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Graves' disease: *in situ* localization of lymphoid T cell subpopulations

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SUMMARY

An *in situ* analysis of immunological features in thyroids from 15 Graves' disease patients has been performed. This study included a search for immune complexes visualized near the follicle's basement membranes with fluorescent rabbit antisera to human IgG, IgA, IgM, C1q, C3 and C9. Cellular immunity was investigated on the humoral side by visualization of B cells and plasma cells. Two series of monoclonal antibodies (OKT3, 4 and 8, Leu 1, 2a and 3a) were used to label the infiltrating cells' membrane. These studies demonstrated the prevalence of T cells, a majority of them with OKT8/Leu 2a suppressor/cytotoxic phenotype. No correlation was found between this observation and peripheral blood T cell subsets analysis.

INTRODUCTION

Interfollicular lymphoid infiltration is regularly observed in thyroid glands from patients with Graves' disease. Although some studies (Totterman, 1978; Skoldstam, Anderberg & Norrby, 1978) have demonstrated that both B and T cells are included in these infiltrations, little is known about the characteristics of these cells.

The recent development of monoclonal antibodies to T cell membrane antigens (Reinherz *et al.*, 1980) opened new immunohistological possibilities. Numerous studies have already been successfully performed with these new tools to characterize T cell subsets *in situ* in various organs' lymphocytic infiltrations (Janossy *et al.*, 1980; Berger *et al.*, 1981; Selby, Janossy & Jewell, 1981; Faure *et al.*, 1982; Meijer *et al.*, 1982; Platt, Le Bien & Michael, 1982). In this paper, we report such a study, performed in immunofluorescence on thyroid specimens from 15 patients, and correlated with other immunological features such as immune complex deposits and peripheral blood lymphocyte subsets.

MATERIALS AND METHODS

Patients. Thyroids were obtained from 15 patients (14 females and one male) with Graves' disease. This diagnosis had been established according to the American Thyroid Association criteria. All patients presented with hyperthyroidism and a diffuse goitre. In all cases, thyrotoxicosis was demonstrated by high levels of circulating tri- and tetra-iodothyronin, elevated free thyroxine index, low TSH and no response at a TRH test.

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Nine patients showed an ophtalmopathy, and one only had pretibial myxedema. Ten patients had not received any medical treatment at the time of study, and two had been given synthetic anti-thyroid drugs in the past but were in an active phase of the disease after a year or more of treatment interruption. The three remaining patients had received carbimazole until 3 months prior surgery. In all cases, lugol was given for 2 weeks as pre-surgical treatment.

Tissue specimen. A fragment of each thyroid was rapidly frozen in liquid nitrogen. The specimens were kept at −70°C until studied.

Tissue sections. Serial sections, 3·5 μm thick, were prepared at −20°C in a thermostated cryostat (SLEE, London, UK) for each specimen. These sections were collected on glass slides, air dried and placed in a humid chamber. Histological observation was performed in each case after toluidin blue staining of one of the sections.

Antibodies. Heteroantisera to human gamma, alpha and mu heavy chains and human C1q, C3 and C9 complement factors were used to analyse immune complexes. These monospecific antisera (Behring, Paris, France), prepared in rabbits and fluorescein conjugated, had previously been checked in immunofluorescence on various human tissues and in immunodiffusion. Monoclonal antibodies (OKT series, Ortho, Raritan, New Jersey, USA and Leu series, Becton Dickinson, Sunnyvale, California, USA) were used in indirect immunofluorescence to investigate infiltrating lymphoid cells *in situ*. They allowed the visualization of T cells (OKT3, Leu 1) and T cell subsets: helper/inducer (OKT4, Leu 3a) and suppressor/cytotoxic (OKT8, Leu 2a). Fluorescein conjugated rabbit anti-mouse IgG serum (Institut Pasteur, Paris, France) was used as second step reagent. This antiserum was absorbed with liver and human skin powders.

One drop of properly diluted antiserum or antibody was placed on each thyroid section, and the slides were incubated at room temperature. After 30 min, they were washed three times for 5 min in phosphate-buffered saline (PBS). A second incubation was then performed with one drop of diluted fluorescent rabbit anti-mouse IgG serum if the first incubation had been with monoclonal antibodies, followed, after 30 min, by three washings in PBS. All sections were mounted under a coverslip with a PBS/glycerol mixture (3/7) and studied under u.v. light with a Leitz Orthoplan microscope equipped with a Ploem system of epi-illumination.

Controls. As control for the indirect immunofluorescence tests, a section of each specimen was incubated with the fluorescent rabbit anti-mouse IgG serum alone. None of the sections displayed any fluorescence. Irrelevant mouse serum was also used to assess the specificity of the monoclonal antibodies labelling.

Peripheral blood studies. Blood was obtained in eight patients on the day of thyroid removal. Lymphocytes were isolated by density gradient centrifugation on Ficoll-Hypaque. Lymphoid cells were tested for B and T cells markers as follows: fluorescein conjugated goat Fab to human Ig Fab was used to label B cells, and monoclonal antibodies (OKT3, 4 and 8) were used as T cell reagents. Rabbit anti-mouse IgG serum was used as second step for T cell labelling. The percentages of each subset were evaluated under u.v. light with a Leitz Orthoplan microscope. An average number of 200 cells was enumerated in each case.

RESULTS

Histology

Histological observation of the thyroids showed hyperplasic glands with numerous large resting vesicles. An important lymphoid infiltrate could be observed in eight cases. The other glands contained more scattered islets of lymphoid cells.

Immune complexes deposits and Ig secreting cells (Table 1)

Incubation with anti-human Ig sera yielded two observations. Granular deposits were seen around the vesicles basal membrane, which were frequently associated with complement and an infiltration of plasma cells. The latter were present in variable amounts in six cases, and produced IgA or IgM. Deposits of immune complexes containing Ig and/or complement factors were seen in 10 cases. They were located either in vessels' walls or along the outer surface of the vesicles basement membrane.

Table 1. Immune complex deposits and Ig secreting cells

	Basement membrane deposits						
Case No.	IgG	IgA	IgM	C1q	C3	C9	Plasma cells
1	+	−	−	+	+	+	IgA, IgM
2	−	+	−	+	+	+	
3	−	−	−	−	−	−	IgA, IgM
4	−	−	−	−	−	−	
5	−	−	+	−	−	−	
6	−	−	−	−	−	+	
7	−	−	−	−	+	−	IgA
8	−	−	−	−	−	−	
9	−	−	+	+	−	+	
10	−	−	−	+	+	+	IgA, IgM
11	−	−	−	−	−	−	
12	+	−	−	+	+	+	
13	+	+	+	+	+	+	IgA, IgM
14	−	−	−	−	−	−	
15	+	+	+	+	+	−	IgA, IgM

The presence (+) or absence (−) of Ig and/or complement deposits was evaluated after immunofluorescent staining of thyroid sections with monospecific fluorescein conjugated antisera. This method also made visible intracytoplasmic immunoglobulins in plasma cells.

T cells in situ (Table 2)

Studies with monoclonal antibodies showed many T cells in the lymphoid infiltrates where 'pan-T' specific antibodies labelled about 75% of the lymphocytes. In two cases, these lymphoid cells were sparse.

In eight cases, all these T cells belonged to the T suppressor/cytotoxic subset stained with OKT8 and Leu2a antibodies. No fluorescence could be observed after incubation with OKT4 or Leu3a antibodies.

In the other samples, again the majority of T cells belonged to this OKT8/Leu2a$^+$ subset, but significant numbers of OKT4$^+$ and Leu3a$^+$ cells were also detected. It should also be mentioned that OKT4/Leu3a$^+$ cells were mainly observed in large intervesicular infiltrates, while many OKT8/Leu2a$^+$ cells could be individually observed as isolated elements between vesicles or inside the vesicles' epithelium.

Peripheral blood lymphocytes (Table 2)

No significant alteration of B cells numbers could be evidenced in patients' blood compared to controls. T cell markers showed no alteration in the patients. T cell subsets and particularly OKT8$^+$ cells were usually within the normal range. Two patients nevertheless showed OKT4$^+$ percentages at the upper normal level (No. 1 and 8), one (No. 1) with a high percentage of OKT8$^+$ cells, the other one (No. 8) with a lowered number of OKT8$^+$ cells. As in many other autoimmune diseases, three of these patients had double labelled (OKT4$^+$/OKT8$^+$) cells, as showed by the higher sum OKT4$^+$ + OKT8$^+$ than the number of OKT3$^+$ cells in the sample.

DISCUSSION

Graves' disease is an endocrine disease associated with immune abnormalities which have been

Table 2. Evaluation of T cells *in situ* and in peripheral lymphocytes

	Lymphoid infiltrate*			Peripheral blood T cells†			
Case No.	Pan-T	H/I	C/S	T3	T4	T8	T4/T8
1	+	−	+	61%	56%	30%	1·9
2	+	−	+	66%	43%	20%	2·2
3	++	+	++	55%	45%	10%	4·5
4	−	−	−	70%	45%	17%	2·7
5	−	−	−	51%	44%	20%	2·2
6	+	−	+	65%	50%	8%	6·2
7	++	−	++	34%	34%	16%	2·2
8	++	+	++	70%	55%	11%	5·5
9	+	−	+	ND	ND	ND	
10	++	−	++	ND	ND	ND	
11	+++	+	+++	ND	ND	ND	
12	++	−	++	ND	ND	ND	
13	+	−	+	ND	ND	ND	
14	++	+	++	ND	ND	ND	
15	++	+	++	ND	ND	ND	

* Infiltrating lymphocytes were labelled with monoclonal antibodies to T cells: OKT3 and Leu1 (Pan-T), OKT4 and Leu3a (helper–inducer cells H/I), and OKT8 and Leu2a (cytotoxic/suppressor cells C/S). Positively labelled cells were evaluated semi-quantitatively: (−) absence, (+) moderately numerous, (++) numerous, (+++) large numbers.

† Monoclonal antibodies of the OKT series were used to enumerate T cell subsets among isolated peripheral blood lymphocytes. Reference values are OKT3:75^{+}–5%, OKT4:50^{+}–5%, OKT8:25 ± 5%.

found in the peripheral blood (Calder & Irvine, 1975; Totterman, Makinen & Gordin, 1977; Aoki, Pinnamanemi & De Groot, 1979; Sridama, Pacini & De Groot, 1982) as well as on suspensions of thyroid infiltrating lymphoid cells (Skoldstam *et al.*, 1978; Totterman, Anderson & Hayry, 1979).

In this paper, we report *in situ* observations demonstrating the participation of both cellular and humoral immunity disorders in this complex pathology.

Glands from 10 out of 15 patients were found to contain immune complexes deposited along the follicles' basement membrane. Similar findings had already been reported in similar conditions (Fujiwara *et al.*, 1981a, 1982b), and might be related to membrane modifications which favour the deposition of immune complexes containing thyroglobulin (Mariotti *et al.*, 1979; Ohtaki *et al.*, 1981). An alternative interpretation of this finding would be the existence of a local activation of complement components (Fujiwara *et al.*, 1981a, 1981b), but little is known about the antigen involved in such locally formed complexes.

The lack of membrane Ig on thyroid infiltrating cells has already suggested that these lymphocytes were of T cell lineage. The observation of large numbers of OKT3/Leu1 positive cells *in situ* confirmed their T cell nature. Most of these cells carried suppressor/cytotoxic membrane antigens. In fact, this was the only subset observed in eight out of 13 patients presenting heavily infiltrated glands, and the dominant one in the five other patients. These cells constituted the majority of large lymphoid infiltrates as well as isolated elements seen in the interfollicular spaces. Helper/inducer OKT4^{+}/Leu 3a^{+} cells, observed in five patients only represented a minor subset and were exclusively observed amidst large infiltrations.

No correlation was noted between this finding in the thyroid glands and the results provided by

peripheral T cells analysis. In our study, we have not seen the decrease in the suppressor/cytotoxic subset which has been reported by others (Aoki *et al.*, 1979; Okita, Row & Volpe, 1981; Sridama *et al.*, 1982). No homogeneous alteration of blood T cell subsets distribution was seen in this study: the OKT4/OKT8 immunoregulatory index varied from 1·9 to 6·25 (normal value = 2).

These results also raise new questions. What, if any, is the functional role played by the OKT8/Leu 2a positive infiltrating cells? Do they act as cytotoxic or as suppressor cells, or both? Is the cellular infiltration a consequence of local humoral abnormalities? In this case, one might conceive that the suppressor cells may 'attempt' to regulate or control the local formation and/or deposition of immune complexes. Conversely, one can not exclude the possible role of cytotoxic T cells. Such cells could lead to membrane alterations within the follicles. The latter could then favour the deposition of circulating immune complexes and/or induce their local formation by generating the leakage of thyroglobulin. Why are OKT4/Leu 3a positive cells so scarce? Is this relative sparcity related to an attempted control of locally activated humoral mechanisms?

At the moment, little evidence supports any of these hypotheses. Humoral abnormalities have frequently been described in the course of Graves' disease, but their origin is still unknown. Totterman *et al.* (1979) evidenced some functions of infiltrating cells *in vitro*. This does not necessarily infer that the cells act similarly *in vivo*. A better understanding of this complex pathology may be expected from further studies.

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