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PERIPHERAL BLOOD T AND B LYMPHOCYTES IN PATIENTS WITH THYROTOXICOSIS AND HASHIMOTO'S THYROIDITIS AND IN NORMAL SUBJECTS

A COMPARISON OF LYMPHOCYTE SEPARATION METHODS

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SUMMARY

Lymphocytes were separated from the peripheral blood of three groups of subjects (normal controls, untreated thyrotoxicosis and confirmed Hashimoto's thyroiditis) by two separation methods: dextran sedimentation and Ficoll–Triosil gradient centrifugation. It has been shown that there is a selective loss of T lymphocytes (as measured by sheep red cell rosettes) with relative enrichment of B lymphocytes (as measured by surface immunoglobulins) in the Ficoll–Triosil-separated suspensions. This distortion of the T/B ratio was seen to a similar extent in each of the three groups of subjects. Furthermore, the mean percentage T and B lymphocytes of both patient groups were not significantly different from those of the controls when separated by the same method. Optimal E-rosette formation occurred after prolonged incubation at 4°C and in the absence of serum. Direct counts using Toluidine Blue were superior to indirect counts with unstained rosette suspensions.

INTRODUCTION

The availability of cell surface markers for the identification of human T and B lymphocytes (reviewed by Jondal, Wigzell & Aiuti, 1973) has enabled investigators to enumerate these cells in a variety of disease states. We have previously reported that the percentage of T lymphocytes as measured by the E-rosette test is reduced in the peripheral blood of patients with Hashimoto's thyroiditis when compared with a group of age- and sex-matched normal persons (Urbaniak, Penhale & Irvine, 1973b). The converse has been claimed by Farid *et al.* (1973) who showed a substantial increase in circulating T cells in both Hashimoto's thyroiditis and untreated thyrotoxicosis (Graves' disease) using a similar rosetting technique. This latter finding was not confirmed by Wara *et al.* (1973) who showed no

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difference between a small group of adolescent thyrotoxics and controls. However, a relative increase in E-rosettes in thyrotoxicosis was demonstrated by Aoki, Wakisaka & Nagata (1973), but their E-rosette values were much lower than in any of the above studies. It has been suggested that the discrepancies could have been due to technical differences in the preparation of lymphocyte suspensions (Urbaniak, Penhale & Irvine, 1973a; Wara *et al.*, 1973). Farid *et al.* (1973) used a dextran sedimentation method which results in a leucocyte suspension containing only 40–60% lymphocytes with considerable polymorph contamination, whereas the other three studies used some modification of the Ficoll–Triosil gradient centrifugation method (Böyum, 1968; Perper, Zee & Mickelson, 1968). The latter method results in a much purer population of lymphocytes but inevitably some lymphocytes are lost during the enrichment procedure. It has generally been assumed that such losses occur non-specifically, but it is possible that cells are lost in a more selective manner. We therefore decided to investigate the effects of the separation methods mentioned above in normal persons and in Hashimoto's thyroiditis and untreated thyrotoxicosis to see whether the discrepancies reported could be resolved.

MATERIALS AND METHODS

Subjects

Normal controls consisted of thirteen females and four males of mean age 42·3 years (range 19–58 years) who were either normal hospital or laboratory personnel with no family or personal history of autoimmune disorders. Patients with thyrotoxicosis (Graves' disease) were all clinically and biochemically hyperthyroid at the time of testing and were not receiving treatment; they consisted of thirteen females and four males of mean age 43·2 years (range 20–58 years). Patients with proven Hashimoto's thyroiditis were attending the Endocrine Clinic and consisted of one male and ten females, mean age 58·8 years (range 36–73 years). Nine of these patients were on L-thyroxine replacement therapy in dose of 0·1–0·3 mg daily, the period of treatment being 3–15 years. Two were euthyroid and on no medication.

Lymphocytes

Fifty millilitres of heparinized venous blood (10 u of heparin/ml; preservative-free) were divided into equal aliquots, one to be separated by the Ficoll–Triosil (F–T) method and the other by dextran sedimentation (DS). A sequestrene sample was also taken for a differential white blood cell count.

(a) *Ficoll–Triosil separation.* Twenty-five millilitres of blood were carefully layered over F–T of specific gravity 1·077 and centrifuged at 400 ***g*** for 30 min, washed in Eagle's basal medium (EBM) (Wellcome Reagents Ltd) at 400 ***g*** for 15 min, followed by two further washes at 200 ***g***. If platelets were excessive they were removed by a further wash at 50 ***g***. The total number of leucocytes was counted and they were adjusted to 4×10^6/ml. Differential counts at this stage generally showed >80% lymphocytes.

(b) *Dextran sedimentation separation.* Twenty-five millilitres of blood were mixed with 12·5 ml of 3% dextran in phosphate-buffered saline (mol. wt 300, 000; Fisons Ltd, Pharmaceutical Division) and allowed to sediment under unit gravity at 37°C for 1 hr. The leucocyte-rich plasma was centrifuged at 200 ***g*** and excess RBC in the pellet were lysed with approximately 8 volumes of 0·83% ammonium chloride. Leucocytes were then washed

three times in EBM and adjusted to 4×10^6/ml. Differential counts at this stage generally showed 30–50% lymphocytes.

Identification of T lymphocytes by sheep erythrocyte (E) rosettes

Sheep erythrocytes (SRBC) were obtained from the same normal animal throughout the study, stored at 4°C in Alsever's solution and used within 10 days of bleeding. SRBC were washed in EBM and adjusted to a 1% solution; fresh suspensions were made up daily. 0·25 ml of SRBC was mixed with 0·25 ml of leucocyte suspension, incubated for 15 min at 37°C, centrifuged at 200 *g* for 5 min and incubated at 4°C in a constant-temperature water-bath. Aliquots were incubated for either 1–2 hr or overnight (16–18 hr). Aliquots were also incubated in either plain EBM or supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Wellcome Reagents Ltd). After the appropriate incubation time the cell pellets were very gently resuspended with a wide-bore Pasteur pipette; no attempt was made to totally resuspend tenacious clumps of cells. The regular method of counting E-rosettes ($\geqslant$three SRBC/lymphocyte) was by direct counting on Toluidine Blue-coated slides as described previously (Urbaniak, Penhale & Irvine, 1973b). Indirect counts were also done by the method described by Farid *et al.* (1973). In this method E-rosettes are enumerated in unstained haemocytometer preparations and the percentage of rosetting lymphocytes calculated on the basis of a differential count on the leucocyte suspension. Two indirect counts were done, one on the original leucocyte suspension, and the other on the rosetted suspensions.

All samples were coded by an independent observer and read without knowledge of their origin.

Identification of B lymphocytes by surface immunoglobulins

Aliquots of both F–T and DS-separated cell suspensions were examined. An indirect immunofluorescence method was used. 5×10^6 leucocytes were incubated for 30 min at 4°C in 0·1 ml of PBS, pH 7·2, with one drop of rabbit anti-human Ig Fab′ fragment (Behringwerke AG). After three washes in ice-cold PBS the cells were resuspended in 0·1 ml of PBS containing one drop of FITC-conjugated goat anti-rabbit IgG serum (Miles Laboratories 'Pentex') for a further 30 min at 4°C. Both antisera were used at 1/10 dilution. After a further three washes microscope slide suspensions were made and examined with a combination of u.v. epi-illumination and transmitted light. Although samples were coded, in practice it was easy to distinguish the dextran-prepared suspensions because of the contamination with polymorphs.

RESULTS

Total lymphocyte counts

These were calculated from the differential white blood cell counts and were assessed in all seventeen controls, seventeen thyrotoxics and eleven Hashimoto patients. The normal subjects had a mean count of 1787 ± 743 (s.d.) lymphocytes per mm^3, the thyrotoxics 1448 ± 618 lymphocytes per mm^3 and the Hasimoto patients 1689 ± 735 lymphocytes per mm^3. The mean T lymphocyte counts (calculated from E-rosette values after F–T separation and overnight incubation without FBS) were 1253 ± 568 (s.d.)/mm^3 in normal subjects, 963 ± 458/mm^3 in thyrotoxic patients and 1188 ± 624/mm^3 in Hashimoto patients.

The mean B lymphocyte counts (calculated from percentage immunoglobulin-bearing cells after F–T separation) were $394 \pm 206/mm^3$ in normal subjects, $291 \pm 131/mm^3$ in thyrotoxic patients and $366 \pm 124/mm^3$ in Hashimoto patients. The differences between normal subjects and both patient groups are not statistically significant.

T lymphocytes as assessed by E-rosettes

Direct versus indirect counts. Falsely high results were obtained in almost all cases where indirect counts were calculated on the basis of the lymphocyte count from the original leucocyte suspensions. This was seen at its worst with dextran-separated cells where the calculated E-rosette count was >100% in thirty-one out of thirty-four separate measurements. Polymorphs were seen to be clumping and/or dying during the rosetting procedure, giving a higher relative lymphocyte count, and a more realistic result was obtained using the percentage of lymphocytes obtained from the rosetted suspension. In some instances these results agreed very closely with those obtained from direct counts on Toluidine Blue-coated slides, but again the overall trend was towards over-estimation (Table 1).

TABLE 1. Indirect versus direct method of counting E-rosettes showing over-estimation by the indirect method

Indirect count calculated from the percentage of lymphocytes in the original leucocyte suspension*
1/34 <10% higher than the direct count
2/34 >10% but <20% higher than the direct count
31/34 had a higher estimated E-rosette than total lymphocyte count
Indirect count calculated from the percentage of lymphocytes in the rosetting suspension†
6/23 within 5% of the direct count
10/23 >5% but <10% higher than the direct count
3/23 >10% but <20% higher than the direct count
3/23 >20% higher than the direct count but <100% E-rosettes
1/23 had a higher estimated E-rosette than total lymphocyte count

* Thirty-four individual counts.
† Twenty-three individual counts.

This was due to a combination of inaccuracies with the double count, including an unstained preparation and the fact that polymorphs were observed to form a variable number of E-rosettes (usually not more than 5% rosettes). This pattern of over-estimation was seen with all three groups examined and there was no suggestion of a selective increase in any one group. The indirect counting method was rejected as being inaccurate and inconvenient and all results expressed hereafter are on the basis of direct counts of E-rosettes.

Effect of FBS. The presence of 10% heat-inactivated FBS in the incubation medium made no significant difference to the overall mean E-rosette values, other parameters being equal (Table 2), except in the case of the thyrotoxic group where there was a barely significant difference in the presence of FBS after 1–2 hr incubation. ($P = <0{\cdot}1 > 0{\cdot}05$ and $<0{\cdot}05 > 0{\cdot}02$ for dextran and F–T separated cells respectively). This was outside the general trend and the result of a series of low readings in the presence of FBS.

Effect of incubation time. In all the permutations tested, the mean E-rosette values after

TABLE 2. Comparison of dextran sedimentation versus Ficoll–Triosil gradient separation; the effects of various incubation conditions on E-rosette formation

	1–2-hr incubation at 4°C				Overnight incubation at 4°C			
	With FBS		Without FBS		With FBS		Without FBS	
	Dextran	Ficoll–Triosil	Dextran	Ficoll–Triosil	Dextran	Ficoll–Triosil	Dextran	Ficoll–Triosil
Normal controls (17)‡	74·5±5·8 (62·0–82·3)	68·3±6·3* (55·5–76·8)	74·6±5·4 (63·7–82)	68·9±6·0 (54·0–78·8)	77·5±5·6 (66·3–84·3)	69·8±5·3† (59·6–80)	76·2±5·9 (60·0–84·8)	69·3±6·4 (55·2–78·5)
Thyrotoxic (17)‡	(67·8±6·6) (54·5–79·5)	59·2±6·8* (50·0–73)	72·1±6·9 (55·0–81·5)	64·7±5·8 (53·0–75·5)	74·0±5·5 (63·5–81·5)	62·8±6·6† (51·5–73·0)	74·5±5·3 (67·5–85·3)	65·8±6·0 (54·0–78·0)
Hashimoto thyroiditis (11)‡	72·7±6·8 (63·2–82·8)	63·1±6·9 (50·5–75·0)	72·7±5·8 (59·2–80·7)	63·5±7·0 (51·0–76·0)	79·4±5·4 (65·0–85·8)	68·7±8·5 (53·5–78·3)	78·6±4·8 (65·0–84·0)	69·1±9·4 (50·7–79·0)

The results are expressed as mean±s.d. Mean values for thyrotoxic and Hashimoto patients are not significantly different from the normal values unless otherwise stated (Wilcoxon Rank Sum test used); * $P < 0.01$, † $P < 0.01$.

Dextran sedimentation results are not compared with Ficoll–Triosil results in this analysis.

‡ Numbers of subjects.

overnight incubation were higher than those after only 1–2 hr, other conditions remaining constant (Table 2). In most cases the increases were not statistically significant. The increases were not due to selective cell death of non-rosetting cells since viability was not significantly lower after the longer incubation. The differences were not due to aliquot sampling errors since the results obtained with a duplicate sample were in all cases tested very similar to the results when the original 1–2 hr-sample was also incubated overnight.

Effect of dextran versus Ficoll–Triosil separation. When normal subjects' DS-separated cells were compared with F–T-separated cells there was a highly significant correlation for each of the incubation time combinations tested (Fig. 1). It can be seen that the slopes

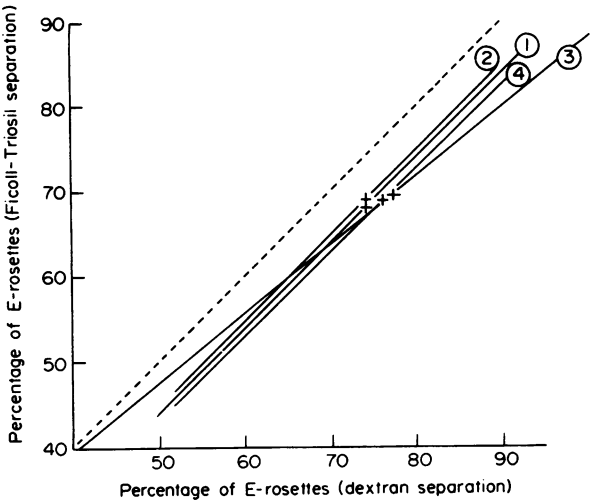

FIG. 1. Regression lines showing the correlation between E-rosette values after dextran and Ficoll–Triosil separation of lymphocytes from normal subjects under different conditions of incubation. (1) 1–2 hr with FBS: $y = 1{\cdot}01x - 7{\cdot}0$; $r = 0{\cdot}94$. (2) 1–2 hr without FBS: $y = 1{\cdot}00x - 5{\cdot}7$; $r = 0{\cdot}89$. (3) Overnight with FBS: $y = 0{\cdot}80x + 7{\cdot}8$; $r = 0{\cdot}85$. (4) Overnight without FBS: $y = 0{\cdot}99x - 6{\cdot}1$; $r = 0{\cdot}92$. (- - -) Theoretical regression line where both separation procedures produce identical lymphocyte populations. (+) = $(\bar{x}, \bar{y})$ for each of the incubation conditions.

of the regression lines are displaced in such a way as to suggest that there is a relative loss of T cells with F–T separation. With the thyrotoxic and Hashimoto groups significant correlations were also seen between DS and F–T separation (Fig. 2). Under some of the incubation conditions tested the correlations were lower than seen with normal subjects.

Under similar conditions, the mean E-rosette values were significantly higher with DS-separated cells than with F–T separation and this pattern was seen with each of the three groups of subjects tested (Fig. 3, Table 2). When the overall mean E-rosette values for normals are compared with the values for the thyrotoxic and Hashimoto patients under similar conditions (Table 2) it can be seen that they are very similar with the exception of the lymphocytes from thyrotoxics separated by F–T in the presence of FBS at both incubation times, which are significantly lower. This is probably due to a series of unusually low F–T results with normal DS values, which were seen only in these two circumstances. It would therefore appear that in the present study we cannot show any significant differences

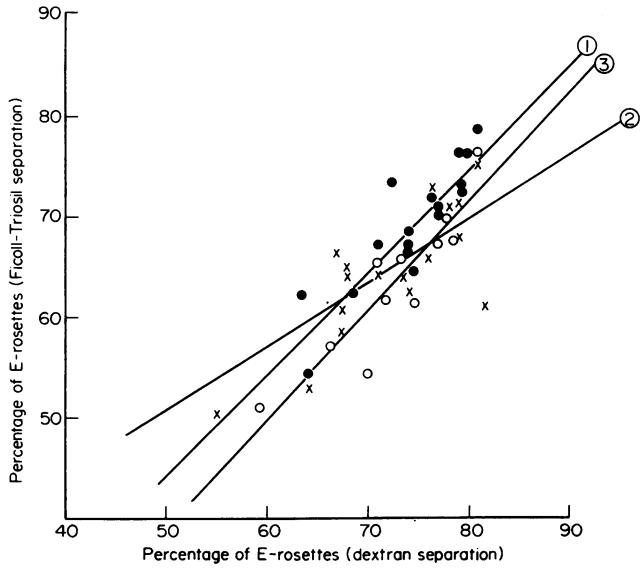

FIG. 2. Regression lines showing the correlation between E-rosette values after dextran and Ficoll–Triosil separation of lymphocytes from normal subjects, thyrotoxic and Hashimoto patients; 1–2 hr incubation at 4°C without FBS. (●), (1) Normal controls: $y = 1{\cdot}00x - 5{\cdot}7$; $r = 0{\cdot}89$. (×), (2) Thyrotoxic: $y = 0{\cdot}62x + 19{\cdot}3$; $r = 0{\cdot}74$. (○), (3) Hashimoto: $y = 1{\cdot}08x - 15{\cdot}0$; $r = 0{\cdot}91$.

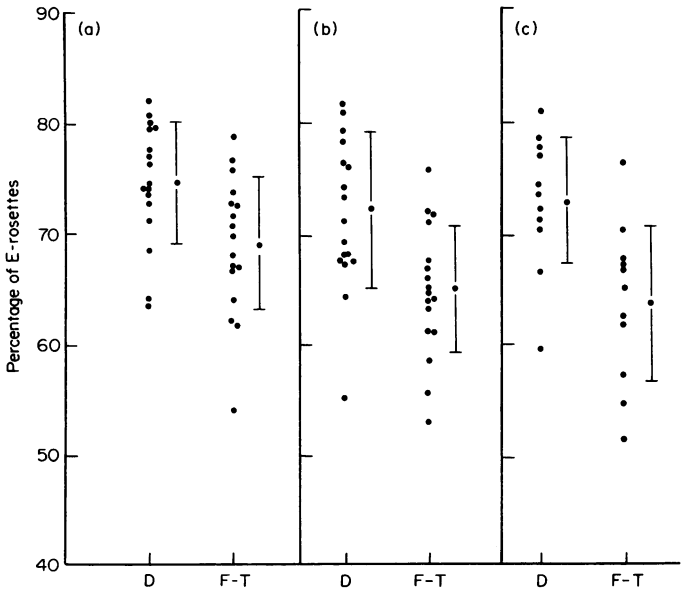

FIG. 3. Comparison between E-rosette values obtained with dextran and Ficoll–Triosil-separated cells in normal subjects, thyrotoxic and Hashimoto patients; 1–2 hr incubation at 4°C without FBS. The bars show the mean ± s.d. (a) Normal controls. (b) Thyrotoxic. (c) Hashimoto. D = Dextran sedimentation separation. F–T = Ficoll–Triosil centrifugation separation.

between the mean E-rosette counts of normal subjects and thyrotoxic and Hashimoto patients whether separated by DS or F–T gradient centrifugation.

B lymphocytes as assessed by surface immunoglobulins

The mean values of Ig-bearing cells in each of the three groups studied is shown in Table 3. There are no significant differences between the mean values for each of the three

TABLE 3. Percentage of peripheral blood B lymphocytes (mean ± s.d.) as detected by surface immunoglobulin. A comparison of dextran and Ficoll–Triosil separation

	Dextran	Ficoll–Triosil
Controls (17)*	15·7 ± 5·3	21·9 ± 5·2
Thyrotoxic (16)*	17·5 ± 5·3	20·8 ± 5·7
Hashimoto (10)*	17·0 ± 3·0	22·8 ± 5·0

* Number of subjects.

groups of subjects with both separation methods but the F–T results are in each case higher than the corresponding dextran result. There is a highly significant correlation between destran and F–T separation (Fig. 4), and it can be seen that the thyrotoxic and Hashimoto

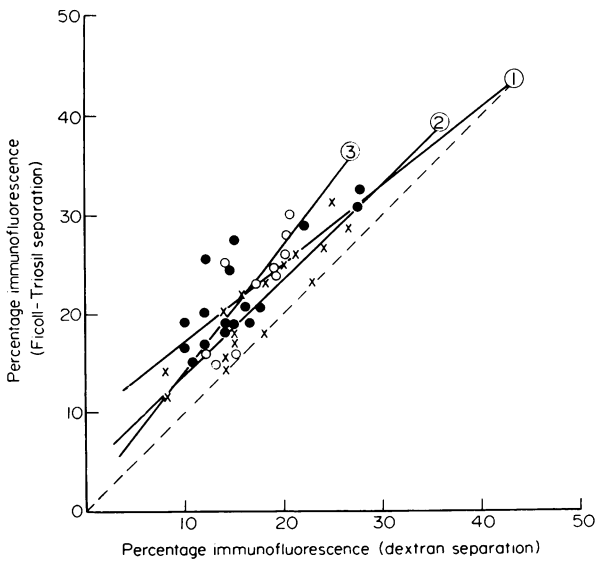

FIG. 4. Regression lines showing the correlation between immunoglobulin-bearing lymphocyte values after Dextran and Ficoll–Triosil separation of lymphocytes from normal subjects, thyrotoxic and Hashimoto patients. (●), (1) Normal: $y = 0{\cdot}79x + 9{\cdot}5$; $r = 0{\cdot}81$. (×), (2) Thyrotoxic: $y = 0{\cdot}96x + 4{\cdot}0$; $r = 0{\cdot}91$. (○) (3) Hashimoto: $y = 1{\cdot}38x + 0{\cdot}6$; $r = 0{\cdot}84$. (– – –) Theoretical regression line where both separation procedures produce identical lymphocyte populations.

groups behave in a very similar manner to the normal controls. The displacement of the regression lines from 'ideal' suggests that there is a relative enrichment of B cells in F–T separated suspensions.

DISCUSSION

The conditions for obtaining maximal E-rosette formation have been examined by several investigators (Lay *et al.*, 1971; Jondal, Holm & Wigzell, 1972; Mendes *et al.*, 1973) and it is generally agreed that the optimal conditions are: an initial incubation between room temperature and 37°C, followed by centrifugation to a pellet and a further incubation in the cold for a minimum of 1 hr. The cold incubation is generally done in ice (0–4°C) and some investigators add serum to the incubation medium. Rosette formation is apparently maximal at 4°C but a higher figure of 10–25°C has been recently advocated (Mendes *et al.*, 1973). The actual temperature may not be critical provided that it does not approximate too closely to 0°C.

Our results indicate that the presence of FBS is not essential for maximal rosette formation. Indeed, in some instances it appeared to interfere in that unusually low results were obtained. This may have been due to the use of FBS which was not pre-absorbed with SRBC. It would also appear that the longer incubation time results in higher values and this may be due to stabilization of the rather fragile rosettes. By maintaining the cell pellet at precisely 4°C rather than over ice, we have obtained higher values for normals than we previously reported (Urbaniak, Penhale & Irvine, 1973b) and in addition, the reduction in E-rosettes in Hashimoto thyroiditis is not consistently seen (Table 2, Fig. 3). This could be due to the examination of a smaller and different group of patients, or possibly due to minor technical differences in the rosette procedure such as the higher incubation temperature.

The indirect method for counting E-rosettes has been previously reported as giving spuriously high results (Hsu & Fell, 1974) and this was shown to be due to polymorphs forming rosettes. We would agree with this finding and have noted as many as 5% polymorph rosettes with Toluidine Blue-stained preparations, albeit infrequently. We would therefore favour the use of direct counts with the use of a stain such as Toluidine Blue where the morphology of the rosetting cell can be readily determined.

Comparison of the results obtained with the two separation methods show that there is a highly significant correlation between them, as might be expected. The divergence of the calculated regression lines from the ideal suggests that there is a loss of T cells with F–T centrifugation of whole blood. The E-rosette counts and immunofluorescence figures are complementary in showing that there is a net increase in the percentage B cells at the same time as a decrease in percentage T cells with an aliquot of the same cell suspension after F–T separation. This confirms that the decrease in E rosettes is real rather than the result of some non-specific interference with rosette formation. It is known that murine T cells are more dense than B cells (Shortman, Cerottini & Brunner, 1972) and if this is the case in humans then it could explain the selective loss of T cells shown here.

Our findings are in accord with those of Brown & Greaves (1974) who have recently shown that separation methods are biased towards the selective loss of T cells. Although our values obtained for T and B cells in the DS-separated cell suspensions approximate quite closely to the 'true' values in whole blood (Brown & Greaves, 1974) it is unfortunately more tedious to enumerate them in view of the contamination by non-lymphocytic cells,

and, of course, in some functional assay systems this contamination may be unacceptable. The distortion of the T/B ratio may or may not be important depending on the parameters being tested, but it does indicate the necessity for comparison with normal controls separated under identical conditions. However, it remains to be seen whether the T-cell losses are selective for a particular type of T cell; this seems unlikely in view of the difficulty of separating even crude T- and B-cell populations by density centrifugation (Shortman, Cerottini & Brunner, 1972). It should be borne in mind that lymphocyte losses during purification are not necessarily nonspecific and the choice of separation method may be crucial to a particular study.

When the mean E-rosette values (with either DS or F–T cell suspensions) for the thyrotoxic and Hashimoto groups are compared with the normal controls, it can be seen that with a few exceptions there is no significant difference between them (Table 2). There are slight reductions in the patient groups which are statistically significant but are not seen consistently with the various aliquots from the same patient and are therefore probably factitious.

The differences between our previously reported results and those of Farid *et al.* (1973) cannot be due to differences in separation technique since we find the same pattern of results with both methods; furthermore, we cannot confirm the increase in E-rosettes in untreated thyrotoxicosis. It is interesting to note that the total lymphocyte counts in thyrotoxicosis ($5400 \pm 673/mm^3$) and Hashimoto's thyroiditis ($6991 \pm 987/mm^3$) reported by these authors is significantly higher than ours ($1448 \pm 618/mm^3$ and $1689 \pm 735/mm^3$ respectively). Although a modest lymphocytosis is sometimes seen in thyrotoxicosis, there are conflicting reports in the literature as to its frequency and whether the increase is relative or absolute (Biström, 1946). A lymphocyte count of $7000/mm^3$ is also uncommon in Hashimoto's thyroiditis (Wallerstein & Castle, 1971). Our own studies (Urbaniak, in preparation) indicate that an absolute lymphocytosis is only occasionally seen in untreated thyrotoxicosis. Aoki *et al.* (1973) found an absolute lymphocytosis in untreated thyrotoxicosis but this was rather modest ($2600/mm^3$ compared with $1673/mm^3$ in controls).

It may be that the discrepancy between the Edinburgh and Toronto results can be resolved on the basis of the differences in total lymphocyte counts in thyrotoxics and Hashimoto thyroiditis. However, the reason for such a marked difference in patients with the same disease is unclear.

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