Clin. exp. Immunol. (1977) **30**, 193–199.

Blood and thyroid-infiltrating lymphocyte subclasses in juvenile autoimmune thyroiditis

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(*Received* 13 *June* 1977)

SUMMARY

We have studied the distribution of T and B lymphocytes in the blood and in the thyroid lymphocytic infiltrates (obtained by fine needle aspiration biopsy) in sixteen patients with juvenile autoimmune thyroiditis (JAIT). The same cell populations were also tested for cell-mediated immunity (CMI) to thyroid antigen in the leucocyte migration test (LMT). The relative and absolute numbers of blood T lymphocytes were normal (71–76%), as were the numbers of blood B lymphocytes (19%). The thyroid infiltrate contained 48% B lymphocytes, whereas only 40–44% of the infiltrating lymphocytes were T cells. Half of the JAIT patients showed a positive CMI to thyroid antigen with blood leucocytes, but when thyroid-infiltrating lymphocytes of these patients were tested in the LMT, they were negative. Thus, in contrast to what is generally assumed, we were unable to demonstrate T cell-dominated lymphocytic infiltrates or the accumulation of specifically sensitized T lymphocytes within the thyroid gland in autoimmune thyroiditis.

INTRODUCTION

Juvenile autoimmune thyroiditis (JAIT) shares most of the features of adult thyroiditis, i.e. Hashimoto's disease and atrophic thyroiditis (Nilsson & Doniach, 1964; Mäenpää, 1972; Rallison *et al.*, 1975). Patients with JAIT develop a goitre at an early age, or the thyroid is atrophic. The clinical course is less fulminant than in adult thyroiditis, euthyroidism is the rule and spontaneous remissions are frequent (Rallison *et al.*, 1975). Circulating thyroid antibodies are demonstrable in most of the patients (Doniach, Nilsson & Roitt, 1965; Loeb, Drash & Kenny 1973; Doniach, 1975). The histological picture of the thyroid is dominated by a diffuse interstitial lymphocytic infiltrate (Nève, Ermans & Bastenie, 1972), frequently organized as lymphoid follicles with germinal centres (Stowens, 1966). Askanazy cells are more sparse than in adult Hashimoto's thyroiditis (Persson & Schnürer, 1968), and signs of epithelial destruction are not obvious.

We have investigated the immunopathogenic mechanisms in JAIT by determining the relative and absolute numbers of T and B lymphocytes in the blood and in the thyroid infiltrates. The same cell populations were also tested for their ability to display cell-mediated immunity (CMI) to thyroid antigen *in vitro*. Normal numbers of T and B lymphocytes were found in the blood of these patients, whereas—in contrast to what is previously assumed (Volpé *et al.*, 1974)—B cells dominated slightly over the T cells in the thyroid infiltrates. Although CMI was readily demonstrable in the blood of approximately half of the patients with JAIT, the thyroid-infiltrating lymphocytes of the same patients were negative in this respect.

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MATERIALS AND METHODS

Patients. Sixteen patients were studied (Table 1). The consent of the parents was obtained for this study. The diagnostic criteria are included in Table 1. The diagnosis of JAIT was based on (a) palpation findings of a firm goitre, (b) elevated circulating thyroid antibody titres, and (c) the cytological findings in a fine needle aspiration biopsy (Nève *et al.*, 1972). In three patients the biopsy was not representative, but as these patients displayed high antibody titres and an enlarged firm gland, they were included in the study.

Collection and preparation of thyroid-infiltrating lymphocytes. For the analysis of thyroid-infiltrating cells, fine needle aspiration biopsies were performed using a 0·7 mm outer diameter needle from both thyroid lobes. No complications from the procedure were observed. One drop of material was immediately spread onto a microscope slide, and the rest diluted in 3 ml of a medium consisting of phosphate-buffered saline (pH 7·2) with 0·5% (v/v) bovine serum albumin, preservative-free heparin (12·5 iu/ml) and 2·5 mM HEPES buffer (Sigma Co., U.S.A.). Mononuclear cells were separated from contaminating erythrocytes and parenchymal cells by density centrifugation over 1·083 g/cm^3 Ficoll–Isopaque (Ficoll–Paque, Pharmacia, Sweden). The yield varied from 0·1 to $4{\cdot}3 \times 10^6$ infiltrating lymphoid cells per sample. The degree of blood contamination was calculated from the blood and thyroid smears according to the following formula:

$$\text{index of blood contamination, IC} = \frac{\text{lymphocytes/polymorphonuclear leucocytes in thyroid aspirates}}{\text{lymphocytes/polymorphonuclear leucocytes in blood}}.$$

Biopsies with an IC less than 3·0 were not considered representative.

Preparation of blood lymphocytes. Blood mononuclear cells were equally recovered by one-step centrifugation over Ficoll–Isopaque (Böyum, 1968). We have earlier established that no subclass-specific cell losses take place during the procedure (Häyry, Tötterman & Ranki, 1977).

Identification of T lymphocytes. T cells were detected histochemically by their expression of the acid α-naphthyl acetate esterase (ANAE) activity, as described by Mueller *et al.* (1975) with some modifications (Ranki, Tötterman & Häyry, 1976). Depending on the cell yields, T cells were also identified by rosette formation with 2-amino-ethyl-isothiouronium-bromide hydrobromide-treated sheep erythrocytes (AET-SRBC-RFC), as according to Pellegrino *et al.* (1975).

Identification of B lymphocytes. The surface immunoglobulin (S-Ig) bearing (B) lymphocytes were detected by rosetting the anti-Ig-treated cells with *Staphylococcus aureus* bacteria, strain Cowan 1 (StaCw-RFC), as described in detail by Ranki *et al.* (1976).

Identification of Fc receptor-carrying lymphocytes. Lymphocytes with receptors for the Fc part of IgG were quantified by rosette formation with antibody-coated human O erythrocytes (EA-RFC), as according to Fröland & Natvig (1973). Most of these cells are probably B lymphocytes (Basten *et al.*, 1972).

All methods using rosette formation with indicator particles were adapted to microscale as described earlier (Ranki *et al.*, 1976). The marker-carrying cells were visualized by staining the smears or cytocentrifuged cell preparations with May–Gruenwald–Giemsa, and it was therefore possible to exactly distinguish between lymphocytes and contaminating non-lymphoid cells, such as monocytes and tissue cells.

Leucocyte migration test (LMT). The leucocyte migration inhibition test of Søborg & Bendixen (1967) was used with minor modifications as an *in vitro* correlate of CMI. The characteristics of our LMT are given elsewhere (Mäkinen *et al.*, 1977). A crude thyroid homogenate of a Graves' disease goitre obtained at operation was used as antigen. The protein concentration in the test was 360 μg/ml of culture medium. The migration areas were measured by planimetry and the results calculated using the formula:

$$\text{migration index (MI) (\%)} = \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}} \times 100.$$

When LMT's were performed with the thyroid infiltrates, the small yield of cells necessitated mixing of the thyroid-infiltrating lymphocytes with blood leucocytes from the same patient at ratios 1:3, 1:6, 1:12, and 1:24.

Controls. The distribution of circulating lymphocyte subclasses was studied in seven healthy control subjects (mean age 20 years; range: 19–23) and the leucocyte migration test in twenty subjects (mean age 33; range: 29–41) with no signs of thyroid disease. Fine needle aspiration biopsies were performed peroperatively in three patients with normal thyroid glands undergoing parathyroidectomy. The consent of the patients was obtained.

RESULTS

Effect of density purification on the lymphocyte subclass distribution

It was important to ensure that the methods used in the preparation of lymphocytes from the thyroid gland did not cause selective depletion of the lymphocyte subclasses. Smears of peripheral blood and thyroid aspirate were stained for ANAE, and the mononuclear cells were separated by density centrifugation over Ficoli–Isopaque. Cytocentrifuged preparations were made from the input cells and from the

TABLE 1. Clinical and laboratory findings and the leucocyte migration test (LMT) in sixteen patients with juvenile autoimmune thyroiditis

Patient	Sex	Age (years)	Goitre	Original clinical state*	Follow-up time (years)	Therapy; thyroxine (μg/day)	S-T4† (μmol/l) (65–160)	S-TSH† (mu/l) (1·6–6·9)	Highest antibody titre‡		Cytology in fine needle aspirate§	LMT¶ (MI, %)
									TgA	MsA		
L.A.	F	17	+	eu.	2	0	66	7·3	20	0	l.t.	*79*
H.H.	F	12	+	eu.	2	150	102	6·0	0	0	l.t.	97
V.M.	F	11	+	eu.	2	0	102	12·3	0	10^4 (F)	n.r.	*73*
A.V.	F	16	+	eu.	5	100	122	2·4	20	64	l.t.	90
T.S.	M	12	+	eu.	1	0	121	13·7	250	10^6 (F)	n.r.	*76*
M.S.	F	9	+	hypo.	0	0	36	76·0	0	0	l.t.	88
S.K.	F	16	+	eu.	0	0	94	1·3	100	0	l.t.	*78*
T.H.	F	14	+	eu.	0	0	117	17·5	0	10^6 (F)	n.r.	*81*
U.T.	F	13	+	eu	0	100	81	7·1	0	0	l.t.	*82*
P.H.	F	15	+	eu.	3	150	120	2·1	40	0	l.t.	*78*
M.R.	F	18	+	eu.	7	0	104	3·3	40	0	l.t.	*86*
S.G.	F	17	+	eu.	7	100	76	2·9	$2{\cdot}5\times10^5$	0	l.t.	92
J.S.	F	17	+	eu.	3	100	152	3·1	1600	0	l.t.	94
T.S.	F	17	+	eu.	3	100	68	5·0	50	4000	l.t.	90
P.A.	F	14	+	eu.	0	0	82	5·6	0	0	l.t.	88
M.J.	F	13	+	eu.	2	0	65	16·5	400	0	l.t.	100
Mean ± s.d.		14 ± 3										86 ± 8

* eu. = Euthyroid; hypo. = hypothyroid.

† S-T4 = serum thyroxine; S-TSH = thyrotrophin. Both determined by radioimmunoassay.

‡ TgA = thyroglobulin antibody; MsA = microsomal antibody. Titres determined according to Roitt & Doniach (1958). F = determined with the Fujizoki kit (Perrin & Bubel, 1974).

§ l.t. = lymphocytic thyroiditis; n.r. = not representative.

¶ Figures in italics = positive in the LMT.

separated cells, and the preparations were also stained for ANAE. In agreement with our previous study on blood lymphocyte separation (Häyry *et al.*, 1977), density purification of thyroid-infiltrating lymphocytes had no effect on the relative number of ANAE-positive (T) lymphocytes under the conditions used (Table 2).

Absolute and relative numbers of blood lymphocyte subclasses

There were no significant differences in the number of T lymphocytes (lymphocytes expressing the ANAE and AET-SRBC-RFC markers) or B lymphocytes (StaCw-RFC) in JAIT and in the control group (Table 3). Neither were significant differences observed in the frequency of Fc receptor-carrying lymphocytes (EA-RFC) between these groups. The absolute number of blood lymphocytes in JAIT was $2{\cdot}48 \pm 0{\cdot}84 \times 10^9$/l (mean ± s.d.) and in the control group it was $2{\cdot}52 \pm 0{\cdot}95 \times 10^9$/l. We therefore conclude that also the absolute number of different marker-carrying lymphocytes is normal in JAIT.

TABLE 2. Lack of subclass selection during density purification of blood and thyroid-infiltrating lymphocytes of patients with juvenile autoimmune thyroiditis

	ANAE-positive lymphocytes (%)			
	Blood		Thyroid infiltrate	
Patient	Before density centrifugation	After density centrifugation	Before density centrifugation	After density centrifugation
U.T.	85	85	42	45
P.H.	81	80	51	49
S.G.	77	79	41	38
J.S.	70	68	25	22
Mean ± s.d.	78 ± 6	78 ± 6	40 ± 9	39 ± 10

Subclass distribution of thyroid-infiltrating lymphocytes

The subclass distribution of thyroid-infiltrating lymphocytes in JAIT is also shown in Table 3. On three occasions the IC was less than 3·0. Due to blood contamination these aspirates were not considered representative (Tables 1 and 3). Depending on the cell yield, one or more lymphocyte markers were determined from the rest of the aspirates. On average, 44 ± 12% of thyroid-infiltrating cells expressed the ANAE marker and 40 ± 14% formed rosettes with AET-SRBC and were considered T cells. 48 ± 8 % of thyroid-infiltrating lymphocytes carried detectable amounts of surface Ig, and 42 ± 13% expressed the Fc receptor and were considered B cells. When fine needle aspiration biopsies were performed on three normal control subjects the IC was always <3·0, and due to blood contamination the percentage of ANAE-positive lymphocytes in these thyroid aspirates was equal to that of blood.

LMT with blood leucocytes

The results of the LMT with blood leucocytes are shown in Table 1. With thyroid antigen, the mean leucocyte migration index (MI) in the sixteen JAIT patients was 86 ± 8% and differed significantly ($P<0{\cdot}01$) from the mean of the twenty healthy controls (93 ± 3%). In eight cases (50%) the MI was below the normal control limit (87%) (mean MI for the controls ± 2 s.d.), and these patients were considered to display a positive LMT. In general, the inhibition of leucocyte migration in JAIT patients was relatively weak. No correlation to the TgA or MsA titres or to the follow-up time was found.

Effect of thyroid-infiltrating lymphocytes in the LMT

The LMT was performed with thyroid-infiltrating cells in three patients (L.A., S.K. and U.T.) showing a significant inhibition in the LMT with blood leucocytes and a high yield of mononuclear cells

TABLE 3. Relative distribution of blood and thyroid-infiltrating lymphocyte subpopulations in juvenile autoimmune thyroiditis

		Blood				Thyroid infiltrate				
		T lymphocytes (%)		B lymphocytes (%)			T lymphocytes (%)		B lymphocytes (%)	
Patient	Sex	ANAE	AET-SRBC-RFC	S-Ig (StaCw-RFC)	Fc (EA-RFC)	IC*	ANAE	AET-SRBC-RFC	S-Ig (StaCw-RFC)	Fc (EA-RFC)
L.A.	F	67	n.d.†	20	24	14·4	50	n.d.	45	36
H.H.	F	72	n.d.	28	25	5·8	55	n.d.	50	56
V.M.	F	76	n.d.	n.d.	n.d.	< 3·0	n.d.	n.d.	n.d.	n.d.
A.V.	F	84	84	20	14	63·0	68	72	36	30
T.S.	M	74	n.d.	n.d.	n.d.	< 3·0	n.d.	n.d.	n.d.	n.d.
M.S.	F	80	87	18	n.d.	14·0	52	42	54	n.d.
S.K.	F	87	80	18	12	3·4	59	45	45	32
T.H.	F	75	73	17	17	< 3·0	n.d.	n.d.	n.d.	n.d.
U.T.	F	85	80	14	16	115·4	42	41	54	37
P.H.	F	81	70	21	22	40·8	51	35	33	34
M.R.	F	71	56	19	22	20·3	30	27	55	59
S.G.	F	77	59	20	24	85·7	38	n.d.	53	n.d.
J.S.	F	70	68	19	12	8·0	25	26	51	40
T.S.	F	60	62	18	15	53·1	32	25	62	67
P.A.	F	73	54	18	n.d.	86·2	31	n.d.	54	n.d.
M.J.	F	78	73	17	20	125·0	44	50	37	32
Mean ± s.d.‡		76 ± 7 (16)	71 ± 11 (12)	19 ± 3 (14)	19 ± 5 (12)		44 ± 12 (13)	40 ± 14 (9)	48 ± 8 (13)	42 ± 13 (10)
Control mean ± s.d.‡		77 ± 4 (7)	71 ± 4 (7)	21 ± 4 (7)	17 ± 3 (7)					
P§		> 0·1	> 0·1	> 0·1	> 0·1					

* IC = Index of blood contamination.

† n.d. = Not done.

‡ The numbers in parentheses show the size of sample.

§ *P* value for differences in means. Student's two-tailed *t*-test.

TABLE 4. Lack of thyroid antigen-induced leucocyte migration inhibition by thyroid-infiltrating lymphocytes

	Migration index (%)				
	Blood leucocytes	Thyroid-infiltrating lymphocytes: blood leucocytes*			
Patient	only	1:24	1:12	1:6	1:3
L.A.	*79*†	n.d.‡	*84*	n.d.	n.d.
S.K.	*78*	n.d.	89	n.d.	n.d.
U.T.	*82*	95	n.d.	98	101

* The ability of thyroid-infiltrating lymphocytes to respond in the LMT was tested by mixing them at the indicated ratios with the patient's blood leucocytes. Control migration without antigen was performed with a cell mixture of 1:24; the addition of the thyroid-infiltrating cells did not alter the migration of blood leucocytes.

† Figures in italics = positive in the LMT.

‡ n.d. = Not done.

in the thyroid fine needle aspiration biopsy. The thyroid-infiltrating cells were mixed with blood leucocytes of the very same patient, and the LMT was performed as described in the Materials and Methods section. As seen in Table 4, mixing of thyroid-infiltrating lymphocytes with blood leucocytes at concentrations 1:3, 1:6, 1:12 and 1:24 did not increase the inhibition. In fact, the mixtures were less inhibited than were blood leucocytes on their own.

DISCUSSION

There is ample evidence that autoimmune mechanisms operate in chronic lymphocytic thyroiditis. Elevated titres of anti-thyroid antibodies are frequently found in Hashimoto's disease and atrophic thyroiditis (Doniach, 1975), and the thyroid gland is infiltrated with lymphoid cells (Nève *et al.*, 1972). The involvement of cell-mediated autoimmunity has been emphasized by the finding that the LMT is positive in most cases of Hashimoto's thyroiditis (Calder & Irvine, 1975), primary hypothyroidism (Amino & de Groot, 1975) and symptomless autoimmune thyroiditis (Tötterman, Mäkinen & Gordin, 1977). The numbers of circulating T and B lymphocytes have been reported to be normal (Urbaniak, Penhale & Irvine, 1974; Calder *et al.*, 1976; Hackenberg, Cohnen & Reinwein, 1976) or elevated (Farid *et al.*, 1973a; Aoki, Wakisaka & Nagata, 1973). The central role of T lymphocytes in the pathogenesis of these diseases has been strongly emphasized (Volpé *et al.*, 1974) although some of the observations, such as the finding of elevated numbers of T lymphocytes in the blood of these patients (Farid *et al.*, 1973a) and in the thyroid infiltrates (Farid, Row & Volpé, 1973b) may be inconsistent (Volpé & Row, 1975).

In agreement with most reports on adult autoimmune thyroiditis, we found a normal distribution of T and B lymphocytes in the blood of JAIT patients. The proportion of B cells was clearly higher in the thyroid infiltrate than in the blood, and in most cases exceeded 50% of the infiltrating lymphocytes. This finding is not surprising when the microscopic features of the disease are considered. In adult Hashimoto's disease especially, but also in JAIT, the gland contains plasma cells and germinal centres. Along with the formation of organized lymphoid tissue in the thyroid, both T- and B-dependent areas are recognized (Söderström & Biörklund, 1974).

Half of the JAIT patients showed a positive LMT with thyroid antigen, indicating the presence of circulating sensitized T cells in the blood. When thyroid-infiltrating lymphocytes were tested in the LMT by mixing them with autologous blood leucocytes, a weaker inhibition of the leucocyte migration was observed. This suggests a lack of thyroid antigen-reactive T cells inside the gland, or alternatively that such cells are present in a functionally inactive state: blocked by, for example, an excess of antigen.

Further functional tests are therefore required to specify possible immunopathogenic roles of the T and B cells infiltrating the thyroid gland.

Finally, we emphasize the usefulness of the fine needle aspiration biopsy in the study of subclasses and functions of immunocompetent cells infiltrating various target organs.

This study was supported by grants from the Sigrid Juselius Foundation and Finska Läkaresällskapet, Helsinki, National Institutes of Health, U.S.A. and DGRST, France.

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