanisms for delivery of cytotoxicity and help. Annu Rev Cell Biol 1991; **7**:479–504.
17 Griffiths GM, Mueller C. Expression of perforin and granzymes *in vivo*: potential diagnostic markers for activated cytotoxic cells. Immunol Today 1991; **12**:415–9.
18 Hammond DM, Nagarkatti PS, Goté LR, Seth A, Hassuneh MR, Nagarkatti M. Double-negative T cells from MRL-*lpr*/*lpr* mice mediate cytolytic activity when triggered through adhesion molecules and constitutively express perforin gene. J Exp Med 1993; **178**:2225–30.
19 McLachlan SM, Pegg CAS, Atherton MC *et al.* Subpopulations of thyroid autoantibody secreting lymphocytes in Graves' and Hashimoto thyroid glands. Clin Exp Immunol 1986; **65**:319–28.
20 Hameed A, Olsen KJ, Cheng L, Fox WM III, Hruban RH, Podack ER. Immunohistochemical identification of cytotoxic lymphocytes using human perforin monoclonal antibody. Am J Pathol 1992; **140**:1025–9.
21 Harlow E, Lane D. Antibodies. A laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1988.
22 McIntosh RS, Tandon N, Pickerill AP, Davies R, Barnett D, Weetman AP. The $\gamma\delta$ T cell repertoire in Graves' disease and multinodular goitre. Clin Exp Immunol 1993; **94**:473–7.
23 Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of Leu-7 (HMK-7) and Leu-11 (NK-15) antigens. J Immunol 1983; **131**:1789–96.
24 Dayan CM, Londei M, Corcoran AE *et al.* Autoantigen recognition by thyroid-infiltrating T cells in Graves' disease. Proc Natl Acad Sci USA 1991; **88**:7415-19.
25 Lichtenheld MG, Podack ER. Structure and function of the murine perforin promoter and upstream region: reciprocal gene activation or silencing in perforin positive and negative cells. J Immunol 1992; **149**:2619–26.
26 Young LHY, Peterson LB, Wicker LS, Persechini PM, Young JD-E. *In vivo* expression of perforin by $CD8^+$ lymphocytes in autoimmune disease. Studies on spontaneous and adoptively transferred diabetes in nonobese diabetic mice. J Immunol 1989; **143**:3994–9.
27 Müller C, Kägi D, Aebischer T *et al.* Detection of perforin and granzyme A mRNA in infiltrating infection of mice with lymphocytic choriomeningitis virus. Eur J Immunol 1989; **19**:1253–9.
28 Young LHY, Joag SV, Zheng L-M, Lee C-P, Lee Y-S, Young JD-E. Perforin-mediated myocardial damage in acute myocarditis. Lancet 1990; **336**:1019–21.
29 Griffiths GM, Namikawa R, Mueller C *et al.* Granzyme A and perforin as markers for rejection in cardiac transplantation. Eur J Immunol 1991; **21**:687–92.
30 Griffiths GM, Alpert S, Lambert E, McGuire J, Weissman IL. Perforin and granzyme A expression identifying cytolytic lymphocytes in rheumatoid arthritis. Proc Natl Acad Sci USA 1992; **89**:549–53.
31 Podack ER, Deng G, Bowen MA *et al.* T-cell mediated cytotoxicity. In: Gergely J, Benczúr M, Erdei A *et al.*, eds. Progress in immunology VIII. Budapest: Springer Hungarica, 1992: 255–9.
32 Lucas-Martin 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 Metab 1988; **66**:367–75.
33 Jansson R, Karlsson A, Forsum U. Intrathyroidal HLA-DR expression and T lymphocyte phenotypes in Graves' thyrotoxicosis, Hashimoto's thyroiditis and nodular colloid goitre. Clin Exp Immunol 1986; **58**:264–72.
34 Ciampolillo A, Napolitano G, Mirakian R, Miyasaki A, Giorgino R, Bottazzo GF. Intercellular adhesion molecule-1 (ICAM-1) in Graves' disease: contrast between *in vivo* and *in vitro* results. Clin Exp Immunol 1993; **94**:478–85.
35 Weetman AP, Cohen S, Makgoba MW, Borysiewicz LK. Expression of an intercellular adhesion molecule, ICAM-1, by human thyroid cells. J Endocrinol 1989; **122**:185–91.
36 Grubeck-Loebenstein B, Buchan G, Chantry D *et al.* Analysis of intrathyroidal cytokine production in thyroid autoimmune disease: thyroid follicular cells produce interleukin-1 alpha and interleukin-6. Clin Exp Immunol 1989; **77**:324–30.
37 Cohen PL, Eisenberg RA. The *lpr* and *gld* genes in systemic autoimmunity: life and death in the Fas lane. Immunol Today 1992; **13**:427–8.
38 Shivakumar S, Tsokos GC, Datta SK. T cell receptor α/β expressing double-negative ($CD4^-/CD8^-$) and $CD4^+$ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. J Immunol 1989; **143**:103–12.
39 Sneller MC, Straus SE, Jaffe ES *et al.* A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. J Clin Invest 1992; **90**:334–41.
40 Wirt DP, Brooks EG, Vaidya S, Klimpel GR, Waldemann TA, Goldblum RM. Novel T-lymphocyte population in combined immunodeficiency with features of graft-*versus*-host disease. N Engl J Med 1989; **321**:370–4.
41 Amano T, Katagiri S, Tominaga N *et al.* Growth inhibition of RPMI 8226 human myeloma cells by peripheral blood lymphocytes. Acta Haematol 1992; **87**:37–44.
42 Mieno M, Suto R, Obata Y *et al.* $CD4^-CD8^-$-T cell receptor $\alpha\beta$ T cells: generation of an *in vitro* major histocompatibility complex class I specific cytotoxic T lymphocyte response and allogeneic tumor rejection. J Exp Med 1991; **174**:193–201.
43 Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell

antigen receptor (TCR) expression by human peripheral blood $CD4^-8^-$ α/β T cells demonstrates preferential use of several $V\beta$ genes and an invariant TCRα chain. J Exp Med 1993; **178**:1–16.

44 Rocha B. Characterization of $V\beta$-bearing cells in athymic (*nu/nu*) mice suggests an extrathymic pathway for T cell differentiation. Eur J Immunol 1990; **20**:919–25.

45 Abo T. Extrathymic pathways of T-cell differentiation: a primitive and fundamental immune system. Microbiol Immunol 1993; **37**:247–58.

46 Okuda M, Pawankar R. Flow cytometric analysis of intraepithelial lymphocytes in the human nasal mucosa. Allergy 1992; **47**:255–9.

47 Groh V, Fabbi M, Hochstenbach F, Maziarz RT, Strominger JL. Double-negative ($CD4^-CD8^-$) lymphocytes bearing T-cell receptor α and β chains in normal human skin. Proc Natl Acad Sci USA 1989; **86**:5059–63.

48 Payer E, Strohal R, Kutil R, Elbe A, Stingl G. Demonstration of a $CD3^+$ lymphocyte subset in the epidermis of athymic nude mice. J Immunol 1992; **149**:413–20.

49 Kägi D, Ledermann B, Bürki K *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 1994; **369**:31–37.