Immunity in Graves' disease at diagnosis: correlation between activated T cells and humoral immune factors

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Abstract. Activated T cells, T-cell subsets, thyrotropin receptor antibodies and immune complexes were evaluated in 31 patients with newly diagnosed Graves' disease. Activated T cells were assayed by monoclonal antibodies against early (4F2) and late activation surface lymphocyte antigens (different epitopes of class II antigens). In comparison with the normal population, Graves' patients showed a significant decrease in the suppressor cytotoxic T-cell subset. Significant increases of 4F2-positive cells (70% of patients studied), class II antigen-positive cells (65%), thyrotropin receptor antibodies (93%), Clq-immune complexes (44%) and conglutinin-immune complexes (37%) were observed. A significant inverse correlation between the increase in 4F2-positive cells and thyrotropin receptor antibody values was also observed. Lymphocytes from Graves' patients were cultured in the presence of thyrotropin receptor antibody-positive or -negative sera, with or without mitogen stimulation. Thyrotropin receptor antibodies were shown not to interfere with the expression of activation antigens in cultured cells. The different patterns of humoral and cellular immune phenomena may indicate the existence of either different stages of Graves' disease or a heterogeneity of the immunopathogenesis in different patients.

Graves' disease is one of the most common autoimmune diseases. Nevertheless, despite the number of immune abnormalities found in patients with this disorder, the aetiology, the sequence of pathogenetic events and the interrelationship of abnormal immune components have not been fully elucidated (Burman & Baker 1985). Thyrotropin receptor antibodies (TRAb), circulating immune complexes (AgAb), changes in the proportion of T-cell subsets in the circulation, and more recently, the presence of activated cells (Jackson et al. 1984; Kennedy et al. 1985; Ludgate et al. 1984) have been reported in patients with recent onset Graves' disease. But still little is known of the significance of lymphocytes in different stages of activation which occur in the early phases of the disease, or of the reciprocal correlations between humoral and cellular immune phenomena.

In order to focus on the possible correlation and mutual influence of humoral and cellular immunity in Graves' disease, abnormalities within T-cell subsets, the presence of activated lymphocytes, and humoral immune phenomena (i.e. TRAb and different types of AgAb) in patients with Graves' disease have been investigated before treatment. In addition, the expression of an antigen indicating early activation (4F2) on lymphocytes cultured with or without the presence of TRAb in the medium has been tested in vitro in order to assess the extent of direct interference by TRAb on the activation surface antigens presented by lymphocytes.
Patients
The criteria for inclusion of patients in this study were as follows: a positive diagnosis of Graves' disease; age range between 14–70 years; no specific treatment initiated. Pregnant or relapsed patients were not included. Thirty-one Graves' disease patients (median age 39 years, interquartile range 29.5–51, eight male and 23 female) were studied at time of diagnosis. In addition to clinical examination, the diagnostic criteria were based on the evaluation of serum triiodothyronine (T3) median 400 ng/dl, range 278–800 ng/dl), thyroxine (T4) (median 18.9 µg/dl, range 11.6–25 µg/dl), and thyrotropin stimulating hormone (TSH) (median 1.1 µU/ml, range 0.5–1.8 µU/ml) values, thyroid scintigrams, iodine uptake (median at 6th h 70.2%, range 36–95%; median at 24th h 60%, range 54–88%) and/or TRH stimulation tests. Subjects were sequentially recruited from patients attending clinics in Rome and Edinburgh.

Four groups of age-comparable control subjects were used: 35 for the study of thyrotropin receptor antibodies, 26 for the study of T-cell subset numbers, 25 for the study of the first component of complement (C1q) binding immune complexes and 25 for conglutinin detected immune complexes. The serum samples used as controls for immune complexes were chosen from a much larger group of normal blood donors and had immune complex values which were statistically representative of the entire blood donor population studied. Aliquots of sera samples were frozen at −20°C, thawed only once and included in all immune complex assays.

T-cell subpopulations
T-cell subsets were evaluated using OKT3, OKT4 and OKT8 antibodies (Ortho Diagnostics) in order to show the total T-cell population, the helper/inducer and the suppressor/cytotoxic T-cell subsets, respectively. The determination of cell surface antigens was carried out using an indirect established immunofluorescence technique (Sensi et al. 1984).

Activated T cells
Activated T cells were enumerated using the following monoclonal antibodies: 4F2, which binds a 120 KD molecular weight glycoprotein present on monocytes and activated T cells and is a marker of early activation (Eisenbarth et al. 1980); L243 (Lampson & Levy 1980), DA6.231 and DA6.164 (Guy et al. 1982; Van Heyningen et al. 1982) which bind different antigenic determinants of the non-polymorphic section of the β-chain of class II surface antigens. T cells bearing class II antigens were labelled using double-staining immunofluorescence (Di Mario & Guy 1984b) and tested with a procedure described elsewhere (Sensi et al. 1984). Since activated T-cell values are positively skewed in the normal population, the 90th percentile was selected as the limit of positivity for these assays.

Thyrotropin receptor antibodies
A commercial kit (RIA Ltd, Tyne & Wear UK) was used based on the ability of TRAb to inhibit the binding capacity of 125I-labelled TSH to detergent solubilised TSH-receptors. Assay results are expressed in terms of the inhibition of TSH binding (TRAb index). Test sera with values greater than 10 were considered positive.

Soluble immune complexes
AgAb were evaluated using two different techniques. The solid phase Clq binding test (Clq-SP) (Di Mario & Guy 1984a) is based on the affinity of the first component of complement, Clq, bound to polystyrene tubes, to the Fc region of complex-bound immunoglobulins. The conglutinin binding test (KgBt) is based on the affinity of conglutinin, adsorbed to the walls of polypropylene tubes, to complex-bound C3 (Casali et al. 1977).

In both methods 125I-labelled staphylococcal A was used to reveal tube bound AgAb containing IgG subclasses 1, 2, 4). Results were expressed as a percentage of the maximum binding of heat aggregated IgG and the 90th percentile was chosen as the limit of positivity (12.8% for Clq-SP and 18% for KgBt).

4F2-POSITIVE CELLS

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<th>GRAVES' PATIENTS</th>
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Fig. 1.
The percentage of 4F2-positive cells in Graves' patients and normal controls. The limit of positivity was 8%.
Influence of TRAb on the expression of activation antigens in T cells: an experiment in vitro

Lymphocytes from Graves' patients and from normal subjects were split into four different aliquots and placed separately in short term cultures in media containing sera (20% v/v) either from strongly TRAb-positive patients with (protocol A) or without (protocol B) the addition of PHA (1:50), or sera (20% v/v) from TRAb-negative patients with (protocol C) or without (protocol D) the addition of PHA (1:50). The expression of 4F2 lymphocyte surface antigens was evaluated in all four cultures.

0.5 ml aliquots of cells (2 x 10⁶/ml) from normal subjects or Graves' patients were placed in the wells of a culture plate (Falcon Multiwell). Each set of cells received 0.5 ml of each of the above media preparations and was cultured for 3 days at 37°C before being removed for immunofluorescence.

Statistical analysis

Comparisons between immunological parameters in Graves' patients and normal controls were made with Student's unrelated t-test (T) for normally distributed data and with non-parametric methods, Kendall's test (K) for ranked categories and Wilcoxon's test (W) for other populations of data; Cox's test for trend in 2xC contingency tables (C) was used in some comparisons. The symbol of the statistical two-tailed test used is indicated in all determinations of probability.

Results

T-cell subsets

The percentages (± sd) of total T cells (OKT3), helper/inducer T cells (OKT4) and suppressor/cytotoxic T cells (OKT8) in normal controls were 57.8 ± 7.2, 41.4 ± 7.3 and 19.3 ± 5.4, respectively, and in Graves' disease patients 53.4 ± 11.2, 41.3 ± 6.4 and 16.4 ± 3.8, respectively. Only the decrease in OKT8-positive cells in Graves' disease was significant (P < 0.02, T).

Activated T cells

Significant differences in activated T cells were noted between the Graves' patients and the normal population. 4F2-positive cells were increased in 71% of patients (P < 0.001 W vs normal subjects) (Fig. 1). 66% of patients showed an increased number of class II-positive lymphocytes with at least one of the monoclonal antibodies used (Table 1). Patients rarely bound L243, Da6.231 and Da6.164 simultaneously at comparable levels.

TSH receptor antibodies

TRAb were present in 93% of the patients studied. All control subjects were negative (P < 0.001, T).

Soluble immune complexes

Clq-AgAb were present in 44% and Kg–AgAb in 36% of the patients studied (P < 0.01 W and P < 0.05 W vs normal controls, respectively). There was no significant correlation between the presence of Clq-AgAb and that of Kg-AgAb.

Comparison between humoral and cellular immunological factors

A significant negative correlation (τ = 0.30) between the level of 4F2-positive cells and TRAb values (P < 0.02, K) was observed (Fig. 2).

A trend for enhanced 4F2-positive lymphocyte numbers in relation to a decreasing level of Clq-AgAb was significant (P < 0.05, C) (Fig. 3).
There was a positive trend for increased TRAb values according to increasing soluble immune complexes as evaluated by the Clq method ($P < 0.02, \text{C}$) (Fig. 4).

No significant correlation between 4F2-positive cells the modifications in percentage of Class II positive cells and suppressor T cells was observed. There was no significant correlation between

**Fig. 2.**
The correlation between the 4F2-positive cells and the thyrotropin receptor antibody index (%) in newly diagnosed Graves' patients.

**Fig. 3.**
The correlation between Clq immune complexes and the percentage of 4F2-positive cells in Graves' patients at diagnosis.

**Fig. 4.**
The correlation between immune complexes detected by Clq, and thyrotropin receptor antibody index in newly diagnosed Graves' patients.
Little is yet known about the functional characteristics of 4F2-positive cells. The antigen is newly synthesized and is one of the first to be expressed on activated T lymphocytes (Sensi et al. 1984). In their resting state, B and T cells bind negligible amounts of 4F2 antibody. The expression of 4F2 antigen seems to be an early marker of an alerted immune surveillance system in a few other autoimmune diseases, such as insulin-dependent diabetes mellitus and Crohn's disease (Jackson et al. 1982; Fais et al. 1985).

A significant increase in class II-positive cells was observed, without a clear concordance between the findings of the three different anti-class II monoclonal antibodies used. Among the possible explanations, a differential recognition of DR β-polypeptides by the three monoclonal antibodies seems most likely. Since the DR locus encodes at least three β-polypeptides, different combinations may be revealed according to the antibody used (Guy et al. 1982; Hurley et al. 1982). On the other hand, different affinities between the monoclonal antibody used and class II alleles should also be considered. The double staining technique, used to detect class II positive lymphocytes, permits to accurately eliminate non-T cells possibly expressing these surface antigens and accounts for minor quantitative discrepancies found with other reports, as far as L243 is concerned (Ludgate et al. 1985; Jackson et al. 1982).

The percentage of suppressor/cytotoxic T cells was significantly decreased in hyperthyroid patients at the time of diagnosis thus confirming the altered function and number of immunoregulatory T cells in many autoimmune diseases (Morimoto et al. 1980; Bach et al. 1980).

The high percentage of patients with detectable levels of TRAb in this project (93%) concords with the results of other studies (Burman & Baker 1985). The increase of circulating immune complexes underlines the fact that immunological phenomena are active during early stages of the disease. The varying levels of circulating immune complexes revealed by the different techniques may be explained by the different principles of the methods used. Apparently only small-medium size complexes in antigen excess, those detected by the Clq method, are correlated with other immunological abnormalities. The clonglutinin binding test or the fluid phase Clq binding test detects larger size complexes, fixing the complement or not, whose characteristics differ from the

Experiments

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<tr>
<th>Protocol</th>
<th>Graves' patients</th>
<th>Normal subjects</th>
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<tr>
<td>TRAb + serum added</td>
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<tr>
<td>A) PHA stimulus</td>
<td>74.3 ± 3.9</td>
<td>78 ± 0.7</td>
</tr>
<tr>
<td>B) no PHA stimulus</td>
<td>44.4 ± 12.2</td>
<td>20.5 ± 3.5</td>
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<tr>
<td>TRAb – serum added</td>
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<tr>
<td>C) PHA stimulus</td>
<td>76.4 ± 5.2</td>
<td>82.6 ± 0.2</td>
</tr>
<tr>
<td>D) no PHA stimulus</td>
<td>42.1 ± 4.7</td>
<td>20.6 ± 3.9</td>
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* Values are given as a percentage of total lymphocytes ± SD.

the levels of soluble immune complexes, as measured by the KgBt method, and the values of either 4F2-positive cells and TRAb.

Discussion

These findings show that lymphocytes in different stages of activation are present in a high percentage of patients in the early phases of Graves' disease, as was suggested by the present authors in a preliminary study on different patients (Kennedy et al. 1985). This reinforces a role for activated lymphocytes in the pathogenesis of the disease (Ludgate et al. 1984; Jackson et al. 1984). 4F2 antigen, a 120Kmol weight glycoprotein, is present in lymphocytes in early stages of activation before the onset of DNA synthesis, while class II antigens (DA6.231, DA6.164 and L243) usually appear in fully activated and mature lymphocytes (Cotner et al. 1983; Haynes et al. 1981; Holter et al. 1985; London et al. 1985).
The presence of TRAb was correlated to the occurrence of Clq complexes. The solid phase Clq method tends to detect medium size complexes in antigen excess or aggregated immunoglobulins (Di Mario & Guy 1984a). Several explanations for this correlation may be put forward. TRAb and Clq complexes may be two independent immune phenomena which are simply temporarily related since both occur in the early stages of Graves' disease. Alternatively, TRAb antibodies may combine in the circulation with antigen component derived from the thyroid cell surface, or TRAb may combine with its own anti-idiotypic antibody. It cannot be excluded that the immunoglobulin complexes detected by Clq are made up of an antibody of an anti-idiotypic antibody comprising the thyrotropin receptor antibody itself.

The most interesting finding of this work is, in our opinion, the negative correlation between thyrotropin receptor antibodies and 4F2-positive cells. The higher the level of TRAb, the lower the number of 4F2-presenting cells. Experiments in vitro show that the presence of TRAb has no direct influence on the expression of 4F2 on stimulated cells. The inverse correlation between TRAb and 4F2 cells is indirectly confirmed by the same type of correlation between Clq complexes and 4F2 cells, whereas TRAb and Clq complexes were positively correlated. No correlation was observed between conglutinin binding complexes and either 4F2-positive cells or TRAb levels. A lack of correlation between conglutinin binding and specific humoral immune parameters has been observed in other diseases. Since the presence of the 4F2 antigen on the surface of lymphocytes is a very precocious marker of activation, it is conceivable that 4F2 and TRAb reflect the early immunological events. Their inverse correlation may be explained either by the fact that they reflect precocious but different stages of Graves' disease, or the heterogeneity of the immunological mechanisms in different patients. Indeed all the patients studied showed either elevated 4F2-positive cell numbers and/or TRAb levels. The evaluation of the 4F2 antigen, in conjunction with the thyrotropin receptor antibody, may provide an accurate and valuable marker of the major immunological phenomena which play a role in the pathogenesis of Graves' disease.

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References


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