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# Increase of peripheral B lymphocytes in Graves' disease

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## SUMMARY

Peripheral T and B lymphocytes were examined in autoimmune thyroid diseases. The percentages of T and B lymphocytes were calculated from the proportions of E and EAC rosette-forming cells and peroxidase-positive cells determined by micromethods. In thyrotoxic Graves' disease, the percentage of T cells was significantly lower, and the percentage of B cells was higher than in normal controls. The absolute count of B lymphocytes was also markedly increased. The serum levels of thyroid hormones showed a significant correlation with the percentage of B cells and an inverse correlation with that of T cells in untreated cases of Graves' disease. Similar abnormalities of lymphocyte subpopulations were observed in patients with thyrotoxic Graves' disease under drug therapy, but the proportions and absolute counts of T and B lymphocytes were normal in euthyroid patients with Graves' disease, either under drug therapy or in remission. No abnormalities in T and B cells were found in Hashimoto's disease. The data indicate that the main feature of the abnormality of the lymphocyte subpopulations in thyrotoxic Graves' disease is an increase of B lymphocytes. The reasons for the discrepancy between our results and those of earlier reports and for the B cell abnormality in Graves' disease are discussed.

## INTRODUCTION

Studies on autoimmune abnormalities in thyroid diseases have progressed rapidly owing to the development of a variety of laboratory techniques (Amino & DeGroot, 1974; Allison, 1976; Volpé, 1977; Doniach, Bottazzo & Russell, 1979). The important role of cell-mediated immunity (CMI), as well as humoral immunity, has been emphasized and evidence for the existence of this activity to thyroid antigens has been reported (Calder *et al.*, 1972; Delespesse *et al.*, 1972; Podleski, 1972; Calder *et al.*, 1973; Lamki, Row & Volpé, 1973; Warternberg *et al.*, 1973; Amino & DeGroot, 1975). T lymphocytes are considered to be responsible for CMI and they have a helper or suppressor function on B lymphocytes which produce immunoglobulins. There are several reports on peripheral T and B lymphocytes in autoimmune thyroid diseases, but the findings are conflicting. In Graves' disease the percentage of T cells has been reported to be increased (Aoki, Wakisaka & Nagata, 1973), unchanged (Urbaniak, Penhale & Irvine, 1974; Mulaisho, Abdou & Utiger, 1975; Calder *et al.*, 1976; Hsu, Chen & Patterson, 1976; Lundell *et al.*, 1976; Maciel *et al.*, 1976) or decreased (Wall, Gray & Greenwood, 1977; Grinblat *et al.*, 1979), while in Hashimoto's disease, it has been reported to be unchanged (Urbaniak *et al.*, 1974; Calder *et al.*, 1976; Tötterman *et al.*, 1977) or decreased (Urbaniak, Penhale & Irvine, 1973). Moreover, in Graves' disease the absolute number of T cells has been reported to be unchanged (Urbaniak *et al.*, 1974; Calder *et al.*, 1976), increased (Maciel *et al.*, 1976) or decreased (Grinblat *et al.*, 1979), and in Hashimoto's disease it has

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been found to be unchanged (Urbaniak *et al.*, 1974; Calder *et al.*, 1976). Furthermore, in Graves' disease the percentage of B cells has been reported to be unchanged (Aoki *et al.*, 1973; Urbaniak *et al.*, 1974; Mulaisho *et al.*, 1975; Calder *et al.*, 1976; Maciel *et al.*, 1976; Lundell *et al.*, 1976) or increased (Hsu *et al.*, 1976), and in Hashimoto's disease to be unchanged (Urbaniak *et al.*, 1973, 1974; Calder *et al.*, 1976, Tötterman *et al.*, 1977). The absolute number of B cells has been reported to be unchanged both in Graves' disease (Urbaniak *et al.*, 1974; Calder *et al.*, 1976; Maciel *et al.*, 1976) and in Hashimoto's disease (Urbaniak *et al.*, 1974; Calder *et al.*, 1976).

In this work we have re-examined peripheral T and B lymphocytes in Graves' disease and Hashimoto's disease, finding a marked increase of B lymphocytes in Graves' disease. The clinical significance of these changes is discussed.

## MATERIALS AND METHODS

*Subjects.* The normal controls consisted of seventeen women and nine men, 21 to 49 years old, who were hospital or laboratory staff with no personal history of autoimmune disorders. The patients with Graves' disease were divided into the following four groups: (1) untreated thyrotoxic patients, eighteen women and three men, 21 to 55 years old; (2) thyrotoxic patients, twelve women under anti-thyroid drug therapy, 20 to 44 years old; (3) euthyroid patients under drug therapy, thirteen women and one man, 20 to 32 years old; (4) Graves' disease in remission, fourteen women, 23 to 34 years old. The patients with Hashimoto's disease consisted of twenty-one women and one man, 22 to 52 years old; eighteen of these were euthyroid without therapy and four had mild hypothyroidism. The patients with other thyroid diseases examined were as follows: eight cases of simple goitre, three cases of thyroid carcinoma after operation and two cases of thyroid adenoma after operation with and without thyroid hormone therapy. Cases with other associated autoimmune diseases were excluded from this study to avoid effects other than those of the thyroid autoimmune abnormality. The diagnoses of Graves' disease and Hashimoto's disease were established on the basis of clinical and laboratory findings. Destruction-induced thyrotoxicosis due to Hashimoto's disease (Amino *et al.*, 1978) was carefully differentiated from Graves' disease, and was not included in this study. The patients with Graves' disease in remission were euthyroid for at least 6 months without anti-thyroid drugs.

*Identification of T and B lymphocytes.* Peripheral T and B lymphocytes were measured quantitatively by a micromethod for the detection of sheep erythrocyte (E) rosettes and sheep erythrocytes–antibody–complement (EAC) rosettes (Tachibana & Ishikawa, 1973), using a commercially available kit (Japan Immunoresearch Laboratories, Tokyo). For preparing EAC, sheep erythrocytes were sensitized with 19S rabbit antibody and coated with human complement instead of mouse complement to obtain more stable EAC rosettes (Tachibana, Yoshida & Takada, 1974). Two millilitres of heparinized venous blood (10 u heparin/ml) was mixed with the same volume of phosphate-buffered saline containing no divalent cations (PBS) and the mixture was carefully layered over sodium metrizoate–Ficoll (SMF) of specific gravity 1·077. The mononuclear cells obtained by centrifugation at 400 ***g*** for 30 min were washed three times with PBS to remove platelets and suspended in PBS at a concentration of $1 \times 10^6$ cells/ml. Then 1-$\mu$l samples of this lymphocyte suspension were introduced into the wells of tissue-typing microplates (Falcon plates No. 3034) which had been coated with poly-l-lysine. Lymphocytes were allowed to adhere to the bottom of the well for 15 min and then each well was filled with 10 $\mu$l foetal calf serum (FCS). After 30 min at room temperature, the FCS was removed. For determination of E rosettes, 10 $\mu$l of neuraminidase-treated E cells at a concentration of $2 \times 10^8$ cells/ml in FCS was introduced into the wells. The plates were incubated for 90 min at room temperature and then stored at 4°C overnight (12–15 hr). They then were inverted for 30 min to detach the unreacted E cells from the bottom, gently immersed in PBS in a plastic box and kept in the inverted position for a further 30 min to remove unreacted E cells. For accurate quantitative measurement of E rosettes, the lymphocytes in each well were fixed and stained with a mixture of 2·5% glutaraldehyde and 0·04% brilliant cresyl blue. For measurement of EAC-binding lymphocytes, after removal of FCS, the wells were washed three times with PBS and then 10 $\mu$l of EAC suspension at a concentration of $2 \times 10^8$ cells/ml in PBS was added. The

plates were incubated in a highly humid incubator at 37°C for 90 min, and then inverted for a further 30 min to stop the reaction. The plates were stored in the inverted position at 4°C overnight and then washed, fixed and stained as described above. For quantitation of E and EAC rosettes, five wells were used for each sample and 500 mononuclear cells were counted. A rosette-forming cell was defined as a cell with at least four erythrocytes. Usually the mononuclear cell fraction separated using SMF contained significant numbers of monocytes and sometimes also granulocytes. Therefore, another plate was stained with peroxidase to differentiate these cells. Finally the percentages of T and B lymphocytes were calculated by the following formula based on the percentages of E rosettes (E%), EAC rosettes (EAC%) and peroxidase-positive cells (P%):

$$T\% = E \times \frac{E+EAC}{E+EAC-P}, \qquad B\% = (EAC-P) \times \frac{E+EAC}{E+EAC-P}.$$

The value of the absolute number of peripheral lymphocytes was calculated as the product of the leucocyte counts measured with a Coulter Counter Model S and the percentage of lymphocytes estimated by differential analyses of smears. To minimize technical error, differential analyses were all performed by the same skilled technician by counts on more than 200 leucocytes. The absolute counts of T and B cells were calculated as the products of the absolute lymphocyte count and the percentages of T and B lymphocytes respectively.

*Serology.* Anti-thyroglobulin antibodies (TGHA) and anti-thyroid microsomal antibodies (MCHA) were measured by the tanned red cell haemagglutination technique as previously reported (Amino *et al.*, 1976).

*Thyroid function tests.* Serum thyroxine ($T_4$), tri-iodothyronine ($T_3$) and resin $T_3$ uptake ($RT_3U$) were measured as previously described (Amino *et al.*, 1976). Free $T_4$ index ($FT_4I$) and free $T_3$ index ($FT_3I$) were calculated as $T_4$ and $T_3 \times RT_3U$/normal $RT_3U$ respectively.

## RESULTS

The percentages and absolute counts of peripheral T and B lymphocytes in normal controls and patients with thyroid diseases are shown in Table 1. The ages of the patients in each group were not significantly different from those of the normal controls. In the group of untreated cases of thyrotoxic Graves' disease, significantly higher values than those of normal controls were obtained for total lymphocyte counts, the percentage of B cells, and the absolute counts of T and B lymphocytes. The absolute B lymphocyte counts were especially high, being about twice those of normal controls. Conversely, the percentage of T cells was lower than normal. Similar changes were found in thyrotoxic patients with Graves' disease under anti-thyroid drug therapy, although the absolute total lymphocyte and T lymphocyte counts were not significantly different from those of normal controls. None of these parameters were different from controls in euthyroid patients with Graves' disease, under anti-thyroid drug therapy or in remission, or in patients with Hashimoto's disease. Fig. 1 shows the values of peripheral B lymphocyte counts in individual patients with Graves' disease in various conditions.

The relations between peripheral lymphocytes and thyroid function, titre of anti-thyroid microsomal antibody (MCHA), the size of goitre and proptosis of the eyes in untreated patients with Graves' disease are summarized in Table 2. The percentage of B lymphocytes was significantly correlated with the serum levels of $T_4$, $FT_4I$, $T_3$ and $FT_3I$. The relation of the percentage of B cells and $FT_4I$ is shown in Fig. 2. The percentage of T cells was inversely correlated with the values of serum $T_4$ and $FT_4I$. No correlation was found between the changes of lymphocyte subpopulations and titre of MCHA, the size of goitre or the degree of proptosis of the eyes.

## DISCUSSION

Reported results on peripheral T and B lymphocytes in autoimmune thyroid diseases and especially in Graves' disease are conflicting. Earlier publications reported increases of T lymphocytes in Graves' disease (Aoki *et al.*, 1973; Farid *et al.*, 1973), although the report of Farid *et al.* (1973) has

**Table 1.** Peripheral T and B lymphocytes in autoimmune thyroid disease

| | Number examined | Age | Total lymphocyte count/mm$^3$ | Percentage | | Lymphocyte count/mm$^3$ | |
|---|---|---|---|---|---|---|---|
| | | | | T cells | B cells | T cells | B cells |
| Normal controls | 26 | 28·4 ± 5·8 | 1,555 ± 244 | 76·7 ± 4·6 | 21·3 ± 5·4 | 1,189 ± 186 | 333 ± 95 |
| Graves' disease | | | | | | | |
| Untreated | 21 | 28·0 ± 10·3 | 2,118 ± 681‡ | 68·0 ± 9·7‡ | 29·6 ± 10·2† | 1,445 ± 525* | 612 ± 242‡ |
| Thyrotoxic on drugs | 12 | 27·6 ± 5·9 | 1,744 ± 447 | 69·8 ± 7·0† | 28·3 ± 5·3‡ | 1,291 ± 347 | 529 ± 183† |
| Euthyroid on drugs | 14 | 25·8 ± 2·9 | 1,660 ± 374 | 76·7 ± 9·5 | 21·6 ± 10·5 | 1,275 ± 333 | 357 ± 160 |
| Remission | 14 | 26·9 ± 3·2 | 1,593 ± 449 | 76·5 ± 4·5 | 21·2 ± 7·6 | 1,218 ± 344 | 335 ± 138 |
| Hashimoto's thyroiditis | 22 | 31·0 ± 8·5 | 1,777 ± 580 | 77·1 ± 6·0 | 21·3 ± 7·4 | 1,362 ± 451 | 376 ± 176 |
| Other thyroid diseases | 14 | 28·6 ± 5·1 | 1,747 ± 520 | 78·3 ± 5·0 | 21·1 ± 6·3 | 1,398 ± 451 | 368 ± 151 |

Results are expressed as mean ± s.d.
* Difference from controls significant at $P < 0.05$.
† Difference from controls significant at $P < 0.01$.
‡ Difference from controls significant at $P < 0.001$.

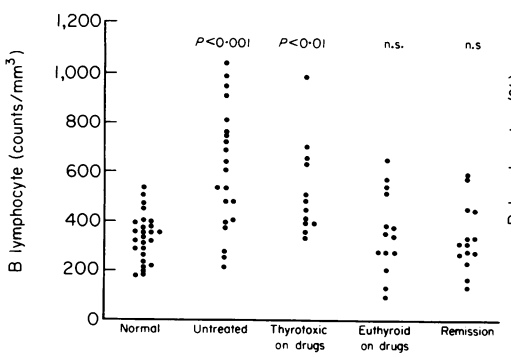


Fig. 1

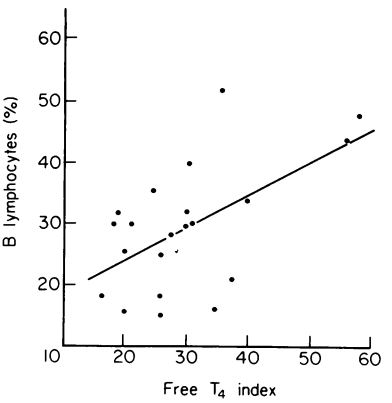


Fig. 2

**Fig. 1.** Absolute counts of peripheral B lymphocytes in Graves' disease with various thyroid conditions.

**Fig. 2.** Correlation of the percentage of B lymphocytes and free $T_4$ index (normal range = 5·0–9·6) in untreated patients with Graves' disease; $n = 21$, $r = 0{\cdot}57$, $P < 0{\cdot}02$, $y = 0{\cdot}54x + 13{\cdot}5$.

**Table 2.** Relation between peripheral lymphocytes and thyroid function, anti-thyroid microsomal antibody, goitre size and proptosis of eyes

| | | Percentage | | Lymphocyte count | |
|---|---|---|---|---|---|
| | Total lymphocytes | T cells | B cells | T cells | B cells |
| $T_4$ | −0·15 | −0·46† | 0·47† | −0·27 | 0·06 |
| $FT_4I$ | −0·33 | −0·43† | 0·57* | −0·41 | 0·10 |
| $T_3$ | −0·36 | −0·37 | 0·55* | −0·40 | 0·06 |
| $FT_3I$ | −0·36 | −0·39 | 0·55* | −0·42 | 0·07 |
| MCHA | 0·35 | −1·34 | 0·79 | −1·34 | 0·25 |
| Goitre size | −0·02 | −0·26 | 0·35 | −0·10 | 0·23 |
| Proptosis | −0·05 | 0·12 | −0·01 | 0·01 | −0·07 |

Values are correlation coefficients.
MCHA = anti-thyroid microsomal antibody.
* Significant correlation at $P < 0{\cdot}02$.
† Significant correlation at $P < 0{\cdot}05$.

been retracted (Volpé & Row, 1975). Later the percentages of T and B lymphocytes in Graves' disease were reported to be normal (Urbaniak *et al.*, 1974; Mulaisho *et al.*, 1975; Calder *et al.*, 1976; Maciel *et al.*, 1976; Lundell *et al.*, 1976). More recently Wall *et al.* (1977) and Grinblat *et al.* (1979) reported that the percentage of T cells in untreated cases of thyrotoxic Graves' disease was significantly lower than normal. Unfortunately, in these studies the changes of B cells were not examined. Hsu *et al.* (1976) reported an increased percentage of B lymphocytes in cases of thyrotoxic Graves' disease, with a normal percentage of T cells.

In the present study the percentage of T cells was found to be significantly lower than normal, and that of B cells to be higher than normal in cases of thyrotoxic Graves' disease, with or without therapy. This discrepancy is probably partly due to the different methods used for detection of lymphocyte subpopulations. Urbaniak *et al.* (1974) studied the possible effect of the method

of lymphocyte purification. They found a selective loss of T lymphocytes with relative enrichment of B lymphocytes in suspensions separated with Ficoll–Triosil. Using this method of separation, they obtained significantly decreased values in the percentage of E rosette-forming cells, but they concluded that reduction in T cells was probably factitious. It is unlikely that our finding of a decrease of T cells and an increase of B cells in thyrotoxic Graves' disease is due to separation of the cells by density centrifugation, since we observed this change only in thyrotoxic Graves' disease and the same method was used in almost all previous studies.

The fraction of mononuclear cells separated by density gradient centrifugation usually contains 10–25% monocytes and sometimes granulocytes. These cells also form EAC rosettes and are usually difficult to differentiate from lymphocytes by conventional microscopic techniques. Thus it is necessary to remove these cells or calculate their percentage accurately. In some previous studies iron powder (Maciel *et al.*, 1976; Lundell *et al.*, 1976) or an adherent technique (Urbaniak *et al.*, 1973; Calder *et al.*, 1976) was used to remove these phagocytic cells. However, in a preliminary study, we found that removal of these cells was associated with loss of B lymphocytes (unpublished data). In many studies little attention has been paid to contaminating monocytes. In this study monocytes and granulocytes were clearly identified by peroxidase staining and an appropriate correction of the percentages of E and EAC rosette-forming cells was made to obtain the true values for the T and B cell populations. B lymphocytes can be identified either as EAC rosette-forming cells or as cells showing fluorescent staining of surface immunoglobulins (sIg). The EAC method may detect a cell population that is sIg-negative and does not form E rosettes but has complement receptors. Our finding of increased B lymphocytes is probably not due to the use of the EAC method, since Hsu *et al.* (1976) also observed an increase in B lymphocytes identified by sIg. Tachibana & Ishikawa (1973) reported that the relative number of EAC-binding lymphocytes was not recovered when a tube was used for rosette formation. Previous reports of failure to detect an increase in EAC rosette-forming cells may be due partly to the effect mentioned above, since the total percentage of T and B lymphocytes was less than 85% in all studies reported (Mulaisho *et al.*, 1975; Maciel *et al.*, 1976; Lundell *et al.*, 1976). Our method using microplates avoids the loss of EAC rosettes. Furthermore the EAC used in this study was fixed with human complement instead of mouse complement to obtain more stable EAC rosettes (Tachibana *et al.*, 1974). As shown in Table 1, the total percentage of T and B cells was near 100% in all groups of patients and in controls. The receptors in activated T cells form sheep erythrocyte rosettes even after a short contact time at 37°C (Wybran & Fudenberg, 1973). Thus EAC rosettes may sometimes be formed by activated T cells, although Wall *et al.* (1977) reported a decrease in active T cells in Graves' disease. This possibility was excluded, however, in this study by the fact that a similar increase of EAC rosette-forming cells was observed when ox erythrocytes were used instead of sheep erythrocytes (unpublished findings).

The absolute counts of circulating T and B lymphocytes were directly affected by the absolute total lymphocyte counts as well as the proportions of T and B cells.

A standard text-book (Herbert, 1978) states that there is absolute lymphocytosis in the peripheral blood of patients with Graves' disease. However, reported results have been conflicting (Aoki *et al.*, 1973; Urbaniak *et al.*, 1974; Mulaisho *et al.*, 1975; Calder *et al.*, 1976; Hsu *et al.*, 1976; Maciel *et al.*, 1976; Irvine *et al.*, 1977; Grinblat *et al.*, 1979). In this work we calculated the total lymphocyte count as the product of the leucocyte count and the percentage of lymphocytes, estimated by differential analysis of smears. Results in differential counts may be affected by slight differences in technique. Thus to minimize technical error in our study, one skilled technician did all the counts, counting more than 200 cells in smears without knowledge of the diagnosis of the patients. Our results confirm that untreated cases of Graves' disease show significant lymphocytosis.

In Hashimoto's disease, the percentages and absolute counts of T and B cells were normal. Previously, an increase in peripheral lymphocytes was observed during post-partum aggravation in a patient with Hashimoto's disease (Amino *et al.*, 1978). Very recently we have consistently observed increases in B lymphocytes during post-partum aggravation in Hashimoto's disease (Amino *et al.*, 1980). These findings suggest that the lymphocyte subpopulation may change, even in Hashimoto's disease at the time of aggravation. Thus the activity of the disease should be considered in Hashimoto's disease, as well as in Graves' disease. The decrease of T cells in

Hashimoto's disease reported by Urbaniak *et al.* (1973) may have been observed because they might have studied patients with more 'active' Hashimoto's disease.

It is important to elucidate the mechanism of increase of B cells in Graves' disease. The increase was only observed in the thyrotoxic state and the B cell percentage was significantly correlated with the serum levels of thyroid hormones, suggesting that thyroid function may directly influence the proportions of T and B cells. In thyrotoxicosis, adrenal function is activated and cortisol secretion is increased (Gallagher *et al.*, 1972). Yu *et al.* (1974) reported that corticosteroids cause a decrease in T cells and an increase in B cells in the blood. It is unlikely, however, that adrenal function has an effect in Graves' disease, since the absolute number of B cells was decreased in spite of an increase in their proportion after corticosteroid administration. Another possibility is that the increase in B cells in Graves' disease may be a primary change related to induction of thyrotoxicosis. As mentioned above, the fact that cases of 'active' Hashimoto's disease also showed an increase in B cells strongly suggests that it is a primary immunological abnormality rather than a secondary change due to thyrotoxicosis. Tötterman (1978) observed predominant infiltration of B cells in the thyroid gland in both Graves' disease and Hashimoto's disease. An interesting possibility is that these infiltrated lymphocytes may migrate into the circulation when the disease is in an active state. In this context it is also interesting that activated lymphocytes, identified by an autoradiographic labelling method, were found in peripheral blood only in untreated cases of Graves' disease (Folb & Bank, 1976). Further study is necessary to determine the clinical significance of increased B cells in active autoimmune thyroid disease.

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