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CIRCULATING LYMPHOCYTE SUBPOPULATIONS IN HASHIMOTO THYROIDITIS

S. J. URBANIAK, W. J. PENHALE AND W. J. IRVINE

Department of Endocrinology, Royal Infirmary and Department of Therapeutics, University, Edinburgh

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SUMMARY

Peripheral blood and T and B lymphocytes and [^{125}I]thyroglobulin-binding lymphocytes were investigated in twenty-two euthyroid Hashimoto thyroiditis patients and in twenty-two age- and sex-matched normal subjects. Although the total lymphocyte count in Hashimoto patients (mean $\pm$ SEM $= 1226 \pm 187/mm^3$) was lower than in normal subjects ($1603 \pm 156/mm^3$) this difference was not statistically significant. There was, however, a statistically significant reduction in the proportion of circulating T lymphocytes in the Hashimoto patients (mean $\pm$ SEM $= 57{\cdot}4 \pm 2{\cdot}5\%$) as assessed by the sheep red-cell rosette method when compared with the normal controls (mean $\pm$ SEM $= 66{\cdot}7 \pm 1{\cdot}8\%$). The proportion of B lymphocytes in the peripheral blood as assessed by indirect immunofluorescence, was not significantly different being $21{\cdot}6 \pm 2{\cdot}1\%$ in the Hashimoto patients and $20{\cdot}2 \pm 1{\cdot}1\%$ in normal subjects.

[^{125}I]thyroglobulin-binding lymphocytes, as assessed by autoradiography were present in the circulation of nineteen Hashimoto patients with a mean frequency of $8{\cdot}37 \pm 1{\cdot}15/10^4$ lymphocytes and in thirteen normal subjects with a mean of $8{\cdot}84 \pm 0{\cdot}93/10^4$ lymphocytes. There was no difference in the degree of [^{125}I]thyroglobulin binding between the two groups as determined by grain count analysis. There was no apparent correlation between age or thyroglobulin antibody titres and the frequency of [^{125}I]thyroglobulin-binding lymphocytes. Thyroglobulin-binding lymphocytes were increased 100-fold in a Hashimoto thyroid biopsy in comparison to the patient's peripheral blood.

INTRODUCTION

The predominant feature of organ-specific autoimmune diseases such as Hashimoto thyroiditis is the infiltration of the target organ with lymphocytes. However, little is known of the origin or turnover of these cells within the lesion, or whether the influx of lymphocytes

Correspondence: Dr W. J. Irvine, Department of Endocrinology, Royal Infirmary, Edinburgh.

is accompanied by corresponding fluctuations in the circulating lymphocyte populations from which these cells are presumably derived.

Recently, techniques have become available for the identification of specifically reactive lymphocytes (Ada, 1970). It is now also possible to distinguish between thymus-dependent T lymphocytes and thymus-independent B lymphocytes on the basis of surface membrane markers (Brain, Gordon & Willetts, 1970; Raff, 1971; Papamichail, Brown & Holborow, 1971; Jondal, Holm & Wigzell, 1972; Fröland, Natvig & Berdal, 1972; Papamichail *et al.*, 1972).

We have studied these lymphocyte subpopulations in the peripheral blood of patients with Hashimoto thyroiditis and in normal subjects.

MATERIALS AND METHODS

Patients studied

Twenty-two female patients with proven Hashimoto thyroiditis, who were attending the Endocrine Clinic of the Royal Infirmary, Edinburgh, were included in this study. Histological confirmation of the diagnosis was available in fifteen of the twenty-two patients. In a further seven patients the diagnosis was made on the basis of a goitre and characterisitic serology (either positive immunofluorescence tests for thyroid microsomal antibody with a complement fixation titre of $>1:32$, or a tanned cell haemagglutination titre of $>1:2500$, or both). All patients were euthyroid at the time of study. Seventeen patients were on L-thyroxine replacement therapy in a dose of 0·2–0·3 mg/day, the period of treatment being 1–14 years. Five patients were clinically and biochemically euthyroid and were on no medication.

Twenty-two control subjects, consisting of medical and laboratory personnel and convalescent hospital patients with miscellaneous conditions not known to be associated with autoimmunity and who were age matched to the patients with Hashimoto thyroiditis, were studied concurrently.

Lymphocytes

25–50 ml of venous blood was collected into heparinized universal containers (Evans preservative-free heparin 10 units/ml) and small samples taken simultaneously for serology and differential white blood count. Purified lymphocyte suspensions were prepared by density centrifugation on Ficoll-Triosil (Calder *et al.*, 1973), washed twice in Eagle's basal medium (EBM) (Wellcome Reagents Ltd) and resuspended in EBM supplemented with 10% Foetal Bovine Serum (FBS). Suspension were then incubated at 37°C for 1–2 hr in flat-sided Falcon plastic tissue culture flasks (No. 3012) to remove remaining polymorphs and monocytes, subsequently washing in EBM and adjusted to 4×10^6/ml. These suspensions consisted of >95% lymphocytes which were >98% viable as assessed by Trypan Blue dye exclusion.

Thyroid biopsy

A thyroid biopsy was obtained from a 32-year-old woman with goitre which confirmed the clinical diagnosis of Hashimoto thyroiditis. This was performed under local anaesthetic by the percutaneous needle method and part of the biopsy specimen was obtained for antigen-binding studies. The tissue was finely teased in EBM—10% FBS and the larger

debris allowed to settle for a few minutes. The resulting cell suspension was layered onto 100% FBS and centrifuged at 400 *g* for 15 mins. The cell pellet was then washed in EBM four times and resuspended in the same medium before being used for thyroglobulin-binding studies.

Identification of T lymphocytes by sheep erythrocyte (E) rosettes

The method used here was essentially that of Jondal *et al.* (1972) as modified by Stjernsward *et al.* (1972). Sheep erythrocytes (SRBC) were obtained from the same animal throughout the study, stored at 4°C in Alsever's solution 1 : 1 and used within 10 days of bleeding. SRBC were washed three times in EBM and adjusted to a 1% solution (approximately 180×10^6 RBC/ml). Fresh suspensions were made up daily. 0·25 ml 1% SRBC (approximately 45×10^6 RBC) and 0·25 ml lymphocyte suspension (1×10^6 cells) were incubated at 37°C for 15 min, centrifuged at 200 *g* for 5 min and incubated overnight at 0–4°C over ice. The upper layers of the pellet were gently resuspended, one drop placed upon slides previously coated with toluidine blue (Coombs *et al.*, 1970) and covered with a coverslip. The stain did not interfere with rosette formation, greatly facilitated the identification of cell types, and enabled morula-like rosettes with a central staining lymphocyte to be distinguished from rare clumps of SRBC. The slides were coded and examined 'blind'; 200 lymphocytes on each of two slides were assessed for % rosettes ($\geqslant 3$ SRBC/lymphocyte) and the results averaged.

Identification of B lymphocytes by surface immunoglobulins

An indirect fluorescence method (Jondal *et al.*, 1972) was used, with polyvalent rabbit anti-human immunoglobulin serum (anti-IgG, IgA, IgM) followed by FITC-conjugated goat anti-rabbit IgG serum. Both antisera (Miles-Seravac; 'Pentex') were used at 1/10 dilution. 5×10^6 lymphocytes in 0·1 ml phosphate-buffered saline (PBS), pH 7·4 were incubated at 0–4°C over ice for 30 min with one drop rabbit anti-human immunoglobulin serum, washed three times in ice-cold PBS, resuspended in 0·1 ml PBS and incubated for a further 30 min over ice with one drop FITC-conjugated goat anti-rabbit IgG. The lymphocytes were then washed three times in ice-cold PBS, resuspended in 0·1 ml, dropped onto cleaned glass slides, covered with a coverslip and sealed with clear nail polish. These slides could then be stored for up to 18 hr without damage at 4°C before microscopy. Slides were coded and read 'blind' under phase-contrast light and epi-illumination u.v. fluorescence. The instrument used was a Zeiss microscope equipped with an Osram HBO–200 mercury vapour lamp and a vertical illuminator. Each field was examined under phase-contrast to enumerate total cells present followed by u.v. epi-illumination to detect fluorescent cells until a total of 200 lymphocytes were counted; two slides were examined per test and fluorescence was observed as a granular ring of fluorescent spots around the surface membrane. The occasional positive granulocyte or monocyte seen was discounted.

Thyroglobulin

Freeze-dried human thyroglobulin was obtained from Wellcome Laboratories and this was further purified by Sephadex G-200 gel filtration. Thyroglobulin was then made up to a concentration of 500 μg/ml, dispensed into small aliquots and stored at −20°C until required. The same batch of thyroglobulin was used for all experiments.

Iodination procedure

The chloramine-T method as modified by Hunter (1967) was used with minor modifications. 500–1000 μCi carrier free ^{125}I(Amersham: IMS30) were reacted with 5 μg thyroglobulin and 10 ml chloramine-T (5 mg/ml) at room temperature for 60 sec and pH 7·5. The reaction was terminated by the addition of 0·75 ml sodium metabisulphite (160 μg/ml) followed by 0·2 ml carrier KI (10 mg/ml). The labelled protein was then separated from unreacted ^{125}I by Sephadex G-50 column fractionation. Pilot studies have revealed that there was no advantage in using G-200 for column fractionation since the thyroglobulin was not detectably damaged by the iodination procedure. Labelled thyroglobulin ([^{125}I]Tg) was stored at 4°C and used within 72 hr of labelling although pilot studies indicated that there was no appreciable loss of label over 6 days. The specific activity of the [^{125}I]Tg varied from 28–116 μCi/μg.

Labelling of lymphocytes with [^{125}I]thyroglobulin ([^{125}I]Tg)

The conditions for antigen binding were as described by Byrt & Ada (1969) and Bankhurst, Torrigiani & Allison (1973). 5×10^6 lymphocytes were incubated with 125–250 ng [^{125}I]Tg for 30 min at 0–4°C over ice in a final volume of 0·5 ml EBM + 0·01% sodium azide. The cells were then washed three times with ice-cold EBM, resuspended in 0·1 ml 100% FBS and smeared onto gelatin coated slides. In blocking experiments lymphocytes were first incubated for 30 min over ice with 50 μg unlabelled Tg (200–400 × excess) in a final volume of 0·5 ml containing 0·1% azide. The lymphocytes were then washed three times with ice-cold EBM before proceeding as above.

Autoradiography

The above prepared lymphocyte smears were air-dried, fixed in methanol: water (90 : 10 v/v), washed in distilled water and subsequently dipped into Ilford K2 nuclear emulsion. Slides were then dried in the dark-room and stored in plastic light-proof boxes and exposed at 4°C for 10–21 days. By varying exposure time inversely with the specific activity of the [^{125}I]Tg one could produce equivalent grain formation with the differing specific activities. Slides were developed with Kodak D19 developer, fixed in 30% sodium thiosulphate, washed and subsequently stained with Leishman's stain. Appropriate controls were included in each batch of slides for latent-image fading and positive and negative chemography (Rogers, 1967).

Antigen-binding lymphocytes

Slides had previously been coded and were examined 'blind' under oil-immersion with a × 56 objective and × 8 eyepiece. 10^4 lymphocytes were counted on each slide and positive cells had to be intact with no obvious damage and not in contact with another cell or debris (Ada, 1970; Bankhurst *et al.*, 1973). Background grains were never more than 5 grains per cell and lymphocytes with 10 or more grains within one cell diameter were considered positive. Under the conditions used, lymphocytes were never so heavily labelled as not to be morphologically distinguishable. Labelling of the cells was arbitrarily classified as light (10–25 grains/cell), moderate (26–50 grains/cell) or heavy (> 50 grains/cell). The more heavily labelled cells occasionally had confluent graining which obviated accurate grain counts. Clumped cells and debris were occasionally heavily labelled.

RESULTS

Total lymphocyte counts

These were calculated from differential white blood counts, and were assessed in fifteen normal subjects of mean age 56·7 (range 50–76), and eighteen Hashimoto patients of mean age 52·3 years (range 32–74). The control subjects had 1603 ± 156 (mean ± SEM) lymphocytes/mm^3 whereas the Hashimoto patients had 1226 ± 187 lymphocytes/mm^3. Although the Hashimoto mean is lower than the control, this difference is not statistically significant.

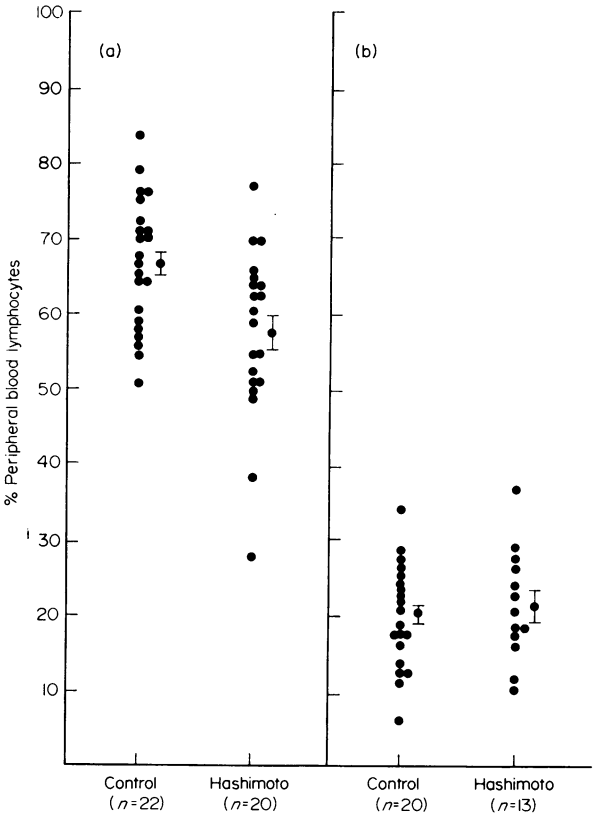

FIG. 1. The relative proportions of circulating T and B lymphocytes in Hashimoto patients and normal subjects. (a) T-lymphocytes (E-rosettes). (b) B-lymphocytes (immunofluorescence). Vertical bar, means ± SEM.

E-rosettes

The peripheral blood of twenty-two control subjects and twenty patients with Hashimoto thyroiditis was tested for the proportion of T lymphocytes as assessed by E-rosette formation. The controls were of mean age 54·6 years (range 30–76) and the Hashimoto group of mean age 53·0 years (range 31–74). The control group formed 66·7 ± 1·8% (mean ± SEM) rosettes with a range of 51·8–83·5%. The Hashimoto group formed 57·4 ± 2·5% (mean ± SEM) rosettes with a range of 20·9–77% (Fig. 1). Although there is considerable overlap between

the two groups, there is nevertheless a significant decrease ($P<0{\cdot}01$) in % T lymphocytes in the Hashimoto group as assessed by the Wilcoxon rank sum test (Wilcoxon, 1945).

Immunofluorescence

Tests were done on twenty controls and thirteen Hashimoto subjects. The controls were of mean age 53·2 years (range 30–76) and the Hashimoto group of mean age 56·1 years (range 32·74). The control group had 20·2±1·1 (mean ±SEM) immunofluorescent positive cells with a range of 6–34·4%. The Hashimoto group had 21·6±2·1 (mean±SEM) immunofluorescent positive cells with a range of 10–36% (Fig. 1). Although there was a slight increase in B lymphocytes in the Hashimoto group to parallel the decrease in T lymphocytes, this difference was not statistically significant.

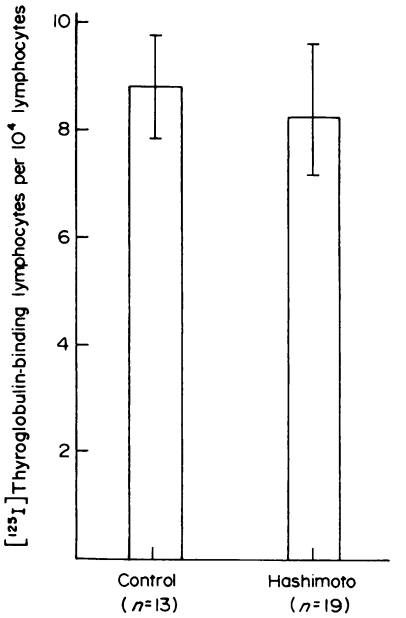

FIG. 2. [^{125}I]thyroglobulin-binding lymphocytes in the circulation of Hashimoto patients and normal subjects. Vertical bar means±SEM.

Thyroglobulin-binding lymphocytes

These were enumerated in thirteen control subjects of mean age 52·8 years (range 37–72) and nineteen Hashimoto patients of mean age 52·7 years (range 31–74). None of the control subjects had significant thyroglobulin antibody titres as assessed by the tanned red cell haemagglutination technique, the maximum reciprocal titre being 5 in three of the thirteen subjects. Twelve of the Hashimoto group had values ranging from 250–25,000 and seven had titres below 250. The control group lymphocytes bound thyroglobulin with a frequency of 8·84±0·93 (mean±SEM) per 10^4 lymphocytes with a range of 3–14/10^4 cells. The Hashimoto group bound [^{125}I]Tg with a frequency of 8·37±1·15 (mean±SEM) per 10^4 lymphocytes and a range of 1–18/10^4 cells (Fig. 2). The difference between these two mean

values was not statistically significant. In order to detect possible differences in the degree of [^{125}I]Tg binding between the two groups, grain counts were done on the positive cells. Arbitrary division of [^{125}I]Tg-binding lymphocytes into those showing light, medium and heavy labelling did not reveal a preponderance in any one group, and there were no statistically significant differences between the control and Hashimoto subjects at any level of labelling. There was no apparent correlation between age or thyroglobulin antibody titres and the frequency of [^{125}I]Tg-binding lymphocytes.

A 200–400-fold excess of unlabelled thyroglobulin produced 60% reduction in [^{125}I]Tg-binding lymphocytes when preincubated with the lymphocytes. This indicates the specificity of the antigen-binding reaction and also suggests that no new antigenic sites were revealed by the iodination procedure.

The lymphocytes in a biopsy obtained from a Hashimoto goitre bound [^{125}I]Tg with a frequency of 96/10^4 lymphocytes. This represents an increase of approximately 100-fold over the corresponding peripheral blood lymphocytes which had only one [^{125}I]Tg-binding lymphocyte per 10^4 cells. The thyroid biopsy lymphocytes were mostly lightly labelled (70%).

DISCUSSION

Recent studies have assigned a possible control function to T lymphocytes whereby they can control and inhibit antibody production by B lymphocytes (Gershon & Kondo, 1970; Baker & Stashak, 1970; Kerbel & Eidinger, 1971). Allison, Denman and Barnes (1971) have drawn attention to the possible importance of such a mechanism in controlling autoantibody production. It is therefore possible that autoimmune phenomena develop as a result of T lymphocyte deficiency within the controlling subpopulation and not as a result of B lymphocyte hyperactivity *per se*. Support for this hypothesis is found in studies which have shown that neonatal thymectomy results in an increased incidence of antinuclear antibodies in mice (Thivolet *et al.*, 1967; Teague & Friou, 1969) and an increase in the incidence of spontaneous thyroiditis in obese chickens (Welch, Rose & Kite, 1973). Also, thymectomy followed by X-irradiation results in the development of spontaneous thyroiditis in rats (Penhale *et al.*, 1973).

In the light of this evidence, the observation reported here that there is a significant decrease in the proportion of circulating lymphocytes in a group of Hashimoto patients as compared with a control group may be highly relevant. It is not known whether control is a feature of T lymphocytes in general or whether this is confined to a subpopulation of T lymphocytes. If the latter is the case then this small decrease in the percentage of total T lymphocytes could represent a more marked decrease in such a subpopulation. It has been recently reported (Aoki, Wakisaka & Nagata, 1973) that the percentage of T lymphocytes is reduced in thyrotoxic patients rendered hypothyroid by overtreatment and that the lymphocyte count returned to normal on treatment. This suggests that thyroid status may directly influence T lymphocytes. It is therefore important to note that our patients were all euthyroid at the time of testing and that the reduction in T lymphocytes found in our studies is therefore independent of thyroid status.

It has also been reported (Farid *et al.*, 1973) that T lymphocytes are markedly raised in Hashimoto thyroiditis; our results indicate the converse. It may be that the reason for the difference lies in the methodology. Although both rosetting methods are not essentially

different Farid *et al.*, separated their lymphocytes by a Dextran sedimentation method (Lamki, Row & Volpé, 1973) and obtain a suspension which contains only 50–60% lymphocytes as compared with our 95%. It may be that we are removing from the Hashimoto suspensions a population of cells which is potentially rosette-forming (these may or may not be T lymphocytes) and that this population does not exist in the normal controls. Whether or not various separation methods alter the original composition of a lymphocyte suspension is a point which needs clarification.

Our study has also shown that normal subjects have circulating lymphocytes capable of binding thyroglobulin with a frequency of 0·09%. Recently, Bankhurst, Torrigiani & Allison (1973) in a smaller group of normal subjects found 0·02% thyroglobulin-binding lymphocytes and also demonstrated that these are probably B lymphocytes. The higher figure obtained by us may be the result of using [^{125}I]Tg of higher specific activity. In addition, our studies have also shown that Tg-reactive cells are present in the circulation of Hashimoto patients. Interestingly, the numbers were found to be similar to those of the control group. A potential rise in circulating Tg-binding lymphocytes may have been masked by sequestration in the thyroid since these lymphocytes were found in higher numbers (100-fold increase) than in the same patient's peripheral blood. Alternatively, these cells may have arisen *in situ* as a consequence of the differentiation of a small number of blood-borne precursors in the absence of a controlling influence.

These possibilities underline the need for further studies on the origin and turnover of auto-reactive cells within autoimmune lesions.

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