E-rosette formation in Graves' ophthalmopathy

Robert C. Sergott, Norman T. Felberg, Peter J. Savino, John J. Blizzard, and Norman J. Schatz

We investigated the lymphocyte characteristics of 77 Graves' disease patients with and without infiltrative ophthalmopathy. Thirteen patients with infiltrative ophthalmopathy without prior antithyroid therapy and 20 euthyroid patients with progressive ophthalmopathy demonstrated diminished percentages of active and total erythrocyte rosette-forming lymphocytes compared to thyrotoxic patients without eye disease and to a control population (p < 0.001). There was no significant difference in rosette-forming cells between untreated thyrotoxic and treated euthyroid patients with ophthalmopathy. No lymphocytotoxic antibodies or rosette inhibitory factor was present in the sera of patients with infiltrative ophthalmopathy. Untreated and treated patients with lid retraction and mild proptosis without extraocular muscle disease had decreased active rosette-forming cells (p < 0.001) but normal total rosette-forming cells. Five patients with infiltrative ophthalmopathy who failed to improve with systemic corticosteroids demonstrated elevated active but normal total rosette-forming cells. Differences in rosette formation between ophthalmic and nonophthalmic Graves' disease may represent an associated cell-mediated abnormality that may explain why control of the thyrotoxic state need not correlate with the ophthalmic manifestations of the disorder.

Key words: Graves' disease, thyroid ophthalmopathy, erythrocyte rosette-forming lymphocytes

Graves' disease may occur in any combination of three clinical syndromes: (1) hyperthyroidism characterized by diffuse hyperplasia of the thyroid gland, (2) infiltrative ophthalmopathy with restrictive ophthalmoplegia and proptosis, and (3) infiltrative dermopathy—"localized pretibial myxedema."1

The pathogenesis of Graves' disease has remained unknown, although many observations have implicated immunologic processes.2-9 Because the ophthalmopathy and thyroid disorder often occur in association, there appears to have been a tacit assumption that the mechanism of the two disorders is similar. It is well recognized, however, that the ophthalmopathy may precede thyrotoxicosis, may occur simultaneously, may occur after treatment of thyrotoxicosis, or may be present without any clinical evidence of thyrotoxicosis. Solomon et al.10 have suggested the possible autoimmune disorder of "isolated" Graves' ophthalmopathy.
Table I. Abridged classification of eye changes of Graves' disease

<table>
<thead>
<tr>
<th>Class*</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No physical signs or symptoms</td>
</tr>
<tr>
<td>1</td>
<td>Only signs, no symptoms (signs limited to upper eyelid retraction, stare, and eyelid lag)</td>
</tr>
<tr>
<td>2</td>
<td>Soft tissue involvement (symptoms and signs)</td>
</tr>
<tr>
<td>3</td>
<td>Proptosis</td>
</tr>
<tr>
<td>4</td>
<td>Extraocular muscle involvement</td>
</tr>
<tr>
<td>5</td>
<td>Corneal involvement</td>
</tr>
<tr>
<td>6</td>
<td>Sight loss (optic nerve involvement)</td>
</tr>
</tbody>
</table>

*Each class usually, but not necessarily, includes the involvement indicated in the preceding class.

Ophthalmic manifestations of Graves' disease may vary from minor and temporary signs to major and permanent complications (Table I). In a study of the immunologic characteristics of Graves' disease patients, we have used active and total rosette-forming lymphocyte determinations as in vitro assays of thymic-dependent (T-cell) immunity and related these values to the manifestations of thyroid ophthalmopathy.

Recognition and enumeration of human T-lymphocyte subpopulations has greatly enhanced the understanding of human immunologic mechanisms. Wybran and associates have identified a subpopulation of T-cells with high binding affinity for sheep erythrocytes (E). Felsburg et al. have shown that a significant rise in these peripheral-blood active rosette-forming cells (A-RFC) occurs in individuals within 48 hours of a positive skin test to several microbial antigens. Skin test–negative individuals demonstrate no increase in A-RFC. In comparison, total rosette forming cells (T-RFC) may not always reflect an individual's immunologic status.

Sera from Graves' disease patients were evaluated for lymphocytotoxic antibodies and for the ability to inhibit rosette formation. This investigation differs from previous studies in that patients were analyzed specifically in relation to their varied ophthalmic manifestations and not merely included under the general heading of 'Graves' disease.'

Our findings indicate that the majority of thyrotoxic and euthyroid Graves' disease patients with progressive ophthalmopathy have decreased percentages of A-RFC and T-RFC when compared to patients without ophthalmopathy or to a control population. In addition, some patients with progressive Graves' ophthalmopathy showed elevated percentages of A-RFC but normal values of T-RFC. These patients had the poorest clinical response to systemic corticosteroid therapy.

Patients and methods

We studied 77 patients with Graves' disease from October 1976 to August 1978. Diagnosis was established by either nonsuppressibility of thyroid iodine uptake following exogenous administration of thyroid hormone or by demonstration of an autonomous pituitary-thyroidal axis with thyroid-stimulating hormone measurement before and after the administration of thyrotropin-releasing hormone. The study population consisted of 54 women and 23 men. Mean age of the group was 41.34 ± 9.23 years.

Patients were classified according to the system of the American Thyroid Association. In addition, the patients' thyroid disease was classified as treated or untreated to see whether thyroid therapy influenced ophthalmopathy and rosette formation. Because of the lack of firm physical diagnostic criteria to separate classes 1 and 2, these patients were considered together (class 1-2). Patients in classes 4 and 5 with recent-onset Graves' ophthalmopathy (less than 3 months' duration) were grouped together because their predominant problem was diplopia and painful restrictive ophthalmoplegia. Restrictive ocular myopathy was documented by positive forced-duction testing. All these patients had punctate corneal staining with fluorescein dye, but the group is referred to as class 4-5 to emphasize their extraocular muscle disease. No patients in class 6 were seen.

Of the 13 untreated patients in class 4-5, seven had Graves' thyrotoxicosis, and six had euthyroid Graves' ophthalmopathy. All 20 treated patients in class 4-5 were euthyroid and had been referred to a neuroophthalmologist because of worsening ocular myopathy. In class 4-5, 11 of 20 were treated with methimazole (Tapazole) or propylthiouracil, and six of 20 were treated with both antithyroid medication and radioactive iodine; three of 20 had undergone partial thyroidectomies, and two of 20 were currently taking systemic corticosteroids (highest dose 20 mg every other day). None of the 77 patients had any other immunologic disorder.

Four patients were studied who had white
Table II. Percentages of A-RFC and T-RFC in Graves’ ophthalmopathy

<table>
<thead>
<tr>
<th>Class</th>
<th>Thyroid state</th>
<th>% A-RFC (No. of patients)</th>
<th>% T-RFC (No. of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Thyrotoxic, untreated</td>
<td>28.40 ± 7.47 (10)(^c)</td>
<td>56.90 ± 7.91 (10)(^c)</td>
</tr>
<tr>
<td></td>
<td>Euthyroid, treated</td>
<td>23.60 ± 4.45 (5)(^c)</td>
<td>58.80 ± 7.56 (5)(^c)</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>26.80 ± 6.86 (15)(^c)</td>
<td>57.53 ± 7.58 (15)(^c)</td>
</tr>
<tr>
<td>1-2</td>
<td>Thyrotoxic, untreated</td>
<td>8.73 ± 6.86 (11)(^A)</td>
<td>52.55 ± 9.77 (11)(^c)</td>
</tr>
<tr>
<td></td>
<td>Euthyroid, treated</td>
<td>9.00 ± 7.87 (6)(^A)</td>
<td>60.17 ± 13.11 (6)(^c)</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>8.82 ± 6.17 (17)(^A)</td>
<td>55.24 ± 11.29 (17)(^c)</td>
</tr>
<tr>
<td>3</td>
<td>Euthyroid, untreated</td>
<td>34.33 ± 1.53 (3)(^A)</td>
<td>51.00 ± 3.00 (3)(^B)</td>
</tr>
<tr>
<td>4-5(^B)</td>
<td>Thyrotoxic, untreated</td>
<td>11.57 ± 6.40 (7)(^A)</td>
<td>34.86 ± 13.93 (7)(^A)</td>
</tr>
<tr>
<td></td>
<td>Euthyroid, untreated</td>
<td>11.17 ± 5.53 (6)(^A)</td>
<td>36.33 ± 13.06 (6)(^A)</td>
</tr>
<tr>
<td></td>
<td>Combined/un-treated</td>
<td>11.38 ± 5.77 (13)(^A)</td>
<td>35.54 ± 12.99 (13)(^A)</td>
</tr>
<tr>
<td></td>
<td>Euthyroid, treated</td>
<td>10.70 ± 7.00 (30)(^A)</td>
<td>40.60 ± 10.09 (30)(^A)</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>10.97 ± 6.45 (33)(^A)</td>
<td>38.61 ± 11.40 (33)(^A)</td>
</tr>
<tr>
<td>4-5(^C)</td>
<td>Combined</td>
<td>36.00 ± 7.81 (5)(^B)</td>
<td>48.60 ± 10.36 (5)(^C)</td>
</tr>
<tr>
<td>4-5(^F)</td>
<td>Euthyroid, treated</td>
<td>34.00 ± 7.07 (4)(^B)</td>
<td>68.75 ± 6.24 (4)(^A)</td>
</tr>
<tr>
<td>Controls</td>
<td>Euthyroid</td>
<td>24.78 ± 10.66 (100)</td>
<td>55.89 ± 10.75 (100)</td>
</tr>
</tbody>
</table>

\(^p\) values vs. controls: \(^A\)<0.001; \(^B\)<0.01; \(^C\)not significant.

\(^a\)Steroid responsive: 60-150 mg of prednisone, orally, daily for 7-14 days.

\(^b\)Steroid unresponsive.

\(^c\)Stable, nonprogressive.

and quiet eyes with stable, nonprogressive proptosis and restrictive ophthalmoplegia for at least 3 years. Because the American Thyroid Association classification does not include provisions for disease activity and since these patients were in clinical remission with fixed ophthalmic findings, we included them in a special "inactive" subdivision of class 4-5.

Separate observers independently performed the ophthalmic, endocrinologic, and immunologic evaluations. Active and total rosette determinations were done without any knowledge of the clinical findings.

The control population consisted of 100 patients seen at Wills Eye Hospital for complaints in which immunologic disorders were not considered in the differential diagnosis. Mean age of the control population was 47.18 ± 11.24 years. After the nature of the investigation was explained, informed consent was obtained from all patients.

Rosette-forming cells. For each determination, 0.10 ml of the lymphocyte suspension of 5 × 10^6 cells/ml prepared by Ficoll-Hypaque gradient centrifugation was mixed with 0.02 ml of absorbed normal human serum and 0.10 ml of 0.5% sheep erythrocytes (E). After 5 min at 37°, the sample was centrifuged at 200 × g for 5 min. Resuspended cells were examined in a hemocytometer for rosette formation (A-RFC). T-RFC were determined in replicate specimens after an additional incubation at 4° for at least 90 min. Ficoll-Hypaque centrifugation yielded a mononuclear population with over 95% lymphocytes. Viability, assessed by trypan blue exclusion test, was over 95%.

**Lymphocytotoxic antibodies.** Patients' sera were assayed for complement-dependent lymphocytotoxic antibodies according to the method of Michlmayr et al. In a 10 by 75 mm test tube, 0.1 ml of heat-inactivated serum was incubated for 45 min at room temperature with 0.1 ml of lymphocytes (5 × 10^6 cells/ml) in RPMI 1640 medium. Then 0.1 ml of either fresh normal rabbit serum as a source of complement or heat-inactivated rabbit serum was added. Following a 2 hr incubation at room temperature, the mixture was centrifuged at 50 × g for 5 min, and the supernatant was removed and replaced with 100 μl of trypan blue in phosphate-buffered saline (pH 7.4) prior to determining the viability.

**Serum inhibition of rosette formation.** To see whether sera from Graves' disease patients were able to inhibit rosette formation, 0.1 ml of lymphocytes (5 × 10^6 cell/ml) was incubated with 0.02 ml of heat-inactivated sera for 2 hr at 37° in a humidified 5% CO₂ atmosphere. Determinations of A-RFC and T-RFC were performed as above after addition of 0.1 ml of 0.5% E. Rosette inhibition and lymphocytotoxic antibody determinations were done with allogeneic and autologous lymphocytes and sera.

**Results**

**Rosette-forming cells.** Table II shows the value of A-RFC and T-RFC in a control population and from patients with different clinical
classes of Graves’ disease. Absolute numbers of A-RFC and T-RFC have been determined in more than half the patients. The absolute and percentage values show similar statistical comparisons between the controls and clinical groups. Only percentages of rosette-forming cells are reported.

Untreated patients in class 0 with active thyrotoxicosis had unaltered percentages of A-RFC and T-RFC when compared by Student’s t test to the control population. Treated euthyroid patients also showed no significant difference in A-RFC and T-RFC when compared to controls.

Patients in class 1-2 (11 thyrotoxic and six euthyroid) demonstrated a statistically significant decrease in A-RFC (p < 0.001) but showed no alteration in T-RFC when compared to controls. Three untreated euthyroid patients with proptosis in excess of 22 mm, isolated from lid retraction and extraocular muscle disease (class 3), had a statistically significant elevation of A-RFC (p < 0.001) and a slight decrease in T-RFC (p < 0.02) when compared to controls.

Steroid-responsive class 4-5 patients (seven untreated thyrotoxic, six untreated euthyroid, and 20 treated euthyroid) had a statistically significant decrease in both A-RFC and T-RFC vs. the control population. Steroid-unresponsive patients in class 4-5 (two untreated thyrotoxic, one untreated euthyroid, and two treated euthyroid) showed elevated percentages of A-RFC compared to controls (p < 0.02) but no difference in T-RFC.

Four patients in class 4-5 with stable, non-progressive extraocular muscle restriction and proptosis for over 3 years had significantly elevated A-RFC (p < 0.02) and T-RFC (p < 0.001) compared to controls.

Cross comparisons of thyrotoxic and euthyroid patients with the same ophthalmic findings failed to reveal a statistically significant difference in rosette-forming cells. However, comparisons of patients without ophthalmic manifestations (class 0) compared to patients in class 1-2 demonstrated a statistically significant decrease in A-RFC (p < 0.001) but no change in T-RFC.

Class 0 compared to steroid-responsive class 4-5 showed a statistical decrease (p < 0.001) in both A-RFC and T-RFC. Between classes 1-2 and steroid-responsive 4-5, there was a difference only in values of T-RFC (p < 0.001). There was a statistically significant difference in both A-RFC and T-RFC (p < 0.001) in patients with stable, nonprogressive proptosis and extraocular muscle disease when compared to patients with steroid-responsive, active, progressive disease in class 4-5. Between steroid-responsive and steroid-unresponsive class 4-5, there was a statistically significant difference in A-RFC (p < 0.001) but no difference in T-RFC.

**Lymphocytotoxic antibodies.** Graves’ disease patients with and without ophthalmopathy failed to show antilymphocyte antibodies by a complement-dependent cytotoxicity assay. Lymphocytes mixed with homologous and heterologous sera retained over 95% viability by trypan blue exclusion. Serum from a patient with systemic lupus erythematosus killed over 75% of heterologous lymphocytes in this assay and served as a positive control.

**Serum inhibition of rosette formation.** Sera from patients with active, progressive steroid-responsive ophthalmopathy and reduced percentages of A-RFC and T-RFC failed to inhibit the ability of autologous lymphocytes and lymphocytes from normal donors to form rosettes.

In addition, lymphocytes from patients were incubated in RPMI 1640 medium supplemented with 20% fetal calf serum and antibiotics for 18 hr at 37° C in a 5% CO₂ humidified atmosphere. Lymphocytes were washed, and E-rosette formation was not altered. Further incubation with autologous sera produced no change in percent of rosette-forming cells (results not shown).

**Discussion**

Much evidence portrays Graves’ disease as a disorder not only of thyroid and eye but also of lymphocytes and antibodies. The interaction between the endocrine and ophthalmic features of this disorder remains obscure. Every form of thyrotoxicosis therapy has been suspected of increasing, of not
affecting, or of decreasing the ophthalmopathy. Previous investigations of peripheral blood lymphocytes have not distinguished between Grave’s disease with and without ophthalmic manifestations and have not measured active rosette-forming lymphocytes.16–19

Since some patients with Graves’ disease have thymic medullary lymphoid follicles4 and since attempts to correlate the ophthalmopathy with humoral factors such as long-acting thyroid stimulators26 and thyroid-stimulating immunoglobulins27 were unsuccessful, it became reasonable to compare cell-mediated (thymic-dependent) immunity in ophthalmic and nonophthalmic Graves’ disease. Unfortunately, the report of thymic abnormalities in Graves’ disease did not classify the patients’ ophthalmic status.

Our assessment of cell-mediated immunity by A-RFC and T-RFC reveals a statistically significant alteration between Graves’ disease with only thyrotoxicosis and Graves’ disease with steroid-responsive infiltrative ophthalmopathy regardless of the thyroid state. Our data indicate that there appears to be an associated cell-mediated abnormality present in Graves’ ophthalmopathy. Our investigation confirms previous reports of normal percentages of T-RFC in Graves’ disease patients with only thyrotoxicosis16,19 and adds the finding of normal percentages of A-RFC in thyrotoxicosis. Therefore each manifestation of Graves’ disease may have a distinct immunologic disturbance for each target organ.

We are acutely aware that other investigators16–19 have reported dissimilar results concerning rosette-forming cells in Graves’ disease. Differences in previous studies arise regarding methodology and patient selection. As pointed out by Urbaniak et al.,16 Farid et al.18 used a dextran sedimentation procedure to isolate lymphocytes as opposed to the Ficoll-Hypeaque technique. This technical difference has been fully commented upon by Urbaniak et al.16 and Mulaisho et al.19 We believe that the crucial difference in our study is the classification of patients with Graves’ disease. Since we explicitly directed our investigation according to the ophthalmic manifestations of this disorder, we believe that our findings should be considered independently from previous efforts. With this “isolation” instead of “unification” of the manifestations of this disorder, it is not surprising to find an immunologic separation to correspond with the well-recognized clinical separation between the endocrine and ophthalmic components of the syndrome identified as Graves’ disease. The simple, and unpredictable, association between Graves’ hyperthyroidism and ophthalmopathy certainly cannot be taken as justification to consider these conditions as a single entity—whether we are discussing their clinical course or their immunologic characteristics.

In addition, we are aware that rosette formation is a nonspecific thymic-dependent assay and by no means completely defines the immunologic status of these patients. Yet, this fact does not detract from our finding of an immunologic dichotomy between the diverse manifestations of Graves’ disease and should encourage further elucidation of these patients’ immune characteristics.

Solomon et al.10 have proposed an immunologic subgrouping within the clinical entity of euthyroid Graves’ disease. Our study has also disclosed an immunologic subdivision of Graves’ ophthalmopathy. Within Werner’s class 4-5, we found that the patients who had the most improvement in their painful restrictive ophthalmoplegia with corticosteroid therapy had statistically significant decreased percentages of A-RFC and T-RFC. The patients with elevated percentages of A-RFC were not benefited by corticosteroid therapy. Whether these patients were in a “steroid-resistant” phase of disease or whether they had a different immunopathologic mechanism of disease remains to be determined. Nonetheless, the difference in A-RFC may be helpful in selecting the mode of therapy for patients with severe ophthalmic Graves’ disease.

Several explanations are possible for detecting reduced percentages of T-lymphocytes in the majority of patients with progressive
Graves' ophthalmopathy: (1) an intrinsic alteration of the T-lymphocytes preventing their usual binding to E; (2) sequestration of T-lymphocytes in another site from the peripheral blood circulation; (3) the presence of increased numbers of nonrosetting lymphocytes (B-lymphocytes, null cells, macrophages, or T-cell precursors); (4) lymphocytotoxic antibodies causing complement-dependent lysis of T-cells; and (5) serum factor(s) interfering with the binding of lymphocytes and E.

The last two possible mechanisms are unlikely in Graves' ophthalmopathy in view of our inability to demonstrate lymphocytotoxic antibodies or rosette-inhibitory factors in patients' sera. In systemic lupus erythematosus, lymphocytotoxicity has shown to be more sensitive to IgM than IgG antibodies28; thus it is still possible that IgG antilymphocyte antibodies could account for the decrease in T-lymphocyte percentages and absolute numbers. Preliminary studies in our laboratory indicate that B-lymphocyte populations are identical to those of controls, suggesting that this cell type is not responsible for an increased number of nonrosetting cells. Likewise, preliminary evidence indicates that EAC rosettes29 (macrophages and some others that bind to erythrocytes coated with antibody and complement) are not responsible for increased numbers of nonrosetting cells. At this time, the decrease in E-rosette formation appears more likely to be from either of the first two mechanisms.

Patients with untreated ophthalmopathy and those with progressive ophthalmopathy despite successful thyroid therapy showed no statistical difference in rosette formation. This finding, though possibly not related to the etiology of the disease, may reflect a related thymus-dependent abnormality that could explain why control of the thyrotoxic state, although obviously beneficial to the patient, may not correlate with or influence the opthalmic manifestations of the disease.

It is interesting, but as of yet unknown significance, that those patients with mild proptosis (less than 22 mm), lid retraction, and no extraocular muscle disease, showed statistically lowered A-RFC and normal T-RFC. During our 20-month analysis of Graves' ophthalmopathy, we encountered only three patients with proptosis isolated from lid retraction or ocular myopathy (class 3). More patients will be needed to determine statistical significance for rosette-forming percentages in this class. Our study also points out the need to incorporate an assessment of disease activity into the American Thyroid Association classification of the eye changes of Graves' disease, since patients with red, inflamed eyes with progressive proptosis and worsening, painful ophthalmoplegia (class 4-5) differ clinically and immunologically from patients with white and quiet eyes with a fixed amount of proptosis and unchanging ocular motility (class 4-5, inactive).

We thank Ms. Sharyn Koch in the laboratory of Jay Federman, M.D., and Diane Fefer for their excellent technical assistance. In addition, we thank Dr. Shalom Leon, A. Einstein Medical Center, Philadelphia, for his gift of lymphocytotoxic serum from a patient with systemic lupus erythematosus. We thank Rose Nagy and Rosemary McFall for their editorial assistance and Ruth Grevious for her secretarial assistance.

REFERENCES