Increase in Autorosette-Forming Lymphocytes in Thyroid Diseases

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Abstract

A subpopulation of lymphocytes forming rosettes with autologous erythrocytes was studied on peripheral blood and thyroid tissues obtained from the patients with various thyroid diseases.

The mean (± S.D.) percentage of autorosette-forming cells (ARFC) was 10.1 (± 5.5)% in the peripheral blood from patients with hyperthyroid Graves' disease, which was higher than that in normal subjects (5.6 ± 2.8%), while the levels of ARFC in the peripheral blood from euthyroid patients with Graves' disease under treatment and Hashimoto's thyroiditis did not significantly differ from the normal level. The mean percentages of ARFC in the thyroid tissues from patients with Graves' disease and Hashimoto's thyroiditis were 14.7 (± 8.5) and 13.3 (± 7.8)%, respectively, which were higher than those in the peripheral blood from the same patients. Most of these cases with abnormally high levels of ARFC were accompanied with the abnormally low T cell to B cell ratios. The microscopic examination of the cytological materials from these patients showed an increased number of large stimulated lymphoid cells or lymphoblasts as compared with those who had few ARFC. These results suggest an increase in an activated T cell subset in the circulation and/or in the thyroid tissue, which is probably caused by active immune response to some stimuli.

The presence of cells which are capable of forming rosettes with autologous erythrocytes (autorosette-forming cells, ARFC) has been reported. ARFC was shown to be a T cell subset of a less mature nature (Charreire and Bach, 1975; Sheldon and Holborw, 1975a; Fournier and Charreire, 1978). Recently, ARFC has been thought to be a suppressor T cell (Kumagai et al., 1981; Sakane et al., 1981) or a post-thymic precursor cell (Palacios et al., 1981). It was also reported that a small number of ARFC were detected in normal peripheral blood, and that the higher levels were observed in thymocytes (Baxley et al., 1973; Kaplan, 1975; Fournier and Charreire, 1978), the peripheral lymphocytes treated with neuraminidase (Sandilands et al., 1975a) and the peripheral lymphocytes stimulated by mitogens (Sheldon and Holborw, 1975a; Fournier and Charrière, 1978) or by mixed lymphocyte culture (Sheldon and Holborow, 1975b). It was further reported that ARFC enriched T cells induced by mitogens could
absorb interleukin 2 (IL-2; TCGF) activity more than ARFC depleted T cells (Ichikawa and Daniels, 1983).

The increased number of ARFC was also demonstrated in the peripheral blood from patients with T cell-proliferative diseases (Sheldon and Holborow, 1975b) and autoimmune hemolytic anemia (Parker et al., 1977).

However, the pathophysiological meaning of the increase in the number of ARFC in these diseases remains obscure. The levels of ARFC in other diseases, especially in organ-specific autoimmune diseases, have not yet been studied. In this paper, we describe the abnormality of ARFC levels in the circulation and thyroid tissues from patients with autoimmune thyroid diseases and discuss its immunological meaning in these diseases.

Materials and Methods

Patients

Patients with Graves' disease (GD) were classified into the following three groups: (I) untreated hyperthyroid patients (6 males and 4 females; 17–49 yr), (II) hyperthyroid patients under thionamide medication (3 males and 10 females; 16–52 yr) and (III) euthyroid patients under thionamide medication (10 males and 28 females; 11–60 yr). The patients with Hashimoto's thyroiditis (HT) consisted of a male and 31 females, 29–68 yr; 23 euthyroid patients under T3 treatment, and 2 hypothyroid and 7 euthyroid patients without treatment. Twenty-nine healthy hospital workers (17 males and 12 females; 21–58 yr) who had no history of autoimmune disorders were studied as a control.

Preparation of peripheral blood lymphocytes

Lymphocytes from heparinized peripheral venous blood were recovered as follows. Three ml of heparinized venous blood was diluted 2-fold with phosphate buffered saline (PBS), and then layered over Ficoll-Metrizoate of specific gravity 1.077 (Japan Immunoresearch Lab., Tokyo). The mononuclear cells obtained by centrifugation at 400 × g for 30 min were washed three times with PBS and suspended in PBS at a concentration of 1.4 × 10^6 cells/ml.

Preparation of thyroid-infiltrating lymphocytes

In 15 cases, thyroid tissues obtained from surgical specimens were washed with PBS and minced with scissors in 3 volumes of PBS. The minced tissue was then filtered through a steel mesh. An aliquot of the filtrates was centrifuged at 200 × g for 10 min, and the pellet was smeared onto a slide glass and the rest was layered over Ficoll-Metrizoate of specific gravity 1.077. The mononuclear cells were obtained by centrifugation at 400 × g for 30 min, washed three times with PBS, and suspended in PBS at a final concentration of 1.4 × 10^6 cells/ml.

In 19 cases, fine needle aspiration biopsies from the thyroid gland were performed by using a 10 ml syringe with a 22 gauge needle. One drop of the aspirated material was spread onto a slide glass, and the rest was diluted in 1 ml of PBS containing 0.5% bovine serum albumin. Separation of lymphocytes from the mixture was performed by density gradient centrifugation over Ficoll-Metrizoate as described above. The degree of blood contamination (index of blood contamination, IC) was calculated from the blood and thyroid smears according to the method of Tötterman (1978), and biopsies with an IC less than 3.0 were not considered to be representative.

Autorosette formation

One μl of cell suspension (1.4 × 10^4 cells) was added into the wells of microplates (Falcon 3034, Oxnard, CA) treated with poly-L-lysine. The lymphocytes were allowed to adhere to the bottom of the plates for 15 min and then 4 μl of fetal calf serum was added to the wells. After incubation for 30 min at room temperature, 4 μl of autologous erythrocytes at 3 × 10^9 cells/ml in PBS was added into the wells. The plates were incubated for 90 min at room temperature and then kept at 4°C overnight. The plates were then inverted for 30 min to detach the unreacted erythrocytes from the bottom, gently immersed in PBS, and kept in the inverted position for an additional 30 min. The lymphocytes which adhered to the bottom of the plates were fixed with 0.25% glutaraldehyde and then the plates were gently washed with distilled water. To differentiate the monocytes and granulocytes from lymphocytes, the plates were then stained with peroxidase using 3-amino-9-ethylcarbazole as a...
substrate. Finally, ten μl of 0.01% brilliant cresyl blue was added to the wells. For quantitation of ARFC, six wells were used for each sample and at least 500 lymphocytes were counted. An ARFC was defined as a cell to form a rosette with at least three autologous erythrocytes.

The results were analyzed for statistical significance by t-test.

Identification of T and B cells

T and B cells were measured quantitatively by using a method of rosette formation with sheep erythrocytes (E) and with antibody-complement-sensitized ox erythrocytes (EAC), respectively. The procedure for rosette formation was the same as that for autorosette formation, except for the use of E or EAC instead of autologous erythrocytes.

Mixed rosette formation

Mixed rosettes were made in a 10×75 mm test tube by adding 40 μl of sheep erythrocytes suspension (1.8×10⁷ cells) to 290 μl of a mixed suspension of lymphocytes (3.5×10⁵ cells) and autologous erythrocytes (1.8×10⁷ cells) already incubated to induce autorosette formation. The mixture was centrifuged at 200×g for 5 min, and kept for 2 h at room temperature and then at 4°C overnight. A cytocentrifuged preparation of the resuspended cells was made and stained with Giemsa.

Identification of activated lymphocytes

 Activated lymphocytes in thyroid-infiltrating lymphocytes were identified by the microscopical observation of thyroid smears, and the following criteria were employed: 1) large in size, 2) increase in nuclear-cytoplasmic ratio, 3) fine structure of chromatin, 4) appearance of nucleolus and 5) basophilic cytoplasm.

Results

Validity of the measurement of ARFC

In preliminary experiments, the reproducibility of the measurement of ARFC was assessed within a day using peripheral blood from 3 normal subjects: the coefficients of variation were 6.2% (mean±S.D., 8.7±0.54; n=5), 7.2% (mean±S.D., 3.9±0.28; n=5) and 9.6% (mean±S.D., 7.6±0.73; n=5).

ARFC in peripheral blood

Auto rosette formation tests were performed on peripheral blood lymphocytes from 29 normal subjects (Fig. 1A), and the mean incidence of ARFC was 5.6% (range, 1.7–11.8%). Some normal individuals had unusually high percentages of ARFC over 10%. The serial tests on 3 normal subjects over a period of 6 months showed relatively large individual fluctuations, and the coefficients of variation between the tests were 21% (mean±S.D., 4.7±1.0%; n=5), 31% (mean±S.D., 3.9±1.2%; n=5) and 50% (mean±S.D., 3.0±1.5%; n=9).

The percentages of ARFC in the peripheral blood from most of the patients with HT was within normal levels, while patients with GD showed a higher mean and range (Fig. 1A). The mean (±S.D.) percentage of ARFC in 22 patients with hyperthyroid GD was 10.6(±5.3)%, which was significantly higher than the normal level. However, the mean level of ARFC in euthyroid patients with GD did not significantly differ from that in normal control, though the increased percentages over the 2 S.D. range of the normal level were observed in 4 of 37 patients.

T and B cells in peripheral blood

The ratios of T cells to B cells (T/B cell ratio) determined in the peripheral lymphocytes are shown in Fig. 1B. The mean T/B cell ratio in the peripheral lymphocytes from normal subjects was 7.6% (range, 5.0–12.4%). The significantly lower values for the T/B cell ratio were observed in the peripheral blood from patients with hyperthyroid GD. In these patients, both the percentages and the absolute counts of T cells were decreased as compared with those in normal subjects, while the percentages and the absolute counts of B cells were increased. The mean T/B cell ratio in the peripheral blood from euthyroid patients
ARFC in the thyroid-infiltrating lymphocytes

ARFC was assessed in the thyroid-infiltrating lymphocytes obtained from patients who had undergone surgery or aspiration biopsy, and the results are shown in Fig. 2A. The incidence of ARFC in the infiltrating lymphocytes from patients with GD and HT were 14.7±8.5% and 13.3±7.8% (mean±S.D.), respectively, which were higher than those in the peripheral lymphocytes from the same individuals. The increased percentages of ARFC over the 2 S.D. range of the normal peripheral level were observed in the infiltrating lymphocytes from 7 of 11 patients with GD and from 8 of 15 patients with HT. As a control experiment, we tried to prepare the lymphocytes from normal thyroid obtained by autopsy, but the yield of the lymphocytes was too low to examine the levels of ARFC and subpopulation.

Some of ARFC observed in the infiltrating lymphocytes from patients with GD and HT were characteristically large in size and had a fine nucleus and distinct nucleoli,

with GD and the patients with HT did not significantly differ from that in normal control.
which formed a bulky rosette with a number of autologous erythrocytes. The large ARFC were demonstrated to be T cells by forming a mixed rosette with sheep E and autologous E.

**T and B cells in the thyroid-infiltrating lymphocytes**

The T/B cell ratios were determined in the infiltrating lymphocytes, and the ratios were $3.8 \pm 2.3\%$ and $4.8 \pm 3.6\%$ (mean $\pm$ S.D.) in the patients with GD and HT, respectively, which were significantly lower than those in peripheral lymphocytes from the same individuals (Fig. 2B). The T/B cell ratios lower than 2 S.D. range of normal peripheral level were shown in the infiltrating lymphocytes from 6 of 11 patients with GD and from 4 of 14 patients with HT.

**Activated lymphocytes in the infiltrating lymphocytes**

The percentages of activated lymphocytes in the infiltrates from patients with GD and
HT are shown in Table 1. In both diseases, the increased numbers of activated lymphocytes were observed in the infiltrates with a high incidence of ARFC.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Level of ARFC</th>
<th>N</th>
<th>Level of activated lymphocytes (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Grave's disease</td>
<td>low*</td>
<td>3</td>
<td>ND**</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>7</td>
<td>ND-10.0</td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td>low</td>
<td>4</td>
<td>0.5-40</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>6</td>
<td>2.0-15.0</td>
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* low, equal to or below 11.2% (mean ± 2 S.D. level in normal control); high, above 11.2%
** not detectable

**Discussion**

The mean and range of the ARFC level in the peripheral blood from normal subjects varied diversely from report to report, such as 0.5-19.5% (mean, 3.4%) (Sandilsands *et al.*, 1975), 5 ± 4.0% (mean ± S.D.) (Kaplan *et al.*, 1975), 2.02% (mean) (Gluckman and Montambault, 1975), below 1% (Fournier and Charreire, 1977), 0.08-7.96% (Parker *et al.*, 1977), and 0-6% (mean, 3.2%) (Sheldon and Holborow, 1975a). In our present study, the normal range of peripheral ARFC was 1.7-11.8% (mean, 5.6%). These variations in ARFC levels among the reports seem to be attributed to the slight difference in the methods used for the measurement of ARFC. However, they agree with one point of view that a small percentage of ARFC was observed in the peripheral lymphocytes from normal subjects.

An increased incidence of ARFC was observed in peripheral blood from patients with hyperthyroid GD. Until now, it has been reported that ARFC belongs to a T cell subset (Baxley *et al.*, 1973; Gluckman and Montambault, 1975; Fournier and Charreire, 1977), and that the number of ARFC in the peripheral lymphocytes increases following stimulation with mitogens such as Con A and PHA (Sheldon and Holborow, 1975b; Fournier and Charreire, 1978). Furthermore, there is an interesting report (Folb and Bank, 1976) showing that activated lymphocytes identified by an autoradiographic labelling method were observed in the peripheral blood from most of the patients with untreated hyperthyroid GD. Recently, it has been also reported that ARFC induced by mitogens were derived from both ARFC enriched T cells and ARFC depleted T cells, and that ARFC enriched T cells could absorb interleukin 2 (IL-2; TCGF) activity more than ARFC depleted T cells (Ichikawa and Daniels, 1983). These reports suggest that, in the peripheral blood of patients with hyperthyroid GD in the present study, the increased ARFC represents an increase in a T cell subset which is stimulated by thyroid-related antigens or lymphokines.

This view is compatible with the observation of high ARFC levels in thyroid-infiltrating lymphocytes. Recently, it has been reported that HLA-DR antigens were expressed by the thyroid cells in sections and cultures of thyroidectomy specimens from GD and HT patients (Hanafusa *et al.*, 1983). Furthermore, it has been also reported that HLA-DR antigens were able to stimulate autologous T cells to lead the activated state (Weksler *et al.*, 1981). In the present study, some lymphocytes in the infiltrates were microscopically found to be activated, which were characteristically large in size and capable of binding sheep E and autologous E. Thus, it is likely that ARFC in thyroid-infiltrating lymphocytes are T cell subsets stimulated directly by HLA-DR antigens expressed on thyroid cells. These infiltrating lymphocytes may migrate from thyroid tissue into the circulation. However, it is unknown at present why, in patients with HT, the high level of ARFC is observed only in the tissue infiltrates.
There is another interesting observation that most of the lymphocytes which had an increased incidence of peripheral ARFC were accompanied by an abnormality in the T/B cell ratio. Mori et al., (1980) reported a similar abnormality in the lymphocyte subpopulation in the peripheral blood from untreated patients with GD, and suggested the possibility that the increase in peripheral B cells in these patients may be a primary immunological abnormality rather than a secondary change due to thyrotoxicosis. Our data on the abnormality of peripheral ARFC levels in patients with hyperthyroid GD confirm their theory.

Furthermore, the decrease in the T/B cell ratio was also observed in the thyroid infiltrates from patients with GD and HT, which was similar to the results reported by Tötterman (1978). These abnormal T/B cell ratios in the thyroid infiltrates are probably the result of active immune responses in the tissue.

Consequently, it is possible that ARFC is employed as a marker for activated T cell subset, i.e. augmentation of the cellular-immunity in the peripheral blood from patients with hyperthyroid GD and in the thyroid tissues from patients with GD and HT.

References


