Re-examination of Peripheral Blood T Cell Subsets in Dysthyroid Orbitopathy

Anna Tyutyunikov,* Radmila B. Railiow,* John S. Kennerdell,* Michael Kazim,† Milton H. Dalbow,* and Deborah Scalise*

Patients with dysthyroid orbitopathy (DO) were grouped according to a multifactorial assessment of disease severity and the rate of disease progression. Using this system and flow cytometric measurements of T cell subsets in the peripheral blood, a significant increase in the percentage of CD4+ lymphocytes correlated with disease severity in DO patients with progressive disease. These observations are consistent with the hypothesis that the CD4+ peripheral blood T helper cells play a significant role in the progression of DO. Invest Ophthalmol Vis Sci 33:2299–2303, 1992

Dysthyroid orbitopathy (DO), also called Graves’ ophthalmopathy, is an autoimmune disease, whose pathogenesis remains obscure. One possible pathogenetic mechanism is an alteration in the percentages of T lymphocyte subsets, ie, a decrease in suppressor cells with or without a concomitant increase in helper cells. Previously reported data bearing on this hypothesis have been conflicting.

In 1974, Volpe1 postulated that DO can be caused by distinct genetic defects of the immune system, probably defects of suppressor T lymphocytes. Using a rosette-formation assay with sheep erythrocytes to mark human T cells, Sergott et al2 demonstrated a decrease of peripheral T lymphocytes in DO patients. Bizarro et al,3 using the same method, however, demonstrated normal levels of T cell populations in these patients. With the introduction of monoclonal antibodies to cell surface markers, a more reliable test method became available,4 but subsequent publications about T cell subsets using monoclonal antibodies continued to report conflicting results. These are summarized in Table 1. Van der Gaag et al5 reported a statistically insignificant decrease of CD4+ and CD8+ levels in DO patients placed in Class 3 and 4 according to the criteria described by Werner.6 In contrast, Felberg et al7 showed increased levels of CD8+ lymphocytes in a similar group of patients. As indicated in Table 1, each investigator used a different approach to categorize the patients. Sridama et al8 based their grouping on the thyroid status of the patients without evaluation of their ophthalmologic symptoms. Van der Gaag et al,9 on the other hand, considered only the Werner classification. Among their 54 patients, only 22 were euthyroid. These major differences in the patient populations and their categorization may account for the divergent results.

In view of these conflicting results, we re-evaluated the distribution of peripheral T cell populations using flow cytometry and a new clinical grouping. In addition to measuring percent of each subset by flow cytometry, we also estimated the absolute number of T helper and T suppressor/cytotoxic cells in the peripheral blood.

Materials and Methods

Subjects

Forty-seven patients treated at the Orbital Center of Allegheny General Hospital between May 1987 and May 1991 were included in this study. Peripheral blood was collected into EDTA vacutainers by venipuncture after the patients signed informed consent. Ten males aged 43 to 70 yr (mean age 53) and 37 females aged 21 to 67 yr (mean age 44) were studied. All patients were euthyroid throughout their history or after successful treatment for hyperthyroidism. None had received immunosuppressive or radiation treatment for at least 6 mo prior to sampling. Each patient was asked whether he or she had experienced any health problems during the month before the blood sample was drawn. No patients with health problems during this period, other than their orbitopathy, were included in this study. All of the patients had ophthalmopathy, which was rated as belonging to

From the *Department of Ophthalmology, Allegheny General Hospital and Allegheny-Singer Research Institute, Pittsburgh, Pennsylvania, and †Edward S. Harkness Eye Institute, Columbia Presbyterian Medical Center, New York, New York.

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Reprint requests: Anna Tyutyunikov, Department of Ophthalmology, Allegheny-Singer Research Institute, 320 East North Avenue, Pittsburgh, PA 15212-9986.
Table 1. Summary of previously published studies of T-cell subsets in dysthyroid orbitopathy patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Quantity</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>18</td>
<td>NS*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>2.</td>
<td>22</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3.</td>
<td>54</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>4.</td>
<td>55</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS means that no statistically significant change from a healthy control group was observed, and I or l means that a statistically significant increase or decrease, respectively, from a healthy control group was observed.

one of three groups described in Table 2. This grouping takes into account disease activity as well as severity. The person who classified patients in these groups had no knowledge of their peripheral blood T cell levels.

Flow Cytometry

We used the fluorescein- and phycoerythrin-labeled Simultest antibody reagent CD4/CD8 (Leu2a/3a; Becton Dickinson, Mountain View, CA) or fluorescein-labeled T4 and T8 antibody (Coulter Inc., Hialeah, FL) in the whole blood technique as described by Coulter. The same results were obtained with either reagent set. Some of the samples also were reacted with fluorescein-labeled anti-CD4 together with phycoerythrin-labeled anti-CD45RA or phycoerythrin-labeled anti-4B4. All three of the reagents used for dual color flow cytometry were purchased from Coulter Inc. All antibodies were used in concentrations recommended by their manufacturers. Cell surface fluorescence was read on a Coulter Epics C flow cytometer with standard settings. Isotype matched control reagents from the same companies were used to determine the thresholds. All of the positive fluorescence reactions were intense enough so no possible ambiguities about the placing of thresholds were encountered.

Other Measurements

Total white blood cell counts were determined with a Coulter Counter (Model Fn), and the percent lymphocytes was determined by a differential count on Wright-Giemsa-stained blood smears.

Statistical Analysis

Initially, mean values for the dependent variables of interest (eg, percent CD4, percent CD8) were compared statistically across the four groups of subjects using analysis of variance. If the analysis of variance was statistically significant, a multiple comparison procedure—Dunnett's test—was used to compare each patient group with the control group.

Additional statistical tests were run on the data of Table 3. Because the dependent variables of interest may not be normally distributed, nonparametric procedures also were employed for group comparisons. A Kruskal-Wallis ANOVA was performed and, if significant, it was followed by a Mann-Whitney U test to determine whether patient groups were significantly different from controls. Bonferroni's inequality was used to adjust the alpha error rate for these pairwise comparisons. The results of the nonparametric analyses were identical to the parametric results. Thus, only

Table 2. Clinical groupings of DO used in this study

<table>
<thead>
<tr>
<th>Group</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stable, mild/moderate DO</td>
<td>Patients had no change in their ophthalmopathy for at least 6 months, with no edema or injection, exophthalmos no greater than 4 mm, mild to moderate restriction of motility, and no evidence of optic neuropathy. Disease severity increased on consecutive visits in past 6 months, with mild edema or injection, exophthalmos 2–4 mm, and no evidence of optic neuropathy.</td>
</tr>
<tr>
<td>2. Active, mild DO</td>
<td>Disease severity increased on consecutive visits in past 6 months, moderate to severe edema of eyelids, moderate to profound chemosis, exophthalmos &gt; 4 mm, restriction of motility moderate to severe, in some cases leading to ophthalmoplegia. All patients with optic neuropathy, even if they showed only minimal proptosis, were included in this category.</td>
</tr>
<tr>
<td>3. Active, moderate/severe DO</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Lymphocyte subset evaluation in Graves' ophthalmopathy patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 27)</th>
<th>DO group 1* (n = 24)</th>
<th>DO group 2 (n = 8)</th>
<th>DO group 3 (n = 15)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %CD4⁺</td>
<td>44.5</td>
<td>43.8</td>
<td>47.6</td>
<td>53.1†</td>
<td>0.0003</td>
</tr>
<tr>
<td>Mean %CD8⁺</td>
<td>25.1</td>
<td>25.1</td>
<td>26.8</td>
<td>20.9</td>
<td>0.086</td>
</tr>
<tr>
<td>Mean (%CD2⁺/CD4⁺)</td>
<td>1.97</td>
<td>1.84</td>
<td>1.87</td>
<td>2.75†</td>
<td>0.0046</td>
</tr>
<tr>
<td>Mean WBC</td>
<td>6421</td>
<td>6357</td>
<td>7019</td>
<td>7803</td>
<td>0.224</td>
</tr>
<tr>
<td>Mean %lymphocytes</td>
<td>29.3</td>
<td>31.2</td>
<td>32.8</td>
<td>26.9</td>
<td>0.333</td>
</tr>
<tr>
<td>Mean absolute CD4⁺</td>
<td>846</td>
<td>846</td>
<td>1099</td>
<td>1110</td>
<td>0.082</td>
</tr>
<tr>
<td>Mean absolute CD8⁺</td>
<td>480</td>
<td>500</td>
<td>610</td>
<td>455</td>
<td>0.417</td>
</tr>
<tr>
<td>Mean (CD2⁺/CD4⁺)</td>
<td>2.01</td>
<td>1.74</td>
<td>1.87</td>
<td>2.72</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Note: analysis of mean WBC, mean %lymphocytes, mean CD4⁺, mean CD8⁺, and mean CD2⁺/CD4⁺ was based on 60 rather than 74 subjects due to missing values.

* Group 1 = stable, mild/moderate DO; Group 2 = active but mild DO; Group 3 = active/severe/severe DO. Table 1 lists the detailed clinical criteria defining these groups.

† Significantly different from control, Dunnett’s test; P < 0.05.

the results from the parametric analyses are reported in Table 3.

Results

The distribution of the major lymphocyte subsets in patients with DO and in the control group are shown in Table 3. We measured the percentage of circulating CD4⁺ and CD8⁺ cells and calculated the CD4⁺/CD8⁺ ratio. As shown in Table 3, the percentages of CD4⁺ and CD8⁺ lymphocytes in the first group of patients with inactive DO were within normal range. In the groups of patients with active DO, there were apparent differences from the control in both T subsets. Even in patients with mild symptoms of active DO, the percentage of CD4⁺ lymphocytes increased relative to the control, but this difference was not statistically significant. However, a significant (P < 0.05) elevation was seen in the group of patients with active and moderate-to-severe DO. This difference also was reflected as a significant difference in the CD4⁺/CD8⁺ ratio. The percentage of CD8⁺ cells remained within the normal range during the inactive and even mild active stages. Only during the active, moderate-severe stage did we see a slight decrease of CD8⁺ lymphocytes. However, this decrease was not statistically significant.

The results of the dual color flow cytometry experiments run on some of the patients are shown in Table 4. The group with active and moderate-to-severe disease showed a marginally significant difference from the control (P between 0.05 and 0.06). The other data of Table 4 were not significantly different from the control.

Because absolute values of T cell subsets may be critical parameters in other contexts, such as immunodeficiency diseases, absolute numbers of CD4⁺ and CD8⁺ lymphocytes were calculated by multiplying the percent subset values by the percent lymphocytes and the total white blood cell counts. As shown in Table 1, the mean absolute number of CD8⁺ cells oscillated within the normal range in the different groups. CD4⁺ cell distributions were normal in inactive patients but increased in parallel with severity in the group of active patients. However, in contrast to the percent subset data, this increase was not statistically significant. Even when the same population of patients was used for the two analyses, statistically

Table 4. Evaluation of the percent of T-cell helpers of suppressors and T-cell helpers of inducers

|                   | Control | DO Group 1* | DO Group 2 | DO Group 3 |
|                   | N       | N           | Mean      | Range    |
| CD4⁺CD5RA*        |         |             |           |          |
| N                 | 14      | 15          | 4         | 8        |
| Mean              | 19.4    | 13.3        | 22.5      | 18.3     |
| Range             | 9-39    | 6-27        | 17-30     | 9-43     |
| CD2⁺4B4⁺ ( Helpers of inducers) |       |             |           |          |
| N                 | 12      | 15          | 4         | 8        |
| Mean              | 28.8    | 28.5        | 28.8      | 35.5†    |
| Range             | 20-41   | 21-38       | 16-39     | 32-42    |

Only some of the patients used to generate the data of Table 3 were also evaluated for these parameters.

* See note to Table 3 for definition of the DO groups.

† This group was marginally significantly different from control (P = between 0.05 and 0.06) by the Dunnett’s procedure.
significant differences were seen only when percent values were compared, not when the absolute values were used, except in the case of the ratios, which were significantly different from the control in the ANOVA test when the percent or absolute values were used.

**Discussion**

In the present study we attempted to detect immunologically significant differences between specifically designated stages of DO using monoclonal antibodies to the major T cell subsets and flow cytometry. In contrast to previously reported findings, discussed in the introduction, we found a significant elevation of the percent of CD4+ cells in the group of patients with active and moderate or severe DO. The CD4+/CD8+ ratio also was significantly increased in the same patient group.

When the calculated absolute values were used, the statistically significant differences between the control and the moderate-severe active group disappeared. This may be explained by our use of two additional measurements to obtain the absolute values. The technical fluctuations of these measurements may have been sufficient to obscure actual differences. We are confident that the differences based on percentages are real because no significant differences could be demonstrated in the total white blood cell counts or in the percent lymphocytes among the groups compared. Our confidence also stems from the significant differences in the CD4+/CD8+ ratio, which provides an internal control for absolute total values within each sample.

Because the level of thyroid hormone itself may affect T cell subsets, all of our patients were euthyroid. Also, they had no prednisone or radiation treatments that may have affected the immune system for at least 6 mo prior to the blood sampling. Despite the existence of numerous different classifications, we found suggesting another one useful. We classified the patients not only by severity of the disease but also according to the activity of their ophthalmologic symptoms. We found the activity of the DO, i.e., whether or not the signs and symptoms were increasing on successive visits, to be a crucial criterion.

A majority of the patients with moderate to severe disease were treated with prednisone after the readings reported here were taken. Therefore, because of our exclusion criterion stipulating no immunomodulating treatments within the past six months, we did not include data gathered serially. Collection of such data is planned. Previous estimates of T cell percentages using E-rosetting techniques and similar patients indicated that T cell parameters may predict the efficacy of prednisone therapy.

According to our data, we postulate that the CD4+ helper cells in the peripheral blood play a major role in the development of DO symptoms. Normal levels of T cell subsets were found in patients with inactive, stable disease regardless of the severity of their disease. When patients displayed active symptoms, such as eyelid swelling and increasing proptosis, chemosis, restriction of motility or optic neuropathy, the percentage of CD4+ helper/inducer cells increased in parallel with the exacerbation of these symptoms. In view of the strongly indicated autoantibody involvement in DO, the CD4+ cells probably help mediate this antibody response.

The CD4+ subset consists of helpers of inducers and helpers of suppressors, and the cell surface phenotypes of these categories have been defined. The helpers of suppressors, defined by the CD4+CD45RA phenotype, have been found to be decreased in autoimmune diseases such as active systemic lupus erythematosus and multiple sclerosis. In the present study, we also used dual label flow cytometry to measure the helpers of suppressors and the helpers of inducers (the latter is defined by the CD4+4B4 phenotype). However, this measurement was made only on a small fraction of the samples used for the estimation of the major T cell categories (Table 4). Despite the limited data, an indication of a significant difference (P between 0.05 and 0.06) was seen in the helpers of inducers between the patients with active, moderate-to-severe DO and controls. This observation supports the hypothesis that the CD4+ cells may be helping to mediate an autoantibody response at the active stage of the disease. Measurement of these subsets by two color flow cytometry is recommended for future studies, because it is possible that expansion of one subset may cancel out a decrease in the other if only the CD4 marker is evaluated.

The T8+ subpopulation also is heterogeneous and appears to contain cytotoxic and suppressor cells, but these functional categories cannot as yet be definitively distinguished according to phenotype. It has been suggested that the suppressor function may be only transiently acquired by T8+ cells and, therefore, clear phenotypic characterization of the T8+ suppressor cells is not possible. Even if this is true, however, total T8+ cells may be decreased if suppressor cell function is defective.

Decreased T8+ cell numbers and defects in suppressor cell functions have been reported in a number of autoimmune diseases, including Graves' thyroid disease. Our data indicate a small, statistically insignificant decrease in T8+ cells in severe active DO. It has been suggested that the development of T suppressor defects in autoimmune diseases may be a secondary phenomenon, which could result from a con-
stant activation of the immune system by autoanti-
gen.21 If this is true, a greater effect on the T8+ suppressor population may occur in patients with long-term chronic disease. Assays of suppressor cell function may help determine whether there is a defect in this subpopulation.22–24 However, the relevance of some in vitro assays of suppressor functions to in vivo phenomena have been questioned.25

Despite that DO is an organ specific autoimmune disease, our data support the hypothesis that an immunologic imbalance in the patients with DO can be reflected by the percent of T cell subsets in the peripheral blood. The division of patients according to the activity and severity of their disease appears to be optimal for these evaluations. Such evaluations may contribute to the understanding of immunologic dysfunction in this disease and could provide us with guidelines for the application of specific immunotherapy, especially when serial readings are taken on individual patients to demonstrate changes in the circulating T cell subset distributions with the progression of their disease.

Key words: dysthyroid orbitopathy, Graves' ophthalmopathy, flow cytometry, peripheral blood, T cell subsets

References