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Autoimmune Thyroiditis

Approaches Towards its Etiological Differentiation

With 67 Figures and 42 Tables

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Preface

Autoimmune thyroiditis may be considered as the archetype of organ-specific autoimmune diseases. Many of the features of such disorders have been elicited from in vivo, ex vivo, and in vitro studies. The past few years have seen exciting new developments in our understanding of autoimmunity in general and of the initiation, expansion, and suppression of immune cell interactions and their effects on target organs at a molecular level. The interaction between the antigen-presenting cell with its major histocompatibility complex molecule, the T-lymphocyte with its α-β-receptor molecule, and the antigen have become much better defined. We therefore felt that it was an appropriate time to discuss new approaches to the understanding and the differentiation of autoimmune thyroiditis.

The topics of this workshop were chosen on the basis of their implications for the above-mentioned aims and of their relevance for future research. It had been known for a long time that the thyroid microsomal antigen is one of the relevant antigenic components in patients with autoimmune thyroiditis. The determination that this antigen is the enzyme thyroid peroxidase has now made it possible to conduct a host of new investigations. It is still not clear how environmental influences become involved in the development of autoimmune thyroiditis. Cytokines certainly play a role in the initiation and expansion of autoimmune thyroiditis.

We are very happy that we were able to assemble many of the world's leading scientists in the field of autoimmune thyroiditis. By including much of the extensive discussion, this volume not only provides recent data obtained on autoimmune thyroiditis, but also gives deeper insight into the subject and its progress.

We wish to thank all the participants at the workshop including the main speakers and those who contributed to the discussion for their help in editing this book. We would also like to thank Henning Berlin for all their support, which made it possible to bring together so many scientists from all over the world. We especially appreciate the kind help of Dr. E. Scheiffele and Dr. G. Decker from Henning Berlin and the secretarial help of Mrs. R. Scheifele from Ulm. We should also like to thank Mrs. Schröder-Djeiran and especially Mrs. Hensler-Fritton from Springer-Verlag, Heidelberg, for all their cooperation and help with the editing of this book.

We hope that this book may both provide an update of the subject to the reader interested in thyroid autoimmunity and stimulate researchers to initiate new projects in this field.

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Immunogenetics

New Insights into Autoimmune Mechanisms and the Pathogenesis of Insulin-Dependent Diabetes Mellitus*

W. A. RUDERT and M. TRUCCO

Today almost everyone agrees that insulin-dependent diabetes mellitus (IDDM) occurs as the result of specific, immunologically mediated destruction of the insulin-producing beta cells of the islets of Langerhans [1, 2]. The immunological pathogenesis of this disease was initially suggested by the presence of a mononuclear cell infiltration of the islets, "insulitis," in patients who had died within a few days of the clinical onset of IDDM [3, 4]. The infiltrating cells have been characterized as T-lymphocytes, most of which were CD8 positive (i.e., cytotoxic T-cells) and some that were CD4 positive (i.e., helper T-cells) [5]. Monoclonal antibodies specific for the interleukin 2 receptor and for HLA (human leukocyte antigen) class II molecules have indicated that these T-cells are activated [5]. Further evidence for the immunological origin of IDDM came from the presence of anti-islet cell antibodies (ICA) in a diabetic's sera [6] and from the success of immunosuppression in increasing remissions in newly diagnosed patients [7, 8].

Except for the attack on the beta cells of the pancreas, there is little evidence of functional abnormalities in the immune system of diabetic patients. This specific immunological attack most likely occurs through the mechanisms which normally direct and restrict the immunological responses to foreign antigens. In the case of IDDM, the peculiarity is that the immunological attack is mistakenly directed against a normal tissue of the same organism (anti-self or autoimmune). An antigen may be taken up by an antigen-presenting cell, and a particular fragment of this antigen becomes closely associated with an HLA class II molecule (Fig. 1). When a T-cell with a specific, complementary T-cell receptor is encountered, a tertiary complex consisting of the antigenic fragment, the HLA class II molecule, and the T-cell receptor forms and the helper T-cell becomes activated. The activated T-cell begins to secrete lymphokines (interleukins) which stimulate the activation and proliferation of other lymphocytes (e.g., cytotoxic T-cells and antibody-producing B-cells) [9]. Generally, these are the cells which are found in insulitis and are believed to be responsible for the destruction of the beta cells of the pancreatic islet [5].

In principle, the immune system's ability to form a particular tertiary complex is dependent on both the genetic presence and expression of a class II

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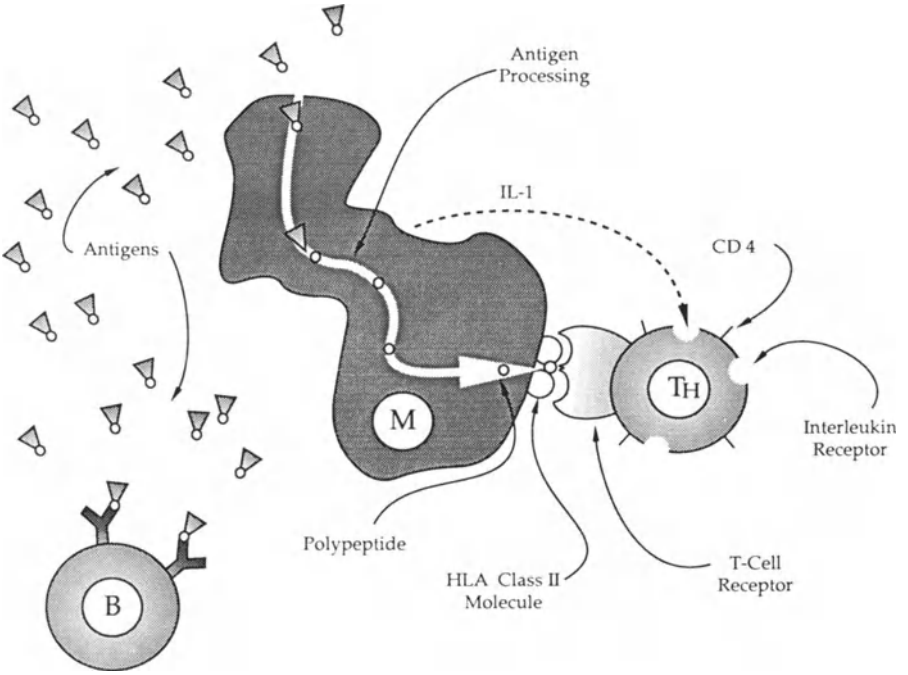

Fig. 1. Helper T-cell *(T_H)* activation mediated by the recognition of the HLA class II molecule-processed antigen (polypeptide) complex via the T-cell receptor. The complex is present at the outer surface of the antigen-presenting cell (e. g., macrophage, *M*). Once activated, the helper T-cell expresses the interleukin receptor, which binds the interleukin-1 *(IL-1)* produced by the macrophage. Only the B-lymphocytes *(B)* producing antibodies specific for the same antigen are also activated. As illustrated, the specific antibodies of the B-lymphocytes bind an epitope which can be recognized on the intact antigenic molecule, and the B cell response might also proceed independently of effective HLA class II presentation. Thus, nondiabetic individuals in whom effective HLA class II molecule-antigen complexes are not formed may still develop ICA. CD4 is a marker present at the surface of the helper T-cells. (From Rudert and Trucco [31])

molecule which allows the optimal positioning of the antigen in its combining site and on the genetic presence and the particular rearrangement of T-cell receptor genes which creates the specific T-cell receptor [10, 11]. The importance of the class II molecule in the autoimmune mechanism which leads to IDDM was suggested by the strong association found between IDDM and HLA class II alleles at different loci [12, 13], and confirmed by the demonstrated relationship which the HLA molecule has with antigen and T-cell receptor [14, 15]. An association similar to the one existing between HLA alleles and IDDM might be expected for IDDM and germ-line T-cell receptor alleles but has not yet been found [11].

In humans, the class II molecules are encoded in the HLA-D region on the short arm of chromosome 6 [16]. The HLA-D region is characterized as a series of linked genes each with a high degree of polymorphism [17]. Three subre-

gions, DP, DQ, and DR, are present, and each encodes α and β chains which compose the corresponding class II molecules. Most of the polymorphisms expressed by the various HLA molecules are limited to the first external domains of both their α and β chains [16, 17].

Approximately 95% of all Caucasoid IDDM subjects were found to have either HLA-DR3 or HLA-DR4 alleles [18]. More recently, attention has focused on the association between IDDM and HLA-DQ alleles [19]. In particular, some DQ β chains were more frequently found associated with diabetes than others. These are the ones that have a noncharged amino acid (alanine, serine, or valine) instead of aspartic acid at position 57 [20]. The importance of the presence of an aspartic acid at position 57 was also suggested by models for the tertiary structure of the HLA class II molecules. In fact, the HLA class I molecule has recently been crystallized and its tertiary structure determined [14, 15]. Modeling of the HLA class II molecules based on the known class I structure has demonstrated that many of the polymorphic amino acid residues – including Asp 57 – are in or near the peptide binding groove, Bjorkman's groove [21]. Presumably, these amino acids determine the specificity of peptide binding by the various class II alleles. In addition, it appears structurally possible for a salt bridge to occur between the aspartic acid in position 57 of the β chain and the DQ α Arg 79 which would possibly occlude a portion of the peptide-binding groove and dramatically modify the peptide-binding specificity (Fig. 2).

In a family study [22], we confirmed the association of IDDM with the non-Asp 57 alleles and determined that DQ molecular alleles are more significant than the serologically determined DR alleles because the association was just as strong for non-Asp 57 haplotypes which were neither DR3 nor DR4. Furthermore, there was a high degree of DQ allele sharing among diabetic siblings. Finally, the presence of at least one Asp 57 DQ allele was almost always associated with a non-diabetic state, and it was determined that the relative risk (odds ratio) for IDDM in non-Asp/non-Asp individuals versus Asp/Asp or Asp/non-Asp was 107.

From the data on Caucasoids we learned that the presence of one allele encoding for an aspartic acid at position 57 of the DQ β chain greatly reduces the incidence of IDDM. Nevertheless, there are several observations which do not allow a simplistic explanation of this result. The DQ α chain must also be involved in this process [23]. Although the α chain is less polymorphic than the β, it may also affect the susceptibility for IDDM albeit to a lesser degree than the β chain. The α/β combination which confers the greatest susceptibility seems to be the one composed of the DQ β generally found associated with DR4 alleles (DQw3.2) and the DQ α which generally associates with DR3 alleles (DQA1.3). This would also explain the association found between serological markers and IDDM. From the same results it has also become clear that not all of the Asp 57 positive alleles have the same effect in protecting against the development of IDDM. In the same way, not all of the non-Asp 57 alleles equally favor the development of the disease. A hierarchical scale seems to exists, which ranges from the most protective (DQw1.2) to the most permissive

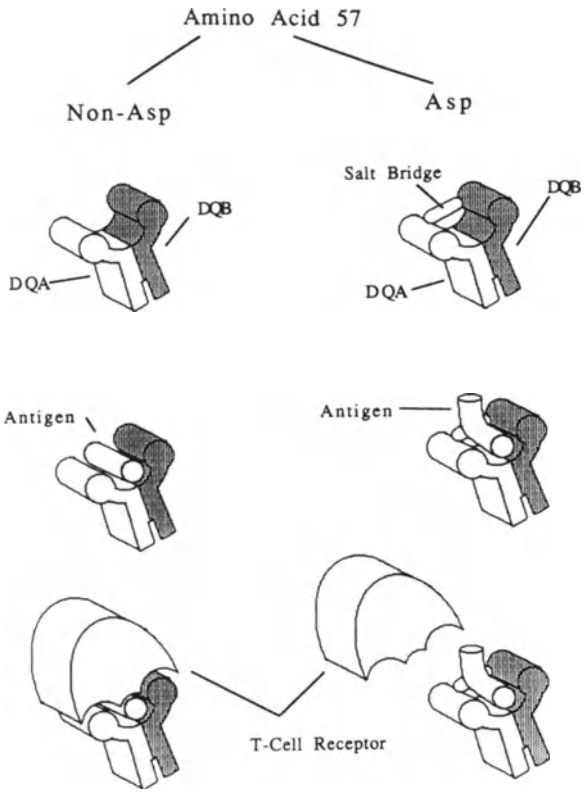

Fig. 2. The possible role played by amino acid 57 of the HLA-DQ β chain in the presentation of the (auto)antigen by this molecule to the helper T-cell at the beginning of the development of the immunological autoaggression against the β cells of the pancreatic islet. (From Trucco and Dorman [2])

(DQw3.2) allele. Most puzzling, however, was the interpretation of the results obtained in the heterozygotes Asp 57/non-Asp 57. It would seem that the heterozygotes should still be able to present the antigen with the HLA class II molecules expressed from the non-Asp 57 allele. Instead, a strong protective effect was evident in the heterozygous individuals. Only 4% of the diabetics in Allegheny County (Pennsylvania, USA) were Asp 57/non-Asp 57 heterozygous, in comparison with 46% of the general population, providing a relative risk of 0.04. Interestingly, the Asp 57 allele in the diabetic heterozygotes is frequently DQw3.1.

When we began to study different Oriental populations, we found that the very limited presence of the non-Asp 57/non-Asp 57 phenotype in the general population may be the basis for the very low incidence of IDDM. The Chinese population is a clear example (Table 1) [24]. Thus, the genetic background of the population seems to be a determining factor for differences in IDDM susceptibility between different countries. The results from the Chinese also demonstrate that the protective effect of Asp 57 alleles is not at all absolute and suggest that if we search more carefully (i.e., test many more cases), we may find diabetic individuals who are Asp 57 homozygous in the Caucasiod population as well. In this case, Asp 57 is an imprecise generalization because, for

Table 1. Comparison between phenotypical frequencies of diabetic patients and controls from Allegheny County, Pennsylvania (USA) and China

Phenotype	Allegheny County		China	
	Diabetics	Controls	Diabetics	Controls
NA 57/NA 57	96	19	6	0
NA 57/A 57	4	46	72	8
A 57/A 57	0	35	22	92

NA = Non-Asp, A = Asp.

example, the Asp 57 effect provided by DQw3.1 or DQw3.3 was less efficient in its protective effect than that provided by the DQw1.2 allele. In fact, the majority of the Asp 57 positive diabetic Chinese were either DQw3.1 or DQw3.3 [24].

The hypothesis suggesting that a salt bridge present between the aspartic acid in position 57 of the β chain and an arginine in position 79 of the α chain of the DQ molecule can be responsible for the inappropriate lodging of the antigen, thus impairing T-cell activation, cannot explain the reduced susceptibility of the Caucasiod heterozygous (Asp 57/non-Asp 57) patients nor the susceptibility of the Asp 57 homozygous Chinese patients. The ability to respond immunologically to a certain foreign antigen is generally found to be restricted to just one or the other allele present in a heterozygous cell without the prerequisite for a special "quantity" of these presenting molecules [25]. If the salt bridge were really the limiting factor, heterozygous cells would not be protected by the suggested mechanism, and the Asp 57/Asp 57 homozygous Chinese cells should not be attacked.

We have thus formulated a new hypothesis which, although a little unorthodox, may quite well explain all of our findings related to the importance of the amino acid in position 57 of the β chain and IDDM susceptibility (Ballou and Trucco, submitted for publication). The hypothesis envisages the modification, or even the destruction, of the critical antigen that primes the T-cell against the pancreatic cells of the islet of Langerhans, when aspartic acid 57 is present inside the combining site of the DQ molecule. The anti-IDDM protective effect can be due to two non-mutually exclusive mechanisms. In the first, the DQ Asp 57 positive molecule itself is active in catalyzing this hypothesized alteration or destruction of the disease-predisposing antigen. The second mechanism considers the binding of the antigen to a particular DQ molecule to be responsible for either protecting (non-Asp 57 alleles), or promoting (Asp 57 alleles) the destruction of the antigen.

The possibility that HLA molecules might function enzymatically is suggested by the overall resemblance of HLA molecule structures to those of the immunoglobulins, and by the discovery that antibodies can actually have catalytic activities. Actually, monoclonal antibodies produced against transition state homologues of certain chemical reactions were proven able to specifically catalyze peptide bond cleavage [26]. The generation of a library of catalytic antibodies specific for amino bonds between different amino acids could be

used to cleave proteins in the same fashion that restriction endonucleases specifically cut DNA molecules. Furthermore, the change of a single amino acid (Tyr for His) in the combining site of one of these catalytic antibodies was recently proven to accelerate 90000 times the hydrolysis of the 7-hydroxycumarin ester of 5-(2,4-dinitrophenyl) aminopentanoic acid, when compared to 4-methylimadozole [27]. Finally, auto-antibodies able to calalyze the hydrolysis of vasoactive intestinal peptides have also been found, thus demonstrating that the enzymatic activity of the antibodies is also present in physiological conditions [28].

If we then take a second look at the standard model of antigen processing mediated by the antigen-presenting cell (Figs. 3, 4), we can imagine that the foreign antigen is brought to the cell surface by a class II molecule which, dur-

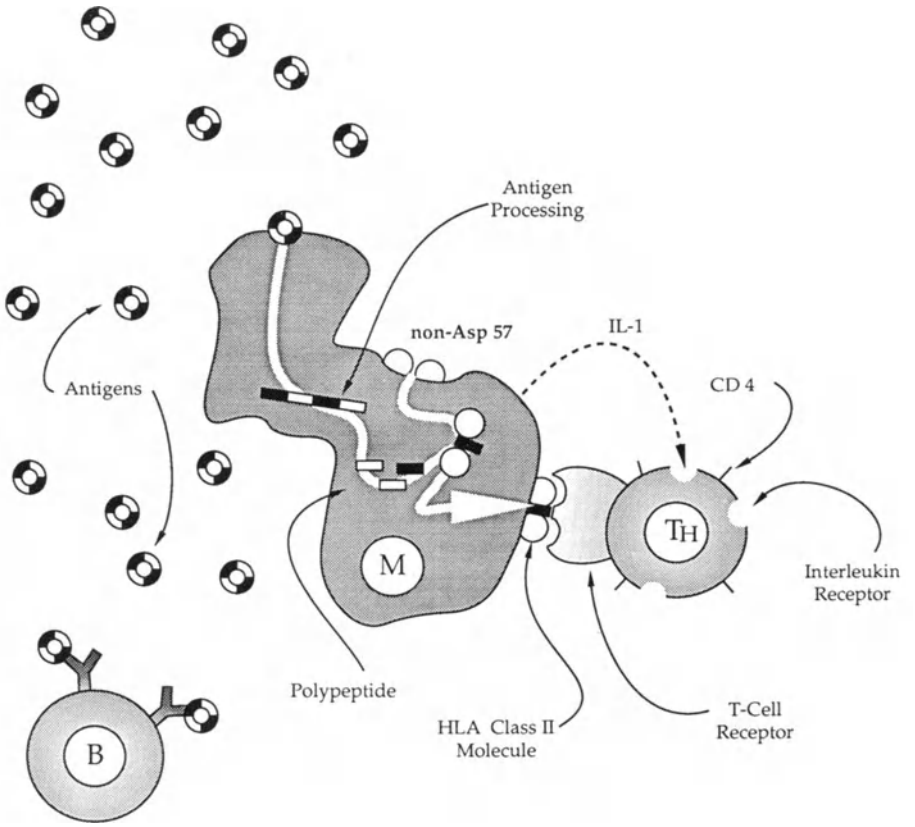

Fig. 3. Helper T-cell *(T_H)* activation mediated by the recognition of the HLA class II molecule-processed antigen (polypeptide) complex via the T-cell receptor. The complex is formed inside the antigen-presenting cell *(M)* where the recycling class II molecule binds one polypeptide derived from the digestion of the original antigen. If the HLA class II molecule is a non-Asp 57 DQ molecule, the antigen is eventually exposed intact at the cell surface. The immune process continues as in Fig. 1

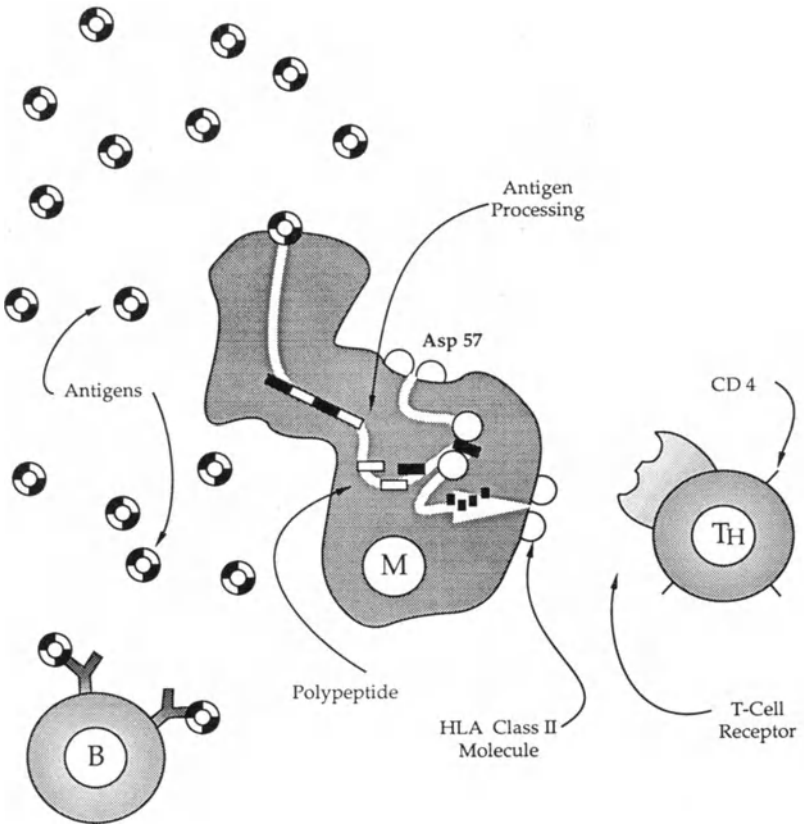

Fig. 4. The helper T-cell *(T_H)* activation does not take place when the processed antigen (polypeptide) binds to a recycling Asp 57 positive HLA DQ molecule which is inside the antigen-presenting cell *(M)*. The hypothesized catalytic activity of this Asp 57 positive molecule actually promotes further digestion of the specific polypeptide so that the resulting fragments are not recognizable by the T_H cell via its T-cell receptor. The absence of an appropriate antigen presentation impairs the T_H cell activation, and by consequence the immunological autoaggression against the beta cells of the pancreas is avoided

ing its recycling, accommodates a fragment of the original antigenic molecule in its groove. The proteolytic cleavage into short peptides of the original antigen has been proven necessary for effective presentation [29]. It was also demonstrated that synthetic peptide fragments corresponding to specific ones generated in vivo, were properly presented without any further processing and were effective in the stimulation of T-cells [30]. We can then argue that during antigen processing, the generated fragments may encounter and combine either with those recycling class II molecules which present it intact, on the cell surface, to the helper T-cell (non-Asp 57 class II molecules; Fig. 3). or with the other type of class II molecules (Asp 57 positive) which actually facilitate the further cleavage of the specific polypeptide (Fig. 4). In the first case, the im-

mune mechanism is activated, and by consequence cytotoxic T-cells are generated which are able eventually to recognize and kill the beta cells of the pancreas. In the second case, the antigens do not reach the cell surface in a form appropriate to activate the helper T-cells. In the absence of activation of these cells no immunological response occurs. We have also foreseen that during the processing of the antigen, DQ molecules from heterozygous alleles compete for the antigenic fragment. Some Asp 57 positive DQ molecules (e.g., DQw1.2) may be able to compete with a non-Asp 57 molecule more effectively than others (e.g., DQw3.1), possibly because of the presence, or the absence, of appropriate amino acids sterically adjacent to position 57. In other words, a sort of competition for the processed antigen takes place between "protective" versus "destructive" molecules in the heterozygous cell. The first type of alleles challenge the potent antigen-specific "scavenger" activity characteristic of the second ones (Ballou and Trucco, submitted for publication). The net result of this competition is determined by the number *and* the type of the Asp 57 alleles genetically present in the various individual phenotypes [24].

Although we cannot yet cite examples for this naturally occurring catalytic class II mediated activity, we are actively working to find scientific evidence for attributing a physiological importance to what we call "Ir-zymes."

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Discussion

Grubeck-Loebenstein:
This is obviously an interesting hypothesis, but I feel a bit uneasy in believing that beta cells process rather than merely present antigen. Marco Londei from Charing Cross Hospital and Franco Bottazzo from the Middlesex have convincingly shown that thyroid epithelial cells present peptides, but not whole viruses, for instance. Do you think that an epithelial cell could be able to process antigen?

Trucco:
In my hypothesis the antigen must be processed. What the HLA class II molecules can do or not is either to further process the antigen, perhaps destroying it, or to protect the antigen from the enzymes that are found in the antigen-presenting cell. The type of cell which presents the antigen is not critical to the hypothesis, but it must be one that expresses HLA class II.

Davies:
I would like you to focus on HLA-DQ, because the evidence for DQ being involved in antigen presentation on its own is not very strong. Dr. Trucco, since the defect in diabetes is exon 2 of DQ only, where is the evidence that DQ in humans can be a major antigen presentation signal in the presence of other class II antigens?

Trucco:
First of all, any class II molecule can be antigen presenting. The association that we found is much higher with DQ than with DR. I don't exclude that DR may be a good antigen-presenting molecule, but in type I diabetes it seems that the influence of DQ is stronger than DR.

Davies:
But can DQ itself, on its own, present antigen?

Trucco:
I would just like to quote some experiments: If you generate transgenic mice with appropriate DQ genes, they develop diabetes, although in transgenic mice the insulitis is not so severe. I also know that transfected DQ molecules (or I-A molecules in the mouse) are capable of activating T cells. I don't think that a

DQ molecule has been demonstrated thus far to be the antigen-presenting molecule for a specific antigen. We are performing some experiments on DQ transfection in my laboratory, and we are about to do this type of experiment, but unfortunately it will be impossible for me to completely answer your question at this time, since we don't know the antigen involved in type I diabetes.

Weiß:
Could one not explain the protective effect of the DQ β-chain with Asp 57 by the hypothesis that the presence of this allele leads to the deletion of possibly autoreactive T cells in the thymus, as it has been shown for IA transgenic NOD mice?

Trucco:
If you are referring to the recent paper of Janeway (*Nature,* 341 : 326, 1989), the deletion was for the I-E molecules and the clones that react with it. I-E corresponds to DR in humans, not DQ. DQ is I-A, but still works perfectly well with my hypothesis. The problem can be seen from three points of view: from the HLA molecule, from the antigen, and from the T cell receptor. So there can be problems related to the T cell receptor; you can have clones that don't exist because they are excluded by the thymus. It can be the antigen that is present or not, correct or not correct; it can be a virus or not – I don't know. My point here was just to try to explain how the HLA molecules may play a role determining the observed correlations. I don't exclude other possibilities.

Thyroid Antibodies and Thyroid Dysfunction in Patients with Type 1 Diabetes and Their First-Degree Relatives

F. BECKER, H. BUSCHLER, R. BRETZEL, and K. FEDERLIN

Introduction

The first evidence that type 1 diabetes mellitus might be a disease of autoimmune origin stemmed from the observation that juvenile diabetes is often associated with other endocrine autoimmune disturbances. To the description of Schmidt's syndrome [1], consisting originally of autoimmune thyroiditis and adrenalitis, diabetes was later added as third component. While the underlying genetic contribution in type 1 diabetes is closely related to class II MHC antigens (DR3/4, DQw8) [2], the situation concerning thyroid and adrenal is less clear, although DR3 [3] as the "autoimmune" haplotype appears a good candidate. This overlap of different autoimmune diseases has led to the concept of polyendocrinopathy [4, 5], with the clinical or subclinical involvement of several organs in the same subject or family.

These observations prompted us to screen type 1 diabetics and their first-degree relatives for the presence of organ-specific autoantibodies. In those with positive antibody results against thyroid antigens the metabolic function was evaluated.

Methods and Materials

Thyroid antibodies against thyroglobulin and microsomal antigens were evaluated by means of a commercially available ELISA (Synelisa, Elias, Freiburg, FRG). Measurements of total triiodothyronine, free thyroxine, and TSH were made with an immunoluminometric assay (Lumitest, Henning, West Berlin). The subjects examined consisted of participants in a prospective family study into the pathogenesis of type 1 diabetes mellitus. The study was started in 1985 in collaboration with the Diabetes Clinic in Bad Oeynhausen (FRG). Blood was taken at 6-month intervals from all family members and stored at $-20\,^{\circ}C$. The study included 233 diabetic patients as index persons, 325 siblings, and 411 parents.

Results

Overall, there was a general increase in thyroid autoimmunity in the population. As has long been realized that thyroid autoimmunity increases with age, parents, siblings, and diabetic patients were considered separately. Antibodies against thyroid antigens were found most often in the older age group (see Fig. 1), and here among the mothers of the diabetics. This increase was highly significant ($p < 0.001$) when compared with an age- and sex-matched control group.

The diabetics and their siblings were of almost identical age (14.8 versus 15.2 years), nevertheless there was a profound difference in the prevalence of thyroid antibodies. While both male and female nondiabetic siblings had antibody levels only slightly above those of the control population, their diabetic brothers and sisters displayed a strong increase in both thyroglobulin and microsomal antibodies. For the sisters, this reached statistical significance ($p < 0.05$).

On the whole, both parents and diabetics showed a female preponderance, and in all groups tested more antibodies against microsomal antigen were found. Only 21.8% of all positive patients tested had antibodies against thyroglobulin.

The evaluation of the functional state of the thyroid antibody-positive individuals was carried out by measuring peripheral thyroid hormones (total triiodothyronine and free thyroxine) and basal TSH. Almost half of all individuals tested had an impaired thyroid function (see Fig. 2), although only a small proportion (6%) had overt disease and clinical symptoms of hypo- or hyperthy-

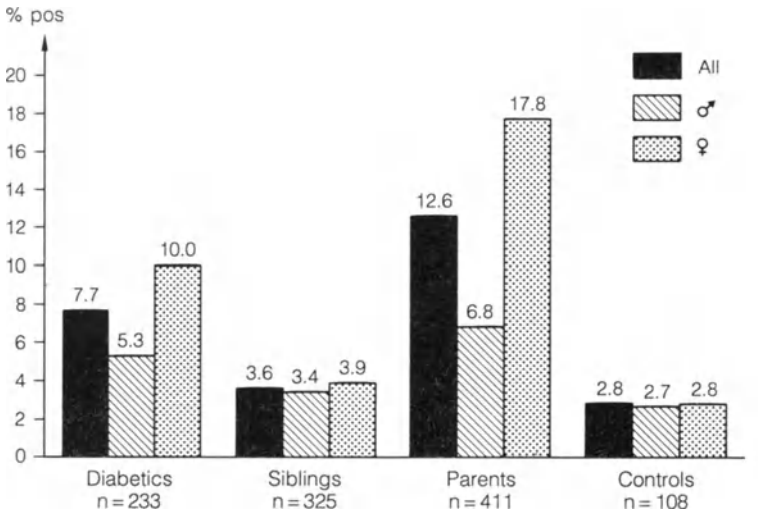

Fig. 1. Thyroid antibodies (thyroglobulin and/or microsomal) in type 1 diabetics, their parents, and siblings

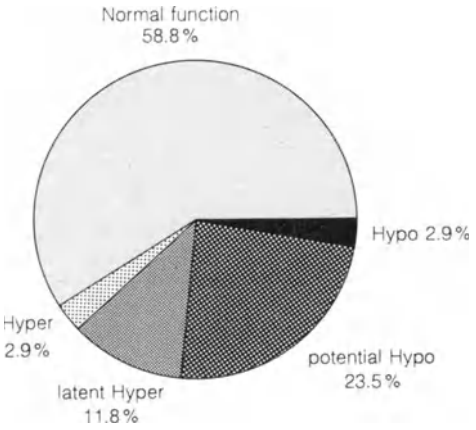

Fig. 2. Thyroid function in thyroid antibody-positive type 1 diabetics and their first-degree relatives

roidism. When the antibody-positive individuals were divided into parents and children (regardless of whether diabetic or not), a clear difference of the functional state could be noted. As opposed to 60% of the parents, only 25% of the children had "chemically" impaired thyroid function. Furthermore, all cases of overt disease and all hyperthyroid states were found in the group of the parents. Here no sex difference was seen.

Discussion

Looking at the functional state of the thyroid in thyroid antibody-positive diabetics and their first-degree relatives has revealed that almost 50% of these individuals show signs of a functional defect of the target organ. Thus, thyroid antibodies appear to be a good marker for the early detection of organ failure. The fact that parents not only have an increased prevalence of autoantibodies but also within the group have more defective states gives further support for the slow progressive destruction of the target organ as it has been postulated for type 1 diabetes mellitus [6]. Investigations in the cohort reported here concerning the endocrine function of the pancreas have shown that with positive antibody results (islet cell antibodies) a progressive loss of organ function could be observed, as demonstrated by intravenous glucose tolerance testing [7]. Similar results were obtained in those individuals with several thyroid function tests in the course of time (data not shown). This would also explain the higher percentage of thyroid disturbances in the parent generation.

Testing for thyroid antibodies in the families of type 1 diabetics and – with positive test results – for thyroid function seems to be an effective measure to prevent thyroid dysfunction in this group of individuals with an increased risk of endocrine autoimmune disease.

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Discussion

Davies:
How did you define potential hypothyroidism?

Becker:
This means, in our hands, an elevated TSH with an overshooting reaction to TRH, with still normal peripheral values for T_3 or free T_4

Davies:
But is the basal TSH normal or raised?

Becker:
The basal TSH is already elevated.

Davies:
So the patient is hypothyroid?

Becker:
As long as the peripheral T_3 and free T_4 are still OK, we call it potential.

Furmaniak:
What method did you use for measuring antibodies?

Becker:
We use the ELISA system from ELIAS in Freiburg, Germany.

Furmaniak:
In the group of patients who were hyperthyroid, have you measured TSH receptor antibodies?

Becker:
Yes, we did, with the TRAK assay from Henning, and two out of four were highly positive, two were borderline.

Furmaniak:
In cases where you are analyzing thyroid function, have you considered measuring the effect of antibodies on TPO enzyme activity?

Becker:
No, we haven't done that yet, because in this study we were mainly considering diabetes.

Association of HLA Class I and Class II Genes and Adrenal Steroid 21-Hydroxylase Gene with Subacute (de Quervain's) and Hashimoto's Thyroiditis: Evidence for an Altered Negative Feedback in Hashimoto's Thyroiditis*

B. O. BOEHM, E. SCHIFFERDECKER, B. MANFRAS, P. KÜHNL, G. HOLZBERGER, C. ROSAK, and K. SCHÖFFLING

Introduction

It is a striking feature of organ-specific autoimmune diseases that they are linked to alleles of class I and class II genes of the MHC gene region [1]; however, until recently the nature of this association was unclear. The hypothesis was then put forward that the MHC molecules per se play a major role in the immune response. They act as restriction elements when antigen-presenting cell and T cell meet in immune recognition either in a physiological milieu or in an autoimmune state [2]. Direct evidence for this hypothesis was deduced from inappropriate expression of MHC molecules on target tissues of an autoimmune attack, i.e., class II expression on pancreatic beta cells in recent-onset type I diabetes mellitus or class I and class II overexpression on thyroid epithelial cells in Graves' disease [3]. Presentation of an antigen or an autoantigen is a prerequisite in organ-specific autoimmunity, but also specific recognition by the T-cell receptor supplemented by helper factors is necessary for an immune response [2]. Since organ-specific autoimmune diseases are chronic diseases, it might he speculated that the helper factors (interleukins), which provide a positive feedback, play an important role in the maintenance of chronic inflammation during target organ destruction. Also, negative feedback mechanisms such as T-suppressor circuits may be impaired [4].

We studied patients with subacute thyroiditis and patients with "classical" Hashimoto's thyroiditis to address the question of whether there is an HLA association between de Quervain's thyroiditis and Hashimoto's thyroiditis. We also wanted to study a non-immune gene, the adrenal steroid 21-hydroxylase, which is physically linked to the class III and class I genes of the MHC on chromosome 6 [5]. Adrenal steroid 21-hydroxylase limits the production rate of cortisol, known to be a strong inhibitor of inflammatory processes. We thus hypothesized that a non-immune gene, due to its functional role, may also be linked to autoimmune diseases.

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Materials and Methods

All individuals studied were of Caucasian origin and were selected from a homogeneous population from the Rhine-Main area in the Federal Republic of Germany. There were 28 patients with subacute (de Quervain's) thyroiditis; these were HLA-typed, and 15 also underwent metabolic tests. Diagnosis was confirmed in all individuals by fine-needle aspiration with cytological examinations. A second group of 54 patients with Hashimoto's thyroiditis were HLA-typed, 44 of whom were further studied by ACTH stimulation tests. In 42 patients of this group diagnosis was confirmed by the presence of lymphocytic infiltration and slight fibrosis in fine-needle aspirate specimen. In addition, 193 local controls with no evidence of thyroidal disease were HLA-typed and were used for the calculation of antigen frequencies. No individual from either disease group was positive for organ-specific autoantibodies against pancreatic beta cells, adrenal gland, and adrenal medulla on an immunofluorescence-test. A total of 30 controls (10 men and 20 women) underwent an ACTH stimulation test to test the overrepresentation of women in both disease groups (female-to-male ratio in de Quervain's and Hashimoto's thyroiditis 5 : 1). HLA-A, -B, and -C typing was performed by the standard microlymphocytotoxicity technique (NIH) according to Terasaki and McClelland [6]. The HLA-DR phenotypes were determined by the two-color fluorescence technique, according to Van Rood et al. [7].

Serum levels of cortisol and 17α-hydroxyprogesterone (17α-OHPG) were determined with commercially available radioimunoassays. The level of steroid hormones is influenced largely by the level of thyroid hormones. Therefore, tests were performed in a phase of euthyroidism defined by normal serum levels of triiodothyronine, thyroxine, and their respective free hormones as well as a normal TSH level in a supersensitive radioimmunoassay (Ciba-Corning, Giessen, FRG). We performed intravenous stimulation tests with a bolus injection of 250 µg tetracosactrid (Synacthen). Basal and stimulated serum levels of cortisol and of its precursor molecule 17α-OHPG were measured.

HLA typing data are presented as antigen fequencies. Relative risks (RR) for the disease were calculated. The data were compared using the χ^2 test with Yates' correction; p values, of 0.05 were taken as significant. For the analysis of stimulation test Student's t and χ^2 statistics were applied.

Results

Class I antigens were positively associated with de Quervain's thyroiditis. Compared to the local control subjects, associations with the antigens HLA-A2 (RR = 1.93), HLA-B35 (RR = 2.42), and HLA-Cw4 (RR = 2.69) were found. All these associations are statistically significant.

Interestingly, class II alleles acted neutrally (DR1, DR4, DR7), while the HLA-DR2 specificity was slightly increased (RR = 1.39). The HLA-DR al-

leles HLA-DR3 and HLA-DR5 were markedly reduced in patients with Quervain's thyroiditis (RR = 0.28 and 0.12, respectively).

The HLA class II antigen HLA-DR3 was positively associated with Hashimoto's thyroiditis (RR = 1.9). The DR specificities HLA-DR2, -DR4, and -DR7 acted neutrally, whereas the alleles HLA-DR1 and HLA-DR5 exhibited reduced frequencies. Both class II specificities defined a preventive fraction for the disease (RR = 0.28 for HLA-DR1; RR = 0.35 for HLA-DR5). The class I specificities HLA-A1 and HLA-B8 were not significantly increased in patients with Hashimoto's thyroiditis.

Basal cortsiol serum levels, determined between 7:30 and 9:30 A.M., are shown in Table 1. The highest levels were found in the control population with 17.4 ± 4.4 μg/dl. Serum cortisol was signficantly reduced in patients with de Quervain's thyroiditis (14.3 ± 4.8 μg/dl) and in patients with Hashimoto's thyroiditis (13.4 ± 5.5 μg/dl). This difference in serum cortisol level persisted after 60 min following an intravenous bolus injection of 250 μg tetracosactid. The highest cortisol levels were observed in the controls, levels in patients with de Quervain's thyroiditis were intermediate, and those with Hashimoto's thyroiditis showed significantly reduced absolute serum levels. Basal levels of the cortisol precursor 17α-OHPG were highest in the control group, intermediate in Hashimoto patients, and lowest in de Quervain patients. Sixty minutes after stimulation, patients with Hashimoto's thyroiditis showed the highest serum levels (5080 ± 2298 pg/ml), which were significantly higher compared to the controls (4482 ± 1352 pg/ml). A calculation of the ratio between 17α-OHPG

Table 1. Serum cortisol and 17α-OHPG levels: basal and stimulated values 60 min after intravenous bolus injection of 250 μg tetracosactid

Group	Basal cortisol (μg/dl)	Stimulated cortisol (μg/dl)	Basal 17 α-OHPG (pg/ml)	Stimulated 17α-OHPG (pg/ml)
Controls	17.4 ± 4.4	31.5 ± 5.9	2377 ± 1300	4482 ± 1352
de Quervain's thyroiditis	14.3 ± 4.8	29.7 ± 6[a]	1130 ± 1243	4030 ± 4632
Hashimoto's thyroiditis	13.4 ± 5.5	26.6 ± 5.2[a]	1455 ± 1031	5080 ± 2298

[a] Differences from control values, significant $p < 0.05$

Table 2. Ratios of basal and stimulated levels of 17α-OHPG to cortisol

Group	Basal	Stimulated
Controls	136	142
de Quervain's thyroiditis	79	135
Hashimoto's thyroiditis	108	191[a]

[a] $p < 0.05$

and serum cortisol levels before and 60 min after the ACTH stimulation test revealed a significantly higher value in patients with Hashimoto's thyroiditis compared to controls and patients with de Quervain's thyroiditis (Table 2).

Discussion

Both subacute (de Quervain's) and Hashimoto's thyroiditis involve inflammation of the thyroid gland. In de Quervain's thyroiditis there is disruption of the follicular structures with extensive epithelial destruction including the formation of histiocytes. In Hashimoto's thyroiditis – the first human autoimmune disease in which an autoantigen-autoantibody reaction was described in 1956 be Roitt and coworkers [8, 9] – a lymphocytic infiltration and slight fibrosis can be seen. It is a striking feature of such diseases that they are associated with certain alleles of the class I and class II molecules of the HLA complex. The HLA molecules are now perceived as antigen-binding molecules which present antigen to the T-cell receptor. It is their important role in immune recognition (formation of the three-molecular complex of antigen/autoantigen, HLA molecule and T-cell receptor) which suggests a functional link of HLA associations with certain diseases [10].

In a homogenous German population we confirmed findings from an earlier analysis of the association between Quervain's thyroiditis and the HLA class I antigen HLA-B35 [11]. The HLA association in Hashimoto's thyroiditis is a matter of controversy. Reports of an association with HLA-DR3 and -DR5 were not confirmed in our study [12]. We detected an association only with the class II allele HLA-DR3, while the frequency of HLA-DR5 was reduced in the disease group. In our series the class I allele HLA-B35 was associated not only with de Quervain's thyroiditis but also with the late-onset form of congenital adrenal hyperplasia [13]. We therefore analyzed the two adrenal steroids cortisol and 17α-OHPG in thyroiditis patients for a possible alteration of steroid metabolism.

Our hypothesis was that due to the physical linkage of the adrenal steroid 21-hydroxylase genes to the MHC class III and class I genes an impaired adrenal steroid 21-hydroxylation can be expected. This hypothesis is based on the strong linkage disequilibrium between alleles from the class I and class III regions. Interestingly, basal and stimulated cortisol serum levels were significantly reduced in both disease groups compared to a population of healthy individuals. On the other hand, although the frequency of the HLA-B35 allele was increased in patients with de Quervain's thyroiditis, the level of the cortisol precursor 17α-OHPG was not indicative of the late-onset form of congenital adrenal hyperplasia [14]. In contrast, in patients with Hashimoto's thyroditis an exaggerated 17α-OHPG response was seen, with a stimulated 17α-OHPG/cortisol ratio that was significantly increased in these patients.

In Hashimoto patients a higher prevalence of Addison's disease is observed. One might argue that the significantly reduced basal and stimulated cortisol

levels are due to a subclinical form of adrenalitis which is not competely ruled out by a negative test for adrenal autoantibodies. However, subclinical adrenalitis does not explain the exaggerated response of the cortisol precursor 17α-OHPG, since both steroids are impaired in adrenalitis and show a blunted response after stimulation. Our data provide evidence that adrenal steroid 21-hydroxylase is impaired in patients suffering from Hashimoto's thyroiditis.

Evidence for an influence of nonimmune genes in autoimmunity also comes from animal models of thyroiditis. In the obese chicken model it was shown that non-MHC genes influence disease susceptibility. First, testosterone treatment reduced the formation of autoantibodies, and second, increased levels of corticoid-binding globulin were detected as another defect in steroid metabolism [15, 16].

Several mediators of an inflammation, interleukin 1, tumor necrosis factor, and other factors, are strong stimulators of the release of corticotropin-releasing hormone [17–20]. These mediators provide help in immune recognition and may play an important role in ongoing autoimmune destruction of the target organ which is due to a chronic inflammation. Therefore, negative feedback mechanisms such as T-cell suppressor circuits or negative feedback provided by steroids which reduce the secretion of mediators may play an important role. Our findings of an altered adrenal steroid 21-hydroxylation in patients with Hashimoto's thyroiditis may therefore be of functional relevance in this organ-specific disease and provide evidence that nonimmune genes, physically linked to MHC, influence expression of a human autoimmune disease.

Acknowledgement. We are indebted to Dr. W. A. Scherbaum for performing immunofluorecence-tests for organ-specific autoantibodies.

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Discussion

Davies:
May I just ask you how you can be sure that your patients were euthyroid. Are these patients on thyroid hormone replacement therapy?

Boehm:
Yes, they are. In each of these patients, the free T_4 and T_3 levels were measured, and baseline as well as stimulated TSH levels were determined by a supersensitive assay.

Seif:
As you know, in Schmidt's syndrome, Hashimoto's thyroiditis may be associated with autoimmune adrenalitis, i.e., Addison's disease. How did you make sure that your patients with Hashimoto's thyroiditis did not have impaired adrenocortical function due to this cause?

Boehm:
In each of the patients, autoantibodies to the adrenal cortex were measured, using both the indirect immunofluorescence test and the highly sensitive indirect ELISA of Dr. Scherbaum. Only patients who were negative in these assays were included in this study. This does not completely rule out subclinical disease.

Seif:
Just another question: If you have a defect of the 21-hydroxylase, you usually get an increase of the dehydroepiandrosterone in these patients. And I remember some publications indicating that DHE would have some effect on the immune system, protecting against some neoplastic diseases. [See: Schwartz AG et al. (1984) Inhibition of tumorigenesis and autoimmune development in mice by dehydroepiandrosterone. In: Cristofalo VJ et al. (eds) Altered endocrine status during aging. Liss, New York] Have you checked on this?

Boehm:
No, we have not checked yet, but what we are doing now is performing a molecular analysis of the 21-hydroxylase genes in these people. I think this is the best way to approach the problem. In collaboration with Massimo Trucco we will probably be able in the near future to look at point mutations of the 21-hydrox-

ylase gene to see if there is an association between the class I allele and a point mutation at the 21-hydroxylase gene. This may give direct evidence for such a hypothesis.

Drexhage:
How sure are you that you are not looking at a sort of counterregulatory mechanism in thyroiditis? We studied patients with thyroiditis who were euthyroid on replacement therapy, and we found homing disturbances of lymphocytes still to be present; so the immune response is going on in the disease. Is this not just a simple regulatory mechanism from the adrenal, and have you also tested family members for this?

Boehm:
We have not yet tested family members, but what we should do is perform a test with interleukin 1, for example, i. v. application, and see what happens, if there is also a decrease in cortisol response which would be a physiological approach with respect to autoimmune reactions.

Strongest Relative Risk for Goitrous Hashimoto's Thyroiditis Conferred by HLA-DQB1 Gene Variation

K. BADENHOOP, G. SCHWARZ, P. G. WALFISH, K. H. USADEL, and G. F. BOTTAZZO

Introduction

Hashimoto's thyroiditis can be found in two forms: the goitrous and the atrophic variants. It is considered to be of autoimmune aetiology closely related to Graves' disease. Both diseases can be observed clustered in families [1]. Furthermore, some patients progress from Graves' disease to Hashimoto's thyroiditis, illustrating that both diseases are part of one spectrum of thyroid autoimmunity. Antibodies to microsomal antigen (thyroid peroxidase) [2] and to thyroglobulin are detected, as well as occasionally antibodies to epitopes of the TSH receptor. Thyroid autoantibodies can also be found in relatives of patients with insulin-dependent diabetes mellitus (IDDM); these relatives show an association with the HLA specificity DR5 [3]. The disease itself has been shown to be associated with the HLA specificity DR5 in its hypertrophic form, with DR3 in its atrophic form and also with DR4 (reviewed in [4]).

All these disease association studies were performed using serological standards of the past International Histocompatibility Workshops (8th or 9th Workshop definitions). Since the 10th Workshop standardized techniques are available to test HLA variation not only with serology but also with molecular approaches using cDNA probes of the HLA-D region [5]. The advantage of the molecular approach is to detect more specificities and to assign homozygotes over former blank typings. These new specificities have led to a new nomenclature [6]. With this nomenclature it is possible to clarify the so far unclear association of HLA-DR specificities in Hashimoto's thyroiditis.

Since both DR4 and DR5 are in linkage disequilibrium with DQw3, we wanted to test which of the DQw3 splits – DQw3.1 (now called DQw7) or DQw3.2 (now called DQw8) – is associated with the disease. Using DNA probes of other gene loci of the short arm of chromosome 6 we intended to characterize additional immunogenetic markers. DQA2 as a DQ-linked marker (former DX α) was analysed as well as tumour necrosis factor (TNF) α. We recently described a novel TNF-α restriction fragment length polymorphism (RFLP) closely linked to extended haplotypes and associated with type I diabetes mellitus [7]. In the present study we have analyzed these immunogenetic markers in patients with goitrous Hashimoto's thyroiditis.

Subjects and Methods

We studied 66 patients with goitrous Hashimoto's thyroiditis, diagnosed by hypothyroidism, presence of antimicrosomal and/or antithyroglobulin antibodies, and with diffuse thyroid enlargement. Of these patients (all Caucasoid) 18 were from Toronto and 48 from London. Controls consisted of 58 randomly chosen Caucasoid subjects from London. Careful history taking excluded thyroid disease in the family. HLA-DR specificities did not differ between a control group in Toronto [8] and the London group. HLA specificities for A, B, C, DR, DRw52/53 and DQ were assigned to 9th International Histocompatibility Workshop definitions.

RFLP studies were performed according to standard procedures for the Southern blotting protocol as recommended by the 10th International Histocompatibility Workshop [9] with some modifications. In brief, DNA was prepared from peripheral blood; 10 µg was digested with 20 units of restriction endonucleases *Taq* I, *Bam* HI, and *Nco* I at temperatures and buffer conditions specified by the manufacturers (Bethesda Research Laboratories, Amersham), and electrophoresed on a 0.8% agarose gel in TAE buffer (40 mmol/l Tris acetate, 1 mmol/l EDTA, pH 8) for 24 h at 45 mA. Molecular weight markers were run on each gel, either *Hin* dIII digested lambda DNA with *Hae* III digested phIX or 10th HLA Workshop reference markers. The gels were then soaked in 0.15 *M* HCl for 10 min, then in 0.4 NaOH for 30 min. DNA was transfered onto Biotrace membranes using the alkaline method (0.4 *M* NaOH overnight). Filters were prehybridized overnight at 42°C and hybridized with a radioactively labelled cDNA probe in the following mixture: 50% deionized formamide, 0.1% Denhardt's solution, 5 × SSPE, 1% SDS, 5% dextrane sulfate and 200 µg/ml salmon sperm DNA. The filters were washed twice in SSPE at room temperature for 5 min then in SSPE 0.5% SDS at 65°C for 15 min and finally in 0.5 × SSPE at 65°C for 15 min. They were then exposed for 3–10 days with intensifying screens on Kodak XAR-5 film at − 80°C. The following probe/enzyme combinations were used: DRB/*Taq* I, DQA 1/*Taq* I, DQB 1/ *Bam* HI, TNF-α/*Nco* I.

RFLP data were correlated with serology. For analysis of 2×2 tabels χ^2 or Fisher's exact test was used as appropriate. Significance *(p)* values were corrected for the numbers of comparisons made (specificities tested in a given probe/enzyme system). Gene frequencies were determined by direct gene counting (for DQA 2 and TNF RFLP).

Results

HLA Serology. Classical serological typing for HLA-A, -B and -C antigens showed no statistically significant difference between patients with Hashimoto's thyroiditis and controls (data not shown). As shown in Table 1, HLA-DR antigens 4 and 5 differed; DR 4 was found in 33 (50%) patients compared to 20 (34%) controls (NS), and DR 5 showed a significant association: 23 (35%) pa-

Table 1. HLA-DR antigen frequencies

	Hashimoto's disease ($n = 66$)	Controls ($n = 58$)
DR 1	6 (9%)	11 (19%)
DR 2	11 (17%)	16 (28%)
DR 3	17 (26%)	18 (31%)
DR 4	33 (50%)[a]	20 (34%)[a]
DR 5	23 (35%)[b]	6 (10%)[b]
DR 6	12 (18%)	13 (22%)
DR 7	16 (24%)	17 (29%)
DR 8	2 (3%)	1 (2%)
DR 9	2 (3%)	2 (4%)
DR10	1 (2%)	–

[a] $\chi^2 = 2.44, p = 0.12$
[b] $\chi^2 = 9.02, p < 0.03$

tients possessed this DR antigen in contrast to 6 (10%) controls (Table 1). No other statistical significant differences were observed. The serological results were confirmed by DNA typing using *Taq* I, DR/DQ α RFLP analysis [10].

DQB1 Polymorphism–DQw Results. RFLP studies with the DQB1 gene probe yielded DQw types according to the novel HLA nomenclature [6], defining old DQw "splits" such as DQw3.1, DQw3.2, DQw1.1, DQw1.2, DQWa. RFLP DQ typing can also discriminate between homozygotes for a specificity and heterozygotes who are blank for the other haplotype, and it can better define the DQw2 type, which is difficult to test by serology. Among the DQ types tested, DQw7 on DR4 and/or DR5 haplotypes differed between patients and controls with the highest statistical association. Of 64 patients tested with Hashimoto's thyroiditis 36 (56%) had DQw7, versus 13 (22%) of 58 controls (significance calculation corrected for all DQ specifities tested, $p < 0.003$). This results in the relative risk of 4.5 (Tables 2, 3). DR 6, 7 and 8 haplotypes, which rarely can be linked to DQw7, were not taken into account.

Table 2. DQB1 polymorphisms in goitrous Hashimoto's thyroiditis

	DQw7[a]	DQwX[b]
Hashimoto's thyroiditis ($n = 64$)	36 (56%)	28 (44%)
Controls ($n = 58$)	13 (22%)	45 (78%)

$\chi^2 = 13.12$; p (corrected) < 0.003; risk ratio, 4.5
[a] DQw7 on DR6, 7 or 8 haplotypes were not taken into account.
[b] DQw7 haplotype excluded.

Table 3. DQB1 Polymorphism in DR4 + Hashimoto's thyroiditis

	DQw7	DQw8
Hashimoto's thyroiditis ($n = 30$)	16 (53%)	14 (47%)
Controls ($n = 17$)	5 (29%)	12 (71%)

$p = 0.2$

Table 4. DQA2 gene polymorphism in goitrous Hashimoto's thyroiditis: heterozygosity/homozygosity

DQA2 *Taq*I	2.1 Kb (U)	2.1/1.9 Kb (UL)	1.9 Kb (L)
Hashimoto's thyroiditis ($n = 51$)	11 (22%)	21 (41%)	19 (37%)
Controls ($n = 46$)	14 (30%)	25 (54%)	7 (15%)

$\chi^2 = 6.00, p < 0.05$

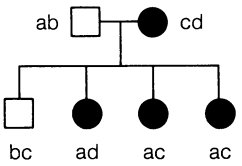
Table 5. DQA2 gene polymorphism in goitrous Hashimoto's thyroiditis: gene frequency

	2.1 Kb	1.9 Kb
Hashimoto's thyroiditis	43 (42%)	59 (58%)
Controls	53 (58%)	39 (42%)

$\chi^2 = 4.02, p < 0.05$

DQA2 Polymorphisms. Hybridization of *Taq* I digested DNA with the DQA1 gene probe results in RFLP patterns that correspond to DR serology and in a diallelic DQA2 polymorphism pattern: 2.1 Kb (U) and 1.9 Kb (L). This DQA2 gene polymorphism is detected because of the high sequence homology between DQA1 and DQA2. One can be either homozygous for either allele (UU, LL) or heterozygous (UL). We have observed a decrease in UU homozygotes and heterozygotes in goitrous Hashimoto's thyroiditis patients: UU, 11 of 51 (22%) versus 14 of 46 (30%); UL, 21 (41%) versus 25 (54%); and an increase in L homozygotes: 19 of 51 (37%) versus 7 (15%; $\chi^2 = 6.00, p < 0.05$). Gene frequences differed accordingly between Hashimoto's thyroiditis patients and controls. The gene frequency of the 2.1 Kb (U) fragment was 42% in Hashimoto's thyroiditis patients in contrast to 58% in controls; the gene frequency of the 1.9 Kb fragment (L) was 58% in patients, but only 42% in controls ($\chi^2 = 4.02$, $p < 0.05$; Tables 4, 5).

Analysis of a Multiplex Family with Goitrous Hashimoto's Thyroiditis. To investigate whether the disease-associated markers are important in the segregation of haplotypes in a family with multiplex cases of Hashimoto's thyroiditis, we analysed such a family in which the disease simultaneously in the mother and three daughters (see Fig. 1). Interestingly, all the children with Hashimo-



a: A 1 B 8 Cw7 DR4 w53 DQw7 DQA2 L
b: A 2 B35 Cw4 DR4 w53 DQw8 DQA2 U
c: A 24 B51 Cw- DR2 DQw6 DQA2 U
d: A 23 B50 Cw6 DR3 w52 DQw2 DQA2 U **Fig. 1.**

Table 6. TNF-α gene polymorphism in goitrous Hashimoto's thyroiditis: homozygosity/heterozygosity of 10.5/5.5 Kb alleles

TNF *Nco* I	10.5 Kb	10.5/5.5 Kb	5.5 Kb
Hashimoto's thyroiditis ($n = 39$)	14 (36%)	20 (51%)	5 (13%)
Controls ($n = 48$)	21 (44%)	19 (40%)	8 (17%)

$p = 0.55$ (NS)

Table 7. TNF-α gene polymorphism in goitrous Hashimoto's thyroiditis: gene frequency

	10.5 Kb	5.5 Kb
Hashimoto's thyroiditis	48 (52%)	45 (48%)
Controls	61 (57%)	46 (43%)

$p = 0.53$ (NS)

to's thyroiditis shared the haplotype DR4, DQw7, DQA2 (L), illustrating the importance of these gene loci on a susceptible haplotype.

Tumor Necrosis Factor α Polymorphisms. As an additional gene locus in the vicinity of HLA-DR and -DQ on the short arm of chromosome 6 we analysed the TNF-α gene. We have described earlier the novel TNF RFLP with *Nco* I in diabetic families [7]. In goitrous Hashimoto's thyroiditis there is a slight decrease in homozygotes for the 10.5-Kb allele and an increase in heterozygotes (NS). On the basis of the numbers available, the gene frequencies do not differ between the patients with Hashimoto's thyroiditis and controls (Tables 6, 7).

Discussion

There has been a long period of controversial findings about susceptibility to goitrous Hashimoto's thyroiditis linked to HLA-D antigens. HLA-DR3, -DR4 and -DR5 were all found to be associated in a variety of studies (reviewed in [4]). The relative risks ranged from 3.08 for DR4 [11] to 4.2 for DR5 [4, 8]. We present for the first time a full analysis of HLA-DR/DQ types, identified according to the standards which have been established after the 10th International Histocompatibility Workshop and defined using the new official nomenclature. We also observe an increase in DR4, albeit not significant, and a significant increase in DR5. The strongest association, however, is observed with DQw7 in association with DR4 or DR5, the former DQw3.1. The increase is due to the linkage disequilibrium displayed between DR5 and DQw7 and between DR4 and DQw7. DR4 is split at the DQ locus into DQw7 and DQw8, the latter being more prevalent (about 70% in the DR4 control population) [12]. The DR4 goitrous Hashimoto's thyroiditis patients possess DQw7 more often than DR4 controls, although due to the small numbers of our DR4-positive controls this does not reach significance. Nevertheless, this DQw7 in-

crease in DR 4-positive Hashimoto's thyroiditis patients contributes to the high relative risk observed with DQw 7. These results demonstrate that DQw 7 accounts for the associations of DR 4 and DR 5 and suggest that the DQw 7 specificity might be the primary susceptibility allele.

Since the DQw 7 specificity is encoded by the DQB 1 gene, the gene region that is distinctively different in DQw 7 from other DQw types is the obvious target to search for sequence variations. This gene region is situated in the first domain of the DQ β chain that has been analysed by DNA sequencing [12, 13]. Particular nucleic acid codons differ in DQw 7 from other DQw types: codon 13, 26, 45 and 57 (see Table 8). A glutamic acid residue at codon 45 is unique to DQw 7 [14], and a recent study using mutational analysis of that epitope underlines the importance of that structure for immune recognition [15]. This epitope is certainly important for the recognition of the monoclonal antibody TA 10 [15], therefore an important serological epitope, also contributing to the sterical structure of the DQ molecule. Thus it would be at the centre of the interaction with an antigenic peptide of the T cell via the T cell receptor and could play a key role in the initiation and/or perturbation of the autoimmune pathogenesis in the thyroid.

We can not exclude, however, that DQw 7 is yet another immunogenetic marker on a susceptible HLA haplotype that is closely linked to another allele of a gene on the short arm of chromosome 6. Since we observe another significant but somewhat weak association with DQA 2, this would point to a contribution to the susceptibility from the DQ region. Although DQA 2 has not as yet been found to be expressed at the protein level, it is not ruled out that DQA 2 might be expressed under certain pathological conditions, perhaps in the target tissue, i.e., the thyroid. DQA 2 has also been implicated in IDDM [16], where the primary association was found with DQ β [17–20]. Therefore we feel that the association is due to the strong linkage with DQ β since DQw 7 is linked to DQA 2 *Taq* I 1.9 Kb in homozygous cell lines and also in family segregation analyses [20]. This points to the DQ β region as the primary susceptibility contributing gene. This assumption is in analogy to association studies in other autoimmune diseases, where DQ β was analysed at the DNA level and susceptibility alleles were identified primarily (IDDM, coeliac disease, phem-

Table 8. Polymorphic residues in the DQB chain at the amino acid level: Hashimoto's thyroiditis associated haplotypes

DR	DQ	Hashimoto's thyroiditis association	Codon			
			13	26	45	57
4	7	Positive	A	Y	E	D
5	7	Positive	A	Y	E	D
4	8	Negative	G	L	G	A
1	5	Neutral	G	G	G	V
2	6	Neutral	G	L	G	D

Amino acid residues were translated from nucleic acid sequences (reviewed in [21]). The one-letter code for amino acids is: A, Ala; Y, Tyr; E, Glu; D, Asp; L, Leu; V, Val.

phigus vulgaris, juvenile rheumatoid arthritis, myasthenia gravis, multiple sclerosis, systemic lupus erythematodes; reviewed in [21]). Having identified a strong susceptibility marker, it will be necessary to pinpoint the molecular contribution of the DQw 7-DQ βstructure to understand the contribution of HLA-DQ β to the disease. Further search for non-HLA-DQ β linked genes, for example, TNF-α or neighbouring genes, is also necessary to understand the multifactorial genetic background of the disease.

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Discussion

Davies:
Your last slide (Table 5), where you showed the sequence on the right, wasn't that some exon 2 again?

Badenhoop:
That was exon 2 again, yes.

Davies:
But there are no RFLPs that can be placed on a change on exon 2.

Badenhoop:
No, but you can define the DQw alleles by RFLP, and since DQw alleles have been sequenced, one can compare the alleles with the published sequences. Nobody has found any exceptions from published sequences. If you sequence a DQw7 sequence yourself, you will confirm the published sequence. Nobody has found a mutation in these sequences so far in patients; if you look at rheumatoid patients or at diabetic patients, they fit the published sequences.

Trucco:
But that is not correct because there exists a RFLP with the enzyme ACYY that is recognized in a patient in position 57 of the second exon recognizing exactly the aspartic acid or not the aspartic acid.

Badenhoop:
Yes, but I just wanted to make the point that the alleles fit to the sequences.

Boehm:
I have some difficulties with your DR4-positive control individuals, because usually in a Caucasoid population the gene frequency of the DQw7 allele is around 40%, so there is no difference, compared to your DQw7 results.

Badenhoop:
The frequency of DQw7 in DR4 Caucasoid controls is 30%; you will find DQw8 in about 70%, this is published.

Boehm:
I am refering to the data from the 10th International HLA Workshop [Dupont B (ed) Immunobiology of HLA. Springer, New York]

Badenhoop:
No, everything that has been published so far finds DQw8 (the former DQw3.2) in the order of 60%–70%. The frequency of the other allele (DR4-DQw7) is 30%.

Davies:
Can I just ask you about your probe? What probe were you using for the DQ?

Badenhoop:
We used the full-length cDNA probe, which was used in the 10th International HLA Workshop.

Bottazzo:
We all know that, initially, conventional HLA serology gave controversial results, but the general concensus is now that Hashimoto's thyroiditis is associated with HLA-DR4 and HLA-DR5. However, by the seriological approach the association has never been too "spectacular." Interestingly, Dr. Boehm has just presented data where he seems to have moved away from chromosome 6, and he has concentrated his efforts on the search for other potentially genetic markers in Hashimoto's thyroiditis outside that gene region. Why did he give up? Was he frustrated? Apparently, you are of a different opinion. But are you convinced that is it worth pursing this path and continue to further dissect the HLA region and hopefully, find a closer association between genes located there and Hashimoto's thyroiditis?

Badenhoop:
I am convinced that in this population with goitrous Hashimoto's thyroiditis, we find the strongest association at the DQ β locus, and I think this is interesting from the past. The first studies were done with DW or DR types and showed rather low relative risks. If you proceed now to modern techniques which come every 4 years with new HLA workshops, you find new markers, and you find better definitions.

Bottazzo:
I agree with you. But, Massimo Trucco has just presented data where the relative risk is 107 when family members of type I diabetics are analyzed for the DQ-B gene product. You have adopted a similar approach and shown now that the relative risk in Hashimoto's thyroiditis is 4.9. Here there ia a tenfold difference between your data in Hashimoto's thyroiditis and those produced in type I diabetes. What is your comment?

Badenhoop:
The data are different, because the relative risk of 107 was constructed on the hypothesis that you can make a genetic formal analysis on the basis of Asp/non-Asp, but this is somehow constructed, because you cannot define an allele as such with the presence or absence of one amino acid. We have defined alleles

with DQw types, and if you use a definition like that in diabetes, the relative risk for DQw 8 is not 107. I do not want to enter into the discussion of Asp 57/non-Asp 57, if this is important or not, because this has been done in the past. Most people believe that the story of the DQ β Asp 57 is somehow out of to the past of immunology. I think it is important to define the alleles at the gene loci and to look for further gene loci in the vicinity, because it is not ruled out that we will find in the future new genes which find an even stronger association, where we can perform additional studies.

Davies:
I think that what amino acid 57 has done is to point out a polymorphic part of the binding fold, and there are other polymorphic signals of the class II binding fold that are likely to be related to other diseases, so that in order to find an association with thyroid disease we probably have to go in and sequence the genes; we are not going to get it by indirect means.

Badenhoop:
You can sequence DQ β chains in Hashimoto's patients, but they will not differ from the DQ β chains which have been published in the past. I would not spend a penny on such an experiment.

Davies:
You are saying there is no polymorphism?

Badenhoop:
No, the sequences are published; you do not need to do that work.

Davies:
There are few sequences published.

Badenhoop:
No, all the DQ β chains of all the DQ β alleles have been published, so you don't need to sequence DQ β chains.

Davies:
It depends whether they are polymorphic or not.

A Search for Linkage Between Phenylthiocarbamide Tasting, the Kell Blood Group Locus and Autoantibodies to Thyroglobulin and Thyroid Peroxidase*

S. LO, A. STEPHENSON, D. PHILLIPS, S. M. MCLACHLAN, G. PYLE, S. MOFFITT, D. F. ROBERTS, E. YOUNG, and B. REES SMITH

Introduction

Using new ultrasensitive assays to measure autoantibodies to thyroid peroxidase (TPO) and thyroglobulin (Tg) in the sera of families with autoimmune thyroid disease, it was recently demonstrated that the inheritance of TPO and Tg autoantibodies was consistent with Mendelian inheritance [1]. The tendency to produce TPO and Tg autoantibodies appeared to be inherited as an autosomal dominant trait fully penetrant in women but with reduced penetrance in men, and therefore a genetic linkage analysis with 28 polymorphic serological markers (located on different chromosomes) was carried out. The analysis revealed that several loci, notably the HLA antigens -A, -B, -DR, -DQ, as well as Bf on chromosome 6, could be excluded while markers for other loci (such as the immunoglobulin heavy chain marker Gm on chromosome 14) were uninformative [2]. However, there was a suggestion of weak linkage between the blood group Kell and TPO autoantibody. Kell has been reported to be linked to the locus governing the ability to taste phenylthiocarbamide (PTC) [3] which in turn may be associated with certain forms of thyroid disease [4]. In order to explore further the genetic basis of autoimmune thyroid disease, the study was extended, using a larger group of families, to determine whether autoimmune thyroid disease, Kell and PTC tasting are linked.

Methods

Blood was obtained from 12 families with autoimmune thyroid disease (seven Graves' disease and five Hashimoto's thyroiditis probands) and 149 of their relatives over 15 years of age. The probands had been identified in endocrine or medical outpatient clinics and their families selected for study if they were locally resident and consisted of two or more generations with evidence of autoimmune thyroid disease in at least two members. Nine families were

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those used in the original investigation [2] and three additional families were studied.

Autoantibodies to TPO and Tg were measured by the ultrasensitive direct assays involving the direct interaction between antibody and ^{125}I-labelled purified TPO or Tg [5] and Kell blood group typed by standard tube and tile methods. PTC tasting was carried out using a series of six solutions prepared by doubling dilutions from a stock solution containing 1.3 g/l modified from the original method of Harris and Kalmus [6]. Individuals were classified as tasters, and therefore either homozygous or heterozygous for the "tasting" dominant gene, if they could detect the bitter flavour of the fifth dilution (81.25 mg/l). Otherwise they were assumed to be homozygous for the recessive, "non-tasting" gene.

Genetic linkage analysis was performed with the LIPED programme [7] assuming an autosomal dominant inheritance of TPO and Tg autoantibodies with a gene frequency of 0.1 and penetrance of 100% in women and 40% in men. For overt autoimmune thyroid disease, the gene frequency was taken as 0.025, with 80% penetrance in women and 20% penetrance in men, as suggested by the segregation analysis of the families [2]. Gene frequencies for Kell and PTC tasting were those in the North-east population, approximately 0.043 and 0.45, respectively, with 100% penetrance in both men and women. Lod scores were calculated for each of the genetic markers at six values of the recombination fraction (0.001, 0.05, 0.1, 0.2, 0.3 and 0.4). Scores of 2 or below were taken as evidence against and scores 3 or higher as evidence for linkage [8].

Results

In contrast to our observations using 9 families which suggested linkage between Kell and TPO autoantibodies, the present analysis using 12 families demonstrated that linkage could be significantly excluded with a maximum lod score of -2.56 at $\theta = 0.001$ (Table 1). However, it should be stressed that

Table 1. Lod scores for Kell blood group, PTC tasting, TPO and Tg antibodies and overt autoimmune thyroid disease

Marker locus		Recombination fraction					
		0.001	0.05	0.1	0.2	0.3	0.4
Kell	TPO antibody	−2.56	−0.84	−0.53	−0.24	−0.09	−0.02
	Tg antibody	−1.08	−0.65	−0.44	−0.20	−0.08	−0.02
	Overt disease	0.32	0.27	0.22	0.13	0.06	0.02
PTC	TPO antibody	−4.17	−0.40	−0.10	0.29	0.18	0.04
	Tg antibody	−2.51	−0.74	−0.43	−0.19	−0.10	−0.04
	Overt disease	−1.08	−0.71	−0.47	−0.19	−0.06	−0.00
Overt disease	TPO antibody	2.62	2.28	1.90	1.15	0.51	0.12
	Tg antibody	3.75	3.28	2.78	1.76	0.85	0.23
Tg antibody	TPO antibody	4.50	4.47	4.08	2.94	1.67	0.59

Kell segregated only in three families, and the negative lod score could be attributed to a crossover in a single family. The analysis for Kell and Tg autoantibody was uninformative, as we observed for the nine families [2], and a similar uninformative result was obtained for Kell and overt autoimmune thyroid disease.

Linkage between the ability to taste PTC and autoantibodies to TPO and Tg could also be excluded, but linkage between PTC tasting and overt disease was inconclusive. However, most of the negative contributions to the lod values for autoantibodies were derived from crossovers in two families. Since it is sometimes difficult to distinguish between tasters and non-tasters, particularly in heavy smokers, it may be difficult to exclude linkage confidently between thyroid autoantibodies and either PTC tasting or Kell.

Overt disease was significantly linked to Tg autoantibody (maximum lod score 3.75) and possibly to TPO autoantibody (lod score 2.62). Furthermore, TPO and Tg autoantibodies were significantly linked to each other (maximum lod score of 4.5 at $\theta = 0.001$).

Discussion

The ability to taste PTC behaves as an autosomal dominant characteristic, tasting being dominant to non-tasting [4]. The possibility of an association with thyroid disease arose when homologies were observed between the active site of the PTC molecule and substances with antithyroid activity [9]. Further, a number of studies showed a significantly higher prevalence of non-tasters in patients with non-toxic, nodular goitre although the results in autoimmune thyroid disease have been contradictory [4, 9].

The linkage originally suggested between the blood group Kell and PTC tasting has recently been questioned [10]. As Kell segregated in only 3/12 families in our study, we were unable to provide evidence for or against linkage. In addition, our study indicates that genetic linkage between TPO or Tg autoantibodies and the ability to taste PTC or the Kell locus is unlikely, However, as the results were almost entirely on cross-overs in two families, it may be prudent to conclude that these data are uninformative.

Overt autoimmune thyroid disease was significantly linked with both TPO and Tg autoantibodies, as was the tendency to produce TPO and Tg antibodies. Since these autoantibodies appear to be inherited as an autosomal dominant characteristic in women, linkage between TPO and Tg antibodies provides further weight to the suggestion that the underlying genetic basis for thyroid autoimmunity is a single gene defect, and it may be, as originally suggested by Adams [11], a specific gene coding for the variable region of the heavy chain.

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Discussion

Davies:
Using the ultrasensitive assay, you showed an improvement or an increased detection in the patients' families. In Hashimoto families you did not find an increased prevalence in the normal controls. Can you explain that?

Phillips:
The increased prevalence among the Hashimoto/Graves families using the ultrasensitive assay was a result of the detection of individuals with low levels of antibody. These borderline cases would be expected to be more common in thyroid families than in the general population. In addition, our control group was fairly small, though we did find an increased prevalence of thyroglobulin antibody with the ultrasensitive assay.

Davies:
Secondly, you based the analysis on the assumption that there are people who do not make thyroid autoantibodies. Is that an inbuilt assumption?

Phillips:
I agree that our analysis depends on this assumption.

Scherbaum:
But there is a good deal of evidence to suggest that almost everybody can make thyroid antibodies if the lymphocytes are appropriately stimulated.

Phillips:
Yes, but there is a difference between spontaneous antibody production and making antibody in response to artificial stimuli. For instance, a limited number of epitopes are recognized in spontaneous antibody production.

Bottazzo:
When a new test for the determination of autoantibodies is introduced, it needs to fulfill certain criteria to validate, for example, its sensitivity, specificity, and predictive value for disease. These are accepted criteria in any protocol design for the standardization of autoantibody assays. I don't think you have tested enough normal controls, especially middle-aged women, in order to be able to precisely calculate these parameters. This is particularly relevant if the test has

to be ultimately introduced in large population screening programs. What is your comment?

Phillips:
The question which you are asking is whether this antibody test is a good way of predicting which people will develop Hashimoto's thyroiditis. The data in these families indicate that while many people have antibodies, a much smaller number go on to develop Hashimoto's thyroiditis or Graves' disease. Possibly other factors – maybe environmental, hormonal, or genetic – are involved in the progression from antibody to disease. So, yes, measuring these antibodies does not predict disease, but it may be useful in understanding the basis of thyroid autoimmunity. I think, therefore, the question of sensitivity and predictive value is irrelevant.

Bottazzo:
This does not answer the question fully. If any autoantibody assay has to be validated, it has to be able to calculate, as I said, its sensitivity, specificity and predictive value for disease.

McLachlan:
I would like to answer Franco Bottazzo's question: The assays are specific because the antibodies can be specifically inhibited by the addition of cold, unlabeled thyroglobulin or thyroid peroxidase, and these data have recently been published [Beever K, Bradbury J, Phillips D, McLachlan SM, Pegg C, Goral A, Overbeck W, Feifel G and Rees Smith B (1989) Highly sensitive assays of autoantibodies to thyroglobulin and to thyroid peroxidase. Clin Chem 35: 1949–1954]. We obviously have to look at larger numbers of women, and we are in the process of such a study.

Bottazzo:
I am not arguing that your assay is not specific for the autoantigen in question. My comment referred to the concept of specificity and sensitivity related to disease predictibility.

McLachlan:
We are not initially looking at disease; we are looking at ability to make antibody.

Bottazzo:
If you are picking up more thyroid antibodies in apparently healthy members of susceptible families, you are somehow biased for the initial material you have chosen. As I said, unless one tests in parallel a large number of normal women between 40 and 50 years of age (workers engaged in the field of autoantibody standardization recommend testing hundreds of controls while engaged in this exercise), we lack knowledge of prevalence of these antibodies in a population known to have raised thyroid antibodies, apparently in the absence of overt clinical disease.

Autoimmune Thyroiditis: Its Implications with Other Organ Systems

F. J. SEIF

Introduction

Syntropic manifestations of autoimmune diseases are found in various organs. Of these pluriglandular syndromes, Schmidt's syndrome – autoimmune thyroiditis associated with autoimmune adrenalitis – is a well-known example of dual organ involvement [18]. The extension of Schmidt's syndrome to chronic insulitis with insulin-dependent diabetes mellitus was described by Carpenter and coworkers in 1964 [4]. As early as 1907 Claude and Gougerot, on the basis of clinical and pathological observations, had coined the concept of "pluriglandular insufficiency" [6], and in 1912 Falta observed the variability of the "multiple sclerosis of blood glands" [8]. In 1930 Ostertag reported the association of calcification in the basal ganglia with tetany, hypothyroidism, pernicious anemia, and Addison's disease [16].

Some patients with pluriglandular insufficiency are prone to chronic mucocutaneous *Candida* infections (candidosis-endocrinopathy syndrome), showing an impaired T cell function [1]. This suggests a pathogenetic link to the Böttiger-Wernstedt-DiGeorge syndrome, the result of defective development of the derivatives of the third and fourth pharyngeal pouches; this syndrome is characterized by thymic hypoplasia or aplasia accompanied by parathyroid hypoplasia in infants.

To evaluate the role of autoimmunity of the thyroid gland, we investigated the reciprocal involvement of the thyroid and adrenal glands [19].

Patients and Methods

We examined two independent groups of patients: 30 patients with idiopathic, nontuberculous Addison's disease and 247 patients with autoimmune thyroiditis. The main presenting clinical signs and symptoms determined the group to which patients were assigned. Both groups were studied with respect to autoimmune manifestations in other organs, especially the adrenals and the thyroid.

Hormones were measured by radioimmune assays. Circulating autoantibodies against thyroid microsomal antigen (thyroperoxidase) and thyroglobulin were detected by indirect hemagglutination or by enzyme-linked immune assays. Anti-TSH receptor antibodies were measured by TSH-binding inhibi-

tion [19]. Cryostat sections of human tissues were used for the detection of antibodies against cytoplasmic antigens of islet B cells, adrenal cells, and gastric parietal cells by immunofluorescence [3].

Results

Of the 30 patients with Addison's disease, 28 (93%) showed autoimmune thyroiditis on the basis of clearly positive antibody titers (Table 1); this is 20 times the prevalence of 4.5%. Of these patients, 13 were hyperthyroid, 3 with Graves-Basedow disease and 10 with Hashimoto's thyroiditis. The other 15 were euthyroid or hypothyroid, 11 with Hashimoto's thyroiditis and one with idiopathic myxedema (Gull's disease), and the remaining three could not be classified by clinical and laboratory features. On the other hand, only 7 of the 247 patients with autoimmune thyroid disease (2.8%) also suffered from Addison's disease (Table 1). This is also 20 times the prevalence of 0.14% [19].

Patients with hyperthyroid bouts in the course of Hashimoto's thyroiditis were more common in the group with Addison's disease ($n = 10$, 33.3%) than in the other ($n = 19$, 7.7%). In both groups there were further associations with other autoimmune diseases such as vitiligo, pernicious anemia, insulin-dependent diabetes mellitus with slow onset, myasthenia gravis, gonadal insufficiency, chronic active hepatitis, and hypoparathyroidism, with a preponderance of these associations in the group with Addison's disease. Chronic

Table 1. Associations of other autoimmune organ manifestations in patients with Addison's disease or with autoimmune thyroid disease (ATD)

Disease	Addison's disease ($n = 30$)	ATD ($n = 247$)
Idiopathic Addison's disease	30 (100.0%)	7 (2.8%)
Autoimmune thyroid disease	28 (93.3%)	247 (100.0%)
Graves-Basedow disease	3 (10.0%)	108 (43.7%)
Hyperthyroid Hashimoto's thyroiditis	10 (33.3%)	19 (7.7%)
Hyperthyroid, unclassified	0 (0.0%)	26 (10.5%)
Nonhyperthyroid Hashimoto's thyroiditis	11 (36.7%)	62 (25.1%)
Idiopathic myxedema (Gull's disease)	1 (3.3%)	19 (7.7%)
Nonhyperthyroid, unclassified	3 (10.0%)	13 (5.3%)
Vitiligo	9 (30.0%)	10 (4.0%)
Pernicious anemia (+ 6)	2 (6.7%)	2 (0.8%)
Insulin-dependent diabetes mellitus (+ 3)	3 (10.0%)	2 (0.8%)
Myasthenia gravis	0 (0.0%)	6 (2.4%)
Gonadal insufficiency	4 (13.3%)	0 (0.0%)
Chronic active hepatitis	2 (6.7%)	0 (0.0%)
Hypoparathyroidism	1 (3.3%)	0 (0.0%)
Mucocutaneous candidosis	2 (6.7%)	0 (0.0%)

(+ n), Additional patients without clinical manifestations but with positive antibody titers.

mucocutaneous candidosis was found only in the group with Addison's disease; these two female patients were classified as having candidosis-endocrinopathy syndrome.

Discussion

The data on the two groups show many aspects of pluriglandular involvements (Table 1) known to be multiple organ manifestations of autoimmune diseases (Table 2). The results indicate that the presence of autoimmune thyroid diseases, Hashimoto's thyroiditis, idiopathic myxedema, and Graves-Basedow disease must be considered a common feature of idiopathic, nontuberculous Addison's disease, the clinical manifestation of autoimmune adrenalitis. The autoimmune processes in Addison's disease are not restricted to the adrenal but also involve mostly the thyroid gland (Schmidt's syndrome). Thus, the isolated idiopathic Addison's disease is a rare single-organ manifestation of the underlying pluriglandular autoimmune syndrome, whereas Schmidt's syndrome seems to be the most common two-organ involvement. However, the data do not support the further conclusion that Hashimoto's thyroiditis is the most frequent single-organ manifestation of the syndrome, since in this setting

Table 2. Clinical manifestations of different organ involvement in pluriglandular autoimmune syndromes

Graves-Basedow disease
Hashimoto's thyroiditis
Idiopathic myxedema (Gull's disease)
Addison's disease
Primary gonadal insufficiency
Hypoparathyroidism
Insulin-dependent diabetes mellitus
Lymphocytic hypophysitis
Chronic sialadenitis
Sjögren's syndrome
Chronic atrophic gastritis
Pernicious anemia
Coombs-positive hemolytic anemia
Idiopathic thrombocytopenia Werlhof
Chronic active hepatitis
Primary biliary cirrhosis
Celiac disease
Mucocutaneous candidosis
Vitiligo
Duhring's dermatitis
Myasthenia gravis

other organ involvements are rare. Moreover, the data indicate that Hashimoto's thyroiditis in association with the pluriglandular autoimmune syndrome predisposes to hyperthyroid spells caused not by TSH receptor antibodies but by phases of hyperreactive autoimmune processes induced by lymphokines and inflammatory tissue lysis, a thyrolytic hyperthyroidism also observed in women after pregnancy in the course of postpartum thyroiditis [12, 17].

Chronic mucocutaneous *Candida* infection is not a feature of this pluriglandular autoimmune syndrome, of which Schmidt's syndrome is a typical example. Rather, it seems to be characteristic of a different pathogenetic entity, the candidosis-endocrinopathy syndrome, showing impaired T cell functions [1] and being pathogenetically related to the Böttiger-Wernstedt-DiGeorge syndrome.

An explanatory theory of the pathogenesis of autoimmune thyroid disease must account not only for its different variants, Hashimoto's thyroiditis, idiopathic myxedema Gull, and Graves-Basedow disease, but also for its association with autoimmune manifestations in other organs, for example, pluriglandular autoimmune syndrome of Schmidt's type, candidosis-endocrinopathy syndrome, and connatal rubella syndrome. Some disposition to these syndromes is associated with genetic HLA markers B 8 DR 3, DR 4, DR 5, DQw 7, and a defective DQ β chain (Badenhoop et al., this volume).

The preferential involvement of certain organs shows that common major pathogenetic factors reside in these organs of immunoreactive attack: (a) the cells of the different organs express MHC class II antigens, as induced by lymphokines, and are antigen presenting [7, 13, 15]; (b) these cells are infected by organotropic lentiviruses [5, 9, 14]; and (c) the different organs contain antigens that show "molecular mimicry" of viral, bacterial, or fungal proteins eliciting antibodies that cross-react with organ-specific cell components [2, 11, 20, 21].

Moreover, the candidosis-endocrinopathy syndrome indicates that a defective T cell system produces a major regulatory disturbance in the immune system not effectively suppressing and inactivating immunoreactive processes. If this were the only pertinent defect, we would expect a generalized autoimmune reaction – a null graft versus host reaction – involving all organ systems. Therefore there must be a local contributing factor that resides in the afflicted organs.

With aging, the incidence of autoimmune diseases increases; the rate of autoimmune thyroiditis rises to 20% in those over 70 years of age [10]. The probability of exposure to a critical viral, bacterial, or fungal infection increases, and the wearing-out of the immune system progresses, as reflected by the involution of the thymus.

It can thus be concluded that autoimmune diseases or pluriglandular autoimmune syndromes stem from an imbalance of immune tolerance. The disturbance is multifactorial in the immune regulatory system, with many positive and negative feedback loops. This may make a search for etiological factors futile since in such feedback systems cause and effect are hardly separable. The genetically fixed factors of disposition of autoimmune diseases can be considered as boundary conditions of the regulatory system. Local contributing

factors at the level of organ destruction and environmental factors, such as viral, bacterial, or fungal infections, xenochemicals, or atopic agents, may overstrain the compensatory capacity of the immune system that is already weakened by suppression defects and inactivation of inflammatory processes. Since regulatory processes in the immune system are nonlinear and autocatalytic, it is also conceivable that by local and environmental factors the regulatory system is driven beyond a critical point, a bifurcation point, where self-tolerance of certain organs is broken, resulting in chronic and persistent autoimmunity.

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Discussion

McLachlan:
It is possible that Addison's patients recognize a T cell epitope on an antigen in the adrenal which cross-reacts with an epitope in the thyroid. This could explain your increased prevalence in the case of the first group.

Seif:
They had mainly both antobodies, but I can't give you the exact figures now.

McLachlan:
It could be a possibility that perhaps in Addison's you have a T cell epitope which is recognized, which is perhaps common to an antigen in the adrenal, and that is, you have a cross-reacting epitope on the thyroid. This could explain your increased prevalence in the case of the first group, but the reverse might not be true. I wonder if this could be reflected in the pattern of thyroid autoantibodies.

Sundick:
Do Addison's patients have an increased immune responsiveness to foreign antigens, for example, to tetanus toxoid, or an increase of gammaglobulin levels?

Seif:
No, they don't have.

Davies:
In vitro, I think Sandra McLachlan has shown that patients with autoimmune thyroiditis have a decreased reponsiveness to tetanus toxoid. Is that right, Sandra?

McLachlan:
What we found was that they were unable to regulate. A normal individual with a low baseline level of antibody to tetanus toxoid would make a large response on boosting, and a normal with a high starting antibody titer would show a small increment on boosting. In contrast, the response of Hashimoto patients to boosting with tetanus toxoid was unrelated to baseline levels.

Davies:
It is probably HLA linked because the DR3 group of patients tend to have a variety of aberrant immune responses.

McLachlan:
You may be correct. In normals it has been shown that DR3 individuals are abnormal in terms of their immune responses.

Davies:
So when you are investigating patients with thyroid disease, you have to use HLA-matched controls. Otherwise you can't say that any abnormality is unique to the disease.

The Genetic Control of Thyroid Cell MHC Class II Antigen Expression*

T. F. DAVIES and D. NEUFELD

The Thyroid Cell as an Antigen-Presenting Cell

MHC Class I Restriction. Thyroid epithelial cells, like almost all cells except red blood cells, express surface antigens coded for by the class I genes of the major histocompatibility complex (MHC). These antigens are recognized by syngeneic T cells expressing CD 8 antigen. The T cell receptor of the thyroid-specific CD 8^+ T cell combines with the MHC class I antigen, which encloses a thyroid-specific antigen within a well-defined cleft, to initiate cytolysis of the thyroid epithelial cell [1]. This phenomenon has been demonstrated in murine and rat models of experimental autoimmune thyroiditis as well as in human autoimmune thyroiditis [2, 3]. In such a situation the thyroid epithelial cell acts as an antigen-presenting cell, and such presentation is prevented by antibodies to MHC class I antigens (MHC restriction).

MHC Class II Restriction. Since thyroid epithelial cells were shown to be capable of expressing MHC class II antigens, it has been assumed that thyroid cells may also act as antigen-presenting cells to CD 4^+ thyroid-specific T cells. The widespread expression of MHC class II antigens in thyroid tissue from animals and humans with autoimmune thyroid disease also suggests a major role for such molecules in the disease process [1, 4]. The CD 4^+ T cell interacts only with MHC class II and not with class I molecules. Crude preparations of human thyroid epithelial cells have been shown to present viral antigen to T cells [5] and T cell cloning studies have also resulted in thyroid antigen-specific human T cell clones [6, 7]. However, all such experiments have used thyroid cell preparations that include a variety of dendritic and monocytic cells which may act as antigen presenters. Only recently have we been able to utilize cloned rat thyroid cells and thyroid-specific T cell clones to demonstrate such thyroid cell autoantigen presentation conclusively (Kimura et al., in preparation).

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The MHC as the Arbiter of Autoantigenic Presentation

Human Disease. Both Graves' disease and autoimmune thyroiditis have been known for many years to be familial, although lacking evidence of Mendelian inheritance [4]. Many families have examples of both diseases within them as well as associated autoimmune diseases such as type 1 diabetes mellitus [8]. Weak associations have been widely reported for autoimmune thyroid disease and specific MHC haplotypes. The highest risk ratios (of approximately 3–5) have been with MHC class II genes HLA-DR 3 and DR 5 [9]. However, the associations are so weak compared, for example, to diabetes that the genes for autoimmune thyroid disease could be well away from the MHC region of the chromosome. Alternatively, the susceptibility genese for autoimmune thyroid disease may be close to the MHC region, but our HLA typing methodology may be inadequate, and we are unable to recognize the susceptible haplotypes with sufficient precision. In this regard the approach using restriction fragment length polymorphisms has been equally unhelpful to date [1].

Animal Models. Inbred strains of animals offer many advantages for the study of the genetics of autoimmunity. Strains which are not truly inbred, however, may confound the situation as much as the study of humans. It is well known that certain strains of animals are susceptible to the induction of autoimmune thyroiditis and/or develop spontaneous autoimmune thyroiditis with age [10]. In the mouse, this susceptibility has been clearly linked to the MHC region [12], thus supporting conclusions from human data. This has not been as clear in the rat [11] although, once again, susceptibility to thyroiditis is highly strain dependent.

How Does the MHC Regulate Antigen Presentation?

The "Goodness of Fit" Approach. A particular MHC molecule may bind to a thyroid antigenic fragment with an avidity that is likely to vary with the MHC molcular structure. Hence, different animals and humans would have MHC antigen molecules which bind different thyroid antigens with differing degrees of success depending upon their particular MHC. The combined MHC antigen/thyroid antigen complex would then be recognized by thyroid-specific T cells with differing degrees of success depending upon how appropriately the complex had formed (or folded).

The "Mass Action" Approach. Since the presentation of thyroid antigen to the T cells of the immune system is MHC restricted, the amount of MHC antigen expression on the surface of the antigen-presenting cell, including the thyroid epithelial cell, would be proportional to the success of its interaction with the T cells. Hence, the greater the degree of MHC antigen expression the greater is the activation of thyroid specific T cells. By this mechanism the MHC may regu-

late the degree of MHC antigen expression by direct, and perhaps indirect, means.

It is likely that both these types of approaches are important regulators of susceptibility to autoimmune thyroiditis. Recently we have begun to explore the importance of this "mass action" approach.

Experimental Evidence for the "Mass Action" Hypothesis

The Thyroiditis-Susceptible Rat. The Fisher strain of rat is resistant to the induction of experimental autoimmune thyroiditis (EAT) while the Buffalo rat is not only susceptible to EAT but develops spontaneous thyroiditis with age [12]. In order to examine whether the expression of thyroid cell MHC class II antigens differs between these two strains we prepared primary thyroid cell monolayers and examined their in vitro response to the cytokine interferon gamma (IFN-γ) [13]. In every experiment performed it appeared that not only did Buffalo thyroid monolayers express more MHC class II antigen, but they expressed it upon more cells, at higher concentration, and more rapidly (Fig. 1). Hence the EAT-susceptible strain was highly sensitive to IF-γinduction of thyroid cell MHC class II antigens. However, such studies utilized crude thyroid monolayer cells. We know from earlier work that such monolayers are highly heterogeneous and contain a mixture of different cells types including fibroblasts. Therefore, comparing experiments is difficult and even more so when using different strains. While it is possible to correct for the number of cytokeratin-positive (i.e., epithelial) cells present, it is not possible to ignore the contribution of lymphocytes and other IF-γ responsive cells which may be present.

The Cloned Thyroid Cell Systems. The Fisher rat thyroid line (FRTL) and its derivatives have been available for over 10 years [14]. However, as stated earlier, the Fisher rat is EAT resistant. Indeed we have extensively characterized a

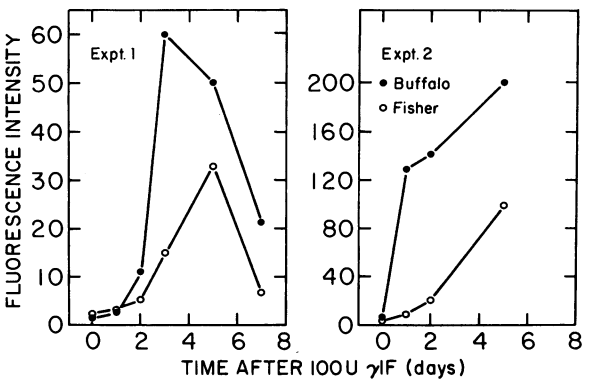

Fig. 1. Expression of MHC class II antigens on thyroid monolayer cells derived from Buffalo and Fisher rats. Two separate experiments are illustrated with data derived after exposure to 100 U/l rat IF-γ. Results are expressed as total fluorescence intensity

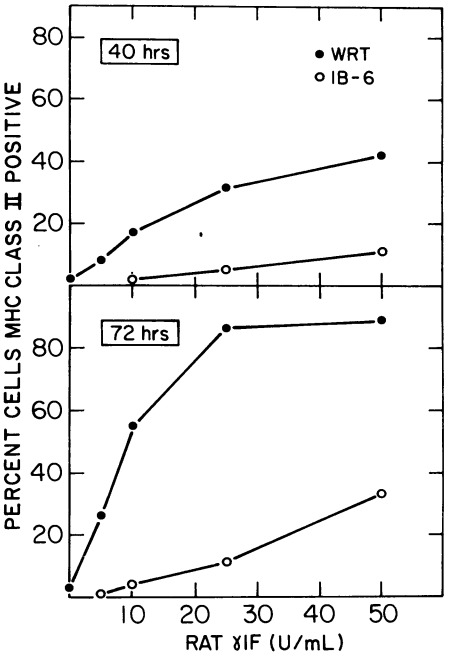

Fig. 2. The kinetics and dose-response to IF-γ of the MHC class II antigen expression on Wistar *(WRT)* and Fisher *(1 B-6)* thyroid cell lines. *Upper panel,* data after 4 h; *lower panel,* data after 72 h

clone (1 B-6) of the FRTL-5 cell line including its MHC class II antigen expression and the regulation of RT1.D α chain mRNA regulation [14, 15]. Recently, an immortalized thyroid cell line from the Wistar rat (WRT) has been developed [16] and found to have similar characteristics to the FRTL-derived cells. However, the WRT is EAT susceptible. Hence, by comparing the WRT and 1 B-6 cells we were able to eliminate the criticism of thyroid monolayer studies. As shown in Fig. 2, the EAT susceptible rat line WRT showed a larger and earlier MHC class II antigen response to IF-γ than the Fisher-derived 1 B-6 cells. Furthermore, because we were dealing with cloned cell lines, we were able to examine the MHC class II α chain mRNA. The mRNA levels in the WRT cells were similarly more sensitive to rat IF-γ than in the 1 B-6 cells, suggesting that such strain-specific differences were regulated at the transcriptional level [17].

Genetic Regulation of Rat MHC Class II Antigen Expression

Little is known of the IF-γ regulatory mechanism of MHC class II antigen genes in the rat. The 5′ region of the MHC class II genes in humans and mice have been more extensively explored, and a number of regulatory regions have been identified (Fig. 3). The IF-γ responsive element is thought to be the W box since IF-γ induces cell proteins which bind to the W box region [18]. Differences in

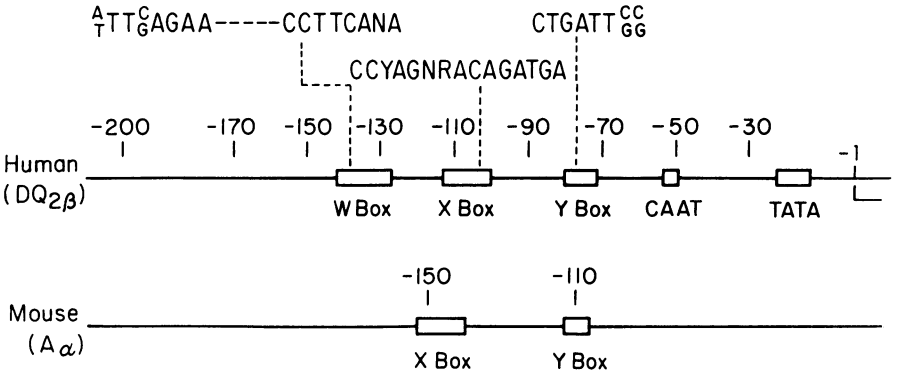

Fig. 3. Schematic representation of the 5′ region of the human HLA-DQ 2 β and mouse IA α genes. The consensus sequences for the W, X, and Y boxes are illustrated. (adapted from [18, 19])

the W box or, more likely, the regulatory DNA-binding proteins produced by different rat strains may greatly influence the expression of MHC class II antigens. Similarly, the IF-γ receptor itself may differ considerably between strains. Clearly these mechanisms need to be explored before we can fully understand the strain-specific differences which our experiments have revealed.

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Discussion

Khoury:
Is there any quantitative difference between class II expression on lymphoid cells from peripheral blood in Buffalo versus Fisher rats?

Davies:
I don't think this is a thyroid-specific phenomenon; this is a whole body phenomenon. We have some data that suggest, for example, with fibroblasts that you can still see the difference between the strains.

Khoury:
Couldn't it be possible that, for instance, the reagent that you are using picks up, apart from the monomorphic component, a polymorphic component in one of the two strains?

Davies:
This is unlikely at the message level, although you could argue at the antibody level.

McLachlan:
It is possible that you are looking at something which enhances the overall immune response?

Davies:
Yes.

McLachlan:
This suggests an irrelevant role for MHC in autoimmune thyroid disease. The primary genetic defect may involve genes from a totally different chromosome.

Davies:
You may be absolutely right. This is clearly just a modulation of the immune response. Maybe the MHC decides which antigen the immune response is going to react to, and which binds nicely in the fold, but the degree of response to that antigen bound to the fold may be determined by a whole set of other genes.

McLachlan:
Alternatively, it could also be determined by variable region genes coding for the T cell receptor or for the antibodies themselves. There is evidence in the literature of antibody-mediated autoimmune diseases in animals in which these genes are involved.

Davies:
I am not presently so sure about the role of antibodies.

Todd:
You may the point very well that the quantity of MHC class II expression is very important. I just wonder to what extent you would agree the quantity and quality are interlinked. For example, in our work we found that if you induce different levels of DR expression on thyrocytes, then the amount of DQ and DP expression obtained is linked to this. Thus, only if you induce high levels of DR expression with fairly high levels of gamma-interferon, do you then see DQ and DP expression as well. So, in terms of the different MHC class II loci, there is a relationship between the quantity and the quality of expression. Here you were obviously detecting the products of rat MHC class II loci with monoclonal antibodies Ox 6 and Ox 17.

Davies:
You raise a number of interesting points that I can't go into now because of limited time, but one of the interesting pieces of data that I think we have analyzed well enough is the fact that in this particular model, where we are concentrating on thyroid reactive rats, the difference in the strains is much greater at the thyroid cell level than at the fibroblast level. In other words, we only just get a p value for the difference using fibroblasts. This is significant. For example, Wistar fibroblasts overrespond to gamma-interferon compared to Fisher fibroblasts, but the difference is nothing like that seen in the thyroid cell lines. Though, you could argue that these are immortalized thyroid cell lines, but the difference suggests that the MHC determining thyroiditis is determining overexpression of class II in the thyroid. But it is much too early to draw a conclusion.

Thyroid Peroxidase: Approaches Towards its Characterization

Clinical Applications of Thyroid Peroxidase Autoantibody Determination*

S. MARIOTTI, P. CATUREGLI, P. PICCOLO, G. BARBESINO, A. PINCHERA

Introduction

In thyroid autoimmune disorders circulating autoantibodies (anti-M Ab) are frequently detected reacting with the thyroid microsomal antigen (M-Ag), a membrane protein associated with the microsomal subcellular fraction and located mainly in the apical cytoplasm and on the plasma membrane of thyroid follicular cells [1–6]. Recent investigations have provided biochemical, immunological, and molecular evidence that thyroid peroxidase (TPO) is the main and possibly the only autoantigenic component of thyroid microsomes [6–13].

Methods currently used in the clinical routine to measure anti-M Ab [14–16] still employ whole thyroid tissue or unpurified microsomal fractions. The availability of highly purified TPO provided the basis for the development of radioimmunoassays (RIA) [11, 17] and enzyme-linked immunosorbent assay (ELISA) [10] methods for anti-TPO autoantibodies (anti-TPO Ab), but the clinical usefulness of these techniques has not yet been fully evaluated.

We have recently developed a RIA for anti-TPO Ab based on competitive inhibition of radioiodinated TPO to an anti-TPO monoclonal antibody coated on plastic microtiter wells [18] or tubes [19] particularly convenient for the clinical routine. In this paper we report our experience of anti-TPO Ab measurements in a large series of sera from normal controls and patients with or without different thyroid diseases. In particular, attention focuses on ascertaining whether and to what extent anti-TPO Ab measurement represents a more accurate diagnostic tool compared to standard anti-M Ab determinations.

Materials and Methods

Control sera were obtained from 119 normal healthy subjects (57 women, 62 men; ages 6–70 years). Pathological sera were obtained from 325 patients with autoimmune thyroid diseases (AITD) and 271 with nonautoimmune thy-

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roid diseases (NAITD). AITD patients included 181 with Graves' disease (GD), 98 with Hashimoto's thyroiditis (HT), and 46 with idiopathic myxedema (IM). NAITD patients included 162 with endemic or sporadic nontoxic goiter, 83 with differentiated thyroid carcinoma, 14 with toxic adenoma, 8 with subacute thyroiditis, and 4 with toxic nodular goiter. The diagnosis was based on common clinical criteria and confirmed by appropriate laboratory tests.

The monoclonal anti-TPO antibody used in this study was obtained as detailed elsewhere [8]. TPO was purified by affinity chromatography using monoclonal antibody from a $(NH_4)_2SO_4$ precipitate of solubilized thyroid microsomes, as previously described [8]. TPO was iodinated by the chloramine-T method [20] to a specific activity ranging between 20 and 40 μCi/μg and purified by gel filtration on a Sephachryl S-300 column.

The assay is based on the competitive inhibition of [^{125}I]-labeled TPO binding to an anti-TPO monoclonal antibody coated on a solid phase. In the method first developed in our laboratory [18], the solid phase was represented by polyvinyl wells of microtiter plates (Cook Engineering, Alexandria VA, USA). We also used a modified assay in which the monoclonal antibody was coated on the surface of polystyrene tubes [19], which have recently become commercially available (Anti-TPO Ab K, Sorin Biomedica, Saluggia, Italy). Aside from the difference between microwells and tubes, the two assays were carried out using the same materials. No significant difference was found between data from the two procedures, and the results obtained were pooled.

Anti-TPO Ab were quantified by adding in each test a standard curve made by progressive amounts of a human IgG preparation calibrated against a positive serum provided by the National Institute for Biological Standards and Control (Hamstead, London, UK) to allow a final expression in terms of U/ml anti-M Ab. After correction for the dilution factor, the minimum detectable amount in the test sera ranged between 5 and 10 U/ml. The coefficient of variation within the assays was 5% ± 3% and between different assays 12% ± 5%.

In almost all cases anti-M Ab and anti-thyroglobulin antibody (anti-Tg Ab) were measured by passive hemagglutination (PH) using commercial kits (Fujizoki Pharmaceutical, Tokyo, Japan). By the latter procedure, antibody titers of at least 1/100 were considered positive. In selected cases (see results) anti-M Ab determinations by PH were also performed in the presence of excess (50 μg/ml) thyroglobulin (Tg) in the assay buffer in order to eliminate the interference of anti-Tg Ab in the anti-M Ab determinations. Tg employed for these experiments was prepared by Sepharose 4 B gel chromatography as previously described [21].

Results

Assay of Anti-TPO Ab in Thyroid Diseases. Preliminary experiments carried out on 50 sera from normal healthy subjects and undetectable anti-M Ab by PH showed anti-TPO Ab by both RIAs consistently ranging from undetectable to

Table 1. Serum anti-TPO Ab levels by RIA (U/ml) in 119 normal subjects and in 441 patients with thyroid diseases

	n	< 10 U/ml	10–99 U/ml	100–999 U/ml	1000–9999 U/ml	≥ 10000 U/ml
Control subjects	119	109 (91.6%)	6 (5.0%)	4 (3.4%)	0	0
Graves' disease	181	46 (25.4%)	48 (26.5%)	46 (25.4%)	32 (17.7%)	9 (5.0%)
Hashimoto's thyroiditis	98	0	11 (11.2%)	22 (22.5%)	41 (41.8%)	24 (24.5%)
Idiopathic myxedema	46	0	12 (26.1%)	16 (34.8%)	15 (32.6%)	3 (6.5%)
Thyroid carcinoma	83	70 (84.4%)	6 (7.2%)	6 (7.2%)	1 (1.2%)	0
Subacute thyroiditis	8	8 (100%)	0	0	0	0
Other NAITD	180	160 (88.9%)	14 (7.8%)	5 (2.8%)	1 (0.5%)	0

10 U/ml. Thus, values exceeding 10 U/ml were considered as positive anti-TPO Ab tests. As summarized in Table 1, positive anti-TPO Ab were observed in the majority (134/181, 74%) of patients with GD, all of the 144 patients with HT or IM, 13/83 (15.6%) with differentiated thyroid carcinoma, none of the 8 patients with subacute thyroiditis, 20/180 (11.1%) of those with other miscellaneous NAITD, and 10/119 (8.4%) normal controls. In general, anti-TPO Ab were found in sera with detectable anti-M Ab, while no correlation was found with anti-Tg Ab (data not shown). It should be noted, however, that some discrepant results were observed, as discussed in more detail below.

Comparison of Anti-TPO Ab by RIA and Anti-M Ab by PH. As shown in Fig. 1, a highly significant positive correlation ($r = 0.979$, $p < 0.001$) was found between anti-M Ab titers by PH and the corresponding mean anti-TPO Ab levels by RIA. With one exception, discrepancies between anti-TPO Ab and anti-M Ab were limited to sera with negative or low (1/100–1/400) titers of anti-M Ab by PH. In particular, positive anti-TPO Ab were found in 25/278 (8.9%) patients with undetectable serum anti-M Ab, while 57/95 (60%) subjects with low circulating anti-M Ab titers were negative for anti-TPO Ab. Further analysis of these discrepant samples (Table 2) showed that in sera with negative anti-M Ab, anti-TPO Ab were found in 22/62 (35.5%) patients with AITD and in only 3/216 (1.3%) sera from NAITD patients and normal controls ($\chi^2 = 69.4$, $p < 0.0001$). Moreover, as illustrated in Table 2, of the 95 sera with anti-M Ab titers ranging from 1/100 to 1/400, 27/32 (81.8%) samples from patients with AITD had detectable anti-TPO Ab, while positive anti-TPO Ab were found only in 11/62 (17.7%) sera obtained from NAITD patients and normal controls ($\chi^2 = 38.9$, $p < 0.0001$). In the remaining serum (Fig. 1, arrow) with anti-M Ab by pH and undetectable anti-TPO Ab by RIA the addition of excess Tg

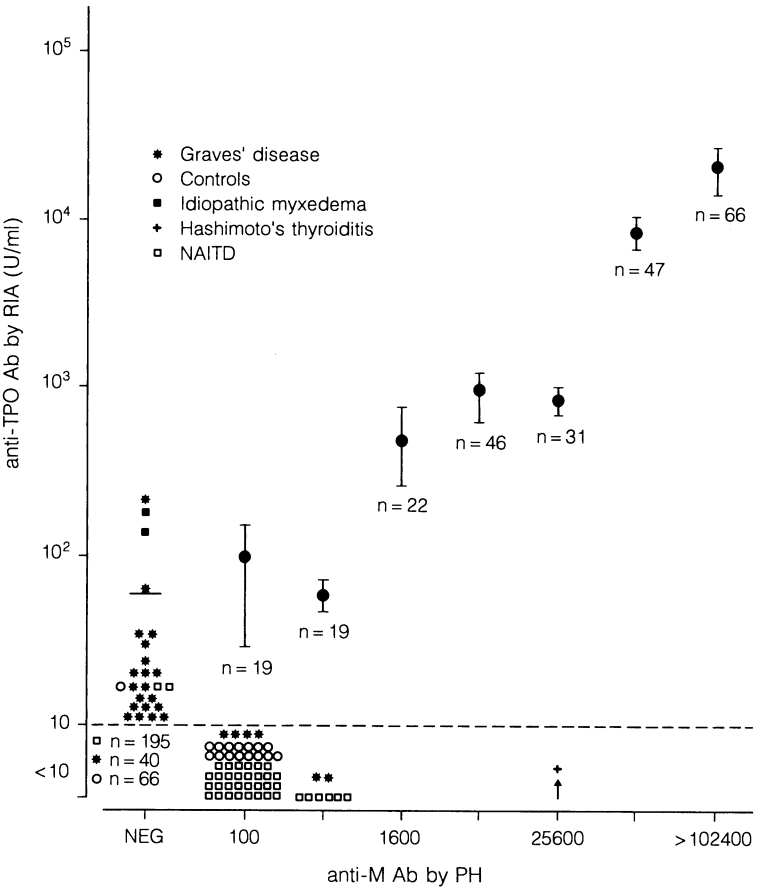

Fig. 1. Comparison of anti-TPO Ab levels (RIA) and anti-M Ab titers (PH) in 633 sera obtained from the patients represented in Table 1. Note that in several instances two or more sera were available from the same patient, making the total number of assays included in this analysis higher than the total number of patients. Positive results for both anti-TPO Ab and anti-M Ab are pooled together and shown as the mean ± SD anti-TPO Ab levels for each anti-M Ab titer by PH (the total number of samples included is indicated below each symbol). Individual diagnoses are depicted by a different symbol only in discordant samples and in sera with undetectable anti-TPO and anti-M Ab. The sample indicated by the *arrow* was negative for anti-M Ab, when the test was carried out in the presence of excess Tg (see text for details)

completely inhibited the agglutination reaction, indicating an interference of anti-Tg antibody in the assay of anti-M Ab. This phenomenon was observed in other occasional sera from AITD patients not included in Fig. 1.

Predictive Value for AITD of Anti-TPO Ab: Comparison with Passive Hemagglutination. The results described in the previous paragraph suggest higher specificity and sensitivity for AITD and anti-TPO Ab measurements when compared to anti-M Ab assays. To further confirm this conclusion, we

Table 2. Incidence of positive anti-TPO Ab by RIA in sera with negative or low titer anti-M Ab by PH: comparison of patients with AITD with those with NAITD and normal controls

Anti-M Ab	AITD			NAITD and normal controls		
	n	Anti-TPO Ab ≥ 10 U/ml	Anti-TPO Ab < 10 U/ml	*n*	Anti-TPO Ab ≥ 10 U/ml	Anti-TPO Ab < 10 U/ml
Negative	62	22 (35.5%)[a]	40 (64.5%)	216	3 (1.3%)	213 (98.7%)
1/100–1/400	32	27 (81.8%)[a]	5 (18.2%)	62	11 (17.7%)	51 (82.3%)

[a] $p < 0.00001$, χ^2 test versus NAITD and normal controls

Table 3. Predictive value of anti-TPO Ab and anti-M Ab for the diagnosis of AITD

	Anti-TPO Ab	Anti-M Ab
Positive results		
True positives	278	248
False positives	30	70
Negative results		
True negatives	269	196
False negatives	47	64
Predictive value of positive tests	90.2%	78.2%
Predictive value of negative tests	85.7%	75.3%
Specificity	89.9%	73.7%
Sensitivity	85.5%	79.4%
Efficiency	87.7%	76.8%

calculated the predictive value of anti-TPO Ab and anti-M Ab for the diagnosis of AITD according to the Galen and Gambino criteria [22]. We considered a true positive any positive antibody assay observed in patients with AITD (GD, HT, or IM), while antibody found in normal subjects or in patients with NAITD was considered a false positive. Conversely, negative antibody tests were considered as true negative when found in normal subjects and in patients with NAITD and as false negative when observed in AITD. The results of this analysis (Table 3) confirm the greater efficiency of anti-TPO Ab measurements in the diagnostic evaluation of AITD for each parameter considered.

Anti-TPO Ab in Different Untreated Autoimmune Thyroid Diseases and Effect of Therapy. Table 4 reports the frequency of positive tests as well as the mean anti-TPO Ab levels observed in patients with different types of autoimmune thyroid diseases, subdivided according to their clinical status. Of the 69 untreated hyperthyroid patients with GD, 55 (79.7%) had serum anti-TPO Ab levels over 10 U/ml, with a mean ± SE of 4680 ± 1468 U/ml. Anti TPO Ab concentrations over 10 U/ml were found in all the untreated patients with hypothyroid or euthyroid goitrous HT or IM. The highest anti-TPO Ab titers (27 970 ± 9188 U/ml) were found in hypothyroid HT patients, while significantly lower levels were found in euthyroid HT (3780 ± 1797, $p < 0.05$) and in IM pa-

Table 4. Anti-TPO Ab levels in patients with untreated and treated AITD

	n	Anti-TPO Ab		
		< 10 U/ml	> 10 U/ml	Mean ± SE
Graves' disease				
Untreated, hyperthyroid	69	14 (20.3%)	55 (79.7%)	4680 ± 1468
Treated, euthyroid	112	38 (33.9%)	74 (66.1%)	2060 ± 932
Hashimoto's thyroiditis				
Hypothyroid, untreated	38	0	38 (100%)	27970 ± 9188[a]
Hypothyroid, on L-thyroxine	25	0	25 (100%)	6950 ± 3011
Euthyroid, untreated	13	0	13 (100%)	3780 ± 1797
Euthyroid, on L-thyroxine	22	0	22 (100%)	2970 ± 1327
Idiopathic myxedema				
Untreated	21	0	21 (100%)	2389 ± 677
On L-thyroxine	25	0	25 (100%)	649 ± 257[b]

*) Number in parenthesis indicate percentages

[a] $p < 0.05$ versus untreated Graves' disease, euthyroid Hashimoto's thyroiditis, and idiopathic myxedema (by unpaired Student's *t* test).

[b] $p < 0.05$ versus idiopathic myxedema (by unpaired Student's *t* test).

tients (2389 ± 677, $p < 0.05$). The mean anti-TPO Ab titer of hypothyroid HT patients was also significantly higher compared to that found in untreated GD ($p < 0.05$). No other significant differences were found by comparing the mean anti-TPO Ab titers observed in untreated conditions, and no relationship was found between thyroid status and serum anti-TPO Ab titers. Positive anti-TPO Ab tests were observed in 74/112 (66.1%) of GD sera obtained during or after methimazole treatment. The mean antibody titer of this group was lower (2060 ± 932 U/ml) than that found in untreated GD, but the difference was not statistically significant. When treated patients were analyzed, anti-TPO Ab levels were also lower during L-thyroxine administration in patients with HT (6950 ± 3011) or IM (649 ± 257). Only in the latter category of patients, however, did the difference in antibody titer versus untreated reach statistical significance ($p < 0.05$ by Student's *t* test).

Discussion

In spite of the recent identification of TPO as the main and possibly the only autoantigenic component of the thyroid M-Ag, few data are available on the usefulness of specific anti-TPO Ab assays in the clinical evaluation of thyroid diseases. We therefore assayed circulating anti-TPO Ab in a large series of normal subjects and patients with different forms of AITD and NAITD using a monoclonal antibody assisted solid-phase RIA for anti-TPO Ab. The results obtained showed the presence of anti-TPO Ab in most subjects with GD and in all patients with HT or IM. Anti-TPO Ab were also found, although generally

at lower levels, in a minority of patients with several NAITD (including thyroid carcinoma), and in 8.4% of apparently normal healthy subjects. When the results obtained in anti-TPO Ab measurements where compared to anti-M Ab determination by PH, in agreement with previous studies [11], a positive correlation was found. Some discordant results were observed, almost exclusively in sera with undetectable or low levels of either antibody. The analysis of these discrepant samples showed that the anti-TPO Ab RIA has several advantages compared to anti-M Ab by PH. When only anti-M Ab negative sera were considered, a significant proportion of positive anti-TPO Ab tests were found in patients with AITD, in keeping with a higher sensitivity of the RIA. Furthermore, the assay of anti-TPO Ab appeared to be more specific for AITD in sera with low antibody titers. This derives mainly from the low percentage of anti-TPO Ab positive tests observed in sera with low anti-M Ab titers obtained from normal subjects and patients with NAITD, while the large majority of patients with AITD and comparably low titers of anti-M Ab also had positive anti-TPO Ab tests. The question of whether the hemagglutination produced by several anti-TPO Ab negative sera from subjects without AITD is due to a still unrecognized autoantibody distinct from anti-TPO Ab or to a methodological artifact has yet to be answered.

In agreement with previous studies [23, 24] we found that anti-Tg Ab may react with Tg present in unpurified M-Ag preparations, giving false-positive results in anti-M Ab determinations unless excess Tg is added in the assay mixture. In contrast, Anti-TPO Ab measurements resulted fully unaffected by anti-Tg Ab and did not require the addition of Tg in the assay mixture.

The assay of anti-TPO Ab in different untreated AITD showed that the highest levels were found in untreated goitrous HT, while no significant difference was observed between the mean anti-TPO Ab levels in GD, euthyroid HT, and IM. The observation might have functional relevance since anti-TPO Ab could interfere with the endogenous TPO, leading to impaired Tg iodination and thyroid hormone synthesis. No direct information on this aspect can be derived from our data, since the RIA employed detects all anti-TPO Ab able to bind TPO, independently of their capacity to interfere with the enzymatic activity. Using different experimental approaches, in vitro inhibition of TPO enzymatic activity by anti-TPO positive sera or IgG has been reported by some [25–28] but denied by other investigators [8, 9].

Treatment of GD with anti-thyroid drugs and of IM or HT L-thyroxine was associated with a significant decrease in anti-TPO Ab. This finding is in keeping with previous observations carried out in this laboratory [29, 30] showing in the same conditions a decrease of anti-M Ab following therapy.

In conclusion, the results of our study show that the behavior of serum anti-TPO Ab in a large series of patients with and without different thyroid disorders is very similar to that of anti-M Ab, further confirming the identity of TPO and M-Ag. Furthermore, the assay of anti-TPO Ab by monoclonal antibody assisted RIA has substantial advantages in terms of sensitivity and specificity compared to anti-M Ab determinations by PH. These characteristics should allow its rapid diffusion in the clinical routine for the diagnostic evaluation of AITD.

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Discussion

Bottazzo:
What happens if you introduce a cut-off?

Mariotti:
To assess this important point, we considered different cut-off levels at 10 U/ml, 50 U/ml and 100 U/ml. At each level the specificity of anti-TPO antibody tests was higher than that of anti-M antibody by passive hemagglutination. The sensitivity of anti-TPO assay was higher at the lowest cut-off level (10 U/ml), while it was similar to that of anti-M antibody when higher cut-offs were considered. In our experience similar results were obtained when thyroid anti-M antibodies were assayed using techniques other than passive hemagglutination, such as RIA or ELISA. It should be pointed out that several commercially available RIA or ELISA kits for anti-M antibodies are less sensitive than passive hemagglutination.

Bottazzo:
Moving to a more practical point, do you see laboratories in the future being in a position to measure anti-TPO antibodies using TPO as the antigen? In addition, can you give us more information about the present availability of commercial kits and how they compare with each other? Do they use the same TPO preparation? Have different kits been exchanged among laboratories and compared in their performance in a blinded fashion?

Mariotti:
We compared our system with a commercial kit provided by Sorin, which employs the same materials (with the exception of tubes instead of microtiter wells) and we obtained comparable results. As far as the standardization is concerned, I would like here to mention that the Italian Endocrine Society has recently organized a collaborative study to standardize endocrine autoantibody measurements, including thyroid antibodies. By the end of next year sufficient data should be available to complete the analysis in terms of specificity and sensitivity.

Davies:
Could I just ask you whether you have looked at any of Pierre Carayon's antithyroglobulin monoclonals, and whether they bind to your TPO preparation?

Mariotti:
I think that Pierre Carayon may answer this question better than I. The anti-Tg monoclonal antibodies that I had the opportunity to test did not react with TPO.

Davies:
They were not directed, but do they bind to the labeled TPO?

Mariotti:
They did not bind.

Scherbaum:
Am I correct in assuming that using this assay you will be measuring IgM antibodies as well as IgG antibodies?

Mariotti:
Yes, this RIA is not immunoglobulin class restricted, since it is a competitive radioassay, detecting any substance interfering with the binding of monoclonal anti-TPO antibody to TPO.

Scherbaum:
Have you investigated the immunoglobulin class?

Mariotti:
Not yet.

Burger:
Do you have any evidence in completely athyreotic patients that T_4 also reduces TPO antibodies?

Mariotti:
We haven't looked specifically at this point. As a rule, however, L-T_4 administration in patients with idiopathic myxedema is associated with a significant decrease of serum anti-TPO antibodies. In most of these cases, however, a small thyroid remnant is detected by echography.

Bogner:
You showed that using TPO radioimmunoassay in all of your patients with Hasimoto's thyroiditis you get a positive result. This is not in our experience because we have some patients with newly diagnosed Hasimoto's thyroiditis and hypothyroidism who don't have any TPO antibody titers. So I am a little bit surprised about these 100% values you presented.

Mariotti:
Were your patients negative also for anti-M antibodies?

Bogner:
Yes, and for TSH receptor antibodies also.

Comparison Between Microsomal Antibodies by Immunofluorescence and Anti-TPO Antibodies in Healthy Individuals and Thyroid Autoimmune Diseases*

U. FELDT-RASMUSSEN, M. HØIER-MADSEN, N. G. RASMUSSEN, L. HEGEDÜS, P. J. HORNNES, and H. PERRILD

Introduction

Thyroid-peroxidase (TPO) ist now well recognized to represent the specific antigen of anti-microsomal antibodies (MAb) [1, 2]. Recently, routine methods for clinical measurement of autoantibodies to TPO (anti-TPO) have been developed [3, 4]. We have therefore found it of interest to investigate the clinical usefulness of replacing measurements of MAb by a commercial method for quantifying anti-TPO. Furthermore, we wanted to compare the anti-TPO measurements with an immunofluorescence method for MAb, which is generally considered rather specific.

Material and Methods

Normal subjects consisted of 88 individuals (38 men, 50 women) with a median age of 52 years (range 17–88 years). None of them had previous or present history of thyroid diseases. All had a normal thyroid gland volume as determined by ultrasound [5] and normal values for triiodothyronine (T_3), thyroxine (T_4), free thyroid hormone indices, and TSH, and none received any medication with known influence on thyroid gland function. Pregnant women examined in the study included 36 with thyroid antibodies (MAb and/or TgAb) in their first trimester (median age 30 years, range 22–40) and 20 randomly selected antibody-negative controls (median age 29 years, range 20–38 years). None had previously known thyroid disease, and none received medication with known influence on thyroid function. All women gave informed consent to participate in the study, which was approved by the Regional Ethics Committee.

The group with Hashimoto's thyroiditis (HT) consisted of 22 patients (21 women, 1 man; median age 61 years, range 26–90) with classical HT (characteristic goiter, elevated serum TSH level, in some cases associated with low

* This study was supported in part by the Danish Hospital Foundation for Medical Research – Region of Copenhagen, Faroe Islands and Greenland, the P. Carl Petersen Foundation, and Helsefonden.

thyroid hormones) and elevated levels of MAb and/or anti-thyroglobulin antibodies (TgAb). The group with primary idiopathic myxoedema comprised 11 patients (9 women, 2 men; median age 62 years, range 32–78) fulfilling the same criteria as HT but without a goiter. The Graves' disease group included 21 patients (18 women, 3 men; median age 56 years, range 17–79); these were studied either untreated ($n = 11$) or after 3–6 months of antithyroid drug treatment ($n = 10$). And finally, the thyroid carcinoma group consisted of 20 patients positive for TgAb and thus with evidence of an autoimmune thyroid reaction (19 women, 1 man; median age 60 years, range 22–84).

Except in the study of pregnant women only one blood sample was obtained from each person. Clinical examination and blood sampling were performed during pregnancy in postmenstrual weeks 18, 24, 30, and 36 as well as 1, 2, 3, 6, and 12 months postpartum. Serum was stored at $-20\,°C$ and analyzed in consecutive assays. Measurement of serum TSH, total T_4, T_3, and free thyroid hormone indices were performed as previously described [5]. TgAb were analyzed by an ELISA method [6], MAb by indirect immunofluorescence both undiluted and at a dulution of 1/10 [7], and anti-TPO by a commercial radioimmunological method (DYNOtest, Henning, West Berlin). The method was based on a principle described by RUF et al. [3]. The within-assay precision (coefficient of variation) was 4% and the between-assay precision was 10%–16% (duplicate determinations of two control sera in 14 consecutive assays). Statistical analyses were performed using Mann-Whitney's U test, Wilcoxon's test for paired differences, and the χ^2test.

Results

Ten of the 88 control subjects had MAb in undiluted serum, and six were also positive in serum diluted 1/10 (Table 1). Four of these were strongly positive for TgAb. These six persons were excluded from further calculations, since they were considered to have asymptomatic autoimmune thyroiditis. Their anti-TPO values ranged from 96 to 4700 U/ml. The values of the remaining 82 controls are presented in Fig. 1, and could be shown to follow a logarithmic normal

Table 1. Number of patients positive for MAb by indirect immunofluorescence in undiluted serum or diluted 1/10

Patient group	n	Anti-microsomal antibodies	
		Undiluted	Diluted 1/10
Graves' disease, untreated	11	7 (64%)	4 (36%)
Graves' disease, treated	10	9 (90%)	5 (50%)
Thyroid cancer	19	12 (63%)	10 (53%)
PPT (1st trimester)	10	7 (70%)	5 (50%)
PPT (3–6 mo)	12	10 (83%)	11 (92%)
Controls	88	10 (11%)	6 (6.8%)

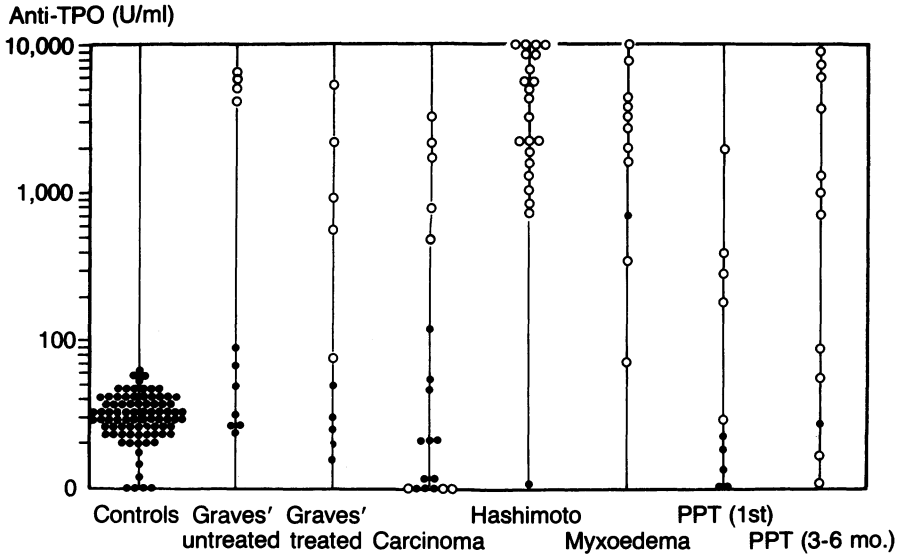

Fig. 1. Serum anti-TPO levels in normal subjects and various antoimmune thyroid diseases. *PPT* Postpartum thyroiditis in the first trimester and 6 months postpartum, respectively. ●, Sera negative for MAb; ○, sera positive for MAb

distribution with a 95% reference range from under 10 to 52 U/ml. No correlation was found between anti-TPO and TSH, thyroid volume, serum Tg, thyroid hormones, sex, or age.

In the various autoimmune thyroid diseases 36%–92% were positive for MAb at a dilution of 1/10 and 63%–90% in undiluted serum (Table 1). For further comparison with anti-TPO a 1/10 dilution was chosen. The anti-TPO values in each disease group are presented in Fig. 1, with a specification of those positive for MAb. In HT 21/22 had anti-TPO levels above 500 U/ml, while the pattern was more scattered in the other groups.

The semiquantitative correlation between anti-TPO and MAb showed that 130 of 133 MAb negative sera had anti-TPO values below 100 U/ml, and 13 of 14 strongly MAb positive sera (+ + +) were above 500 U/ml. However, only 130 of 153 sera with anti-TPO levels below 100 U/ml were MAb negative, and only one of 54 sera over 500 U/ml was MAb negative. In the area between (+ and + + for MAb and 100–500 U/ml for anti-TPO) the correlation was less pronounced.

Of the 36 TgAb and/or MAb positive women in the first trimester 12 developed postpartum thyroid dysfunction (clinical details will be published elsewhere) [8], whereas all 20 women in the control group remained euthyroid. There were thus three groups in this study on anti-TPO: 12 antibody-positive women developing postpartum thyroiditis (PPT; group 1), 24 antibody-positive women remaining euthyroid (group 2), and 20 euthyroid antibody-negative controls (group 3).

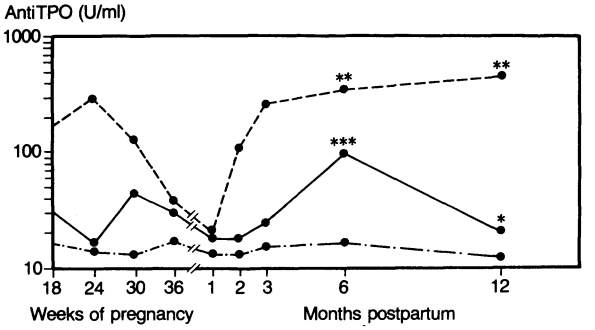

Fig. 2. Median levels of anti-TPO in three groups of pregnant women during pregnancy and up to 12 months postpartum. Group 1 (––), 12 women positive for thyroid autoantibodies in the first trimester of pregnancy and subsequently developing postpartum thyroiditis. Group 2 (—), 24 women positive for thyroid autoantibodies in the first trimester of pregnancy who did not develop postpartum thyroiditis. Group 3 (–.–), 20 healthy women without thyroid autoantibodies remaining euthyroid during and after pregnancy. $*p < 0.05$, $**p < 0.02$, $***p < 0.001$ compared to the first trimester

Anti-TPO was less frequently positive in groups 1 and 2 than MAb, but this difference was not statistically significant ($p > 0.1$). Figure 2 shows that the anti-TPO concentration remained low in the control group throughout the study period. Both groups 1 and 2 exhibited fluctuations during pregnancy and postpartum, the most pronounced changes seen 6 months postpartum with increasing levels. Wide interindividual difference in values were seen, and the Fig. 2 illustrates only median values for each group. The median values at the various sampling periods, however, masked individual variations in the fluctuation pattern, peak values being reached at different times between 1 and 12 months postpartum.

Discussion

Anti-TPO has in previous clinical studies been shown to correlate well with MAb and is considered a more specific method [4], but larger clinical studies remain to clarify whether anti-TPO measurements can replace MAb for routine clinical use. In the present study a new commercial method for anti-TPO was evaluated in comparison with MAb by immunofluorescence. Anti-TPO exhibited low values in normal persons without clinical or biochemical evidence of thyroid disease except in six persons with positive MAb and in four of these cases also TgAb. The anti-TPO method was able to distinguish clearly high levels in HT with a high correlation to MAb. In other autoimmune thyroid diseases the number of patients positive for both anti-TPO and MAb was lower, and without a clear correlation of values in the middle range of both methods. The reason for this discrepancy is not quite clear. Possible explanations include differences in sensitivity, different epitopes recognized by the

two methods, and interference in the immunofluorescence assay from other autoantibodies.

In a group of pregnant women at risk of developing PPT anti-TPO was less frequently positive than MAb, but the difference was not statistically significant. Neither of the antibodies were good predictors of PPT, since only 7/12 women developing PPT had MAb in the first trimester compared to 6/12 positive for anti-TPO. These results are in disagreement with those of LEJEUNE et al. [9], but the two studies are not directly comparable.

Anti-TPO was, however, able to demonstrate fluctuations in the levels corresponding to those previously reported for TgAb and MAb [10] and may thus serve as a quantitative marker of the immune processes.

In conclusion, the new commercial method for measurement of anti-TPO seems in this preliminary evaluation to be promising as regards the clinical diagnosis of HT, with a clear distinction between their values and those of normal subjects. The results further showed a good agreement with MAb by immunofluorescence when evaluating very high and low values. Further studies will be needed to clarify the cause of the discrepancy in the middle range of both methods.

Acknowledgements. The excellent technical assistance of BENTE FRISS MIKKELSEN, LISBETH KIRKEGAARD, and LENE OVERGAARD is gratefully acknowledged. Henning, West Berlin, is thanked for providing the anti-TPO kits. Thanks are due to other members of the Copenhagen postpartum thyroiditis study group (K. BECH, MD, and J. HERTEL, MD) for their cooperation and support. Finally, Dr. PIERRE CARAYON is thanked for valuable discussions.

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Discussion

Davies:
I didn't quite get the technical details of the system. Are you using dyna-beads or beads with fluorescent label?

Feld-Rasmussen:
No, it is a coated-tube radioimmunoassay system.

Davies:
Is the generation of the fluorescence label, dependent on the antibody-antigen interaction or not?

Feldt-Rasmussen:
No, the immunofluorescence method for microsomal antibodies is a conventional technique using thyroid slices.

Davies:
Why do you call it the dyna-fluorescence technique?

Feldt-Rasmussen:
No, the dyna-test ist the anti-TPO assay.

Davies:
But what are you using as a label?

Feldt-Rasmussen:
Radiolabeled TPO. It is exactly the same principle as Dr. Mariotti uses.

Davies:
The point is that for mass screening, in large populations, what we need is not a radioactive test; we need one of the test systems that have been developed now that allow total automation, and I thought you were describing one of these new systems where you get only the fluorescence emission or the chemiluminescence emission in the presence of antibodies.

Feldt-Rasmussen:
The purpose of this study was to compare the radioactive anti-TPO method with microsomal antibodies not measured by hemagglutionation inhibition,

but by immunofluorescence, which should be more specific, and not liable to the same interferences as the inhibition of hemagglutination.

Drexhage:
When you talk about immunofluorescence on sections, is it not better to speak about cytoplasmic antibodies, because (as was shown by Deborah Doniach and others) there is a difference between antimicrosomal and anticytoplasmic antibodies.

Feldt-Rasmussen:
Sure, it is not clearly specific, you are right; it is cytoplasmic.

Banga:
You mention that, using immunofluorescence and the DYNOtest Anti-TPO as assays to measure TPO antibody, there was no discrepancy using normal human serum containing anti-TPO antibody while sera from PPT patients clearly showed discordant results. You allude to one reason for this difference in the sensitivity of these two assays. It is equally possible that the anti-TPO antibody in normals and PPT patients recognizes different epitopes on the TPO molecule. If some of the epitopes on TPO recognized by the PPT sera are not accessible by immunofluorescence testing, but using purified TPO in DYNOtest these epitopes are accessible, this would explain some of your results.

Feldt-Rasmussen:
It is certainly a possibility. I think it would need further studies trying to displace the activity and so on.

Scherbaum:
Perhaps one point has to be added to the last question. It would be interesting to retest the sera which were negative on thyroid sections by indirect immunoflourescence at higher dilutions and see whether they remain negative. I am saying this because of the known "prozone phenomenon" which is responsible for giving negative reactions with undiluted serum.

Feldt-Rasmussen:
That is actually being done right now.

Longitudinal and Cross-Sectional Studies on Anti-Thyroid Peroxidase Antibodies in Thyroid Disorders Compared to Conventional Anti-Microsomal Antibodies

K. WEBER and H. SCHATZ

Introduction

Determination of autoantibodies directed against microsomal antigen has become an important diagnostic procedure, as their presence in serum is closely associated with autoimmune thyroid disease [1, 2]. Because of its unknown nature it has not been possible to develop a specific assay for antibodies against microsomal antigen. Current systems use preparations from thyrotoxic glands isolated by high-speed centrifugation, which may contain undefined antigens [3, 8]. Recently, microsomal antigen was identified as thyroid peroxidase (TPO) [4–7]. After immunopurification of TPO and anti-TPO antibodies highly specific assays were developed [3, 8]. Mariotti et al. compared anti-microsomal antibodies and anti-TPO antibodies by competitive binding assay and immunoradiometric assay using antigen-coated wells [8]. In our study we compared anti-microsomal antibodies measured by newly introduced methods (passive hemagglutination and radioligand assay) with a newly developed radioimmunoassay for anti-TPO antibodies using the inhibition of binding of labeled TPO to a solid-phase bound monoclonal antibody by autoantibodies [3].

Materials and Methods

A total of 361 serum samples were obtained from 245 persons, including a control group ($n = 48$), cases of Graves' disease ($n = 88$), autoimmune thyroiditis ($n = 37$), de Quervain's disease ($n = 4$), euthyroid diffuse ($n = 21$) and nodular ($n = 43$) goiter, and connatal hypothyroidism ($n = 4$). For longitudinal observations 30 patients (24 with Graves' disease, 4 with autoimmune thyroiditis, and 2 with euthyroid diffuse goiter were followed for up to 4 years. Anti-microsomal antibodies were measured by passive hemagglutination (Thymune M, Wellcome) and radioligand assay (Promak, Henning Berlin). For determination of anti-TPO antibodies we used a new radioimmunoassay (DYNOtest Anti-TPO, Henning Berlin). Statistical analyses were performed by calculating the correlation coefficients. Normal values were defined as following: DYNOtest Anti-TPO below 100 U/ml, Promak below 500 U/ml, Thymune M below 1:100.

Results

Cross-Sectional Studies. Among 48 normal persons all were negative in DYNOtest Anti-TPO (range 1–94 U/ml, mean 35.6 ± 19.2 U/ml) and Promak (1–417 U/ml, 80.6 ± 72.5 U/ml). Using Thymune M, hemagglutination was negative in 45 cases; three persons showed low titers of 1:100. In 88 cases of Graves' disease a correlation was seen in 78; divergent results in ten cases are shown in Table 1. Evaluating 37 serum samples of autoimmune thyroiditis, 32 showed a correlation; differing results obtained in five cases are presented in Table 2. Among four patients with de Quervain's disease all were negative in Promak and Thymune M; one patient was positive in DYNOtest Anti-TPO only. In euthyroid diffuse goiter there was a correlation in 20 of 21 patients. Only one who was negative in DYNOtest Anti-TPO and Promak showed a titer of 1:400 in Thymune M. Of 43 patients with euthyroid nodular goiter 40 were negative in all three assays. Two persons were positive in DYNOtest Anti-TPO and Thymune M, and one in Promak only. In four patients with connatal hypothyroidism no autoantibodies could be detected.

Longitudinal Studies. We followed 30 patients over a period of up to 4 years. Of these, 28 (22 Graves' disease, 4 autoimmune thyroiditis, 2 euthyroid goiter) showed an identical development in all assays. The course of two patients suffering from Graves' disease is demonstrated in Figs. 1 and 2. In these two cases DYNOtest Anti-TPO and Promak seem to be more sensitive to detect changes in antibody concentrations.

The highest correlation was seen between DYNOtest Anti-TPO and Promak ($r = 0.917$). Results in comparing DYNOtest Anti-TPO and Thymune M ($r = 0.804$) and Promak and Thymune M ($r = 0.830$) showed lower correlations.

Table 1. Divergent results in ten patients with Graves' disease

	n			
	3	1	3	3
DYNOtest Anti-TPO	+	+	+	−
Promak	−	+	−	+
Thymune M	+	−	−	+

+, Correlation; −, no correlation.

Table 2. Diverging results in five cases of autoimmune thyroiditis

	n			
	2	1	1	1
DYNOtest Anti-TPO	+	−	−	+
Promak	−	+	−	+
Thymune M	+	+	+	−

+, Correlation; −, no correlation.

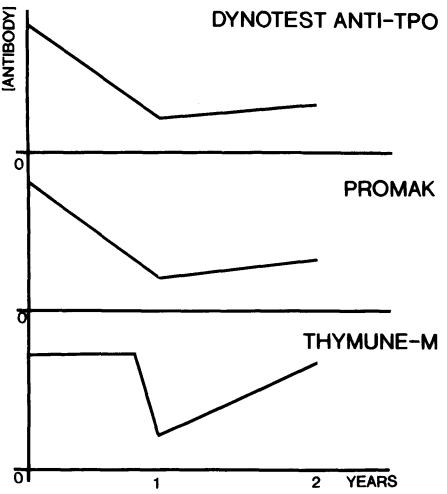

Fig. 1. Comparison of antibody determinations in a case of Graves' disease. DYNOtest and Promak are more sensitive in detecting decreases in antibody concentrations

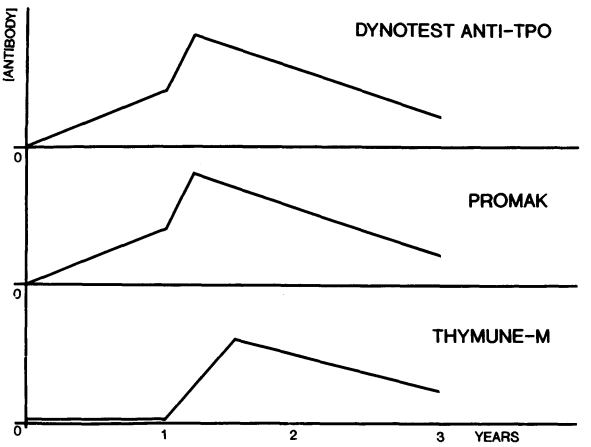

Fig. 2. Comparison of antibody determinations in a case of Graves' disease. Titers in Thymune M rise with a delay of 4 months. DYNOtest and Promak are more sensitive for indicating the onset of autoimmune thyroid disease

There is thus a high correlation between these assays in about 90% of all cases, however we cannot confirm the close relationships found by MARIOTTI et al. [8]. DYNOtest Anti-TPO seems to be the most sensitive assay concerning antibody fluctuations, followed by Promak and Thymune M.

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Discussion

Davies:
Have you taken one serum that ist positive for thyroid antibodies in serial dilutions and compared the sensitivity in the three assays? And which assay is more sensitive, and does that reach significance?

Weber:
We obtained corresponding results by comparing DYNOtest Anti-TPO and Promak in serial dilutions of sera with antibody concentrations exceeding our standard curves. Antibody determination by passive hemagglutination is based on the principle of serial dilution anyway.

Phillips:
Were the people who were detected with one test and not the other borderline cases?

Weber:
Persons positive in one and negative in another assay are of special interest, and we look at them at short-term intervals. It is too early now to comment on their development of autoimmune thyroid disease.

Comparison of New and Conventional Assay Techniques for Measuring Thyroid Microsomal Antibodies

R. HOERMANN, B. SALLER, A. FATEH-MOGHADAM, and K. MANN

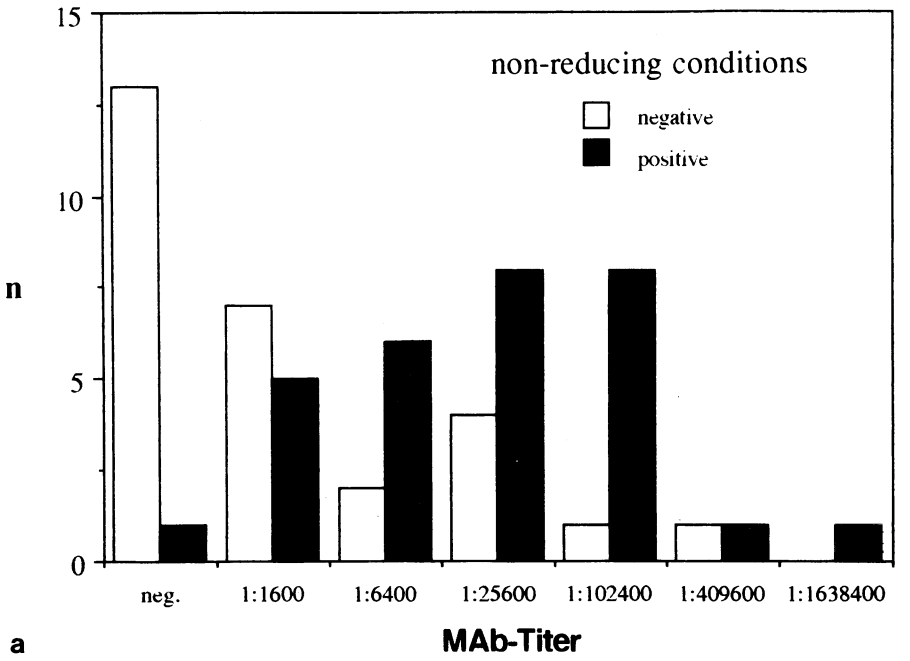
Introduction

The determination of microsomal antibodies (MAb) has a long tradition in the diagnosis of thyroid autoimmune disease [1]. The antigen, however, against which those antibodies are directed has only recently been identified and appears to be closely related to or identical with thyroid peroxidase (TPO) [2–5]. These advances in defining the microsomal antigen offer a chance to develop new assay techniques for measuring MAb. Hence, new assays rely on the use of molecularly well-defined antigens instead of crude microsomal fractions being employed in current routine assays for MAb. In the present study we were interested from a clinical point of view to investigate the interaction of MAb with microsomal antigen by three different techniques: (a) a conventional hemagglutination test (HAT), which measures MAb reaction with a crude thyroid microsomal preparation; (b) Western blot analysis, which shows the reaction of MAb with distinct microsomal proteins; and (c) anti-TPO radioimmunoassay, which determines the reaction of MAb with purified human TPO.

Materials and Methods

The study group consisted of 28 patients with Graves' disease and 9 patients with Hashimoto's thyroiditis. From the Graves' disease patients serum samples were obtained in the hyperthyroid state before initiation of treatment ($n = 28$) and during the course of antithyroid medication ($n = 21$). Diagnosis of autoimmune thyroid disease was confirmed in all cases by clinical evaluation, ultrasonography, ^{99m}Tc scintigraphy, hormone analysis, and determination of TSH receptor antibodies, microsomal antibodies and thyroglobulin antibodies.

A detailed description of SDS-PAGE and Western blot analysis is included in SALLER et al. (this volume). Briefly, human thyroid microsomal proteins were resolved by SDS-PAGE using a 6%–22% polyacrylamide gradient gel under nonreducing and reducing conditions. For Western blot, proteins were electrophoretically transferred on nitrocellulose membranes and incubated with patients' sera at a 1:50 dilution for 3 h at 20 °C. Antibodies bound were visualized after incubation with an alkaline phosphatase conjugated $F(ab')_2$ fragment of goat anti-human IgG(H + L) (1 h, 20 °C, 1:750) by nitroblue tetra-



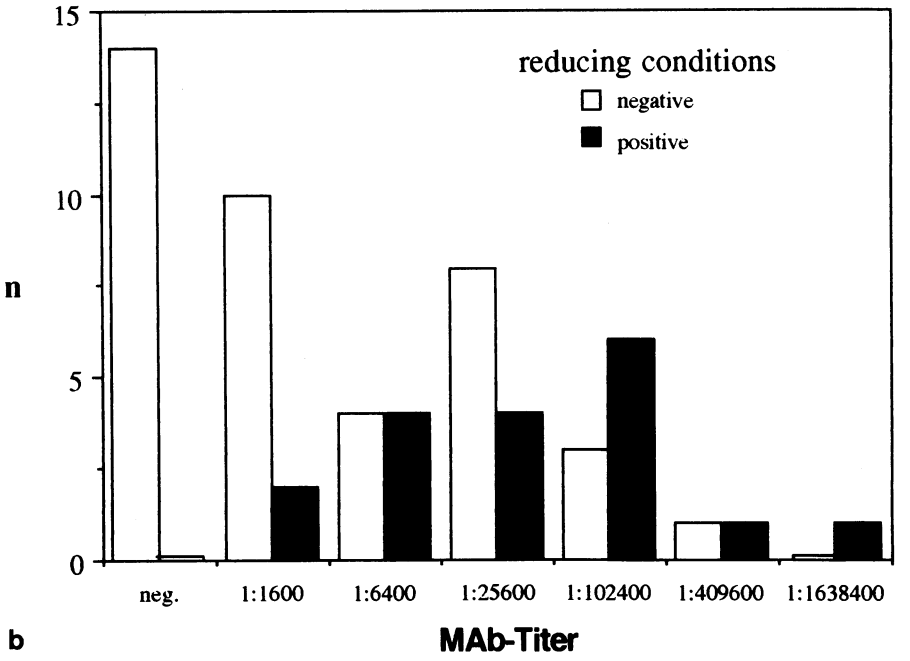


Fig. 1 a, b. Relationship between results of Western blot analysis and MAb titers in hemagglutination test. **a** Nonreducing conditions. **b** Reducing conditions

zolium and 5-bromo-4-chloro-3-indolyl phosphate. When serum samples stained proteins in the 80 to 125-kDa region under nonreducing conditions or two distinct protein bands of 96 and 103 kDa under reducing conditions, reactivity in the Western blot was regarded as positive.

Antibodies against purified TPO were determined by a radioimmunoassay using radiolabeled human TPO and a monoclonal anti-TPO antibody coated to polystyrene tubes. The assay (DYNOtest anti-TPO) was kindly provided by Henning corporation (Berlin) and performed according to the protocol described by RUF et al. [6]. In this assay the range of normal values was under 100 U/ml.

For measurement of MAb by hemagglutination test, Thymune-M-HAT (Wellcome Reagents, UK) was used. The assay was performed according to the manufacturer's instructions. Antibody titers of at least 1 : 1600 were considered positive.

Results

Out of 58 serum samples of patients with Graves' disease or Hashimoto's thyroiditis, 44 (75.9%) showed positive results on HAT. On Western blot the rate of positive reactions with MAb was less than that on HAT: 30/58 (51.7%) under nonreducing and 18/58 (31.0%) under reducing conditions. Generally, a positive reaction on Western blot was associated with a high titer of MAb as measured by HAT (Fig. 1). However, there were some sera which apparently failed to react with the 96- and 103-kDa proteins on Western blot despite considerably elevated MAb titers. In addition, all sera were assessed for their ability to react with purified TPO by radioimmunoassay. In this assay, positive

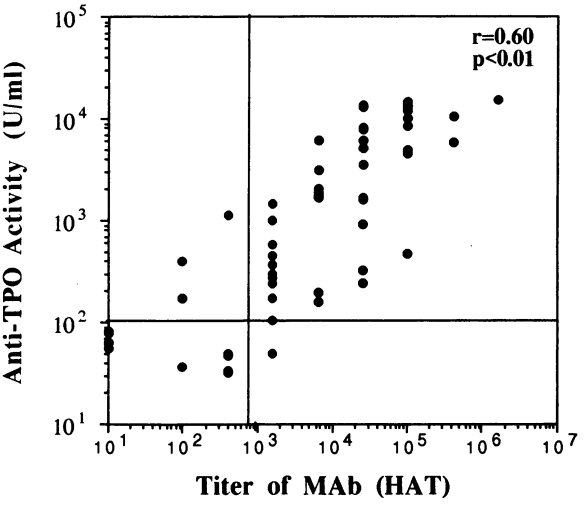


Fig. 2. Relationship between anti-TPO activities measured by radioimmunoassay and MAb titers estimated by hemagglutination test ($n = 58$; $r = 0.60$; $p < 0.01$)

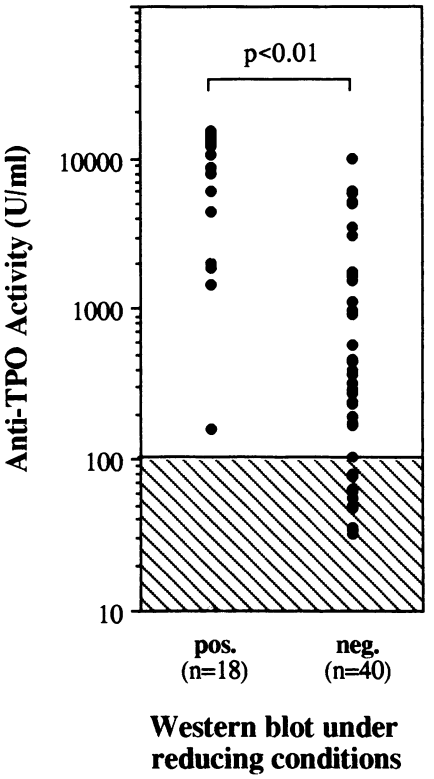


Fig. 3. Relationship between anti-TPO activities and results obtained by Western blot under reducing conditions (Mann Whitney *U* test)

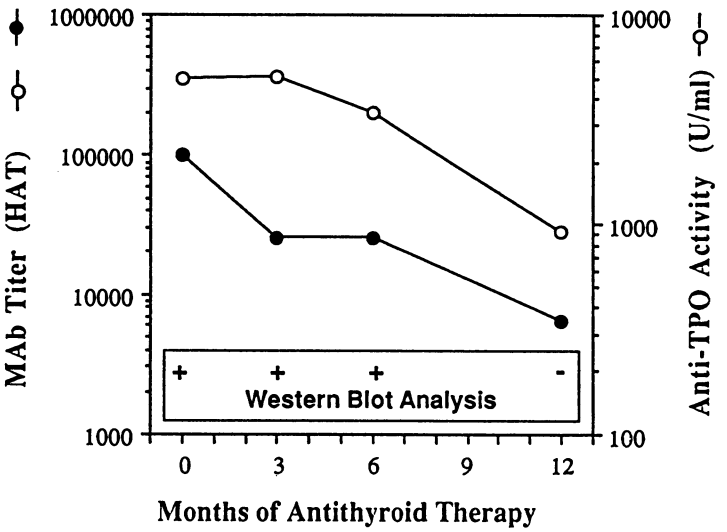


Fig. 4. Course of MAb titers, anti-TPO activities, and results of Western blot during the follow-up of one patient with Graves' disease

results were found in 46/58 sera (79.3%), and there was a close correlation between anti-TPO activities and MAb titers measured by HAT (Fig. 2). When anti-TPO activities were compared to the results obtained by Western blot analysis, sera staining the 96- and 103-kDa bands revealed significantly higher values than sera not reactive on Western blot (Fig. 3). From seven patients with Graves' disease, serum samples were obtained after 3, 6, and 12 months of antithyroid therapy. Overall, there was a close relation between the course of MAb titers, anti-TPO acitivities, and the results of Western blot analysis (Fig. 4).

Discussion

In the present study we evaluated two new techniques for the measurement of MAb that employ well-defined thyroid microsomal antigens in comparison with a conventional HAT method using crude microsomal fraction. With the first method, Western blot, the interaction of MAb with distinct proteins of the microsomal fraction was analyzed. As expected, the sensitivity of Western blot analysis was lower than that of HAT, under both reducing and nonreducing conditions, although it was considerably higher with the technique that we used than has been reported by others [7]. With respect to differentiation of the reactivity of MAb with various antigenic proteins, however, this approach may be advantageous. Generally, there was a close relation between positive results on Western blot and high MAb titers measured by HAT. Some exceptions, however, should be noted, as they add further support to the concept of a heterogeneous composition of MAb [7, 8].

The second new method employs purified human TPO as a labeled antigen for the determination of MAb by radioimmunoassay. The anti-TPO activities evaluated by this approach showed a high correlation with both HAT titers and results on Western blot. This was also the case in patients studied during the course of antithyroid therapy.

The data are in good accord with the current view of TPO being the major thyroid microsomal antigen [2–5, 8]. As regards the sensitivity for detection of MAb in patients with autoimmune thyroid disease, the new TPO assay appears at present to have no advantage over conventional HAT.

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Discussion

Furmaniak:
I don't think we should consider the Western blot technique as a routine method for measuring antibodies in patients' sera. That is just a point that I wanted to make. It is a very useful and powerful technique for studying the antigen structure, and possibly epitopes of the differently treated antigen, but really as a method for screening patients, it is not to be recommended.

Hörmann:
I would certainly agree. It was not our aim to recommend this as a routine technique. It was just of interest to see if there might be any differences which could be of clinical relevance. I would not advocate using this as a routine technique.

Bogner:
I got from your slides the impression that the higher the microsomal antibody titers, the lower was the detection in the Western blot. Is that right?

Hörmann:
No, the lower the titers in hemagglutination test, the lower was the detection by Western blot.

Bogner:
But there were some exceptions?

Hörmann:
Yes, but those were exceptions.

Further Clarification of the Significance of Thyroid Peroxidase Activity-Inhibiting Immunoglobulins in Autoimmune Thyroid Disease

Y. OKAMOTO, N. HAMADA, J. NOH, K. ITO, and H. MORII

Introduction

Antibodies against human microsomal antigens are often present in autoimmune thyroid disease and have been found to be antibodies against thyroid peroxidase (TPO) [1–3], which catalyzes the synthesis of thyroid hormone. We have reported [4] that some anti-TPO antibodies inhibit the activity of TPO in vitro, and that these TPO activity-inhibiting immunoglobulins (TPII) seem to inhibit thyroid function in some patients, but the relationship between TPII index and thyroid function is not simple. In this study, we examined whether TPII can inhibit the activity of TPO present in thyroid tissue.

Materials and Methods

Effects of TPII on TPO Activity in Cryostat Sections. The thyroids of two women suffering from Graves' disease were used for this study. Immunoglobulin G (IgG) from a patient with Hashimoto's disease and from a healthy subject was purified by protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography. The TPII index of the patient's serum, assayed by a method previously described [4], was 0.71 (positive TPII > 0.38). Cryostat sections 4 and 10 μm thick were washed in phosphate-buffered saline for 15 min and incubated with 0.5 mg/ml IgG from the patient or healthy subject overnight at 4 °C. Sections were washed twice, and those 4 μm thick were stained for IgG by being incubated with rabbit anti-human IgG conjugated with fluorescein (1:80 dilution) for 2 h at room temperature. The sections 10 μm thick were stained for endogenous TPO activity by being incubated with 0.05% 3,3'-diaminobenzidine and 0.0025% H_2O_2 for 30 min at room temperature.

Effects of TPII on TPO Activity in Cultured Human Thyroid Slices. Organ culture was done as previously reported [5], the IgGs described above were used. A Graves' thyroid slice was cultured in Ham's F-10 synthetic culture medium (Flow Laboratories, Mclean VA, USA) containing 5 mg/ml TPII-positive or normal IgG for 2 h at room temperature. After incubation, 4-μm and 10-μm cryostat sections, respectively, were stained for IgG and endogenous TPO activity by the methods described above.

Effects of Goitrous Hashimoto's IgG on TSH-Stimulated ^{125}I Uptake in Cultured Human Thyroid Slices. We observed the effects of IgG from two hypothyroid patients with goitrous Hashimoto's disease on iodine uptake by thyroid slices. One IgG (H1-IgG) was positive for microsomal antibody as measured by a particle agglutination test, and the other (H2-IgG) was negative for microsomal antibody. Human thyroid slices were cultured in medium containing 1 μCi ^{125}I, 5 mg/ml IgG from patients or a healthy subject, and 10 mU/ml bovine TSH (Calbiochem-Behring, San Diego CA, USA) for 5 days. After incubation, thyroid slices were washed three times with saline and weighed. The radioactivity of each slice was measured.

Results

Effects of TPII on TPO Activity in Cryostat Sections. When the cryostat sections from two Graves' thyroids were incubated with normal IgG, no IgG deposits were seen by direct immunofluorescence. For both patients' thyroids, endogenous TPO activity was seen as brown lines on the apical border and as intracellular staining. When cryostat sections were incubated with TPII-positive IgG, IgG deposits were seen as lines of stain on the apical border and as intracellular staining, for both patients' thyroids. TPII inhibited endogenous TPO activity diffusely in these patients' thyroids.

Effects of TPII on TPO Activity in Cultured Human Thyroid Slices. In the thyroid slices cultured with normal IgG, immunofluorescence staining showed no IgG deposits, and endogenous TPO activity was present. In the thyroid slices cultured with TPII, there was no difference in endogenous TPO activity compared with the slices cultured with normal IgG.

Effects of Goitrous Hashimoto's IgG on TSH-Stimulated ^{125}I Uptake in Cultured Human Thyroid Slices. TSH-stimulated ^{125}I uptake was significantly lower ($p < 0.01$) in slices incubated with either H1-IgG (7388 ± 3113 cpm/mg) or H2-IgG (7388 ± 2611) than in slices incubated with normal IgG (15380 ± 5180).

Discussion

To understand why the relationship between TPII index and thyroid function is not simple, we examined whether TPII can inhibit TPO activity in thyroid tissue. TPII bound to TPO and inhibited TPO activity in cryostat sections. Therefore, if TPII have access to TPO in vivo, TPII should inhibit its activity. However, IgG deposits on the apical border of complete follicles were not found, and TPO acitivity was not inhibited when thyroid slices were cultured with TPII. Therefore, TPII may not reach TPO in vivo.

IgG from hypothyroid patients with goitrous Hashimoto's disease inhibited TSH-stimulated ^{125}I thyroidal uptake compared to normal IgG. This result was obtained with the use of IgG that included microsomal antibody or not. Therefore, IgG that inhibits ^{125}I thyroidal uptake is different from microsomal antibody.

In conclusion, TPII may reach its antigen only with difficulty. This is probably why no simple relationship is observed between the TPII value and thyroid function.

References

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Discussion

Carayon:
How do you explain this effect on iodine incorporation?

Okamoto:
I think, one possibility is, that this antibody may be directed against the iodine pump in the thyroid cell membrane. This effect was observed by IgG with or without antimicrosomal antibody. We also examined the organification of iodine in this organ culture system. This antibody did not inhibit the organification. Therefore, this antibody is not directed against TPO.

Sundick:
It is well known that Hashimoto's patients have a defect in organification. Many have felt that the defect in organification may be secondary to either lymphoid damage or TPO antibodies. So I think that you have at least clearly ruled out the likelihood that these TPO antibodies are responsible for the organification defect. Of course there is still the possibility that lymphocyte-mediated damage might impair organification.

Okamoto:
I agree with you. Anti-TPO antibody may inhibit TPO of follicles that were first destroyed by some mechanism.

Mariotti:
My question is related to the immunoglobulin of Hashimoto's thyroiditis inhibiting iodine uptake, but unrelated to immunoglobulins blocking thyroid growth and function. Was it observed in selected IgG preparations, or was it a general phenomenon?

Okamoto:
We examined the effect of IgG from 13 hypothyroid patients with Hashimoto's disease. The TSH-stimulated iodine uptake was inhibited by IgG from seven patients.

Scherbaum:
Were these iodine-uptake blocking IgG always unable to block TSH-dependent cAMP production and/or growth?

Okamoto:
We have not examined all the patients' IgG yet, but IgG from at least two patients were always unable to block TSH-dependent cAMP production.

Banga:
Using your cryostat and organ culture system, it is interesting that nearly 50% of your sera (7/13) containing autoantibodies to TPO showed varying degrees of inhibition of enzymatic activities of TPO at the iodide catalytic site. This agrees very well using thyroid microsome preparations as a crude source of TPO to assay inhibition of iodide catalytic activity using sera containing anti-TPO antibody. Approximately 60% of the sera show this inhibitory activity by this procedure [DOBLE et al. (1988) Immunology 64: 23].

Okamoto
In this presentation, I showed two kinds of effects of patients' IgG. First, anti-TPO antibody from one Hashimoto's patient inhibited TPO activity in the cryostat sections from three Graves' thyroids. We have reported in a previous paper that 24% of the sera from Graves' patients and 40% of the sera from Hashimoto's patients had positive TPO activity-inhibiting immunoglobulin using Graves' thyroid microsomes. In an organ culture system, anti-TPO antibody did not inhibit TPO activity. Second, 7 of the 13 patients with hypothyroid patients with goitrous Hashimoto's thyroiditis, either with or without microsomal antibody, inhibited TSH-stimulated iodine uptake using organ culture system.

Banga:
Can I just ask another related question in order to clarify: In your organ cultures, did you say that if you have open follicles, then your immunoglobulin is accessible, but in closed follicles in organ culture you do not get any TPII activity?

Okamoto:
I said that anti-TPO antibody did not inhibit TPO activity in closed follicles in organ culture.

Carayon:
Just a comment: I really think that depending on the preparation of TPO or microsomes or cells the TPII activity is different. In our lab, for instance, we were unable to show any TPII activity using highly purified TPO. Using microsomes, I think that you were able to show a higher prevalence of TPII activity.

McLachlan:
Were the Hashimoto sera positive only for microsomal antibody, or were some of them also positive for thyroglobulin antibody?

Okamoto:
In 13 sera from Hashimoto's patients used in the experiment of iodine uptake, none of them were positive for thyroglobulin antibodies and eight of them were positive for microsomal antibodies.

McLachlan:
Then my second question is: Did you observe any effects of damage in those cultures of thyroid epithelial cells?

Okamoto:
I did not observe any damage in thyroid epithelial cells.

McLachlan:
I am just suggesting that the possibility exists that you may have been looking at an effect of both microsomal and thyroglobulin antibodies damaging thyroid cells, because we have observed that thyroglobulin antibodies are, in fact, able to mediate an antibody-dependent cell cytotoxic damaging mechanism. So it may be Hashimoto's sera containing thyroglobulin antibodies. You may have something too complex to analyze directly in this system.

Bottazzo:
When referring to the functional activity of autoantibodies to target cells, in this case cytotoxic effects, one should not forget that, in the case of the thyroid, we are dealing with a well-defined anatomical follicular structure, as it appears in its organization in vivo. Emilio Khoury can appropriately comment on this. In my view, none of the experimental systems set up in vitro and which use conventional thyroid cell preparations, i. e., cultured monolayers, sections, or isolated microsomes, takes into full account the "physiological" reality. None of them, in fact, reflect the in vivo situation. It would therefore be particularly interesting to see if all the "cytotoxic" data produced so far using sera containing thyroid antibodies and conventional thyrocyte preparations can be reproduced by substituting them with reconstituted thyroid follicles.

Davies:
The investigator used a thyroid slice. That is an intact system.

Bottazzo:
We keep forgetting that the thyroid microsomal antigen, now TPO, is apparently not expressed on the vascular pole of the thyroid follicular cells only on the internal apical pole, which faces the colloid space. So, to finally assess cytotoxicity by antibodies in a thyroid system one has to use reconstituted follicles, otherwise it is difficult to have exact comparison with the in vivo situation.

Davies:
That is an expression of faith; it is not a fact.

Bottazzo:
"Faith" is the vital support for religions, "facts," hopefully, for understanding thyroid autoimmunity! Unless you produce convincing evidence that the TPO antigen is expressed on the vascular pole of the thyroid follicle, my "fact" will continue driving the argument. I personally doubt that the autoantibodies have a direct cytotoxic effect in vivo. Until now, passive transfer experiments of thyroid antibodies have failed to produce disease in animals. Sandra McLachlan, I believe, can confirm this, because she has worked quite intensively in this particular aspect of experimental thyroid autoimmunity. In addition, mothers with high titers of anti-TPO antibodies apparently give birth to euthyroid babies, and the antibodies, we know, have crossed the placenta during fetal development. If the antibodies were acting in vivo producing the same cytotoxic affect as they exert in vitro, they should cause tissue damage. Why do they not do that? The most likely explanation is that the antibodies do not "see" the antigen which is internalized in the thyroid follicles and therefore not exposed on the vascular pole, so precluding its accessibility to the autoantibodies.

Biochemical Dissection of the Reactivity of TPO Antibodies

B. SALLER, R. HÖRMANN, and K. MANN

Introduction

In patients with autoimmune thyroid disease the presence of autoantibodies against several distinct antigens including the TSH receptor, thyroglobulin, and the microsomal antigens (MAg) is well established. The MAg is a glycoprotein of an apparent molecular weight of about 100 kDa [1–3], and its major antigenic component is thyroid peroxidase (TPO) [4–7]. Recently published studies have suggested a heterogeneous nature of autoantibodies directed against TPO (anti-TPO-Abs) [8] and reported that anti-TPO-Abs not only bound to TPO but also inhibited its enzymatic activity [9–12]. However, the important question of whether the antigenic determinants of TPO include its catalytic sites is presently controversial, since another study failed to find any inhibition of TPO activity by anti-TPO-Abs [4].

The present study was undertaken to characterize the reactivity of microsomal antibodies (MAb), i.e., anti-TPO-Abs, with their antigen in patients with autoimmune thyroid disease by comparatively applying different techniques. In this respect, it was of particular interest to reevaluate whether MAb/anti-TPO-Abs could directly inhibit the enzymatic activity of TPO, and whether such an inhibitory effect would be related to the clinical stage or presentation of the underlying autoimmune thyroid disease.

Materials and Methods

Serum samples were obtained from 37 patients with autoimmune thyroid disease selected because of positive titers of MAb in a routine hemagglutination test (Thymune-M-HAT, Wellcome Reagents, UK). These included 13 patients (11 women and 2 men; aged 35–68 years, mean 49 ± 8 years) suffering from overt Graves' disease and 13 patients (11 women and 2 men; aged 22–72 years, mean 48 ± 13 years) with a history of Graves' disease but who had been clinically euthyroid for a period of 3–21 years (mean 7 ± 4 years), five of whom had been in remission after antithyroid drug treatment, three after radioiodine therapy, and five after subtotal thyroidectomy. All these patients had thyroid hormone levels within the normal ranges, but two had a subnormal and four an exaggeratd TSH increase after intraveneous TRH administration. The third

group consisted of 11 patients with Hashimoto's thyroiditis (7 women and 4 men; aged 37–74 years, mean 52 ± 14 years) who were hypothyroid ($n = 7$) or had normal thyroid hormone levels but an exaggerated TSH increase after TRH administration ($n = 4$).

All sera selected for investigation were additionally tested for MAb by a quantitative enzyme linked immunosorbant assay (ELISA; elias, Freiburg, FRG).

All steps of preparation and solubilization of thyroid microsomes were performed at 4 °C. Homogenates from surgical specimens of thyroid tissue from patients with Graves' disease were prepared in a Teflon glass homogenizer with about 10 vol. 10 m*M* Tris HCl buffer, pH 7.4, containing 0.25 *M* sucrose and passed through a double layer of gauze. The supernatant of two centrifugations at 800 *g* for 10 min and 9000 *g* for 20 min was sedimented by ultracentrifugation at 105 000 *g* for 60 min. The pellet was washed twice and suspended in a minimal volume of 15 m*M* phosphate buffer with 150 m*M* NaCl, pH 7.4. Protein concentration was determined by the method of Bradford [13] with bovine serum albumin as standard.

Solubilization of thyroid microsomes was carried out in a modification of the method described by NEARY [14]. To remove loosely bound and soluble proteins, the 105 000-*g* pellet was homogenized in 1 *M* NaCl, 10 m*M* Tris HCl, 0.1 m*M* KI, pH 7.4, stirred gently for 60 min and centrifuged at 105 000 *g* (60 min). The pellet was resuspended in 0.2% Triton X-100, 10 m*M* Tris HCl, pH 7.4 (about 2 mg protein/ml), mixed gently overnight, and centrifuged at 105 000 *g* (60 min).

SDS polyacrylamide gel electrophoresis (PAGE) was based on the method of GOERG et al. [15]. Thyroid microsomes were heated for 10 min at 70 °C in 50 m*M* Tris HCl containing 1% SDS and 2.25 *M* urea with or without 4% 2-mercaptoethanol (reducing/nonreducing conditions). Each sample applied contained about 4–6 µg total protein in 5 µl and was resolved on horizontal ultrathin-layer (0.5 mm) gels containing an exponential pore gradient (6%–22%) in 0.375 *M* Tris glycine, pH 8.3.

Proteins resolved by PAGE were electrophoretically transferred onto a nitrocellulose (NC) sheet (0.2 µm, Biotec-Fischer, FRG) using a semidry horizontal blotting system consisting of two graphite electrode plates (0.8 mA/cm^2, 60 min) as recently described by KYSHE-ANDERSON [16]. A discontinuous buffer system with 0.3 *M* Tris, 20% methanol (v/v), pH 10.4 (anode solution 1), 0.025 *M* Tris, 20% methanol (v/v), pH 10.4 (anode solution 2), and 0.04 *M* 6-amino-*n*-hexanoic acid, 20% methanol (v/v), pH 7.6 (cathode solution) was used as blotting medium. Protein staining on NC membranes was performed according to the method of HANCOCK [17]. After transfer, NC sheets were washed three times with 10 m*M* PBS containing 0.05% Tween 20, pH 4.0 (PBS-T). The proteins were stained overnight with 0.1% india drawing ink (Pelikan, FRG) in PBS-T. For immunostaining procedures the NC membranes were incubated after transfer of proteins for 10 min (20 °C) with 50 m*M* Tris, 150 m*M* NaCl, pH 10.3, containing 0.5% Tween 20 (TBS-T) to block the remaining binding sites. The NC sheets were incubated for 3 h at 20 °C with patients' sera

(usually 1:50, diluted in TBS-T) and washed three times with 50 m*M* Tris, 150 m*M* NaCl, pH 10.3. After incubation (1 h, 20 °C, 1:750) with an alkaline phosphatase conjugated $F(ab')_2$ fragment of goat anti-human IgG(H + L) (Zymed Laboratories, USA), bound antibodies were visualized by nitroblue tetrazolium (NBT, Sigma Chemical, USA) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma), modified according to BLAKE et al. [18]. The reaction solution was prepared with 3.5 mg BCIP dissolved in 25 ml O. 1 *M* $NaHCO_3$, 1 m*M* $MgCl_2$, mixed with 7 mg NBT in 200 µl *N,N*-dimethylformamide (Sigma) and added to the washed NC membranes.

Peroxidase activity in thyroid microsomal preparations was determined using guaiacol or iodide as the second substrate as previously described by HOSOYA et al. [19]. For the guaiacol assay, the reaction mixture (3.0 ml) contained 33 m*M* guaiacol, 0.27 m*M* H_2O_2, 33 m*M* Na_2HPO_4 (pH 7.4), and for the iodide assay it contained 4.95 m*M* KI, 0.135 m*M* H_2O_2, 33 m*M* Na_2HPO_4 (pH 7.0). The reaction was started at 20 °C by the addition of H_2O_2 (20 µl) and followed spectrophotometrically (Spectrophotometer, model 24, Beckmann, USA) at 470 nm (guaiacol) or 350 nm (iodide). Preparations of thyroid microsomes were added at an amount resulting in a linear increase of absorbance for at least 30 s. The amount of enzyme which gave a change of 1.0 absorbance unit/s was taken as 1 unit and expressed as guaiacol units (GU) or iodide units (IU). The intra-assay coefficient of variation was 3.9% for the guaiacol assay ($n = 5$) and 13.9% for the iodide assay ($n = 5$).

A quantity of 60 µl solubilized thyroid microsomes (60 µg protein) was incubated for 1 h at 37 °C with 10 µl patient's or normal control serum. A quantity of 40 µl ice-could swollen protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden) suspended 1:1 in 0.14 *M* Na_2HPO_4, pH 9.0 was added and mixed for 60 min at 4 °C. After centrifugation at 500 *g* for 5 min the supernatant was tested for TPO activity in the guaiacol assay. The relative precipitation of TPO was calculated as:

$$\text{Immunoprecipitation of TPO (\%)} = \left(1 - \frac{\text{TPOactivity (supernatantfromtestsample)}}{\text{TPOactivity (supernatantfromnormalcontrol)}}\right) \times 100\%$$

To analyze the influence of autoantibodies in patients' sera on TPO acitivity, solubilized thyroid microsomes (15 µg protein) were incubated for 1 h at 37 °C with 2–64 µl patient's or normal control serum. At the end of incubation aliquots of the reaction solution were tested for TPO activity using the guaiacol or the iodide assay. To directly compare the effect of control and patients' sera on TPO activity, all sera were tested in a single assay after incubation of 60 µl solubilized thyroid microsomes with 16 µl control or patients' sera (1 h, 37 °C).

Statistical analysis was performed with the Mann-Whitney *U* test and with calculation of the correlation coefficient.

Results

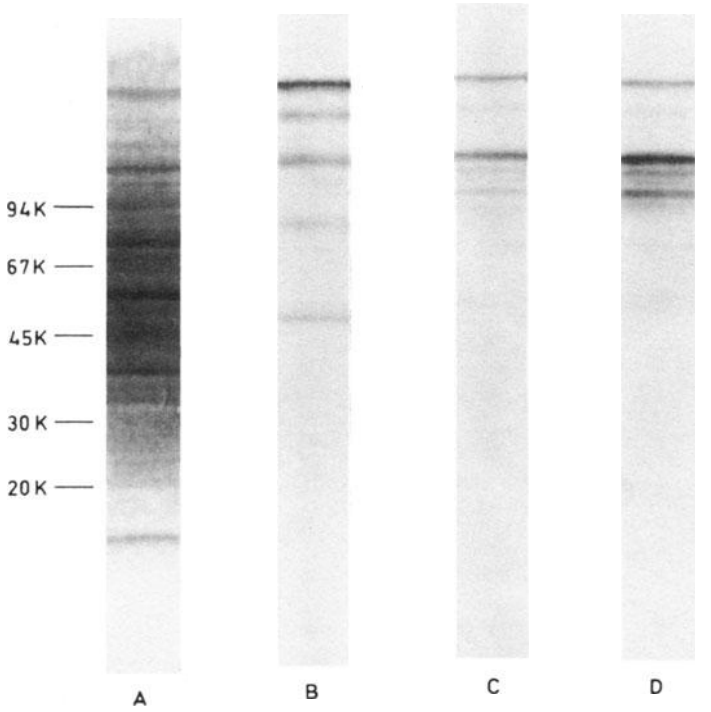
MAb values measured by the quantitative ELISA ranged from 264–22920 IU/ml in sera from patients with active Graves' disease ($n = 13$), from 251 to 9340 IU/ml in sera from patients with Graves' disease in remission, and from 695 to 60992 IU/ml in sera from patients with Hashimoto's thyroiditis.

Immunoblot Analysis. To characterize the reactivity of autoantibodies with distinct microsomal proteins, immunoblot studies were carried out using human thyroid microsomes separated by SDS electrophoresis under nonreducing and reducing conditions. Under nonreducing conditions, all sera recognized various microsomal proteins in the 80 to 125-kDa region, but showed two prominent bands at about 96 kDa and 118 kDa (Fig. 1). A weak staining of the 118-kDa band could also be observed when the NC membranes were incubated with pooled normal serum or sera from patients with autoimmune thyroid disease but negative titers of MAb ($n = 10$). Under reducing conditions only 9/13 sera from patients with active Graves' disease, 4/13 sera from patients with Graves' disease in remission, and 8/11 sera from patients with Hashimoto's thyroiditis reacted with two microsomal proteins of about 96 kDa and 103 kDa (Fig. 1). Pooled normal serum and sera from patients with autoimmune thyroid disease but negative titers of MAb did not show any reactivity under these conditions ($n = 10$). The values of MAb were significantly higher in sera recognizing the 96-kDa and 103-kDa proteins under reducing conditions (range 562–60992 IU/ml versus 251–4082 IU/ml; $p < 0.05$; Fig. 2). In contrast, positive reactivities in the immunoblot were not related to the presence of thyroglobulin antibodies or TSH receptor antibodies. The spectrum of protein bands recognized by patients' autoantibodies under nonreducing and reducing conditions did not differ between the three groups of patients investigated.

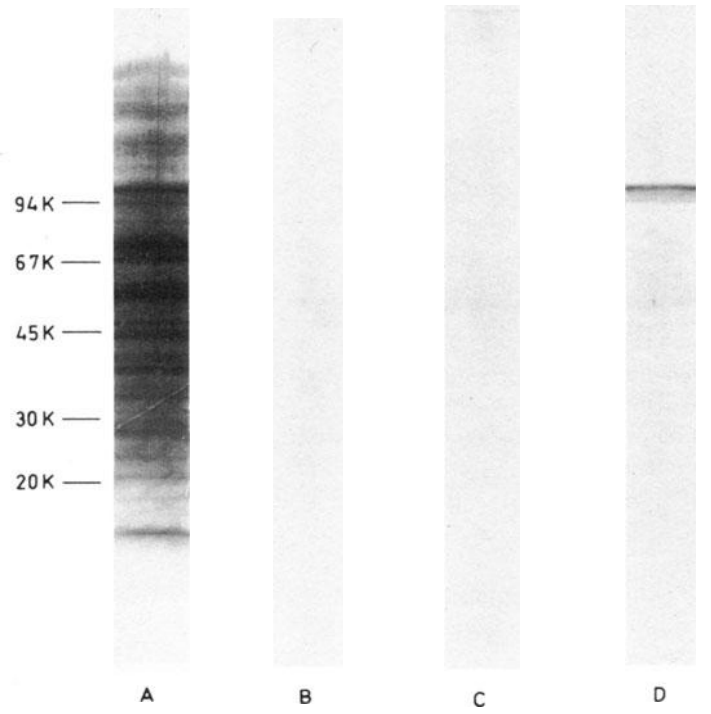
Immunoprecipitation of Thyroid Peroxidase by Autoantibodies. To investigate the interaction of patients' sera with native, nondenatured TPO, immunoprecipitation of TPO activity was measured in solubilized thyroid microsomes. In the case of 12/13 sera from patients with Graves' disease, 9/13 sera from patients with Graves' disease in remission, and 11/11 sera from patients with Hashimoto's thyroiditis, the serum could significantly immunoprecipitate TPO activity, resulting in a 6%–71% decrease in TPO acitivity in the supernatant. In all groups of patients studied, relative immunoprecipitation values were highly correlated to the titers of MAb ($r = 0.77, n = 37, p < 0.001$; Fig. 3). There were no significant correlations with the titers of thyroglobulin antibodies ($r = 0.28$), NS) or TSH receptor antibodies ($r = 0.04$, NS). Sera with a positive reactivity in

Fig. 1. Reactivity of normal control serum *(lane B)* and sera from patients with autoimmune thyroid disease *(lanes C, D)* with microsomal proteins in the immunoblot. *Lane A* shows the india ink protein stain of thyroid microsomal proteins resolved by SDS electrophoresis under nonreducing and reducing conditions

non-reducing conditions



reducing conditions



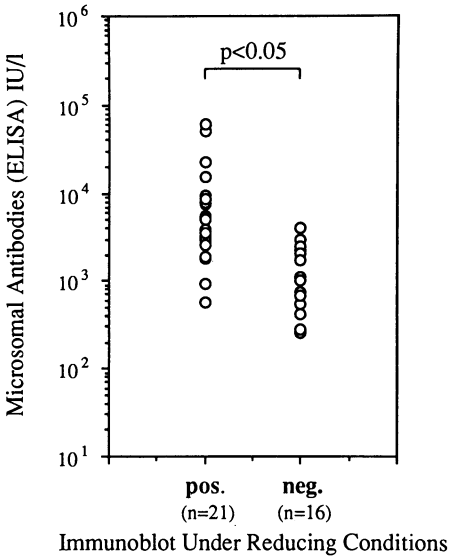


Fig. 2. Values of microsomal antibodies measured by ELISA in sera with positive and negative results in the immunoblot under reducing conditions

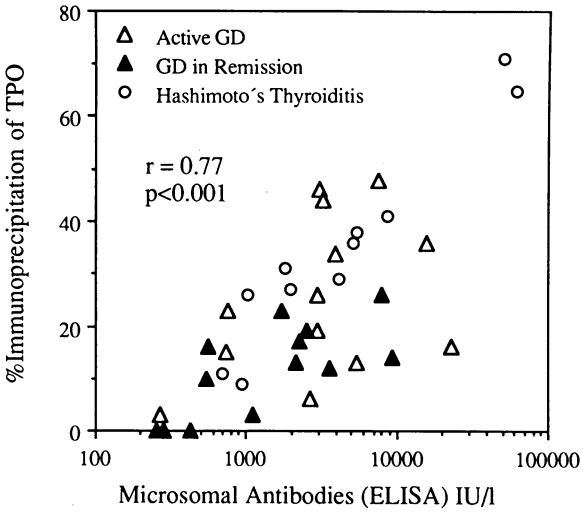


Fig. 3. Correlation between immunoprecipitation of TPO activity and values of microsomal antibodies (ELISA) in sera from 37 patients with autoimmune thyroid disease

the immunoblot under reducing conditions revealed a significantly higher mean immunoprecipitating activity than sera with a negative reactivity (range 6%–71% versus 0%–29%), $p = < 0.01$; Fig. 4). However, in some sera significant immunoprecipitation of TPO activity could be observed in spite of negative immunoblot results and vice versa; positive immunoblot reactions could be associated with very low immunoprecipitating activities.

Influence of Autoantibodies on Thyroid Peroxidase Activity. As a prerequisite of the studies on the direct influence of patients' sera on TPO activity, we estab-

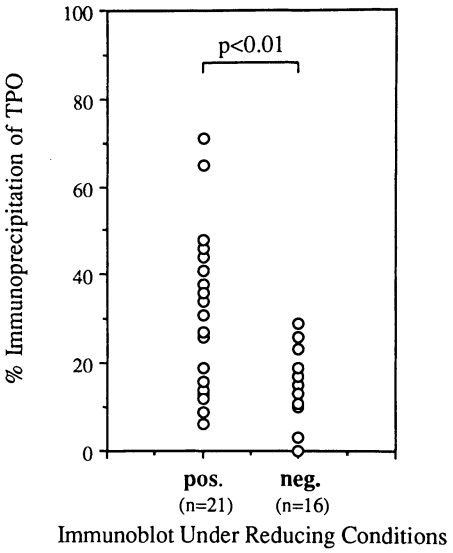


Fig. 4. Values of relative immunoprecipitation of TPO activity in sera with positive and negative results in the immunoblot under reducing conditions

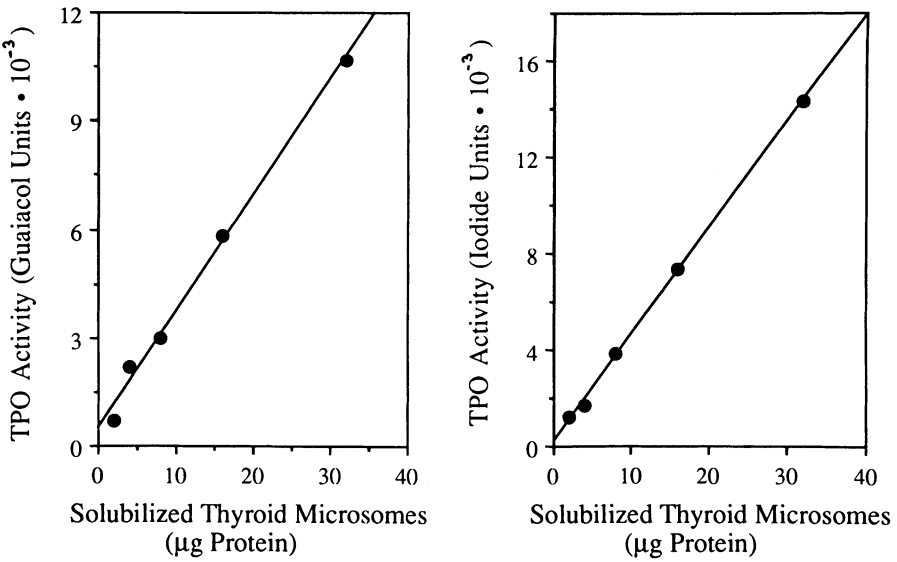


Fig. 5. Effect of increasing concentrations of TPO in solubilized thyroid microsomes on TPO activity measured by the guaiacol *(left panel)* and iodide assay *(right panel)*

lished well-reproducible and quantitative assays for determination of TPO activity. Since TPO presents at least two catalytic sites, one for the aromatic donor and another one for iodide, the enzymatic activity was estimated by two different methods, one of which measures the oxidation of guaiacol and the other the oxidation of iodide. After experiments with various concentrations of Tri-

ton X-100 for the solubilization of thyroid microsomes, a final concentration of 0.2% was selected. This procedure gave a sixfold purification of TPO compared with nonsolubilized thyroid microsomes (specific activity, 180 GU/g protein versus 30 GU/g protein. When increasing amounts of TPO in solubilized thyroid microsomes were added to the reaction mixture of the guaiacol or iodide assay, a linear increase in the enzymatic activity was observed (Fig. 5). For the TPO inhibition experiments, an amount of thyroid microsomes that was on this linear part of the curve was selected (15 µg protein). The addition of various amounts of normal human serum to the reaction mixture of the guaiacol or iodide assay resulted in a dose-dependent decrease in TPO activity (Fig. 6).

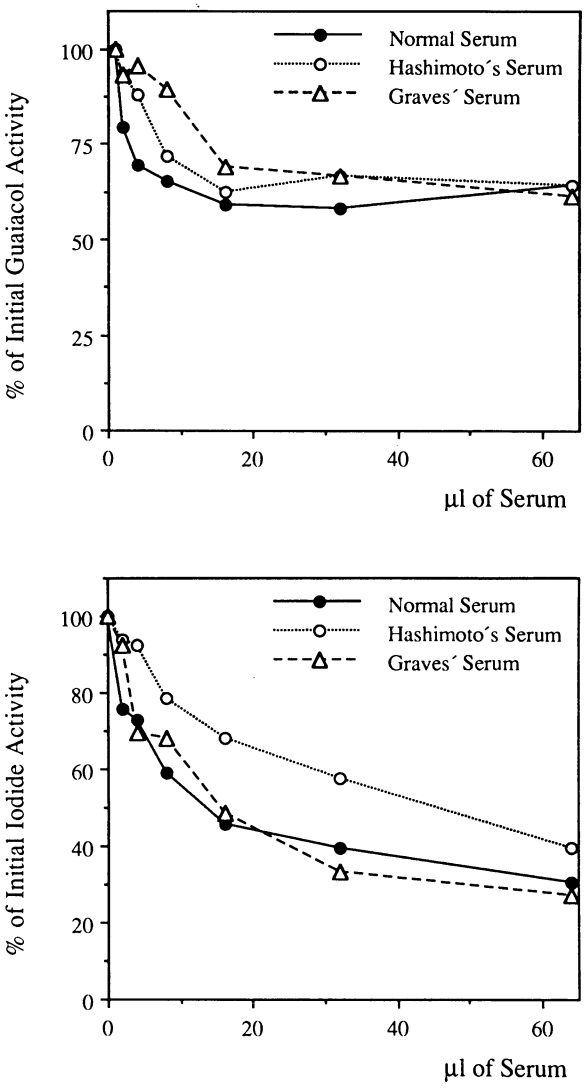


Fig. 6. Effect of increasing amounts of normal control serum or serum from patients with Graves' disease and Hashimoto's thyroiditis on TPO activity measured by the guaiacol assay *(upper panel)* and by the iodide assay *(lower panel)*

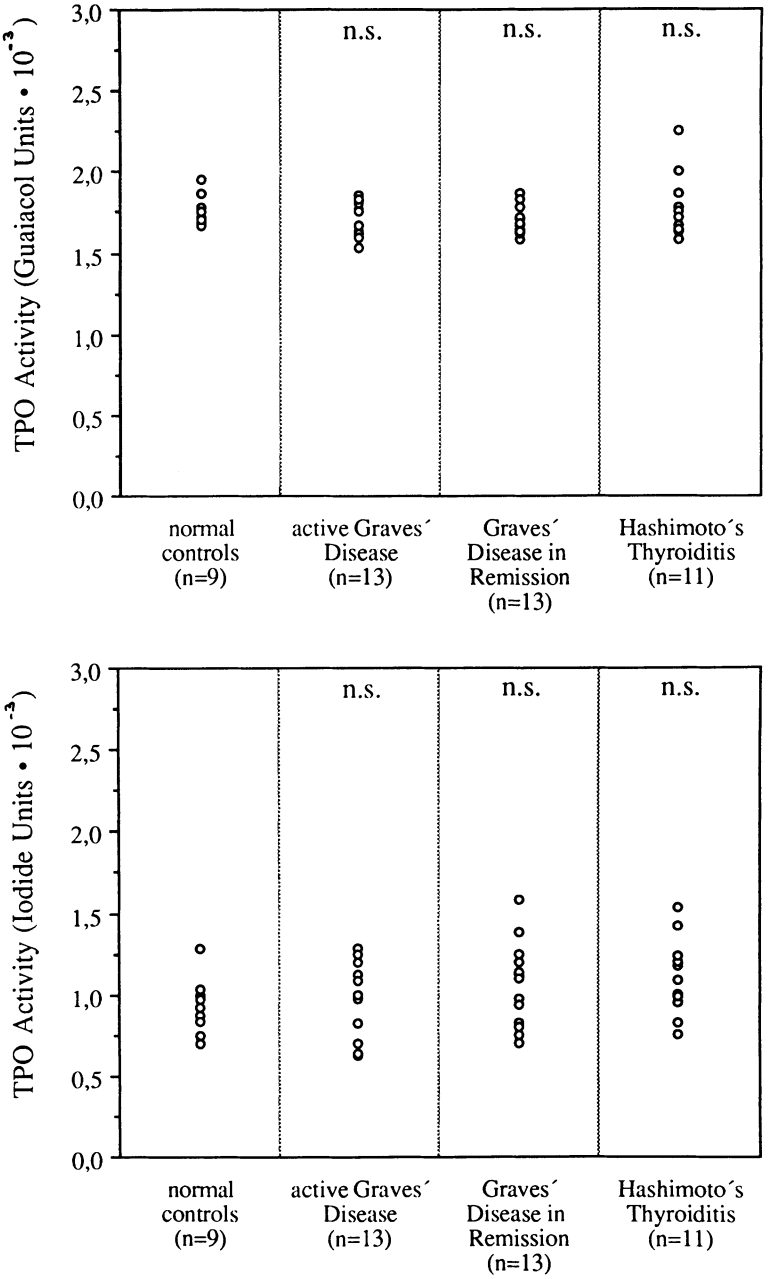


Fig. 7. Influence of sera from normal controls and from patients with autoimmune thyroid disease on TPO activity measured by the guaiacol assay *(upper panel)* and by the iodide assay *(lower panel)*

This inhibitory effect was not significantly different when serum samples from patients with Graves' disease or Hashimoto's thyroiditis were tested and could also be seen with increasing amounts of protein A purified IgG preparations (data not shown). None of the sera from patients with active Graves' disease, with Graves' disease in remission, or Hashimoto's thyroiditis could significantly inhibit TPO activity in the guaiacol or in the iodide assay (Fig. 7).

Discussion

It ist now well established that the MAg is closely related to, if not identical with TPO [4–7]. Although anti-TPO-Abs can be detected in most patients with autoimmune thyroid disease, only few data exist about the significance of these antibodies during the course of Graves' disease or autoimmune thyroiditis. Thus, it is unclear whether these antibodies represent an immunological epiphenomenon in autoimmune thyroid disease or whether they act as causal agents like TSH receptor antibodies. In this respect, the most important issue whether the antigenic determinants of TPO include its catalytic sites remains unsettled, since anti-TPO-Abs have been reported either to inhibit [9–12] or not to affect the enzymatic activity of TPO [4]. Theoretically, if anti-TPO-Abs influence the enzymatic activity of TPO, they could contribute to the pathophysiological process either by altering thyroid hormone biosynthesis during the acute phase of Graves' disease, or their presence could play a role in maintaining a biochemical remission of Graves' disease despite the persistence of the underlying immunological process. Additionally, these antibodies could be important factors in producing thyroid dysfunction in patients with Hashimoto's thyroiditis. To assess these possibilities, we characterized the reactivity of MAb/anti-TPO-Abs in different groups of patients, i. e., in patients with active, hyperthyroid Graves' disease, in patients with Graves' disease in clinical remission with an euthyroid hormonal status, and in patients with Hashimoto's thyroiditis.

The first part of our studies aimed at the characterization of the reactivity of MAb/anti-TPO-Abs with human microsomal proteins by immunoblot technique. When studies were conducted under nonreducing conditions, a not entirely specific staining of various proteins in the range of 80–125 kDa was observed with all sera investigated. Under reducing conditions, immunoreactivity of Graves' sera with thyroid microsomes was restricted to two bands with apparent molecular sizes of 96 and 103 kDa. Both bands were stained by 9 out of 13 sera from patients with active Graves' disease, 4 out of 13 sera from the remission group, 8 out of 11 sera from patients with Hashimoto's thyroiditis, but none of the control sera. These data indicate a higher immunoreactivity of autoantibodies with the nonreduced proteins and strongly suggest a heterogeneity of MAb/anti-TPO-Abs, since they are best explained by the presence of different antigenic determinants which are partially destroyed by the use of reducing agents. Nevertheless, the antigenic epitopes that are recognized under

nonreducing and reducing conditions are apparently identical, as evidenced by immunoabsoption studies which revealed a loss of staining of the reduced proteins after prior absorption of autoantibodies to nonreduced antigens (data not shown).

On the basis of the known association between the MAg and TPO, it was of particular interest to evaluate how the interaction of MAb/anti-TPO-Abs with various antigenic epitopes within the microsomal fraction would relate to the structure and function of TPO. As expected, we found a close relation between the interaction of Graves' sera with MAg in the immunoblot and their ability to precipitate TPO activity. TPO precipitation was best related to the staining of the 96- and 103-kDa bands, which are in the molecular range known for TPO. These findings underline and extend previous data by demonstrating a close relationship of TPO precipitation by MAb/anti-TPO-Abs and distinct constituents of the microsomal fraction. In some sera, however, significant immunoprecipitation of TPO activity was observed in the absence of binding to microsomal proteins in the immunoblot under reducing conditions. This observation adds another line of evidence for a heterogeneous nature of MAb/anti-TPO-Abs, since it suggests that the MAg presents a broad spectrum of antigenic determinants which are in part destroyed by the application of denaturing and reducing agents. As a result, only sera containing high titers of antibodies against antigenic determinants resistant to denaturing and reducing agents would show a positive result in the immunoblot.

During the course of Graves' disease, the heterogeneity of MAb/anti-TPO-Abs has received scant attention so far. Only one study from HAMADA et al. [8] has addressed this question and reported a higher percentage of antibodies against the denatured, reduced MAg in patients who had either a long-lasting disease or developed hypothyroidism after radioiodine treatment, as opposed to patients with active untreated Graves' disease. We could not confirm this observation in the present study. On the contrary, we found a higher reactivity of MAb/anti-TPO-Abs with reduced MAg in patients with active Graves' disease (9/13) as compared to patients in clinical remission (4/13). Further, the spectrum of protein bands which were recognized by MAb/anti-TPO-Abs did not differ in the two groups of patients with Graves' disease and in patients with Hashimoto's thyroiditis. A possible explanation for this discrepancies between the present findings and those of HAMADA et al. may be the use of different methos for determination of MAb/anti-TPO-Abs. While HAMADA et al. used autoradiography with ^{125}I-labeled protein A and ELISA technique, we employed an alkaline phosphatase conjugation method which, we believe, in superior in terms of sensitivity as well as specificity of antibody detection. From our data, we conclude that the existing heterogeneity of MAb/anti-TPO-Abs ist not significantly altered during the course of Graves' disease. This conclusion is further supported by a lack of differences among the three groups of patients with respect to TPO immunoprecipitation.

The most important question concerning the pathophysiological significance of MAb/anti-TPO-Abs is whether they have a direct impact on the enzymatic activity of TPO. To obtain further data about this controversial question,

we established sensitive and quantitative assays for the determination of TPO activity, one using guaiacol and the other using iodide as the second substrate. When increasing concentrations of TPO in solubilized thyroid microsomes were added to the guaiacol and iodide reaction mixtures, linear increases of the enzymatic activity were observed. Thus, as a prerequisite for further studies the methods used allow the precise quantification of TPO activity.

As already described by other authors [9, 12], normal human serum and also normal human IgG preparations partially inhibited the enzymatic peroxidation of guaiacol and iodide. This effect is most likely due to nonspecific interactions of IgGs with solubilized TPO. The degree of nonspecific inhibition is similar to that reported earlier [9, 12]. In our study, the TPO activity measured in the presence of sera from patients with autoimmune thyroid disease, whatever their clinical stage or presentation, was not significantly different from that measured in the presence of normal controls. These results were obtained under a very rigorous methodological regimen in that we tested normal controls and patients' sera over a wide concentration range and in single guaiacol and iodide assays to avoid high interassay variabilities.

The lack of a significant inhibitory effect is in good accord with a former report from PORTMANN et al. [4]. In contrast, KOHNO et al. [9] reported on TPO inhibition by autoantibodies from patients with Hashimoto's thyroiditis. More recently, DOBLE et al. [12] and YOKOYAMA et al. [10] described an inhibition of TPO activity by the majority of sera from patients with autoimmune thyroiditis or Graves' disease. OKAMOTO et al. [11] also found TPO inhibition, but only in a minority of the serum samples from patients with Graves' disease or Hashimoto's thyroiditis.

The differences among these studies can hardly be explained by different methodological approaches, since all authors used very similar assay systems. However, in our experience, these assays show high interassay variabilities and a considerable variability among the results of different normal control sera. This must be taken into account when calculating the significance of TPO inhibitory effects.

In conclusion, the present studies strongly indicate a heterogeneous nature of autoantibodies directed against the MAg/TPO molecule. Comparative studies with sera from patients with active Graves' disease, with Graves' disease in clinical remission, and from patients with Hashimoto's thyroiditis suggest that the spectrum of antigenic determinants which are recognized by MAb/anti-TPO-Abs is constant during the course of autoimmune thyroid disease. Since in our study MAb/anti-TPO-Abs failed to exert a significant inhibitory effect on the enzymatic activity of TPO, the antigenic determinants of the TPO molecule do not apparently include its catalytic sites. These data argue against a major pathogenetic role of MAb/anti-TPO-Abs in the metabolism of thyroid hormones and, as a result, in the regulation of thyroid function during the course of autoimmune thyroid disease. This is in good accord with the great variability of the clinical presentation among patients with highly positive anti-TPO-Abs, which ranges from hypothyroidism to overt hyperthyroidism.

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Discussion

Schleusener:
If you prepare your TPO, how about the conformational structure of the TPO after the preparation? I could imagine that this very complicated tertiary structure is somehow changed by your preparation, and that an antibody initially binding to closely adjacent epitopes can no longer do so after unfolding of the structure due to in vitro preparation. Thus, different antibodies are probably binding to these epitopes after preparation.

Saller:
This is right, we cannot exclude nonspecific effects because we used crude thyroid microsomal preparations and not the purified and intact TPO molecule. But I think our Western blot results concerning the molecular weight of the corresponding antigen and our data about the immunoprecipitation of TPO activity strongly indicate that the antibodies really bind to epitopes of TPO.

Furmaniak:
I understand you to be saying that the differences which you found between immunoprecipitation and immunoblotting results imply that there is a heterogeneous group of autoantibodies. I would disagree with that, because in immunoprecipitation you can pick up other groups of antigen and antibody systems. You did not explain how you carried out your immunoprecipitation. If you are using the serum and a crude preparation of microsomes, you could be precipitating different systems.

Saller:
Let me first tell you the method we used for immunoprecipitation. We added serum or IgG preparations to solubilized thyroid microsomes. After an incubation period, protein A-Sepharose was added, and the antigen-antibody complexes were removed by centrifugation. The decrease in TPO activity in the supernatant indicated the degree of TPO immunoprecipitation. What I would say is that in immunoprecipitation studies we used native thyroid microsomes and in immunoblot studies we used thyroid microsomes after denaturation with SDS or even after application of reducing agents. This means that if we have different results with these methods, we can conclude that we also have different antigenic determinants and a heterogeneous group of autoantibodies reacting with these antigens.

Bottazzo:
I don't believe you have fully answered the question. The question is: When the antigen is manipulated in a Western blotting assay, does it maintain the same native configuration or is this altered?

Furmaniak:
What I was saying is that if you had a crude preparation of microsomes, other antigens would be present also, such as cytoskeletal antigens, and then, if you have the serum from patients who may have antitubulin or antidesmin antibody, and precipitate it down, the positive result of immunoprecipitation does not mean that you are studying only TPO antibodies.

Bottazzo:
Can I throw the question back to Paul Banga. Could you comment on my previous point?

Banga:
For Western blotting, antigen is denatured by SDS prior to gel electrophoresis, whereas for immunoprecipitation your antigen is still native in the sense that it is solubilized in a nondenaturing detergent like Triton X 100 or sodium deoxycholate.

Bottazzo:
And what about the possibility that the antigen can change its configuration by these manipulations?

Carayon:
Whatever you do with an antigen, you are denaturing it, even coated TPO is denatured in some way.

T Cell Epitopes and Thyroid Peroxidase*

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Introduction

Investigations of immune responses to exogenous antigens suggest that antibodies tend to interact with conformational, non-linear regions of polypeptides whereas T cells recognize short, lenear amino acid sequences of denatured antigen in association with MHC molecules. Further, detailed analyses of peptides stimulatory for T cells have indicated that such T cell "epitopes" have characteristic patterns of amino acids which may be predicted according to the algorithms of ROTHBARD [1] or BERZOFSKY [2].

A series of peptides (10–17 amino acids in length) predicted on the basis of the amino acid sequence of thyroid peroxidase (TPO) to be potential T cell epitopes, were synthesized and tested for their ability to stimulate T cell proliferation in culture. No consistent, substantial proliferative responses were observed in blood, thyroid or lymph node lymphocyte cultures from patients with Hashimoto's thyroiditis or Graves' disease [3]. However, other parts of the molecule might be immunogenic, and one region of particular interest is an 8 amino acid sequence (Leu-Ser-Glu-Asp-Leu-Leu-Ser-Ile) in the amino-terminal region of human TPO which contains a Rothbard epitope and has a high degree of homology with a sequence at the carboxy terminus of human thyroglobulin (Tg) [4]. If this potential epitope common to TPO and Tg was antigenic in man, the responsive T cells could in turn drive secretion of both TPO antibody and Tg antibody by T cells specific for TPO and Tg antibodies. Consequently, we have tested the ability of lymphocytes from individuals with TPO and/or Tg autoantibodies to respond to this potential TPO/Tg epitope.

Methods and Materials

Two peptides were synthesised by solid-phase f-moc amino acid residue coupling [5] using the RaMPS Multiple Peptide synthesis system (DuPont-NEN Products, Boston MA, USA). Peptide 116, extending from residue 116 to residue 131 at the amino terminus of TPO, included the potential shared epi-

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tope (residues 119–126). Peptide 9 (residues 277–286), which lacks amino acid sequences corresponding to Rothbard or Berzofsky epitopes, provided a control peptide. The quality of the peptides was confirmed by amino acid composition. Tg was purified from Graves' thyroid tissue as previously described [6]. TPO was not used as in the previous study [3] we had been unable to observe proliferative responses to human TPO prepared from deoxycholate solubilised thyroid microsomes using affinity chromatography based on monoclonal anti-porcine TPO antibodies.

Lymphocytes were extracted as previously described [7] from peripheral blood by density centrifugation, from lymph nodes draining the thyroid by mechanical disaggregation, and from thyroid tissue by digestion and purification of the non-adherent cell population (after overnight incubation at 37 °C on density gradients. Thyroid tissue and lymph nodes were obtained from one patient with Hashimoto's thyroiditis and two with Graves' disease and blood from four with Hashimoto's thyroiditis and one with Graves' disease, and three euthyroid controls. Serum levels of TPO and Tg autoantibody levels were determined by ELISA [6, 8]. Since TPO antibodies were measured using thyroid microsomes, the assay results are referred to as TPO (mic) antibody.

T cell responses, measured in terms of [^{3}H]-thymidine ([^{3}H]TdR) uptake, were investigated using unfractionated lymphoid suspensions in conventional 96-well plate microcultures as follows: 200 µl aliquots of 1×10^5 cells in culture medium (RPMI 1640 containing 10% A + human serum and antibiotics) were incubated at 37 °C in the presence of 5% CO_2 with or without peptide 116, the control peptide or Tg. The mitogen concanavalin A was used as a positive control. Antigens or mitogens were tested in triplicate, and three to six replicates were set up in medium alone to determine levels of background incorporation. The control peptide was soluble in medium alone, but peptide 116 was dissolved in dimethylformamide, and therefore responses to this peptide were assessed in comparison with control cultures containing 0.25% and 0.025% dimethylformamide, which corresponded to the final concentrations in medium containing 25 and 2.5 µg/ml peptide 111, respectively.

[^{3}H]TdR was added after 5 days, and cultures were harvested at 6 days. Proliferative responses were expressed as the ratio of [^{3}H]TdR uptake in the presence of antigen to [^{3}H]TdR uptake in culture medium alone. The significance of differences between cultures with and without antigen was assessed by Student's *t* test.

Results

T cell responses, expressed as a stimulation index, are shown in Table 1 for individuals with TPO and/or Tg antibody detectable in serum diluted 1:1000, individuals with low levels of TPO and/or Tg antibody detectable in serum diluted 1:100, and individuals without detectable levels of these autoanti-

Table 1. Proliferative responses expressed as a stimulation index in cultures of lymphocytes from blood, thyroid and lymph node incubated for 6 days with a peptide containing the potential shared TPO/Tg epitope

Lymphocyte source	Donor	TPO/Tg Ab	Stimulation index in response to:								
			Peptide 116 (μg/ml)		Control peptide 4 (μg/ml)		Tg (μg/ml)			Concavalin A (μg/ml)	
			2.5	25	2.5	25	3	10	30	3.8	38
High serum levels of TPO (mic) or Tg Antibody											
Blood	A1	TPO, Tg	0.2	1.4	1.5	1.5	2.3[a]	1.6	2.2[a]	11.2[a]	121.9[a]
	A2	TPO, Tg	0.5	0.6	1.6[a]	1.6[a]	1.5[b]	1.6[b]	1.7	21.7[a]	268.7
	B	TPO	0.6	0.6	2.4[c]	0.8	0.6	0.6	0.5	24.5	37.2
	C	TPO, Tg	0.5	0.9	1.3	2.6[b]	2.2	3.6	2.0[a]	43.8[a]	100.2[a]
	D[d]	TPO	1.8	1.0	2.4[b]	1.5	0.8	1.0	1.0	27.3[a]	139.6[a]
	E	TPO, Tg	1.1	0.7	1.0	0.8	1.3	1.1	1.4	8.0	86.9[a]
Thyroid	E	TPO, Tg	0.8	0.6	0.9	0.8	0.9	1.0	0.8	11.4[a]	31.8[a]
Very low levels of TPO (mic) or Tg autoantibody											
Blood	F	TPO	1.4	0.6	1.1	1.1	1.2	1.0	1.2	13.6[a]	67.9[a]
Lymph node	G[d]	TPO	1.3	1.4	1.2	1.2	3.0[a]	1.6	1.3	811.4[a]	1057.9[a]
Undetectable serum levels of TPO (mic) or Tg autoantibodies											
Blood	H		1.9	1.2	1.6[c]	1.1	0.8	1.3	0.9	33.3[a]	268.3[a]
	I		0.8	0.5	0.6	0.6	0.9	1.0	1.0	4.7	70.8[a]
Thyroid	J[d]		3.2[c]	2.3[b]	1.5[c]	0.5	0.5	0.4	0.1	2.1	2.5
Lymph node	J[d]		1.6	1.1	1.5[c]	1.5[c]	3.6[b]	ND	3.2[a]	32.3[a]	179.8[a]

Donors F, H and I had no evidence of thyroid dysfunction. Patient A was studied on two occasions (1 and 2).
Significance refers to cultures in which [^{3}H]TdR uptake was greater in the presence of peptide, Tg, or concanavalin A than in medium alone.
[a] $p < 0.01$; [b] $p < 0.05$; [c] NS; [d] Patient with Graves' disease.

bodies. Background levels of proliferation were 142–942 cpm for blood and lymph node lymphocytes and 1365–3451 for thyroid lymphocytes; similar values were obtained for cells cultured with medium containing 0.25% and 0.025% dimethylformamide. High levels of proliferation occurred in the presence of one or both concentrations of concanavalin A (stimulation indices > 50.0) in all cultures except for thyroid lymphocytes from patient J (Table 1). In contrast, responses to peptide 116 or to Tg were small (maximum stimulation index 3.6), sometimes without relation to antigen dose. Further, responses were observed in individuals with high levels of autoantibodies as well as in thyroid and lymph nodes lymphocytes from a carbimazole-treated patient with Graves' disease (J) whose serum was negative by ELISA for TPO or Tg antibodies (Table 1). Responses to control peptide 9 occurred in some individuals (patients A, B and C), and the maximum response (stimulation index 2.3) was comparable with that observed for peptide 116.

Discussion

In conventional microwell cultures, no clear-cut responses to a peptide containing the potential shared TPO/Tg epitope or to purified Tg were observed. Further, observations in some patients without detectable levels of TPO/Tg autoantibodies of small responses to the peptide containing a potential T cell epitope, as well as responses to a control peptide, suggest that the proliferation was unrelated to a *specific* thyroid autoimmune response. These results are similar to those that we obtained with a further 12 linear peptides predicted to be T cell epitopes on TPO and nine control peptides [3].

Using miniaturised "hanging drop" cultures, it has been shown that dendritic cells can present Tg to blood lymphocytes from patients with Hashimoto's thyroiditis measured in terms of Tg antibody synthesis [9] and T cell proliferation [3]. Preliminary experiments using this system indicate that autologous B cells are effective antigen-presenting cells, possibly more efficient than dendritic cells. In addition, we have observed that denaturation of Tg reduces T cell proliferation, and this T cell proliferation seems to be associated with cultures containing plasma cells secreting Tg antibody. Overall, these observations suggest that B cells specific for Tg (or TB) may be involved in processing and/or presenting Tg (or TPO) to T cells in autoimmune thyroid disease as demonstrated for antigens such as tetanus toxoid by LANZAVECCHIA [10].

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Discussion

Banga:
Sandra, it is not surprising that you failed to obtain any proliferative responses in primary cultures using synthetic peptides simply because you have an extremely small number of antigen-specific populations of T cells that will respond to a particular epitope present on your synthetic peptide. I wonder whether you have looked at or proposed to examine the proliferative responses in cloned T cell populations rather than peripheral blood cells with the synthetic peptides, where you should almost certainly get higher SI values.

McLachlan:
We have looked at intact TPO and cannot see any responses using these conventional cultures. I think there are two problems: firstly, sufficient numbers of the specific precursor T cells in lymphoid cell suspensions; secondly, the presence of the antigen-specific B cell. It is difficult to achieve simultaneously the right number of T cell precursors and B cells carrying on their surface the specific receptor for the thyroid peroxidase. When we started this work we did not envisage how difficult it would be to get the right antigen-presenting cell. We thought that as the antigen is complex, an alternative would be to give the cell some cut-up ("processed") TPO. We did similar experiments with Tg before and after denaturation, denaturing it by boiling. To our surprise, we observed less efficient presentation after denaturation. It looks as though the antigen-specific B cell is needed to bind antigen – perhaps to protect certain epitopes. I agree that we should look at T cell clones, but we do not have any at present.

Banga:
It is interesting that in another organ-specific autoimmune disease, myasthenia gravis (MG), John Newsom-Davis' group in Oxford have shown that significant proliferative responses are obtained using peripheral blood lymphocytes from MG patients using recombinant preparations of alpha subunit or short synthetic peptides corresponding to several regions on the alpha subunit. Starting with peripheral blood populations, they get SI indexes between 5 and 12, and as they clone the T cell population these SI indexes get higher (SI > 100), showing enrichment of the antigen-specific T cells as they clone these populations.

McLachlan:
Perhaps I could remind you that it was observed that of the clones which were highly specific for acetylcholine receptor, many of them did not respond to the peptides, and, conversely, clones which responded to the peptides did not respond to the acetylcholine receptor. I wonder whether cloning in this way produces some clones which have limited significance for the human autoimmune response to the acetylcholine receptor.

Davies:
I though that this idea had not been shown to be true for any large protein; there was only experimental evidence to support it for small peptides?

McLachlan:
The peptides of the acethylcholine receptor (which consists of five subunits) were predicted from sequence data using the Rothbard/Berzofsky approach. I think there is a very big problem because recently, at the 7th International Congress of Immunology in West Berlin, Jonathan Rothbard showed that some peptides which were predicted to be T cell epitopes bound to many MHC types. I wonder whether this is reflected in nonspecific proliferation.

Davies:
Could you just tell me again why you thought the peptides would work when the intact microsomal antigen preparation didn't?

McLachlan:
This is based on our work with thyroglobulin (Tg). We found originally that intact Tg in conventional cultures did not induce satisfactory proliferative responses. As Tg is a very large protein, perhaps we did not have the right antigen-presenting or -processing cells. Therefore it might help to have some form of degraded protein. We tried to overcome this initially by giving artificially processed antigen in the term of potentially stimulatory peptides.

Wenzel:
Do you know if your epitopes have sequence homologies to heat shock proteins (HSP)? In that case the unresponsiveness would be understandable. The HSP are highly conserved regions, and it might depend on your T cells whether they respond or not. This would also explain why a large molecule would respond and not this peptide.

McLachlan:
Referring to the shared epitope, the Tg/TPO epitope?

Wenzel:
You were also mentioning the *E. coli* sequence, but it could also be inducible HSP sequences.

McLachlan:
No it is not. This sequence has been compared with protein sequence data in the Swiss protein data bank (10008 proteins). There was significant homology between two *E. coli* proteins, *E. coli* stringent starvation protein, and *E. coli* aminopeptidase N (McLachlan SM and Rapoport B, 1990, Evidence for a potential common T cell epitope between human thyroid peroxidase and human thyroglobulin with implications for the pathogenesis of autoimmune thyroid disease. Autoimmunity 5: 101–106).

Pujol-Borrell:
I like very much the idea of B cells being the presenters. I think there is a bit of a warning; there is evidence coming that B cells cannot be very good presenters to virgin T cells.

McLachlan:
Yes, I agree with you.

Pujol-Borrell:
It is basically memory cells which can respond well to antigens presented by B cells, and at some point thyroid autoimmunity must have been set in motion – back to the famous argument on the chicken and the egg. At the moment it seems that the process may be driven by memory T cells; it is then when your presentation by B cells comes in, maybe even in the maturation of the response and in the final selection of epitopes, more than during the initial selection.

McLachlan:
You are quite right. We don't know what activated the original T cells. What the T cell recognizes may not be on either thyroglobulin or TPO; it may only need to be on the surface of the thyroid cell.

Carayon:
We should not be too dogmatic about this because there are still a lot of things to know.

Thyroid Microsome/Thyroid Peroxidase Autoantigenic Epitopes Recognized by Autoantibodies Map to the Amino Terminal, the Central Core and the Carboxyl Terminal of the Molecule*

J. P. BANGA, P. S. BARNETT, D. L. EWINS, M. J. PAGE, and A. M. MCGREGOR

Introduction

The occurrence of autoantibodies (aAbs) to thyroid proteins is a feature of autoimmune thyroid disease (AITD) and a common serological marker for disease activity [1]. Although their precise role in the aetiology of AITD remains speculative, considerable evidence has accumulated to the effect that aAbs to the thyroid microsome/thyroid peroxidase antigen (TMA/TPO) may be pathogenetic [2]. TPO is a well-characterized enzyme involved in the generation of thyroid hormones from thyroglobulin and comprises a 105-kDa protein [3] which has recently been cloned [4]. Two forms of TPO cDNA have been reported, termed TPO-1 and TPO-2, with exon 10 coding for 57 amino acids is spliced from the coding sequence of the shorter TPO-2 cDNA [5].

We have recently shown that several autoantigenic sites are present on the TMA/TPO molecule that are recognized by aAbs from AITD patients [6, 7]. In order accurately to map the autoantigenic sites, we have now used recombinant DNA technology in conjunction with polymerase chain reaction (PCR) [8, 9] to prepare recombinant preparations of TPO [10]. By expressing selected segments of TPO as fusion proteins together with immunoblotting, we show that a minimum of six independent autoantigenic sites are present on the autoantigen.

Materials and Methods

Cloning of Human TPO cDNA. A human Graves' thyroid cDNA library in lambda gt10 was screened with a complementary 40 mer synthetic oligonucleotide probe corresponding to TPO nucleotide sequence 2600–2640, under hybridization conditions described previously [10, 11]. Two TPO cDNA clones were isolated, plaque-purified and fully sequenced after subcloning in M13. TPO-18 cDNA comprises nucleotides 1011–3066 of TPO, whilst TPO-21 cDNA comprises the complete TPO cDNA of 2845 nucleotides coding for the alternatively spliced TPO-2 [10, 12].

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Amplification by Polymerase Chain Reaction. The cloned TPO cDNA templates of TPO-18 and TPO-21 were used to amplify different segments of TPO cDNA by PCR in a Cetus thermal cycler. Oligonucleotide primers were designed for PCR, with the forward primer containing an in-frame *Bam* HI restriction site with the glutathione S-transferase (GST) gene in the expression plasmid pGEX-2T [13] and a second reverse hybrid primer containing a restriction site for *Eco* RI in-frame with pGEX-2T [10]. Six different fragments of TPO, termed R1a + R1b, R1c, R2b, R3a, R3b and R3c, and one large fragment, termed R3, encompassing the three regions R3a, R3b and R3c were amplified. For PCR, 10 ng cDNA template was mixed with 45 pmol oligonucleotide primer in Gene Amp DNA amplification reagent kit and 2.5U Taq polymerase in a total volume of 100 µl. Each PCR cycle consisted of 1 min of denaturation at 95°C, 2 min of annealing at 37°C and 3 min of chain extension at 72°C. Following 25 cycles, 10 µl sample was electrophoresed in an agarose gel in ethidium bromide to visualize the PCR products. The remainder of the amplified DNA was extracted with phenol/chloroform and digested with *Bam* HI and *Eco* RI.

Expression of Recombinant Protein. The *Bam* HI/*Eco* RI digested amplified DNA was ligated into *Bam* HI/*Eco* RI digested pGEX-2T [13] and used to transform library efficiency DH5 competent cells (BRL). Expression of recombinant fusion proteins and preparation of inclusion bodies were performed as already described [10].

Patient's Sera and Immunoblotting. Sera from AITD patients containing aAbs to TMA were used. The sera were further selected for use in this study based upon our previous observations that these sera react with TMA in thyroid microsome preparations following SDS polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions and were also known to contain a spectrum of different aAbs to TMA/TPO based upon the cross-reactivity patterns with other related peroxidase [7]. SDS-PAGE on the recombinant TPO preparations and immunoblotting were performed as already described [7]. Sera were absorbed with bacterial inclusion bodies containing non-recombinant pGEX-2T, prior to immunoblotting to remove any antibody reactivity with *Escherichia coli* proteins. Affinity purified, ^{125}I-labelled rabbit anti-human IgG was used following immunoblotting with aAbs and autoradiography to visualize aAb binding.

Results

Using either TPO-18 or TPO-21 cDNA as templates and oligonucleotide primers for PCR, the region of TPO cDNA that have been amplified and expressed as recombinant protein are shown in Fig. 1. It was necessary to use two different TPO cDNA templates for PCR since R1a + R1b and R1c could be

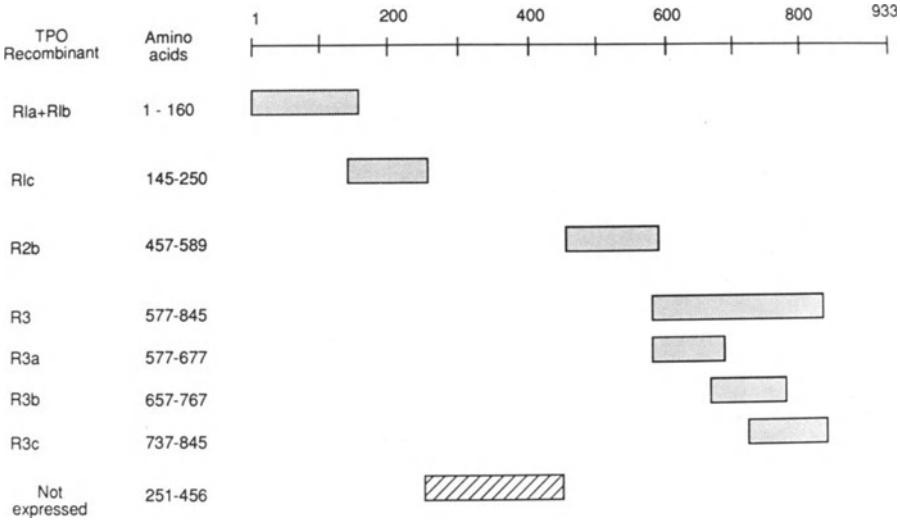

Fig. 1. The location of regions of the TPO molecule which have been amplified by PCR and expressed as recombinant fusion proteins *(shaded areas)*. Amino acid residues 251–456 have not been expressed as recombinant protein *(striped area)*

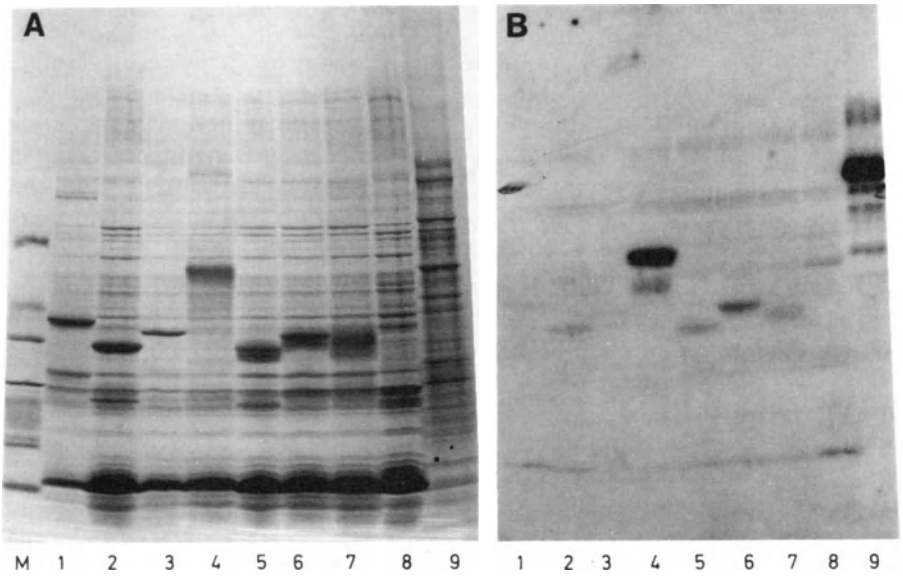

Fig. 2 A, B. SDS polyacrylamide gel electrophoresis of inclusion body preparations of various TPO recombinant polypeptides (**A**) and autoradiograph of immunoblotting with AITD serum of patient number 10 (Table 2; **B**). The protein stained gel (**A**) contains the following marker *(M)* proteins: lane 1, R1a + R1b (43 kDa); lane 2, R1c (33 kDA); lane 3, R2b (39 kDa); lane 4, R3 (56 kDa); lane 5, R3a (32 kDa); lane 6, R3b (38 kDa); lane 7, R3c (36 kDa); lane 8, non-recombinant plasmid expressing GST protein (26 kDa); lane 9, human tyroid microsome preparations

amplified only on the full-length TPO-21 cDNA; R2b could be amplified only with TPO-18 cDNA since this part of the sequence is spliced out in the TPO-21 cDNA [12]. Amplification of R1a-R1b, R1c, R2b, R3, R3a, R3b, and R3c produced DNA fragments close to the expected size of 480, 318, 399, 807, 303, 333, 327 base pairs respectively (not shown) [10].

Following ligation of amplified DNA into pGEX-2T and transformation of *E. coli*, the bacterial colonies expressing recombinant polypeptides upon induction with isopropyl-β-D-thiogalacto-pyranoside were grown and inclusion bodies prepared [14]. Analysis of inclusion body protein components by SDS-PAGE is shown in Fig. 2 A. All inclusion body preparations showed a strong protein staining band representing the GST-TPO fusion protein of the expected size. Hence recombinants from R1a + R1b, R1c, R2b, R3, R3a, R3b and R3c produce TPO fusion polypeptides of approximately 43, 33, 39, 56, 32, 38 and 36 kDA in size, respectively (Fig. 2 A, lanes 1–7; Table 1).

The TPO fusion polypeptides were analysed for aAb binding by SDS-PAGE under reducing conditions followed by immunoblotting. Ten different AITD sera that had previously been shown to contain a spectrum of aAbs to TPO [7] showed binding to various recombinant TPO fusion proteins (Table 2). The autoradiograph of aAb binding from patient 10 is shown in Fig. 2 B. Serum from patient 10 showed binding to all seven TPO recombinant preparations (Fig. 2 B, lanes 1–7) with the strongest reactivity to R3 and its subfragment R3b (Fig. 2 B, lanes 4 and 6, respectively); the reactivity with other recombinant preparations was weaker. Non-specific binding to several other polypeptides, which are also present in the non-recombinant bacterial preparations was also apparent (Fig. 2 B, lane 8); the binding to all the recombinant TPO preparations was specific since the co-migratory positions of all the positive bands (Fig. 2 B) are parallel to the protein stained co-migratory positions (Fig. 2 B). Immunoblotting on thyroid microsomes showed binding of the aAbs to TMA at 105 kDA (Fig. 2 B, lane 9); the other weaker bands represent immunoglobulin and their heavy and light chains which are found in human thyroid microsome preparations [6, 7] and are recognized by the radiolabelled rabbit anti-human IgG. A systematic analysis of the aAbs binding to different recombinant TPO preparations of ten AITD sera is shown in Table 2. Different sera

Table 1. PCR amplification of different regions of TPO cDNA: size of amplified DNA and approximate molecular weight of the generated GST-TPO fusion proteins

Recombinant TPO	Amino acid position	Length of PCR amplified DNA	Approx. mol. wt. of GST-TPO fusion protein
R1a + R1b	1–160	450 bp	43 kDa
R1c	145–250	318 bp	33 kDa
R2b	457–589	399 bp	39 kDa
R3	577–845	807 bp	56 kDa
R3a	577–677	303 bp	32 kDa
R3b	657–767	333 bp	38 kDa
R3c	737–845	327 bp	36 kDa

Table 2. Immunoblotting analysis under reducing conditions

Patient number	Thyroid microsomes	TPO recombinant (amino acid residues)						
		R1a + R1b (1–160)	R1c (145–250)	R2b (457–589)	R3 (577–845)	R3a (577–677)	R3b (657–767)	R3c (737–845)
1	+	–	–	–	+	+	+	–
3	+	–	–	–	–	–	–	–
4	+	–	–	–	+	–	+	–
5	+	–	–	–	–	–	+	–
8	+	–	+	–	+	–	–	–
9	+	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+
12	+	–	+	+	+	+	+	–
16	+	–	+	–	+	+	+	+
18	+	–	+	–	+	–	+	–
NHS	–	–	–	–	–	–	–	–
Rabbit anti-porcine TPO	+	–	+	+	+	+	+	–

NHS, Poded normal human sera, showing no reactivity with thyroid microsomes; +, positive reaction, –, negative reaction. Patient numbers are those in [7].

showed binding to different recombinant TPO preparations. Interestingly, a rabbit polyclonal antiserum to porcine TPO [15] recognized four of the six different recombinant segments of TPO (Table 2), with strongest reactivity with the R1c segment of TPO (not shown).

Discussion

In this study the autoantigenic sites on the TPO molecule recognized by aAbs' present in AITD patients have been mapped. The use of the recently introduced technique of PcR has allowed amplification of selected regions of TPO cDNA for expression as recombinant fusion proteins. Different segments of TPO representing 80% of the extracellular region of TPO have been expressed as recombinant proteins.

Using ten AITD sera which were known to contain a different and heterogeneous species of aAbs to TPO [7], a minimum of six autoreactive, sequential antigenic determinants on the TPO molecule have been identified. These determinants reside in the amino-terminal region of TPO (R1a + R1b and R1c), the central region (R2b) and the carboxyl terminus of the molecule (R3 and its subfragments R3a, R3b and R3c). It is possible that one recombinant preparation may contain more than one sequential autoantigenic epitope, and that one or more of the epitopes identified may reside on the overlapping areas of two recombinant preparations.

Multiple autoantigenic epitopes are clearly present on the TPO molecule that are recognized by aAbs in AITD patients. Different specificities of aAbs are present in different AITD patients, confirming our earlier studies on the polyclonality of the autoimmune response to TMA/TPO [6, 7]. This may explain the observations of some Hashimoto's or post-partum thyroiditis patients suffering destructive thyroid disease perhaps due to the presence of aAb to a site on the TPO molecule that is pathogenetic. Further accurate mapping will allow characterization of the precise regions on the TPO molecule which induce pathogenetic aAbs which lead to disease.

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Discussion

Ludgate:
Your approach is very ingenious. Could I just clarify one point: you say that you are using your peptide fragments as a fusion protein, and you described thrombin cleavage. Did you actually do that before you put them on a gel and blotted them?

Banga:
What I described here were fusion proteins.

Ludgate:
So, in other words, they have not been cleaved?

Banga:
They have not been cleaved yet, because we need to purifix these recombinant proteins first before cleaving them with thrombin. That is something which we are just now doing. Purifying the recombinant proteins is incredibly difficult, and we are using HPLC and preparative gel electrophoresis techniques.

Ludgate:
The reason for asking is, as you yourself have shown, antibodies to TPO can cross-react with a variety of other peroxidases.

Banga:
One of the controls in the Western blot experiments is the bacterially expressed nonrecombinant protein glutathione-S-transferase (GST). None of the anti-TPO containing sera shows any reactivity with the GST protein. Thus, when fusion proteins comprising GST TPO fragments react with the anti-TPO sera, it gives compelling evidence in favor of reactivity with the TPO moiety of the fusion proteins.

Davies:
There are only two segments which did not have autoimmune activities, am I right?

Banga:
No, all the different segments we looked at showed autoantibody binding with different sera.

Davies:
How many segments did you look at?

Banga:
We looked at six different segments representing 80% of the extracellular region of TPO.

Davies:
And all six that you looked at had immunoreactivity?

Banga:
With one patient, all six different segments of TPO showed reactivity; other patients' sera show reactivity with one or two of those TPO fragments, again arguing the heterogeneous nature of the autoantibody response to TPO of these patients.

Davies:
So, I thought now, you have not defined an area of TPO which is not an epitope.

Banga:
So far, we have not defined an area, but don't forget that the average size of the recombinants which I have shown is 120 amino acids, so they are still fairly large.

Davies:
Why don't you use a cell expression system?

Banga:
The eukaryotic expression system?

Davies:
Yes.

Banga:
That is just what we are going on to next.

Davies:
I don't understand the reason to work with the fusion proteins rather than just transfecting and expressing.

Banga:
The reason for working with the procaryotic expression system is that it is relatively easier to establish than the eukaryotic expression system. Secondly, the GST fusion protein protocol in the recently introduced pGEX vectors provides a simplified procedure to purify the recombinant protein by a single-step affinity purification method.

Carayon:
Working with fusion proteins you miss the carbohydrate.

Banga:
One of the disadvantages of the prokaryotic expression system is that the recombinant protein synthesized in bacteria folds incorrectly and also is not glycosylated. If carbohydrate residues are playing a role in influencing antigenicity of TPO, then we will not be able to assess that with our recombinant proteins. However, in the eukaryotic expression system, where posttranslational modifications such as glycosylation and heme incorporation occur accurately, would lead to an enzymatically active recombinant protein for immunological analysis.

McLachlan:
Can you show that the reactivity present in the serum against these fragments is specific by absorbing out with purified TPO?

Banga:
Those experiments require large amounts of highly purified recombinant TPO. At the moment we do not have sufficient recombinant TPO to couple to Sepharose for the absorption studies.

McLachlan:
What about simply purifying TPO?

Banga:
We can do that. The specificity of our immunoblots is shown by the fact that there is no reaction in the nonrecombinant TPO, there is no reaction with normal human serum, and the specificity is also shown by the comigrating patterns of the recombinant. TPO preparations on the Coomassie stains and the Western blots.

Comparison of Circulating Thyroid Microsomal, Cytotoxic and Thyroid Peroxidase Antibodies

P. WADELEUX, J. RUF, P. CARAYON, and R. WINAND

Introduction

Thyroid microsomal antibodies, recently identified as anti-thyroid peroxidase (anti-TPO) antibodies [1], are frequently present in patients with autoimmune thyroid disease and have been thought to be implicated in thyroid cell damage observed in autoimmune thyroiditis. In support of this concept, antibodies that are cytotoxic for thyroid cells in vitro have been found in the sera of patients with autoimmune thyroiditis and exhibiting antimicrosomal antibodies. These cytotoxic antibodies are active both directly in the presence of complement [2, 3, 4] and in association with K cells [5].

Most clinical laboratories measure microsomal antibodies in the various thyroid diseases, and recently a routine assay for anti-TPO autoantibodies has been developed [6]. In a recent report [7] we have described an assay for the quantitation of complement-dependent thyroid cytotoxic antibodies. In the present study we used this assay to correlate the level of these antibodies with the level of antimicrosomal and anti-TPO antibodies.

Material and Methods

Sera were collected from patients with various suspected thyroid disorders; they were obtained in the outpatient clinics of the hospital. To test all sera in identical conditions with respect to complement activity, they were first heat-inactivated (30 min at 56 °C). This procedure has also been shown to destroy some nonspecific cytotoxic activity present in some sera [8].

The cytotoxic assay was performed essentially as described previously [7]. Porcine thyroid cells were isolated by using the dispase digestion technique [9] and unless otherwise stated plated in 24 well-plates (0.75×10^6 cells/well) and incubated in the presence of test substance in 0.6 ml 199 culture medium containing 1 μCi (L-4-N^3H) leucine (60 Ci/mmol; 1 mCi = 37 MBq). Assays were performed in duplicate. The cell layer was washed 48 h after plating, removed, and processed for DNA [10], protein [11], and [^{3}H]leucine incorporation determination. As control of specificity, hepatocytes isolated from fetal rats by the technique of KREMERS et al. [12] were treated in strictly identical conditions.

Antimicrosomal antibody determination was performed by the Promak assay (Henning, West Berlin). This test is based on a solid-phase technique using tubes coated with microsomal antigen and the ability of ^{125}I-labeled protein A to bind to the previously fixed autoantibodies.

The anti-TPO antibody assay was based on autoantibody inhibition of the binding of labeled TPO to a solid-phase bound monoclonal antibody to TPO [6]. Antibody titer against thyroglobulin was determined with a commercially available kit (Techland, Belgium). Gammaglobulins were purified from serum by QAE Sephadex chromatography [13].

Results

When thyroid cells are incubated in the presence of normal human serum, they form a monolayer of epithelial cells 48 h after plating. In the presence of cytotoxic serum from a patient with autoimmune thyroiditis, very few cells stick to the bottom of the dish. Quantitation of the cell layer may be performed by using DNA determination, protein measurement, or [^{3}H]leucine incorporation. Figure 1 illustrates the results obtained using increasing amounts of a cytotoxic serum. For these three parameters, the dose-response curves are identical.

As already reported [7], this effect requires the presence of complement. It is specific for thyroid cells since no cytotoxic effect could be obtained by using fibroblasts [7] or hepatocytes instead of thyroid cells. The cytotoxic effect was associated with the gammaglobulin fraction isolated from the cytotoxic sera. A dose-response curve was obtained analogous to that obtained by addition of unfractionated serum (not illustrated).

We investigated on a large scale the possible correlation between antimicrosomal and cytotoxic antibodies. Figure 2 shows the relationship between cytotoxic antibodies and microsomal antibodies in one representative of 13 experiments. In these 13 experiments, subjects visited the outpatients clinics, and no attempt was made to establish a clinical diagnosis. When [^{3}H]leucine incorporation, which decreased proportionally to the cytotoxic effect, is plotted versus microsomal antibody level (on a logarithmic scale), a statistically significant correlation is obtained ($p < 0.05$). For a relation of the type $Y = A + B \log_x$ the following values are: $A = 176 \pm 40$, $B = -35 \pm 8$, and $r = -0.57 \pm 0.08$. Clearly however, some discrepancies were observed. Some sera with high titer of antimicrosomal antibodies had no cytotoxic effect, and some sera with low titer had a marked cytotoxic effect. Moreover, in 17/240 (7%) of these antimicrosomal antibody negative patients, a cytotoxic effect was also observed.

We next investigated whether anti-TPO antibody is a better index of the presence of cytotoxic antibodies. Sera of 130 patients with thyroid diseases were tested in the anti-TPO assay. A close correlation was observed both in the absence and the presence of added thyroglobulin, confirming that antithyroglobulin antibodies do not interfer in this test (data not shown). Results of the antimicrosomal antibody assay and the anti-TPO test showed a highly significant

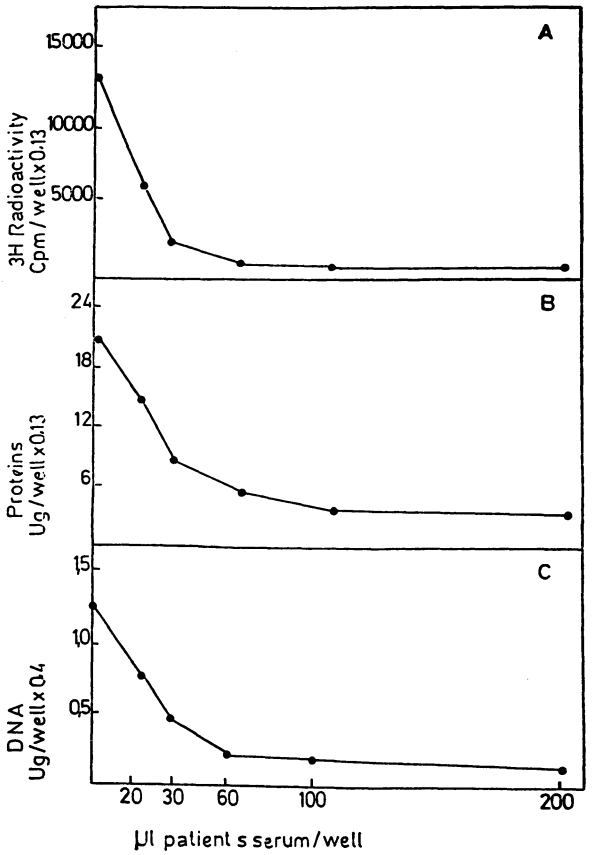

Fig. 1a–c. Cytotoxic assay with increasing amounts of sera from patients with autoimmune thyroiditis. In this experiment we used six well-plates (3×10^6 cells/9.6 cm^2). The total amount of serum was adjusted to 200 µl with normal human serum. After 48 h of incubation, cell layer was washed and recovered in 1.5 ml physiological buffer. Cells were homogenized by a sonicator (30 s). **a** [^{3}H]leucine incorporation determined with 200 µl cell homogenate. **b** Protein test performed with 200 µl cell homogenate after solubilization by 100 µl 0.1 *M* NaOH (30 min at 100 °C). **c** DNA content determined with 600 µl cell homogenate

correlation. Conversely, the relationships between cytotoxic effect and both antimicrosomal antibodies level and anti-TPO level were similar, exhibiting a significant correlation but also some clear descrepancies (data not shown).

Discussion

While a significant relationship exists, between cytotoxic effect and antimicrosomal antibody level, some discrepancies were also detected. Differences in the two methods with respect to the implicated immunoglobulins could partly ex-

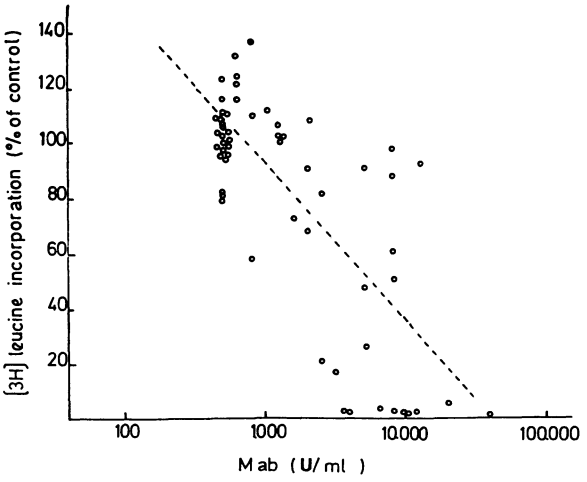

Fig. 2. Correlation between thyroid cytotoxic activity and microsomal antibody level in patients visiting the outpatient clinics

plain this phenomenon. Thus protein A, used for the detection of antimicrosomal antibodies, binds to the various classes of gammaglobulins (IgG1, IgG2, IgG4) with the exception of IgG3 [14] but does not bind to immunoglobulins A, M, or E. Complement binds to immunoglobulins M and G. The binding ability of immunoglobulin G varies in the order Ig3 > Ig1 > Ig2 > Ig4 [15]. Several reports indicate that the subclass distribution of antimicrosomal antibodies [16] and of anti-TPO gammaglobulins [17] varies in the various thyroid diseases and is not necessarily identical to the distribution of total gammaglobulins. The clinical significance of such subclass distribution is suggested by the association between IgG1 antimicrosomal antibodies (and not IgG4) and the development of postpartum thyroiditis in patients who are clinically euthyroid and exhibit antimicrosomal antibody positive sera in early pregnancy [18]. An identical phenomenon could explain the unexpectedly high cytotoxic effect that we observed in one case of Hashimoto's thyroiditis.

The high correlation between anti-TPO and antimicrosomal antibody levels as well as that between cytotoxic effect and both anti-TPO and antimicrosomal antibodies confirms the concept that TPO is identical to the microsomal antigen. The lack of cytotoxic effect of several antimicrosomal antibody positive sera could not be explained by an absence of anti-TPO antibody.

In several instances we also observed a cytotoxic effect for thyroid cells in sera of patients in the absence of antimicrosomal antibodies. We have not yet studied the specificity of this effect. Nevertheless, the presence in these sera of antibodies directed against a surface antigen without relationship with microsomal or TPO antigen [19, 20] could explain this phenomenom.

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Discussion

Drexhage:
I am a little bit puzzled. I am an ordinary immunologist, and immune cytotoxic assays mostly make use of chromium release, etc. You are describing a new type of assay, but how confident are you that it is cytotoxicity that you measure, and not, for instance, nonadherence or bad culture conditions?

Wadeleux:
We have tested several other parameters such as the trypan blue exclusion test and we have observed that all the cells that do not adhere to the petri dish are killed cells. In addition, these cells which are in suspension do not incorporate ^{3}H-leucine, which also indicates that they are killed cells.

Are TPO Antibodies and Cytotoxic Thyroid Antibodies Identical?*

U. BOGNER and H. SCHLEUSENER

Introduction

Since the detection of microsomal antibodies (Mab) in Hashimoto's thyroiditis (HT) and the association of cytotoxic antibody activity with Mab, it has been generally accepted that the presence of these antibodies is an indicator of thyroid cell destruction [1–3]. Nevertheless clinical follow-up studies showed that in some patients Mab are present for many years without development of hypothyroidism [4, 5]. Preliminary data from our laboratory suggested that no correlation exists between cytotoxic antibody activity and the titers of Mab [6]. In this paper we add further evidence for the diversity of cytotoxic and microsomal/thyroid peroxidase antibodies (TPOab).

Patients and Methods

A total of 67 patients, 57 women and 10 men, aged 21–83 years (mean 55 years), with autoimmune thyroiditis were studied. The diagnosis was established by findings of elevated TSH, significant titers of thyroid microsomal antibodies ($\geq 1:400$), thyroglobulin antibodies ($\geq 1:100$), typical hyporeflexive image by ultrasonography, and in some cases, by biopsy evidence of lymphocytic infiltration. Controls were 61 normal subjects, 53 women and 8 men, aged 27–64 years (mean 45 years), with no history of thyroid disease and absence of significant titers of thyroid antibodies.

Thyroid tissue obtained from surgical specimens of patients with multinodular goiter (blood group 0) was finely minced and enzymatically isolated by incubation in 0.5% collagenase (Boehringer, Mannheim, FRG). After 45 min of incubation at 37 °C, the supernatant was decanted through a screen and mixed with Iscove's modified Dulbecco's medium (Seromed, West Berlin) containing 10% fetal calf serum (FCS; Seromed), 100 U/ml penicillin, and 100 µg/ml streptomycin (Seromed). After three washes the cells were diluted to 5×10^6/ml in Iscove's medium containing 10% FCS, antibiotics and 7.5% dimethylsulfoxide (Sigma, Munich, FRG). The cell solution was transferred to cryovials, frozen, and stored in liquid N_2 until used for the experiments.

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Antibody-dependent cell-mediated cytotoxicity (ADCC) was determined as previously described [7]. The frozen cells were replated by rapidly warming the vials at 37 °C and transferring them into preheated Iscove's medium in 75 cm^2 flasks. After 3 days of culturing the cells had reached confluency and were transferred in suspension by incubation with 10 ml trypsin/EDTA solution (0.05%/0.02%; Seromed) for 5 min at 37 °C. After several washes, 2×10^6 thyroid cells were incubated for 1 h with 100 µCi $Na_2{}^{51}CrO_4$ (Behring, Marburg, FRG), washed twice, and diluted to 10^5 cells/ml. Then 50 µl radiolabeled cells (5×10^3/well) were incubated in triplicate with 100 µl 1:10 diluted heat-inactivated serum in microtiter plates (Flow Laboratories, McLean VA, USA) at 37 °C and 5% CO_2 in a water-saturated incubator. After 1 h of incubation the supernatant was removed, and the cells were washed once with medium. Then 100 µl effector mononuclear cells (1.25×10^5) from a normal subject were added, giving an effector : target cell ratio of 25 : 1. After 18 h of incubation an aliquot of the supernatant was aspirated, and the radioactivity was measured in a gamma counter (cpm_{exp}). Samples for the determination of the 100% value (cpm_{max}) and unspecific lysis (cpm_{unspec}) contained medium instead of lymphocytes. The non-specific release was determined by counting an aliquot of the supernatant, and maximal release was determined from an aliquot of the incubation mixture. Specific lysis was then calculated according to the following formula:

$$\% \text{ specific lysis} = \frac{cpm_{exp} - cpm_{unspec}}{cpm_{max} - cpm_{unspec}} \times 100$$

Cytotoxicity was considered positive if the specific lysis of the patient's serum was above the 95th percentile of the control sera determined in the same assay.

The IgG fraction was isolated from serum samples by means of affinity chromatography on columns of protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Peak fractions detected by spectrophotometry (280 nm) were combined and protein concentration adjusted to 1 mg/ml.

Fresh mononuclear cells were obtained from heparinized blood of a normal adult by means of Ficoll (Seromed) density centrifugation. The mononuclear cells were washed and diluted with medium to 1.25×10^6/ml.

Serum microsomal antibodies were determined by passive hemagglutination technique (Thymune M, Wellcome Diagnostics, Kent, UK) and by an enzyme immunoassay in which purified microsomal antigen was bound to a solid phase (Thyr Mic-IgG EIA, Pharmacia). Thyroid peroxidase antibodies were measured by a radioimmunoassay against solid-phase-bound monoclonal anti-TPO antibodies (DYNOtest, Henning, West Berlin). Mab titers of $\geq 1:400$, > 16 U/ml, > 100 U/ml, determined by passive hemagglutination, ELISA, and TPOab radioimmunoassay, respectively, were defined as positive. Thyroglobulin antibodies (Tab) were measured by passive hemagglutination technique (Thymune T, Wellcome Diagnostics). Tab titers of at least 1:100 were considered positive.

Data were assessed for significance using the unpaired Mann-Whitney U test; $p < 0.05$ was considered significant.

Results

ADCC was positive in 42 (63%) of 67 investigated patients with HT. The median of the specific lysis was 20.2% (range 2.1%–58.8%) compared to 8.1% (range 0%–19.6%) in the normal controls ($p = 0.00001$). Values above the 95th percentile ($\geq 15.4\%$) were regarded as positive. The values of the specific lysis did not correlate with the duration of the disease.

Significantly increased titers of microsomal antibodies were found in 72% of the patients with HT (range 1:400–1:25 600). The enzyme immunoassay for Mab determination yielded positive results in 37 of 47 (79%) patients, and 38 of 47 (81%) patients were positive applying the radioimmunoassay for the measurement of TPOab. No correlation could be found between the titers of Mab/TPOab, determined either by passive hemagglutination technique, enzyme immunoassay method, or TPOab radioimmunoassay technique, and the activity of the cytotoxic antibodies ($r = 0.2$, $r = 0.16$, and $r = 0.02$, respectively). There was likewise no correlation between the cytotoxicity and the titers of thyroglobulin antibodies ($r = 0.00$).

To exclude an influence of unspecific serum components on thyroid cell lysis, IgGs were isolated by protein A Sepharose chromatography and added to the cytotoxicity assay instead of serum. IgGs from 16 sera with positive and 4 sera with negative Mab and Tab titers were simultaneously tested in the ADCC assay. In both groups (Mab positive and negative) the cytotoxic activity was found to be located in the IgG fraction, which proves that cytotoxic activity in Mab-negative sera is also mediated by an IgG (Fig. 1).

To investigate in more detail whether the cytotoxic antibody is different from the TPOab, sera with positive and negative titers of TPOab but positive results for cytotoxicity were preincubated with purified TPO for 2 h and then

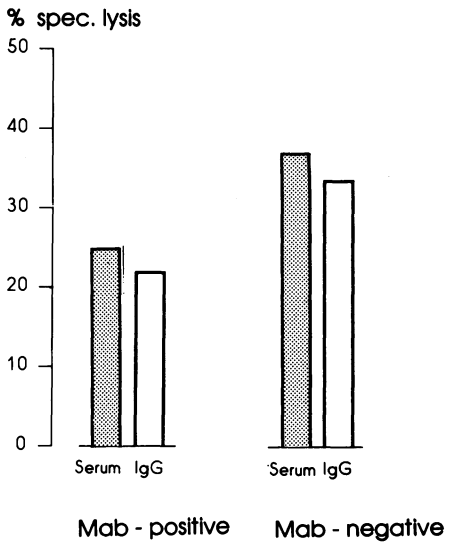

Fig. 1. Cytotoxicity determined by addition of serum and IgG (1 mg/ml) in the ADCC assay. The median specific lysis in the Mab/Tab-positive group ($n = 16$) was 25% with addition of serum and 21.6% with addition of IgG. In the Mab/Tab-negative group, specific lysis was 36.9% in the serum assay and 33.6% in the IgG assay

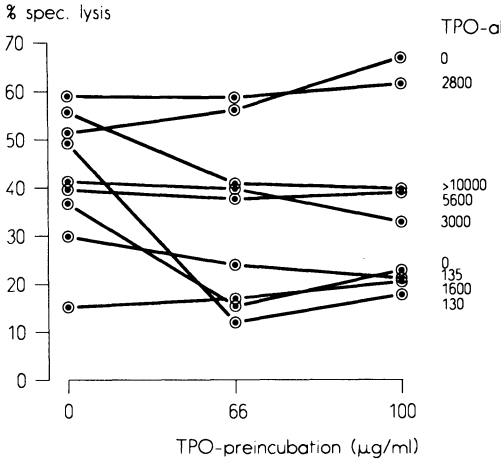

Fig. 2. Influence of preincubation with increasing concentrations of thyroid peroxidase for 2 h on percentage specific lysis in sera of patients with HT. Values at the curves indicate the TPOab levels

added to the ADCC assay. Interestingly, the cytotoxic activity remained unchanged in seven of nine sera, irrespective of whether they had a positive or negative result for TPOabs (Fig. 2). Only two cases showed a significant decrease in cytotoxicity after preincubation with TPO.

Discussion

Some years after the detection of the autoimmune pathogenesis of HT, PULVERTAFT et al. [1] described a cytotoxic factor in sera from patients with HT which induced lysis of cultured thyroid cells. Further investigations showed that this factor is located in the Mab fraction, that the effect is abolished by heat-inactivation to 56 °C, and that it can be reinstalled by addition of fresh complement [2, 3]. Absorption experiments with thyroglobulin proved that cytotoxic and thyroglobulin antibodies are not identical [1, 8]. Although some studies did find a cytotoxic effect in the absence of elevated titers of microsomal antibodies as well as a lack of correlation between cytotoxicity and microsomal antibody titer [1, 8, 9], it has been generally accepted that the cytotoxic factor is located in the Mab fraction.

Studies analyzing cytotoxic effects by ADCC assay showed a higher frequency of positive results in patients with HT; unfortunately, there are no data on the presence of Mab [10–12]. Earlier data from our laboratory indicated a simultaneous occurrence of cell lysis, determined by ADCC assay, and Mab [7]. Including a higher number of patients, however, we could no longer confirm a correlation between cytotoxicity and Mab acitivity. No simultaneous analysis has been published for a comparison of the results between antibody-dependent complement- and cell-mediated cytotoxicity. This is mainly due to different experimental procedures, since the determination of complement medi-

ated lysis requires fresh thyroid cells, whereas cytotoxicity measurements by ADCC procedures yield optimal results using cultured thyrocytes.

The microsomal antigen has now been identified as TPO [13, 14]. To exclude the influence of false-negative Mab determinations by the insensitive passive hemagglutination technique, we used an ELISA technique and a TPOab radioimmunoassay and determined the antibody concentrations in the same sera. Nevertheless, there was still no correlation between cytotoxicity and Mab/TPOab.

We demonstrated that cytotoxicity in the Mab/TPOab-negative sera is mediated by an IgG. The results of the experiments with TPOab-negative and -positive sera showed that preincubation with purified TPO did not exhibit any influence on the cytotoxic effect in the majority of tested sera. These data suggest that cytotoxic IgGs and TPO antibodies are induced and directed against different antigen structures on the thyroid cell surface. Further studies are in progress to confirm the hypothesis of the diversity of cytotoxic and thyroid peroxidase antibodies.

Acknowledgement. We thank Dr. Carayon for the generous gift of purified thyroid peroxidase.

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Discussion

Mariotti:
Do you have any experience with sera from patients with other autoimmune diseases, either endocrine or nonendocrine, in order to ascertain whether this antigen which is so subtle to demonstrate, is really thyroid specific.

Bogner:
ADCC in lupus erythematosus, was found positive in 5%, and in rheumatoid arthritis in 10%. So there was a difference between these groups.

Khoury:
It was not clear to me whether you were using an autologous system. The cells and the serum were from the same patient?

Bogner:
No, it was not autologous. We use one thyroid preparation for the determination of ADCC.

Khoury:
Did you absorb out the sera with blood group A and B red cells?

Bogner:
We didn't absorb out the sera, but we used blood group 0 thyroid target cells.

Khoury:
In any case, if the system is not autologous, the possibility always exists that some alloantigens are reexpressed in culture, like the ABH blood group antigens, and those antigens may be the reason for the discrepancy between the cytotoxic effect and the presence or titer of microsomal antibodies. The ADCC system is a very, very sensitive system, which may also be a reason for some discrepant results, especially if you are using a not very sensitive system to measure microsomal antibodies; perhaps that may explain a negative result for microsomal antibodies and a positive one for ADCC.

Bogner:
I cannot rule out completely your argument, but we have to find out whether other antigens are present on target cell.

Khoury:
I think that in order to be sure that there is no reexpression of alloantigen, as a reason for the discrepancies, you should use an autologous system.

Bogner:
I agree, but, as you know, to get targets from Hashimoto patients is a problem.

McLachlan:
I noticed that you used your serum at a dilution of 1/10. We have different results from yours. We use our sera at a dilution of 1/1000 – and under those conditions we observed a very big difference between normal and Hashimoto's sera, and the cytotoxicity correlated with microsomal antibody results. You may be observing nonspecific killing because you are using a high concentration of serum containing a wide range of antibody against a variety of antigens present on the thyroid cells. Have you tried the sera from normals and patients at higher dilutions?

Bogner:
We have done these experiments when we established this assay system. We got positive results even at higher dilutions, but we did not investigate this systematically.

Pujol-Borrell:
I am sure you are aware of the many variables you have in your system, and it is very difficult to control.

Bogner:
For all these experiments the same target was used. We tried to standardize the system, as far as possible.

Weiss:
Is the cytotoxic effect thyroid specific?

Bogner:
I can't tell you; we did not use liver or other organs as targets.

Weiss:
Blood cells or fibroblasts from the patient?

Bogner:
We have not done these experiments.

Studies with Recombinant Autoepitopes of Thyroid Peroxidase*

M. LUDGATE, R. ELISEI, S. SWILLENS, and G. VASSART

Introduction

In a previous study [1] we characterized a major epitope of thyroid peroxidase (TPO), C2, which was recognized by 66% of patients with autoimmune thyroid diseases (AITD), who also had antibodies to TPO as measured by radioimmunoassay (RIA). Despite the fact that the recombinant antigen preparation was not recognized by sera from normal individuals or the sera from most patients with nonthyroidal autoimmune diseases (NTAID) an exception was those having antibodies to the gastric parietal cell antigen (PCA); six of nine such sera were positive for C2.

In the first part of this study we sought by affinity purification of C2 antibodies to observe whether they are able to bind to the native protein. We then investigated the incidence of C2 reactivity in sera positive for PCA. We also used a second fragment of TPO, C21 [2], which overlaps C2 by 20 amino acids in the part of the peptide which contains a sequence of 11 residues which is similar to a fragment of the H^+ K^+ ATPase enzyme [3]; this was recently shown to be a major component of the PCA [4].

Material and Methods

Preparation of Affinity-Purified Antibodies. Lysis plaques from approximately 30000 identical phages (either C2 or an unrelated control) were transferred to nitrocellulose filters soaked in isopropylthio-β-D-galactoside. The filters, saturated with either C2 or control fusion proteins, were incubated in phosphate-buffered saline (PBS) containing 1% bovine serum albumin to block unoccupied sites and then incubated in sera, diluted 1.40 in PBS containing 0.25% gelatin and 0.1% NP40 (wash buffer) plus 100 µl *Escherichia coli* extract to reduce background staining, at 4 °C overnight with agitation. A small wedge was cut from each filter and stained by standard procedures [5] to determine whether the serum had bound to the fusion protein. The remainder of the filter

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was washed three times in wash buffer and once in PBS. Bound antibody was eluted using 2.5 ml per filter of 200 m*M* glycine HCl pH 2.5. The eluates were immediately neutralized by the addition of 100 µl 2 *M* Tris and dialysed against 100 volumes of PBS. The filters were subjected to staining to demonstrate that all the bound antibody had been eluted. Eluates were assayed for antibodies to C2 in an enzyme-linked immunosorbent assay (ELISA) and for antibodies to TPO in an RIA as previously described [1]. Sera from two normal individuals and nine patients with AITD (three with Hashimoto's thyroiditis, four with Graves' disease, and two with idiopathic myxedema), as assessed by established clinical and laboratory data, were subjected to this protocol.

ELISA for Antibodies to C2 and C21 Fragments of TPO. C2 (TPO 590–675) and C21 (TPO 655–755) fragments [2] were used as β-galactosidase fusion proteins to coat microtiter plates at 5 µg/ml, as previously described [1]. To permit comparison of values obtained in different assays, results are expressed as a percentage of a standard reference serum. In a typical experiment in which C2 was the antigen, the mean OD_{405} of 20 normal sera was 0.175 ± 0.045, corresponding to 21% ± 6% of the reference. Thus values greater than OD_{405} 0.280 or over 33% of the references, i. e., exceeding the mean of the normals by 2 standard deviations, were considered positive. Similarly, when C21 was the antigen, the mean OD_{405} of 17 normal sera was 0.340 ± 0.096, corresponding to 28% ± 8% of the standard. Values greater than OD_{405} 0.530 or over 44% of the reference were considered positive. Antibodies to C2 (C21) were measured in 20 (17) normal individuals and 11 (16) patients with NAITD. These 31 (33) sera were all negative for antibodies to TPO and to the PCA; their results are considered together as a group PCA negative. Similarly antibodies to C2 (C21) were measured in 15 (7) patients with AITD and 30 (21) patients having a variety of NTAID. All 45 (28) sera had antibodies to PCA but varied in their reactivity to TPO (see below). Their results are considered together as a group PCA positive.

Results

When C2 and control eluates were tested for binding to the C2 peptide (in an ELISA) and native TPO (in an RIA) only C2 eluates from sera having antibodies to both C2 and TPO were positive in the two assays. Representative examples are shown in Table 1.

Antibodies to the C2 peptide were not detected in 31 PCA-negative sera, but were present in 69% of 45 PCA-positive sera. Sera from 25 patients in the PCA-positive group did not contain antibodies to native TPO, but almost 50% of them had detectable levels of antibody to C2.

Antibodies to the C21 peptide were present, at a low level, in five patients in the PCA-negative group and in almost 100% of the 28 patients in the PCA-positive group (21 with NTAID, 7 with AITD). Individual results are shown in Fig. 1.

Table 1. Effect of C2 affinity purification on levels of antibody to C2 and TPO in various sera

	Unfractionated Serum	C2 Eluate	Control Eluate
Serum 1: Hashimoto's thyroiditis			
TPO Ab	4800	17	< 1
C2 Ab	79	80	14
Serum 2: idiopathic myxedema			
TPO Ab	1347	8	< 1
C2 Ab	46	39	15
Serum 3: Graves' disease			
TPO Ab	30	< 1	< 1
C2 Ab	11	11	11
Serum 4: normal individual			
TPO Ab	< 1	< 1	< 1
C2 Ab	13	21	18

Sera were incubated with filters saturated with C2 or control fusion proteins and bound antibodies eluted and tested for antibodies to thyroid peroxidase (TPO Ab) in a RIA and for antibodies to C2 (C2 Ab) in an ELISA. In the TPO RIA, values greater than 1 are positive, and for the C2 ELISA values greater than 33 are positive, both defined using a panel of sera from normal individuals.

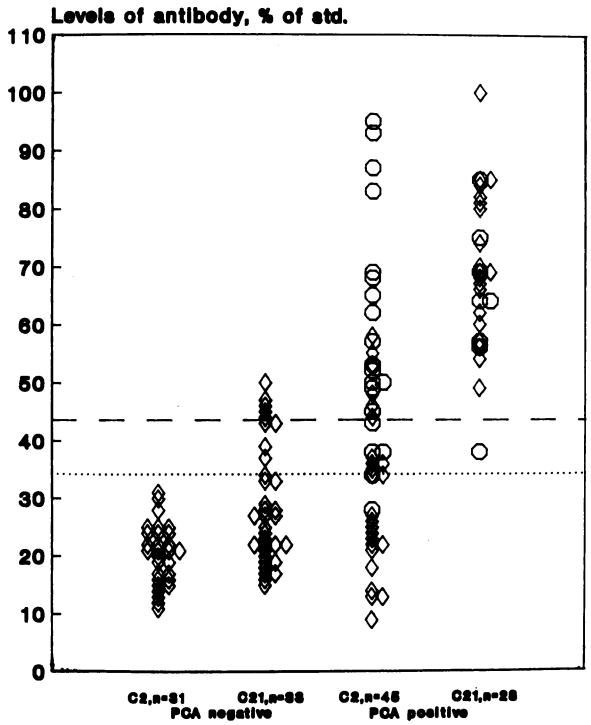

Fig. 1. Levels of antibody to C2 and C21 peptides of thyroid peroxidase in patients with and without antibodies to the gastric parietal cell. *Dotted line,* cut-off point for C2 (33%); *dashed line* that for C21 (44%); both defined as the mean + 2 standard deviations of a panel of normal sera. ◊, Sera negative for antibodies to TPO; ○, sera positive for antibodies to TPO

Discussion

In the affinity purification experiments in which 11 sera were absorbed onto filters saturated with C2 or control fusion proteins, only C2 eluates from patients whose unfractionated sera contained antibodies both to the C2 peptide and native TPO were positive for antibodies to C2 and TPO. This indicates that antibodies which bind to C2 are able to bind to the native TPO enzyme. The fact that control eluates were negative for the presence of both C2 and TPO indicates that nonspecific carry-over of antibodies to TPO could not explain the result observed in C2 eluates. Thus we have demonstrated that antibodies which recognize a peptide fragment are also able to bind to the protein in its native state [6], thereby reenforcing the evidence that the C2 fragment represents a major epitope of TPO.

In a previous study we noted that six out of nine patients with NTAID who had antibodies to PCA also had antibodies to C2; when these patients were assayed for antibodies to TPO, three were found to be positive, indicating subclinical thyroiditis, but of the remaining six 50% were positive for C2. In the present work we have extended and confirmed this initial observation and have shown that almost 50% of 25 patients with NTAID who are negative for TPO but positive for PCA also have antibodies to the C2 peptide.

The H^+ K^+ ATPase enzyme has recently been shown to be a major component of PCA [4], and comparison of its sequence [3] with that of C2 [2] reveals an 11-residue fragment in which six amino acids are identical, and three residues are conservative substitutions (Fig. 2). The fragment is found in both C2 and C21 peptides of TPO but seems to be more accessible in C21 since binding was observed in 100% of 18 patients with NTAID who were PCA positive but TPO negative.

This is strong evidence for an epitope in common between TPO and H^+ K^+ ATPase. Taken together, the data suggest that antibodies to the C2 fragment of TPO are heterogeneous. One subset recognizes a conformational epitope, and these antibodies are able to bind to native TPO. A second subset recognizes a sequential component, which is also present in C21, and which is found in the H^+ K^+ ATPase. Sera from AITD patients may contain either or both subsets of autoantibodies in varying proportions, which might explain the coincidence of antibodies to tissue-specific antigens.

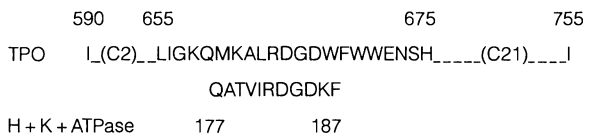

Fig. 2. Amino acid sequence of thyroid peroxidase in the region of C2 and C21 peptides showing the 11-residue fragment found in the H^+ K^+ ATPase enzyme of the gastric parietal cell

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Discussion

Bottazzo:
Obviously, the samples that you have found to cross-react with the gastric parietal cell antigen were negative for gastric parietal cell antibodies by conventional indirect immunofluorescence technique, were they?

Ludgate:
No. The antibodies that we tested were all positive for antibodies to gastric parietal cells.

Scherbaum:
I just wonder, if you state that they recognize the same epitope, did you look at the time course in these patients? Do the antibodies really move in a parallel fashion up and down? Such an observation would suggest that you are looking at cross-reactivity.

Ludgate:
No, I have not tested this. The data which I have shown represent one serum from one patient. We have not looked at any kind of variation of these antibodies during the course of either disease, autoimmune thyroid disease or gastric disease.

McLachlan:
Are these antibodies present in high concentration, or are they very low-level antibodies? What serum concentrations were you using?

Ludgate:
I used them at 1/100. Certainly in terms of antibodies to C_2 these we have titrated out to 1/1000, and some sera go beyond that. I have not done that yet with these sera.

McLachlan:
Can you be sure that when you do the elution study, you have removed all the antibody binding to the C_2? Perhaps what you have left on the C_2 affinity chromatography column is the very high affinity antibody that is difficult to remove?

Ludgate:
That is possible, and in fact, I have data in which I did a staining to show the binding of the antibodies before elution and after elution, and it looks quite clear that we are managing to elute off all those antibodies from the C_2 filter.

Furmaniak:
Talking about shared epitopes: Did you try to find out if any of C_2 or C_{21} is in high homology with some of the fragments of myeloperoxidase?

Ludgate:
Certainly C_2 is virtually intact in myeloid peroxidase, and obviously then C_{21} as well.

Environmental Effects on the Development of Autoimmune Thyoiditis

Mechanisms by Which Iodine Induces Autoimmunity*

R. S. SUNDICK, N. BAGCHI, and T. R. BROWN

Introduction

The evidence that iodine may be a causative agent in autoimmune thyroiditis is substantial. Persuasive data derive from studies of the epidemiology of thyroid autoimmunity in human populations as well as experimental manipulations of animal models. These are summarized in this report. We then discuss several hypothetical mechanisms by which excess iodine may induce thyroid autoimmunity. Finally, we propose a number of experiments using animal models and patients with Hashimoto's thyroiditis to test our hypotheses.

Epidemiological Evidence

Three epidemiological approaches have shown that when individuals increase their intake of iodine they exhibit an increased incidence of thyroid autoimmune phenomena. The first approach, a retrospective examination of thyroid histological specimens in the midwestern United States before and after the introduction of iodized salt, demonstrated a large increase in lymphocytic infiltration [1]. The second approach was a prospective study of endemic goiter patients in Greece. The subjects were tested for thyroid autoantibodies, then either injected with iodized oil [2] or provided KI orally [3], and again tested for thyroid autoantibodies. In both studies the incidence of thyroid autoantibodies increased in those individuals provided with exogenous iodine. Finally, cardiac patients treated with the iodine-containing drug amiodarone exhibited an increased incidence of thyroid autoantibodies and thyroid abnormalities (either hyperthyroidism or hypothyroidism) [4, 5]. It should be noted that in the first four of these five studies [1–4] the subjects lived in endemic goiter regions, and that during the course of the investigations they received excess iodide. It is possible that long-term adaptation to a low-iodide diet that occurred in the goiter region might have increased their susceptibility to high or even moderate iodide supplementation. However, in the fifth of these

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amiodarone studies [5] subjects from both goiter and nongoiter regions were treated with amiodarone, and in both populations the incidence of thyroid autoimmunity increased.

Animal Models

A number of animal studies have provided more direct evidence that iodide administration induces thyroid autoimmunity. We administered KI to cornell-strain (CS) chickens, a strain susceptible to late-onset spontaneous autoimmune thyroiditis. This induced a more rapid onset of disease, characterized by autoantibodies to thyroglobulin (Tg), triiodothyronine (T_3), and thyroxine (T_4) and lymphocytic infiltration [6].

We then performed a series of experiments to determine whether depletion of iodide from the diet of the severely afflicted Obese strain chicken would reduce or prevent spontaneous autoimmune thyroiditis. We provided them at hatching with a normal diet supplemented with $KClO_4$ (to block transport) in the drinking water (0.1%) and T_4 (to suppress TSH) in feed (50 μg T_4/100 g). This reduced the incidence of antibodies to Tg, T_3, and T_4, but it did not reduce lymphoid infiltration [6]. Hypothesizing that the failure to reduce infiltration may have been due to the transfer of large amounts of iodide into the egg and subsequently the embryonic thyroid gland, we repeated this experiment with a group of Obese strain chicks that had been derived from hens provided a lower iodide diet. These chicks were treated with the same $KClO_4$ and T_4 regimen as previously reported and then tested for Tg antibody by a radiobinding assay and lymphoid infiltration (greater than 15% of the gland infiltrated by mononuclear cells; 20 chicks/group). The results (Table 1) show that this treatment caused a dramatic reduction in autoantibodies and infiltration. Subsequent experiments on several batches of Obese strain chickens with the same regimen has yielded variable results, and we are currently testing whether the accumulation of iodide in the embryonic thyroid gland is already sufficient to initiate severe autoimmune thyroiditis within 2 weeks of hatching.

We have tested two other antithyroid drugs, propylthiouracil (PTU) and aminotriazole, for their ability to prevent autoimmune thyroiditis in Obese strain chickens. Both drugs allow active transport of iodide into the gland, but

Table 1. Effects of $KClO_4$ and T_4 on spontaneous autoimmune thyroiditis in Obese strain chickens

Diet	Percentage of chicks with:	
	Tg antibody	Thyroid infiltration
Normal	68	89
$KClO_4$ (0.1% in water) + T_4 (50 μg/100 g feed)	10	10

Table 2. Effects of antithyroid drugs administered from hatching on thyroiditis in Obese strain chickens

Diet supplement	Antibody to Tg (% specific binding)	Thyroid infiltration (mean ± SEM)
None	18.1 ± 5.9	88.5 ± 8.9
Propylthiouracil (0.1% in water)	5.6 ± 3.0	22.2 ± 7.6
Aminotriazole (0.2% in feed)	8.2 ± 4.7	23.8 ± 11.9

Antibody ot Tg: the amount of ^{125}I-labeled low-iodine Tg specifically precipitated by 1 µl of serum; 9–11 chickens per group.

they interfere with the thyroid peroxidase catalyzed iodination of Tg. We administered each drug in the food or drinking water from the day of hatching. At 5 weeks of age both drugs had caused a severe drop in serum T_4 and reduced Tg antibodies and infiltration (Table 2).

Similar studies have been performed on BB/WOR rats that spontaneously develop autoimmune thyroiditis and diabetes. The findings of these studies are as follows (a) CS chickens fed KI exhibited an increased incidence of Tg, T_3, and T_4 antibodies and lymphocytic infiltration [6]. (b) $KClO_4$, PTU, and aminotriazole treatments of Obese strain chickens reduced the incidence and/or magnitude of Tg antibody and lymphocytic infiltration. (c) BB/WOR rats, intact or hemithyroidectomized, and fed KI exhibited an elevated incidence of lymphocytic infiltration [7–9]. (d) Treatment of BB/WOR rats with methimazole reduced the incidence of lymphocytic thyroiditis [10]. (e) Buffalo strain rats fed KI exhibited an increased magnitude of Tg antibody and lymphocytic infiltration [11]. In general the effects of iodine depletion and iodine supplementation parallel the effects on lymphoid infiltration in the chicken model. However, Tg antibody produced by the BB/WOR rats is not affected by changes in intrathyroidal iodine. Interestingly, another rat strain with spontaneous autoimmune thyroiditis, the Buffalo strain, when fed KI developed more Tg antibody and lymphocytic infiltration [11].

Genetic Susceptibility to Iodide-Induced Thyroiditis

It is appropriate at this time to emphasize the importance of genetic factors in the susceptibility to iodide-induced thyroiditis. While we do not know the location or the function of the relevant genes, it is clear from studies of human populations, chickens, and rats that only certain individuals or animal strains respond to high doses of iodide by developing autoimmune thyroiditis. CS chickens, for example, but not four normal chicken strains developed iodide-induced thyroiditis (unpublished observations). Very high doses of iodide (amiodarone, KI tablets for nuclear fallout victims, radiopaque con-

trast media) induced autoimmune thyroiditis in a very low percentage of individuals [5, 12, 13]. Also, the BB/W and Buffalo strain rats but not normal rats respond to KI [7, 8].

Hypothetical Mechanisms by Which Iodide Induces Autoimmune Thyroiditis

Two mechanisms are of current interest to us. The first is based on the widely known fact that the iodine content of Tg is approximately proportional to the thyroidal intake of iodide. By variations in the iodide content of the diet Tg can be isolated from individuals that contains 50 or more atoms of iodine per molecule Tg or as little as 5 atoms per molecule. These extremes in the I/Tg ratios are known to affect the stability of Tg [14] and its tertiary structure [15]. Our first hypothesis was that the intake of excess dietary iodide resulted in a highly iodinated Tg molecule which was highly immunogenic. We tested this hypothesis by placing CS chickens on high (20 mg KI/dl drinking water) or low (0.1% PTU in drinking water) iodide regimens for 1 month [16]. Tg was prepared from the respective thyroid glands, analyzed for its I/Tg ratio, and then injected intravenously into normal chickens. The sera were tested for antibodies to high-iodine Tg (HI-Tg), low-iodine Tg (LI-Tg), and to the thyroid hormones T_3 and T_4. In two separate experiments HI-Tg elicited more antibody than LI-Tg to Tg. Furthermore, the antibody that was produced reacted with HI-Tg but not with LI-Tg. Many of the antibodies elicited in response to HI-Tg also reacted with T_3 and T_4. Interestingly, the few chickens that did respond to LI-Tg produced antibodies that reacted much better with HI-Tg than with LI-Tg.

The importance of the iodine content of the Tg molecule for Tg-specific T cells and for the initiation of thyroid infiltration was recently determined by analysis of experimentally induced thyroiditis in mice [17]. Champion et al. [17] demonstrated that murine T cell hybridomas responded to Tg in direct proportion to the iodine content of the Tg. They also determined that immunization of mice with LI-Tg plus LPS adjuvant was ineffective in inducing thyroiditis, while Tg of normal iodine content plus lipopolysaccharide caused thyroiditis.

Despite the inherent simplicity of the above hypothesis, and the possibility that it plays a role in some cases of Hashimoto's thyroiditis, there are three lines of evidence that the creation of a highly iodinated Tg molecule is not a prerequisite for autoimmune thyroiditis. First, Obese strain chickens (in contrast to iodide-fed CS chickens) produce antibodies that react at least as well, if not better, with LI-Tg than with HI-Tg [16]. Second, patients with Hashimoto's thyroiditis do not have Tg with a high iodine content. In fact, several studies have shown that the Hashimoto's thyroid gland has an unusually low amount of iodine [18, 19]. Furthermore, a significant amount of the iodine present in the Hashimoto's thyroid gland is inorganic, i. e., it is dischargable by $KClO_4$ with or without exogenous iodide [20]. Finally, a number of Hashimoto's thyroiditis

patients have a strong antibody response to the thyroid microsomal antigen but no detectable response to Tg.

An additional hypothesis was therefore needed to explain those instances in which elevated iodide intake resulted in autoimmune thyroiditis through a mechanism in which the immune response was not directed specifically to highly iodinated Tg. We were impressed by the studies of FOLLIS [21] who treated hamsters for at least 37 days with PTU to stimulate secretion of endogenous TSH and then gave KI in the absence of PTU. These animals displayed acute and severe leukocytic infiltration of the thyroid gland. This work was confirmed and expanded by MAHMOUD et al. [22], who showed that the iodide transported into the stimulated thyroid gland of mice was organified, and that it induced rapid thyroid cell necrosis. Lipofuscin granules were observed in the epithelial cells, which is an indication of free radical damage.

The above studies suggested the following hypothesis. The thyroidal intake of excess iodide under certain environmental or genetic conditions leads to the production of reactive oxygen intermediates or iodine radicals that either damages membrane proteins or lipids of thyroid epithelial cells or iodinates membrane proteins other than Tg. These events may lead to leukocyte infiltration and eventually sensitization of autoreactive T and B cells (Fig. 1.).

Our hypothesis may raise more questions than it answers, but there is considerable circumstantial evidence for it. First, it is well established that at the apical (follicular) surface of the thyroid epithelial cell the thyroid peroxidase enzyme catalyzes the iodination of Tg in the presence of H_2O_2 (produced by the thyroid cell). Second, it is postulated that reactive iodine and oxygen species are generated during this process. Third, several studies indicate that patients with Hashimoto's thyroiditis transport iodide into the thyroid gland, but that their total thyroidal iodine content is low [18, 19], the I/Tg ratio is low [23], and significant amounts of iodide remain in their thyroid glands as inorganic iodide [20]. This is compatible with the idea that in the presence of excess iodide, the peroxidase enzyme of the Hashimoto's thyroiditis patient may iodinate membrane proteins (e. g., tubulin; [24]) and may cause peroxidation of membrane lipids. By extrapolation from in vitro experiments [24] it appears that peroxidase enzymes, in the presence of peroxides and iodide, can iodinate cell membranes,

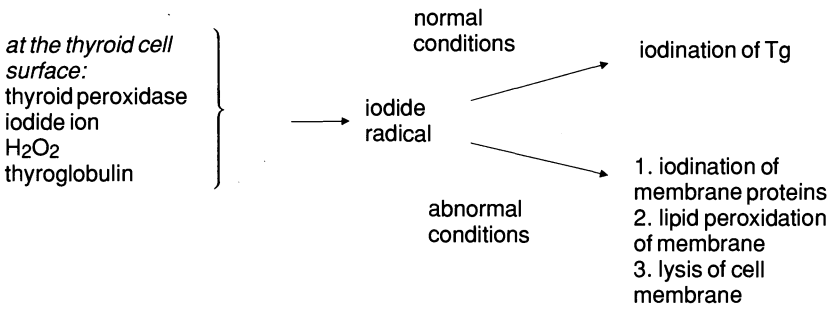

Fig. 1. Hypothesis to explain iodide-induced damage to thyroid cells

Table 3. Effects of antioxidants on thyroid autoimmunity in newly hatched Obese strain chickens

Additions to diet	Percentage of chicks with antibody to: Tg	T_3 or T_4	Percentage of chicks with infiltration > 25%
None	93.3	80.0	92.8
BHA (1% in feed)	30.8[a]	15.4[a]	46.1[b]
PTU (0.1% in water)	13.3[a]	13.3[a]	13.3[a]

significance, χ^2 test, treated versus controls.
[a] $p < 0.001$; [b] $p < 0.01$

cause peroxidation of membrane lipids (analyzed by the generation of malondialdehyde), and induce cell lysis. In the in vitro system PTU blocked all three phenomena. The antioxidant butylated hydroxyanisole (BHA) blocked lipid peroxidation and cell lysis, but it did not prevent iodination of membrane proteins.

We decided to test one aspect of the above hypothesis, using Obese strain chickens as a model. In particular, we tested whether BHA could prevent disease. We had already demonstrated that depletion of iodine from the thyroid gland of Obese strain chickens (by the use of $KClO_4$, PTU, or aminotriazole) reduced the severity of their disease. Accordingly, chicks at hatching were provided a diet supplemented with BHA (1%). They were tested at 5 weeks for autoantibodies and thyroid infiltration.

They were bled and sacrificed at 5 weeks. Serum was considered Tg antibody positive if 1 µl specifically precipitated more than 3% of the radiolabled LI-Tg. There were 13–15 birds per group. BHA reduced autoantibodies and thyroid infiltration (Table 3), which suggested that oxidative damage plays a role in this disease. In other studies (manuscript in preparation) we learned that BHA must be given to the chicks prior to infiltration, since administration after the onset of disease had no effect. BHA did not appear to act at the level of the immune system since it had no effect on antibody responses to the injected antigens, sheep red blood cells, or *Brucella abortus.* BHA was also studied for its effects on normal thyroid function, and it had no demonstrable effects.

Additional Experiments Needed to Test the Above Hypothesis

As already stated, our hypothesis raises at least as many questions as it answers. However, some questions can be experimentally tested. For example, can one detect elevated levels of oxygen radicals (or the result of oxygen radical damage) in the thyroid glands of Obese strain chickens or BB/WOR rats prior to lymphocyte infiltration? Does excess iodide increase free radical generation? Does one find unusual iodinated proteins in the thyroid cells of Obese chickens or patients with Hashimoto's thyroiditis? Do the T cells of Hashimoto's thy-

roiditis patients (or Obese chickens) respond more strongly to thyroid cells in vitro in the presence of high concentrations of iodide? To determine whether the defect in iodine organification of patients with Hashimoto's thyroiditis is a primary event or secondary to immune-mediated cell damage or the presence of antibodies to peroxidase one could test the young female children of Hashimoto's thyroiditis patients for their total thyroidal iodine content (using the noninvasive flourescent scanner; [18]) and for microsomal antibodies.

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Discussion

McLachlan:
The Obese chicken is born with a thyroid which is going to be destroyed very quickly because the antibody is transferred in egg. If you had actually bred from chickens which had been treated with your antioxidant, I wonder whether, in fact, you would see an even lower incidence in the Obese chicken of thyroiditis in the offspring. Because, if you do it in a chicken which is hatching, you are actually a little bit late, aren't you? I wonder if you had done these experiments.

Sundick:
You bring up two points: First, there is some thyroglobulin antibody transferred into the egg of some of the Obese strain chickens, so some newly hatched chicks already have antibodies, but when we have measured this antibody, it was present in low incidence so we don't think that applies generally to the Obese chicken. Concerning your second point, that the initiation of treatment at hatching may be too late to completely prevent disease, we completely agree. While we can show that antioxidants and antithyroid drugs can reduce thyroiditis when mixed in the food, even though the chicks begin eating about 2 days after hatching, we do have concerns that we are administering these drugs too late. There is a trial going on now in my laboratory, in which we have administered perchlorate into the chick embryo, because there is a tremendous incorporation of iodine into the embryonic thyroid starting at 14 days of embryonic life. So the experiment is in progress, and I don't know whether that is a more effective regimen than treatment commencing at hatching.

Burger:
You used aminotriazole in some experiments; aminotriazole, besides inducing hypothyroidism, is antioxidant. Could you speculate on this as a side effect? Do you know acatalatic animals have more antibodies?

Sundick:
You make a very good point. That is one reason we tested aminotriazole, for it has the two effects. Since it so effectively prevented autoimmune disease we would hypothesize that it acts primarily by reducing iodine, and even if it is increasing the catalase within the thyroid, the net effect is the reduction of disease. As far as acatalytic animals or people, I have no information about that.

Höfer:
Could you quantitate the expression "iodine excess with respect to inducing thyroiditis?" What do you mean by excess?

Sundick:
The doses we are using to induce disease in C strain chickens are very high doses of iodine. They are not as high as Allen and Apple used in the BB/W rats, but they are much higher than normal levels. The other side of the story is the Obese strain chicken, which develops a severe thyroiditis on a normal iodine level, but if we prevent iodine from getting into their thyroid or being put into thyroglobulin by use of any of these antithyroid drugs, we can reduce the amount of infiltration and antibody.

Goiter and Autoimmune Thyroiditis in Iodine-Sufficient Areas*

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Introduction

Goiter continues to occur, despite adequate iodine supplementation, in the coal-rich Appalachian area of eastern Kentucky in the United States [1–3] and in many shale- and coal-rich localities of western Colombia [4]. The incidence of lymphocytic autoimmune thyroiditis (AT) has steadily increased in the United States during the past five decades, and this has been attributed to iodine prophylaxis since 1924 and to excessive iodine intake [1, 5–7]. In western Colombia, where iodine prophylaxis was instituted in 1955, a similar trend has been documented, both in autopsy (1959–1972) and surgically (1953–1973) removed thyroid glands [8]. However, lymphocytic infiltration was of high degree in all the cases in the United States [7] while it was minimal in 71.4% of the Colombian cases [8]. Urinary iodine excretion values from 1960 to 1974 ranged between 108 and 633 μg/gCr in schoolchildren and between 229 and 758 μg/gCr in adults living in the areas of western Colombia where the autopsy and surgical materials had been obtained [4, 9–12], However, retrospective studies of this kind have differences in methodology and design which limit the ability to make valid comparisons. Thus, cross-sectional and prospective studies designed to reassess the problem of goiter and AT in areas of eastern Kentucky and western Colombia, where goiter persists despite iodine supplementation, have been conducted since 1983. Nearby localities where goiter has not been a problem were similarly investigated.

Subjects and Methods

Cross-Sectional Study. Studies using identical methodologies [4] were conducted in 1983–1984 in 1331 children (equal number of each sex, aged 7–17 years) in Bourbon, Breathitt, and Owsley Counties of Kentucky and in five of the localities previously surveyed in western Colombia [4] (Table 1). In-

* This work was supported by grants from the Veterans Administration Medical Research Service of the United States and the National Foundation for Science and Technology of Colombia (COLCIENCIAS).

formed consent was obtained from parents or guardians of these children. All subjects were examined by at least two physicians. Thyroid size was graded by the modified WHO classification [13] and measured using the thyroid surface outline method [14]. Coefficient of variation between observers was 6%–14%. Blood samples were collected from 35%, and spot urine samples (between 9 A.M. and 1 P.M.) from 40% of the children were examined. Thyroid hormone measurements consisted of TSH using Serono Diagnostics immunoradiometric assay kit (Norwell MA, USA) which incorporates two high-affinity monoclonal antibodies providing increased levels of specificity and sensitivity (detection limit, 0.02 mU/l); Thyroxine (T_4) and triiodothyronine (T_3) using competitive radioimmunoassay procedures performed on antibody-coated tubes from Clinical Assay (Travenol, Cambridge, MA) and thyroid hormone binding ratio (THBR-T_3) for calculation of free thyroxine index using a competitive binding assay from commercially available kits (Clinical Assay, Travenol, Cambridge, MA). Serum antithyroglobulin (TgAb) and thyroid antimicrosomal-peroxidase (TMAb) antibodies were measured by passive hemagglutination using Thymune-T and Thymune-M kits (Wellcome Diagnostics, Dartford, UK). The test was considered positive when the titer was equal to or higher than 1:10 for TgAb and 1:100 for TMAb. Urine samples were analyzed for iodine and creatinine as previously described [4, 9]. Fine-needle aspiration cytology was obtained by experienced surgeon (H. G.) and pathologist (E. D.) from both thyroid lobes of 50 Colombian individuals with goiter (grades 1 b, 2, and 3; 32 children and 18 immediate family members, 21–63 years old) in the localities of Alaska, Vijes, and Darien.

Prospective Study. A prospective study using the same methodology described above was conducted in a cohort of 173 (69 goitrous and 104 nongoitrous) children from Breathitt and Owsley Counties in Kentucky, and among 62 (45 goitrous and 17 nongoitrous) children from Alaska, Vijes and Darien in Colombia.

The results were analyzed statistically by one-way analysis of variance, the Student-Newman-Keuls multiple range test, and the χ^2 test after adjustment for age, sex, and percentage with and without goiter [15].

Results

Results show significantly higher goiter prevalence ($p < .001$) in the eastern coal-rich Appalachian area of Owsley and Breathitt counties than in the central limestone-rich area of Bourbon County in Kentucky (Table 1). Similarly, goiter prevalence was significantly higher ($p < .001$) in the localities of Alaska, Vijes, and Darien (predominantly sedimentary rocks: organic-rich shales, silstones, and cherts) than in La Cumbre (predominantly igneous rocks: organic-free basalts and granite) in western Colombia [4, 16]. Results also indicate that goiter and AT are intimately related in Kentucky children, as demonstrated by

Table 1. Goiter and autoimmune thyroiditis among schoolchildren in Kentucky and Colombia (1983–1984)

Localities	Population (1984)	Number of children examined	Goiter[a] (%)	TSO (cm^2, mean)	TgAb/ TMAb[b] positive (%)	Urinary iodide (μg/gCr, mean)[c] ± SD
Kentucky		(735)				
Bourbon Co.	19203	(262)	8.0	8.8	3.0	237 (±81)
Breathitt Co.	16569	(273)	22.8[d]	8.9	7.0[e]	268 (±100)
Owsley Co.	5656	(200)	35.5[d]	9.8	13.0[d]	268 (±121)
Colombia		(596)				
La Cumbre	13000	(156)	5.1	7.4	0.1	267 (±91)
La Habana	4330	(65)	13.8[d]	7.7	0	345 (±115)
Vijes	10524	(163)	20.8[d]	8.9	0.4	310 (±112)
Darien	8014	(155)	22.4[d]	9.2	0	282 (±109)
Alaska	250	(57)	43.9[d]	8.8	0	333 (±197)

TSO. Mean thyroid surface area of goiters in each locality. Significance, as compared to Bourbon Co. and La Cumbre.
[a] Grades 1b and 2 (WHO classification).
[b] Thyroid autoantibodies.
[c] To convert μgI/gCr to μmol I/nmol Cr, multiply by 0.00089.
[d] $p < 0.001$.
[e] $p < 0.05$.

presence of positive thyroid autoantibodies (TgAb mean titer 1:100, range 1:10–1:400; TMAb mean titer 1:2750, range 1:100–1:10000) in one-third of children with goiter. Of positive thyroid autoantibodies, 70% were found in children with the largest goiters (grade 2) and only 6% in nongoitrous individuals. Goiters with negative TgAb and TMAb were distributed equally between boys and girls, both in Kentucky and in Colombia, while positive thyroid autoantibodies were three times more frequent among girls.

In contrast with the Kentucky children, low prevalence of AT was documented in the Colombian goitrous children by negative TgAb and TMAb ($p < 0.001$ as compared to those in Breathitt and Owsley Counties) and fine-needle aspiration cytologies. Results are summarized in Table 2. Material was

Table 2. Results of thyroid fine-needle aspiration cytologies of 50 goitrous subjects from western Colombia

	n	Percentage
Adequate material	45	90
Colloid	32	71
Cellular pleomorphism	27	60
Cytoplasmic enlargement	23	51
Pyknosis	22	49
Cytoplasmic melanin-like granules	11	24
Macrophages	10	22
Lymphocytes	2	4

Histologic diagnosis: diffuse colloid goiter, 32 (71%); lymphocytic thyroiditis, 2 (4%).

Table 3. Thyroid hormones in serum of children in Kentucky and Colombia (1983–1984)

Locality	*n*	TSH (mU/l)[a]	TSH (mU/l; % > 5.5)	Free thyroxine index[a]	Triiodothyronine (nmol/l)[a]
Kentucky					
Bourbon Co.	112	2.6 (0.8–5.4)	0	8.1 (4.9–11.8)	2.7 (1.8–3.5)
Breathitt Co.	88	2.7 (0.1–15.6)	3.4	7.4 (4.4–17.2)	2.3 (1.7–4.8)
Owsley Co.	73	6.2 (0.1–182.0)	11.0	8.1 (2.8–12.4)	2.4 (1.8–3.0)
Colombia					
La Cumbre	43	3.1 (1.4–7.9)	4.6	9.5 (6.0–12.6)	2.5 (1.6–3.6)
La Habana	23	3.5 (0.3–6.1)	4.4	8.1 (6.3–10.8)	2.8 (2.3–3.4)
Vijes	59	3.7 (1.6–12.3)	8.5	9.3 (5.3–12.4)	2.4 (1.4–3.3)
Darien	50	2.6 (1.1–5.1)	0	9.1 (4.8–12.0)	2.3 (1.6–3.2)
Alaska	12	3.8 (1.5–6.8)	8.3	8.7 (6.7–11.7)	2.7 (2.0–3.4)
Normal range		0.2–5.5		4.0–13.0	1.2–3.0

[a] Student-Newman-Keuls multiple range test for differences in Kentucky and Colombian data, nonsignificant.

adequate in 45 (90%) individuals, providing histologic diagnosis of diffuse colloid goiter in 32 (71%) and lymphocytic thyroiditis in only two children (4%) who had positive TgAb and TMAb.

Urinary iodine excretion values (Table 1) which indicate more than adequate iodine intake, were similar among the eight study groups in Kentucky and Colombia. Furthermore, no difference was found between goitrous and nongoitrous subjects.

Thyroid hormone measurements in serum were comparable between the Kentucky and Colombia groups (Table 3). However, the highest percentage (11%) of TSH values above normal level (> 5.5 mU/l) and the highest TSH concentrations were observed in Owsley County with the highest prevalence of goiter and AT. Thyroid autoantibodies (TgAb and TMAb) were positive in 44% of individuals with above-normal TSH levels, while TgAb and TMAb were positive in only 2.5% of those with normal TSH ($p < 0.0001$).

The prospective study indicated in Kentucky an incidence rate of 8% of goiter development and 11% of goiter disappearance over a 12-month period; the thyroid situation was unchanged in 91% of these children, including those with AT. Ten out of 11 goitrous subjects with positive thyroid autoantibodies (TgAb and/or TMAb) remained the same at 2–5 years in the follow-up study; elevated TSH levels was present in six of these children, two of them requiring treatment with L-thyroxine. In Colombia an incidence rate of 12% of goiter development and 4% of goiter disappearance was observed for a 24-month period; the situation was unchanged in 94% of the children examined. Urinary iodine values in Kentucky ($n = 113$; mean 234 ± 146 µg/gCr) and Colombia ($n = 40$; mean 255 ± 119 µg/gCr) were similar to those found in the initial study.

Discussion

This study demonstrates significantly different prevalence rates of goiter and AT in nearby localities equally supplemented with iodine. Results also show that goiter and AT are intimately related in certain community areas while in others with equal goiter frequency and iodine intake AT is not prevalent.

These findings agree with previous observations by other investigators indicating persistence of goiter despite adequate iodine intake in Owsley [2] and Breathitt [3] Counties of eastern Kentucky and high prevalence of positive TMAb (20%) and elevated TSH (28%) in schoolchildren with goiter from Breathitt County [3]. Similarly, persistently, high titers of TMAb and TgAb, with elevated TSH levels in a significant number of goitrous children in our study fulfills the diagnostic criteria of AT. Actually, the presence of thyroid autoantibodies (TMAb and TgAb) constituted a risk marker of clinical or subclinical hypothyroidism. The increased frequency of AT in goitrous children of eastern Kentucky is also consistent with histologic findings demonstrating that lymphocytic AT has steadily increased in the United States during the past five decades [1, 5–7]. Our finding of low goiter prevalence in Bourbon County is also in agreement with earlier observations indicating that central Kentucky is not part of the endemic goiter region [17], with most cases of goiter coming from the mountainous area in eastern Kentucky.

Results in Colombia also agree with our previous observations on the persistence of goiter in certain areas where sufficient iodine supplementation has been in effect for more than three decades [4]. The low incidence of AT demonstrated by negative thyroid autoantibodies (TMAb and TgAb) and Fine-needle aspiration cytologies among children in the study areas of western Colombia is in full agreement with results obtained in the whole population of the same areas [18] and with previous findings in the city of Cali in western Colombia [11]. Recently a low incidence of lymphocytic AT (3.8%) using histologic criteria was documented by other investigators in a group of 103 goitrous children and young adults in northern Colombia [19]. Our results are also consistent with histologic findings in surgical and autopsy material from western Colombia [8]. It is of interest that on Hokkaido Island in Japan colloid goiter was the dominant histopathologic type of the endemic coast goiter attributed to exceedingly high dietary iodide, and that histologic findings were not consistent with lymphocytic thyroiditis [20–22].

Finally, our results clearly demonstrate that Kentucky and Colombian goiters are not due to iodine deficiency, and that iodine supplementation is not the primary environmental cause of AT. Other region-specific environmental factors (i. e., organic and microbial water pollutants) [2, 4, 23–33], host and immunogenetic predisposing conditions [34–37] working in concert with adequate or increased iodine intakes, appear to be responsible for the significantly different prevalence rates in goiter and AT among nearby localities as well as between the Kentucky and Colombian populations.

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Discussion

Orgiazzi:
This is a methodological question: I have been surprised by the high prevalence of goiter. Did you include in the goitrous population the 0b stage of the WHO classification?

Gaitan:
We have used the modified WHO classification with stages 0, for nonpalpable thyroid gland, and 1 a, 1 b, and 2, which are goiters in increasing order of size. We have included only the stages 1 b and 2 to calculate goiter prevalence rates. Those of stage 1 a, which were not included, are small, palpable, but not visible thyroid glands with an area of less than 7 cm^2 using the thyroid surface outline (TSO) method. We have correlated the WHO classification with this latter, more objective method of calculating thyroid size. Therefore, thyroid glands reported as goiters in this study are definitely enlarged, visible, and easily palpable.

Höfer:
You say that it is not iodine deficiency, but as far as I could see from your slides, you speak of the iodine content in the urine per microgram of creatinine. But this does not imply that there isn't possibly an iodine deficiency, as you said yourself in your last slide, because the iodine was not biologically available to the thyroid gland. Have you looked at the iodine in the thyroid?

Gaitan:
Yes, we have done extensive studies in Colombia for several years, which are already published [World Rev Nutr Diet (1973) 17: 53–90]. We performed not only direct measurements of iodine in the thyroid glands of children, but also kinetic studies. Although the concentration of iodine (mg/g wet tissue) is decreased in goitrous glands, its content for the whole gland is equal to that of normal thyroid glands. The histology also is not consistent with that of an iodine-deficient gland. Microscopically, these diffuse goiters are characterized by areas of focal hyperplasia surrounded by dilated follicles filled with dense colloid, suggesting an adequate iodine intake. The serum TSH and free thyroxine index (FT_4I) values in most of these children are within the normal range, with median TSH and FT_4I values being not significantly different between goitrous and nongoitrous individuals. The thyroid response to exogenous TSH is

equally similar in both groups. Therefore, most goiters occur in these children in the presence of normal parameters of thyroid function. Now, dietary iodine intake ranged between 100 and 600 μg per day as indicated by urinary iodine excretion values. Changes in thyroid function should probably be more apparent if this iodine was not available to the gland, for instance, by the action of potent blockers of iodide utilization. Although antithyroid organic pollutants have been isolated from water supplies of the Kentucky and Colombia study areas, the data support the concept that besides TSH, thyroid-growth promoting effects from other factors may also be involved in goitrogenesis of these children.

Phillips:
Presumably you are going to plan nutritional studies on these schoolchildren?

Gaitan:
Yes, we have already done two types of studies. First, goiter prevalence among schoolchildren in the town of Candelaria in western Colombia was found to be high or low according to the district where these children lived. However, detailed studies indicated that the dietary composition, calorie and iodine intakes were similar in both groups, permitting us to conclude that these nutritional variables could not account for the significant difference in goiter prevalence. Second, the body mass index (wt/ht^2) which is a simple and reliable indicator of nutritional status was measured in the present study. The values obtained were not significantly different in children with and without goiter both in Colombia ($p = 0.530$) and the United States ($p = 0.668$). Furthermore, results of previous studies among 929 schoolchildren from 37 localities in western Colombia [Ecol Dis (1983) 4: 295–308] had similarly shown that none of the nutritional variables, namely height, weight, arm skinfold thickness, or body mass index were significantly related to goiter prevalence.

Phillips:
Wasn't this area somewhere near that where the outbreak of thyrotoxicosis from hamburgers occurred?

Gaitan:
No that was in Minnesota and South Dakota, located to the northwest of Kentucky. The Appalachian area of eastern Kentucky, where we conducted our study, has been known to be an endemic goiter area for many years. The whole length of the Appalachian range and the states bordering on the Great Lakes are parts of the "goiter belt," one of the areas with the highest goiter prevalence in the United States before the iodization of salt was introduced in 1924. Vought and collaborators in the 1960s and Hollingsworth and collaborators in the 1970s demonstrated persistence of goiter in Owsley and Breathitt counties of Kentucky, respectively, in spite of more than adequate iodine intake. Thyroid autoantibodies were documented in one-third of the goitrous children in Breathitt County at that time. These are reasons why we selected this area for

the present study. Similarly, since 1960 we have performed cross-sectional and prospective studies in 41 communities of western Colombia. Because of the follow-up data from previous years we selected from them the five localities for the present study. Thus, for sure we are dealing with an endemic situation, where goiter has persisted in spite of iodine prophylaxis, and not with epidemic or episodic outbreaks as it was the case with hamburger thyrotoxicosis.

Bottazzo:
What was the interval between iodine implementation, especially in Colombia, and the time when you conducted the study. How many years went by?

Gaitan:
Colombia introduced the iodization program in 1955, and we conducted the present study from 1984 to 1987, or after 30 years.

Bottazzo:
So the iodine was there in an optimal amount for many years?

Gaitan:
Yes, no question.

Scherbaum:
What is your opinion on the possibility that iodine itself could be the potential environmental factor responsible for triggering thyroid autoimmunity?

Gaitan:
I think there is good circumstantial evidence in the United States of a progressive increase in incidence of lymphocytic thyroiditis after the iodization program was begun in 1924. This trend was documented using clinical and histological criteria in studies conducted by the Mayo Clinic in Olmsted County, Minnesota, from 1935 through 1967, and also by Weaver and collaborators in the State of Michigan. In western Colombia a similar trend was documented both in autopsy and surgically removed thyroid glands after iodine prophylaxis was instituted in 1955. However, lymphocytic infiltration was of high degree in all U. S. glands, namely grades 3 and 4, while it was minimal in 70% of the Colombian cases. Roughly, lymphocytic infiltration was ten times less frequent in the Colombian thyroid glands. So I think that iodine is certainly a predisposing factor causing autoimmunity, but host and other environmental factors appear to play also a primodial role in development of lymphocytic autoimmune thyroiditis.

Prevalence of Postpartum Thyroiditis in two Iodine-Deficient Regions of Germany

H. LÖBIG, W. BOHN, J. MAU, and H. SCHATZ

Introduction

In the past decade, disorders of thyroid function during pregnancy and especially in the postpartum period have been the subject of several studies in Japan [1], Scandinavia [2, 3], the United Kingdom [4] and North America [5]. Detection of antibodies against different thyroid antigens, studies of lymphocyte subsets in blood and thyroid tissue, HLA studies and histological methods have led to the conclusion that these disorders are also influenced by an autoimmune process [6].

It was the aim of our study to evaluate the prevalence of thyroid antibodies and thyroid dysfunction in pregnancy and postpartum for two regions of Germany.

Methods

As sufficient iodine supply is considered to be associated with a higher prevalence of autoimmune thyroiditis [7–10], two different regions of Germany were compared: Kiel on the Baltic Sea, an area which has been thought to be well supplied with iodine, and the Giessen area in central Germany, a recognized endemic goitre region with iodine deficiency.

Two practising gnyaecologists from each area sent us blood samples of all women attending their practice, in pregnancy and postpartum, from January 1987 to April 1989. In this randomized study 983 sera of 110 women from Kiel and 317 women from Giessen were determined for total thyroid hormones, thyroxine (T_4) and triidothyronine (T_3) by means of commercial kits (T4-RIA, T3-RIA, H. Biermann, Bad Nauheim, FRG), for free tyroxine (fT_4; two-stage assay RIA-gnost FT4, Behringwerke, Marburg, FRG) and for basal TSH (TSH-IRMAclon, Henning Berlin, FRG; detection limit 0.03 mU/l). In addition, antibodies to microsomal antigen (MAb; PROMAK, positive > 300 U/l), to thyroglobulin (TAb; THYRAK, positive > 100 U/l) and to the TSH receptor TBIAb (TRAK, positive > 13% factor) were measured (all with kits from Henning, West Berlin).

Following the time patterns of routine examination of both gynaecologists, four study periods were distinguished: first and third trimena of pregnancy, 1–

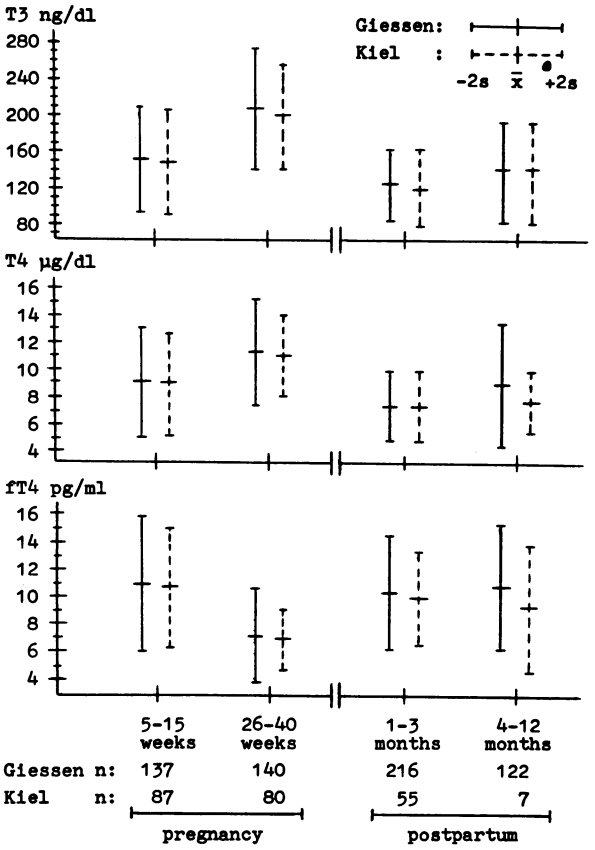

Fig. 1. Normal ranges for T_3, T_4 and fT_4 established for four study periods (means ± 2 standard deviations)

3 months postpartum and 4–12 months postpartum. For each period normal ranges (mean ± 2 standard deviations) for T_4, T_3 and fT_4 were established. There was scarcely any difference between the two study regions (Fig. 1). While the mean total hormone values increased clearly during pregnancy and decreased again after delivery, the fT_4 values showed the opposite changes, presenting a significant decrease during pregnancy with a postpartum increase. As to the changes in TSH values, there was a significant increase during pregnancy. Hypothyroidism was defined as latent with TSH over 3 mU/l and peripheral thyroid hormones values within normal range, as manifest with TSH over 3 mU/l and thyroid hormones below the normal range. Latent hyperthyroidism was diagnosed with TSH under 0.1 mU/l and thyroid hormones still withn the normal range, as manifest with TSH under 0.1 mU/l and thyroid hormones clearly above the normal range.

Cases of autoimmune thyroiditis or Graves' diseases known before pregnancy were excluded from the study. Besides cases of intake of thyroxine tablets showing suppressed TSH and/or elevated thyroid hormone values and cases of intake of oral contraceptives postpartum showing spuriously

high total T_3/T_4 values were considered during the evaluation of thyroid function.

The iodine supply in the two regions was compared by means of a small sample of volunteers (Kiel, $n = 23$; Giessen, $n = 26$), whose morning urine was determined for iodine concentration using the Cer-Arsenit method [11].

Results

Table 1 presents the frequency of each kind of antibody at four different periods in Giessen and Kiel. The antibody prevalence was high during the first trimenon, decreased during pregnancy and rose again in the postpartum period. MAb were most often found, and more distinctly in Kiel. TAb occurred less frequently and TBIAb in only a small number of cases. There were no major differences in antibody prevalence between Giessen and Kiel. Unfortunately, owing to local reasons, blood samples of only a few selected women were available from Kiel in the late postpartum period; thus no conclusions can be drawn from these figures.

Figure 2 shows the median (and first and third quartiles) of the concentrations of MAb in a cross-section of only those women who were positive for MAb. It can be seen that not only the prevalence of antibodies (Table 1) but also their concentrations decreased during pregnancy and rose sharply some months after delivery.

Table 1 also demonstrates the prevalence of thyroid dysfunction in our sample during pregnancy and postpartum. Hypothyroidism, especially in manifest form, was rare during pregnancy. It occurred more frequently in the late postpartum period (up to 3.5% in Giessen). Most cases were mild and were often overlooked clinically. Hyperthyroidism, mainly latent, was observed more frequently during the first trimenon of pregnancy. There was no difference in the prevalence of thyroid dysfunction in Giesen and in Kiel. Latent hyperthyroidism, however, was more often found during the first trimenon in Giessen.

The prevalence of thyroid antibodies in cases of thyroid dysfunction depended very much on the kind and the moment of occurrence of a dysfunction. Thus, the prevalence of MAb and/or TAb in women who showed a dysfunction during pregnancy was hardly higher than in the total sample. The later a dysfunction occurred after delivery the more often antibodies could be found. In hypothyroidism the prevalence of thyroid antibodies was about twice as high than in hyperthyroidism. Altogether MAb and/or TAb were positive in about 40% of cases of postpartum thyroid dysfunction (PPTD) (latent and manifest, hypo- and hyperthyroidism).

It has been suggested that one could determine MAb as a risk factor for the possible event of PPTD [3, 4, 12]. In this study approximately one-third of women who had MAb and/or TAb developed – as far as examined – a latent or manifest dysfunction during the postpartum period. The incidence of PPTD

Table 1. Prevalence of thyroid antibodies and thyroid dysfunction in pregnancy and postpartum period

		Antibodies against			Hypothyroid		Hyperthyroid	
		Microsomes	Thyroglobulin	TSH receptor	Latent	Manifest	Latent	Manifest
	n	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Kiel								
1st Trimenon	140	7.9	4.3	0	0.7	0	7.9	2.9
3rd trimenon	145	5.5	1.4	0	2.8	0	2.8	0.7
1–3 months postpartum	229	4.8	3.0	0.4	0.9	0	2.2	1.8
4–12 months postpartum	141	12.1	6.4	1.4	2.8	3.5	1.4	1.4
Giessen								
1st trimenon	88	11.4	3.4	1.1	1.1	1.1	3.4	2.3
3rd trimenon	85	5.9	1.2	0	2.4	1.2	1.2	0
1–3 months postpartum	69	7.2	1.5	0	2.9	0	0	1.5
4–12 months postpartum	7[a]	57[a]	28[a]	0[a]	14[a]	14[a]	0[a]	14[a]

[a] Selected cases.

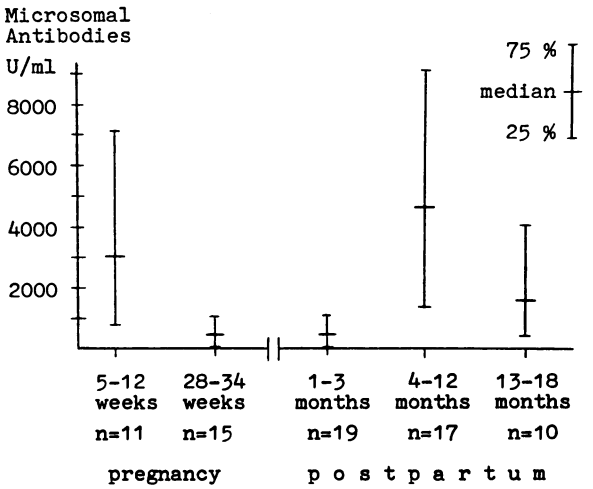

Fig. 2. Concentrations of MAb in women with MAb during and/or after pregnancy (cross-sectional, medians), ranging from first (25th percentile) to third (75th percentile) quartile

rises to 67% if high concentrations of MAb over 1000 U/l were measured during the early postpartum period (1–3 months postpartum). There were, however, also cases of MAb concentrations up to 20000 U/l which never showed a dysfunction.

Discussion

Owing to a complicated system of immune modulation and suppression during pregnancy, the fetus in utero has been called "nature's most successful transplant" [13]. Some of these changes in pregnancy can be measured, such as the decrease in total lymphocyte count [14], the decrease of the T-helper/T-suppressor ratio [14, 15] and lowered IgG and IgA serum levels [16], but the suppressive factor itself is still unknown [13, 17, 18].

For the treatment of many autoimmune diseases [19–21], and thus also Graves' disease, autoimmune thyroiditis and postpartum thyroiditis (PPT) [22–25], the immunological changes are important, for these disorders often undergo remission during the second and third trimena but exacerbate a few months after delivery. During this "rebound" cases which have so far been subclinical can become manifest if certain predisposing factors such as a certain HLA type [26–29] or a positive family history of autoimmune diseases (contradictory reports) [2–4, 30] are present.

For different countries, different HLA-DR antigens have been described as being associated with the PPT syndrome [6, 7, 27, 29]. The existence of such an HLA type is thought to be combined with the defect of suppression of those B cell clones which are responsible for the production of MAb [7, 31, 32]. These MAb have destructive character and can cause hypothyroidism after short hyperthyroid phases [3, 6, 29]. Owing to self-regulation the dysfunction is most-

ly transient, but it can persist in few cases (up to 13% or 17% [2, 26]) if the process of destruction perpetuates itself.

While hyperthyroid hormone values were not unfrequently found during the first trimenon, hypothyroidism was predominant in the late postpartum period. The prevalence of thyroid dysfunction was lowest in the third trimenon and in the early postpartum period. As both the infertility rate and the abortion rate are higher in thyroid dysfunction [33, 34], we were surprised to find such a high prevalence of biochemical hyperthyroidism during the first trimenon, especially in Giessen. Mori et al. [35] found that increased fT_4 and hCG serum levels and decreased TSH levels in early pregnancy correlated highly with the severity of morning sickness, which is not unusual in early pregnancy. The authors assessed the tendency to increased fT_4 and decreased TSH levels during the first trimenon as a sign of a thyroid gland which is physiologically activated – possibly by hCG – without pathological significance.

If one compares our results with other cross-sectional studies on PPT (1–3, 5] (Table 2), the postpartum prevalence of MAb and TAb lies approximately within the same range of 6%–12%, while the prevalence of biochemically manifest PPTD of 2% (1–3 months postpartum) to 4.3% (4–12 months postpartum) in our study is lower than in other countries. This difference can be explained partly by the failure to notice short, transient PPTD episodes in our study. On the other hand, there is a very high iodine supply in some parts of the countries compared, which is regarded as intensifying autoimmune thyroiditis [7–10]. The percentage of antibody-positive cases among PPTD (33% and 67%) is also lower than in the studies compared.

Hayslip et al. [12] recently reported findings 7% MAb on the 2nd day postpartum among 1034 cases studied. Of those women with MAb titers of at least 1:400, 86% showed a dysfunction until 6 months postpartum, and 54% needed therapy. The authors pointed to the significance of MAb measurement as a screening test for PPTD. This can be supported only with reservation by our study for the regions examined. Firstly, the sensitivity of MAb/TAb measurement concerning the occurrence of PPTD was only one-third and 67%, respectively, if MAb concentrations over 1000 U/l were measured 1–3 months postpartum. Secondly, the observed dysfunction reached mostly only mild stages and rarely needed therapy. However, one must keep in mind the possibility of PPTD when MAb have been found.

Table 2. Studies of postpartum thyroiditis

Reference	*n*	Study-period months postpartum	MAb/TAb	PPTD	MAb/TAb in PPTD
Amino et al. 1982	507	3–8	12%	5.5%	89%
Jansson et al. 1984	460	2–7	10%	6.5%	76%
Nicolai et al. 1987	238	2–3	8%	6.7%	56%
Lervang et al. 1987	591	3	6.5%	3.9%	87%
Giessen + Kiel	298	1–3	6.4%	2.0%	33%
Giessen only	141	4–12	14%	4.3%	67%

In 1975, a goitre prevalence was reported for the regions of Kiel and Giessen of 4% and 19%, respectively, suggesting a difference in iodine supply with a north-south gradient [36]. Unexpectedly, no major difference was found between our two study regions, neither in the prevalence of thyroid dysfunction nor in the prevalence of thyroid antibodies. During the course of our study it was reported by Gutekunst et al. [37] that northern Germany is also an area of moderate iodine deficiency. Determination of iodine excretion in urine and ultrasound measurements of the thyroid gland showed that the suspected north-south gradient of endemic goitre and iodine supply no longer exists [37, 38].

With a small sample of volunteers from Kiel ($n = 23$) and Giessen ($n = 26$) we were able to confirm these findings. Means and medians of iodine excretion in morning urine ranged for both regions between 60 and 70 μg iodine per gram creatinine, corresponding to an iodine deficiency of the first degree in the WHO classification [39]. These findings can be explained partly by a change in consumer habits, such as increased consumption of cafeteria food and ready-cooked meals of supermarket chains instead of fresh products bought on the (fish-) market.

In conclusion, manifest thyroid dysfunction during pregnancy and especially postpartum can be found in Germany but with a prevalence 2%–4% less than in other countries. The use of MAb measurement as a screening test can be supported for Germany only with reservation, particularly since the PPT syndrome is mostly transient and mild. The same degree of PPTD in the north and centre of Germany can be explained by similar consumer habits, resulting in the same extent of moderate iodine deficiency in both areas.

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Discussion

Drexhage:
May I ask you, what was the rationale to perform this study? Have you hoped for a difference between the two areas in Germany because there was a difference in iodine intake, but did it turn out later that the two areas were similar?

Löbig:
The aim of our study was to evaluate the prevalence and significance of postpartum thyroiditis in two region of Germany which were originally considered to be different in iodine supply. The basis of this assumption was an extensive study in 1975 which reported a goiter prevalence of 4% and 19% for the areas where Kiel and Giessen are located. As iodine deficiency is the major cause of goiter development in Germany, it was concluded that there is also a difference in iodine supply. Thus we checked the prevalence of postpartum thyroiditis for these two regions, as an association of iodine intake with the development of autoimmune thyroiditis was often reported. Later, both regions turned out to be insufficiently supplied with iodine.

Hüfner:
Several authors have reported on a hyperthyroid phase during development of hypothyroidism. Have you noticed such a hyperthyroid period before the hypothyroid status?

Löbig:
Generally the phases of dysfunctions were mild in most cases, and we could not confirm the results from Japan, where extreme hyperthyroid phases were seen before the hypothyroid phase. In our study the hyperthyroid cases were mainly latent, which means a TSH below 0.1 U/ml and peripheral hormones within the normal range and these being biochemically manifest, with elevated peripheral hormones showing only few symptoms.

Phillips:
It is extremely difficult to compare these studies of postpartum thyroiditis because it depends on how you do the study. We find particularly in our own work in Cardiff that the hyperthyroid phase can be very very short indeed and will only be detected if you do the thyroid function tests very frequently. How did you actually define thyroid dysfunction?

Löbig:
We used merely biochemical definitions. The TSH limits were 0.1 and 3 mU/l, and whether peripheral hormones were within or beyond the normal ranges determined latent or manifest dysfunction. But you are absolutely right: differences in study periods and intervals make it very difficult to compare the prevalence of postpartum thyroid dysfunction, as short phases of dysfunction can be overlooked easily.

Phillips:
Is this from a single estimation? When you find one raised TSH in a patient, do you define that as postpartum thyroiditis?

Löbig:
You must differentiate between postpartum thyroiditis, which generally means that the woman has thyroid antibodies, and postpartum thyroid dysfunction. In our study not all women with postpartum thyroid dysfunction also showed thyroid antibodies.

An Immunosuppressive Factor Sharing Homology with the p15E Protein of Leukomogenic Retroviruses is Present in the Serum of Patients with Graves' Disease

M. TAS, M. DE HAAN-MEULMAN, and H. A. DREXHAGE

Introduction

Approximately 30% of $CD14^+$ and nonspecific esterase-positive cells from the blood monocytic pool are able to differentiate into cells with a morphology and marker pattern of dendritic cells when cultured under nonadhering conditions. An exposure of the cells to metrizamide or thyroid hormones prior to the culture period enhances this differentiation step, and 40%–60% of cells with a dendritic morphology and marker pattern are obtained. The induced dendritic cells are functionally active, which is indicated by the fact that the cell population containing the dendritic cells has an enhanced stimulator capacity in mixed leukocyte reactions and an increased cluster capacity [1].

The term dendritic cell has been used to describe a group of large nonlymphocytic, class II MHC-positive mononuclear cells that appear to act as accessory cells in immune response. This group comprises the Langerhans' cell in the epidermis and dermis of the skin, the interdigitating cell in lymphoid organs such as lymph nodes, spleen, and thymus, and the veiled cell in lymph [2].

The dendritic cell is of crucial importance in the presentation of antigen during the elicitation of primary immune responses. Secondary responses can probably be sustained by any cell having class II determinants, such as macrophages, B cells, and T cells, as well as epithelial cells expressing aberrant class II determinants [2]. The most characteristic feature of the dendritic cell in comparison to other class II positive cells is its capability to actively form cellular clusters with immunocompetent cells in its vicinity and thus to create a microenvironment suitable for optimal antigen presentation and T and B cell activation [2].

In Hashimoto's goiter, Graves' disease, and sporadic nontoxic goiter (which we consider an autoimmune thyroid disease) the numbers of intrathyroidal dendritic cells are higher compared to those in the normal gland, and these dendritic cells are clearly positive for RFD1 and L25, markers for active dendritic cells. The cells are often seen in contact with a few intrathyroidal lymphocytes, forming small lymphoid cell clusters. They are also found in the T cell zones of larger, well-organized intrathyroidal lymphoid structures (focal thyroiditis). Moreover, on ultrastructural examination the dendritic cells in Graves' glands, Hashimoto's goiter, and sporadic nontoxic goiter are similar to the interdigitating cells present in secondary lymphoid organs. This suggests an active involve-

ment of dendritic cells in the immune process in the thyroid of patients with autoimmune thyroid disease [3].

We recently found that dendritic cells isolated from the peripheral blood of patients with Graves' disease, primary myxedema, and sporadic nontoxic goiter showed a decreased capability to form cellular clusters with allogeneic lymphocytes, while the recovery of viable dendritic cells was not different from controls. Simultaneously, three Graves' disease patients showed an absent expression of RFD1 on peripheral dendritic cells. Moreover, the chemotaxis of monocytes in Grave's disease, as measured with the polarization, was also decreased (in press).

These findings are seemingly contradictory to our findings on thyroid-infiltrated dendritic cells. There are a few possibilities to explain these results. One is that the function of dendritic cells in thyroid autoimmune disease is intrinsically disturbed, and that the high numbers of dendritic cells infiltrated in the thyroid represent the very active dendritic cells. Another explanation is that because of exaggerated immune activity in the thyroid gland, counterregulation mechanisms keep the autoimmune process localized and exert an immunosuppressive effect on peripheral blood dendritic cells.

Chemotaxis disturbance of peripheral monocytes (i. e., lowered monocyte polarization) and also a lowered cluster capability of dendritic cells have been well documented in AIDS [4, 5], in immunodeficiencies accompanying various types of malignancies [6–8], and in chronic purulent rhinosinusitis [9]. The presence in serum of factors capable of inhibiting the function of both lymphocytes and monocytes has been reported, particularly in malignancies. Factors molecular weight under 25 kDA (low molecular weight factors, LMWF) capable of inhibiting interleukin-2 production and monocyte chemotactic responsiveness were detected in serum, urine, and cancerous effusions of cats [10], mice [11], and man [12, 7, 13]. These factors appeared to share structural homology with the feline and murine retroviral transmembrane protein p15E [12], as could be shown by adsorption studies using monoclonal antibodies against this immunosuppressive viral protein.

The hydrophobic transmembrane protein p15E is highly conserved among many type C and type D retroviruses. A structural homology between p15E and transmembrane components of other retroviruses has recently been described [14]. A number of human cell lines derived from lymphocytic and monocytic neoplasms as well as normal PHA-stimulated lymphoblasts were found to produce p15E-like factors [14]. Apparently p15E-like factors can be produced endogenously by lymphocytes, monocytes, and squamous epithelial cells; moreover the factors could also be detected by immunohistochemical methods in epithelial cells overlaying areas of inflammation [15] and in normal thymic epithelial cells, monocytes, and spleen macrophages (unpublished observations). This suggests that the p15E-like factors play a role in normal immune regulation.

This paper describes the presence of p15E-like factors (influencing monocyte polarization and dendritic cell clustering) in the serum of patients with Graves' disease.

Patients and Methods

Eleven patients with Graves' disease (aged 17–65 years) had on first clinical presentation clinical hyperthyroidism, raised serum thyroxine and triiodothyronine levels, decreased serum TSH or absent TSH responses to TRH, and a diffuse non-nodular appearance of the thyroid on palpation, scan, or ultrasonography. Controls were 34 healthy laboratory personnel (aged 22–43).

Sera were collected from the patients and controls by venapuncture and diluted 1 : 1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 centriflo cones (Amicon, Danvers, USA) for 15 min at 700 g (molecular weight cut-off point 25 kDa). The residues were resuspended and stored at − 70°C until further use.

To validate the p15E-like character of the LMWF's adsorption experiments were carried out by incubating the serum fractions with a combination of two p15E-specific monoclonal antibodies (see below) in a final dilution of 1 : 200 at 4°C for 16 h, followed by Amicon ultrafiltration to remove formed complexes; this adsorption procedure was carried out twice [13]. The monoclonal antibodies used were a combination of 4F5 and 19F8 (anti-p15E isotypes IgG2a and IgG2b; kindly provided by Dr. G. J. Cianciolo, Genentech, South San Francisco CA, USA).

Donor blood monocytic cell fractions were obtained via counterflow centrifuge elutriation. In brief, mononuclear cells were separated from 450 ml whole blood via Percoll (Pharmacia Diagnostics, Uppsala, Sweden) centrifugation (20 min, 1000 *g*, room temperature). Thereafter the mononuclear cells were injected into an elutriation centrifugation system (Beckman J21 centrifuge with a JE-6 elutriation rotor). The elutriation medium was PBS with 13 m*M* trisodium citrate and 5 mg human albumin per milliliter. To separate the different cell populations, the flow rate was kept constant at 20 ml/min, while the rotor speed decreased from 4000 to 0 rpm. The fraction collected at 2500 rpm contained 93%–97% monocytes, as judged by positivity for nonspecific esterase activity. This fraction was used in further experiments after storage in liquid nitrogen.

The Cianciolo and Snyderman [6] assay was performed with slight modifications [8]. Aliquots (0.2 ml) of suspensions containing 0.2×10^6 monocytes were added to 12 × 75 mm polypropylene tubes (Falcon Labware Division, Becton Dickinson, Oxford CA, USA) containing 0.05 ml either RPMI 1640 (Gibco, Glasgow, UK) or *N*-formyl-methionyl-leucyl-phenylaline (FMLP; Sigma, St. Louis MO, USA) in RPMI, to reach a final concentration of 10 n*M*. All experiments were carried out in duplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml icecold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification 250 ×). The test was read "blindly" by two persons; 200 cells were counted from each tube. A cell was considered "polarized" if any of the following occurred: elongated or triangular shape, broadered lamellopodia, membrane ruffling. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\text{ total cells polarized}}{\%\text{ NSE positive cells}} \times 100\%$$

The ability of the serum fractions to inhibit FMLP-induced polarization of healthy donor monocytes was determined by incubating the monocytes (1×10^6/ml) for 15 min at 37°C either with FMLP alone or with FMLP in combination with a serum fractions (final dilution 1:60). Addition of serum fractions alone to donor monocytes did not affect the polarization. The polarization test was performed as described above, and the percentage of inhibition of FMLP-induced polarization minus spontaneous polarization caused by addition of the serum fractions was calculated.

Cells from the elutriation-purified monocytic fractions were exposed to metrizamide (Serva, Heidelberg, FRG) for 30 min at 37°C and 5% CO_2 in polypropylene tubes. Thereafter, the cells were washed (culture fluid added slowly to prevent osmotic lysis of the cells), and further cultured for a period of 16 h in polypropylene tubes (5% CO_2 and 37°C, 100% humidity). The population contained 60% cells (on average) with a dendritic morphology and were more than 90% viable (trypan blue).

A quantity of 50×10^3 dendritic cells were allowed to cluster with 5×10^3 allogeneic lymphocytes (4 h, 37°C and 5% CO_2) in 250 µl flat-bottomed wells. Formed clusters were counted using an inverted microscope and expressed as the number of clusters per six microscopic fields (× 200). A cluster was defined as containing 4–20 cells. Control lymphocytes/monocytes/PBL never clustered.

The capability of LMWF's to inhibit the cluster capacity of dendritic cells was determined by incubating the metrizamide-treated, elutriator-purified monocytes (dendritic cells: 5×10^5/ml) with a serum fraction (final dilution 1:6; 4 h, 37°C, 5% CO_2). The percentage of inhibition (i) was calculated as follows:

$$i = 1 - \frac{F \times 100\%}{N}$$

where F is the number of clusters in presence of LMWF's and N is the number of clusters in presence of medium.

Results and Discussion

Figure 1 shows the effects of serum fractions of molecular weight under 25 kDa on the polarization of healthy donor monocytes and on the cluster capability of healthy dendritic cells with allogeneic lymphocytes. The LMWF's of patients with Graves' disease clearly inhibited the polarization (57% ± 8% versus 14% ± 6%; $p < 0.01$) and also had an inhibitory effect on the cluster capability of dendritic cells (30% ± 13% versus 12% ± 5%; $p < 0.01$). The inhibitory effects were neutralizable with a monoclonal antibody directed against p15E (see fig. 1). A monoclonal antibody with the same isotype against an unrelated antigen had no effect (data not shown).

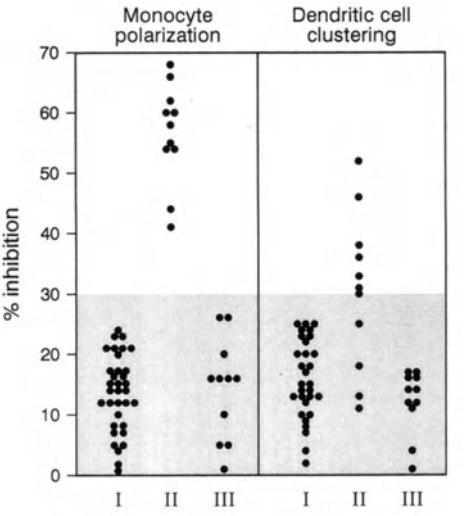

Fig. 1. The effects of serum fractions under 25 kDa on the polarization of healthy donor monocytes and on the cluster capability of healthy dendritic cells with allogeneic lymphocytes. *I,* Healthy control serum fractions; *II,* Graves' serum fractions; *III,* Graves' serum fractions after adsorption with anti-p15E

The origin of the p15E-related factors is speculative. The possibilities range from exogenous infection with an as yet unknown retrovirus possessing envelope substances that share structural homology with p15E to an endogenous production of the factors; it is known that p15E-related factors can be produced by monocytes and by thymic and diseased epithelial cells [15].

Recently the expression of retroviral sequences in thyrocytes and lymphocytes has been implicated in the pathogenesis of thyroid autoimmune disease. Chickens of the Obese strain develop a hereditary spontaneous autoimmune thyroiditis which closely resembles human Hashimoto's disease. Analysis of the genome of these animals revealed a new endogenous avian leukosis virus sequence (ev22), which was suggested to have a modulatory role in the development of the disease [16]. Ciampolillo et al. [17] found that on Southern blotting of DNA extracted from thyroid glands of five patients with Graves' disease, probes for *gag* HIV gave a positive hybridization signal. Their findings suggested the presence of a novel retrovirus in Graves' disease, and the retrovirus-like sequences seemed to be closely associated with thyroid autoimmunity.

The relationship between the p15E-like factor described here and the genomic expression of the ev22 and HIV *gag* retroviral sequences needs further clarification.

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Discussion

Sundick:
You say that this immunosuppressive factor is also present in increased incidence. Is this correct?

Drexhage:
Yes.

Sundick:
But yet, as far as I know, there is no general immunosuppression occurring in Graves patients.

Drexhage:
Well, this factor suppresses monocyte chemotaxis and dendritic cell clustering and has also some effects on NK cell activity. It does not necessarily mean that the patient is generally immunosuppressed. This factor suppresses certain functions of certain cells.

Atkins:
Is there any homology between this p15E-like factor and tumor necrosis factor, which certainly is a factor that can be produced by tumors, and which has been shown to inhibit neutrophil chemotaxis in vitro?

Drexhage:
There is no hemology between p15E and tumor necrosis factor. It turned out that there was a partial homology between p15E and gp41 of HIV, P21, and an endogenous human DNA sequence, clone 4-1.

Scherbaum:
Have you looked for immunosuppression by parameters other than monocyte chemotaxis, for example, proliferation?

Drexhage:
Yes, there are extensive studies going on regarding this topic. The factor suppresses monocyte chemotaxis, dendritic cell clustering, and NK cell activity; some investigators report on IL-2 production suppression, and the factor also

has effects on DTH reactivity. Hence there is a whole body of effects, but no one actually knows the mechanisms of action.

Bottazzo:
Were your Graves patients treated and euthyroid, or were they untreated, newly diagnosed cases?

Drexhage:
These Graves patients were newly diagnosed. We only collected the few that you saw.

Bottazzo:
Do you think that high circulating levels of thyroid hormones could somehow influence the expression and production of this endogenous factor?

Drexhage:
This could well be. We do not know what actually triggers the production, where the factor comes from. One might think it could be the Graves thyrocyte because normal thyrocytes are capable of producing it; at least we have shown immunoreactivity of normal thyrocytes with anti-p15E antibodies. However, in Graves glands we found a lower expression of the factor. We would like to verify this biochemically, but it is unlikely that the Graves thyrocytes produce it. Another thing I want to say is that in sporadic goiter we had hoped to find the factor, but it is not there. So p15E production might have something to do with the thyroid status, but we also find p15E-like factors in the other diseases I mentioned.

Bottazzo:
Did I recall correctly that, when you presented this data in London a few months ago, you mentioned that p15E was overexpresed in normal thyroids, but that its content was reduced in Graves glands.

Drexhage:
Correct, it is still like that. We have extended these studies with more Graves thyroid samples, but, as I said, we need to verify our immunohistochemical data with biochemical isolation procedures.

Hashimoto's Thyroiditis and Enteropathogenic *Yersinia enterocolitica**

B. E. WENZEL, R. GUTEKUNST, and J. HEESEMANN

The-main stream interest in autoimmune thyroid diseases (AITD) is focused on hypotheses such as clonal deletion, the network theory or the cellular regulatory circuit of helper and suppressor cells.

At times, the dogmatic view has prevailed that autoimmunity would be identical with auto-aggression, implying that autoimmunity would reflect a pathological dysregulation of the normal "healthy" immune system. The frequent reference to "aberrant" MHC class II expression by epithelial cells is part of the bias; it considers the MHC class II expression of epithelial cells as a consequence of a primary immune defect rather than a manifestation of a secondary effect of an immune mechanism which could have a physiological role.

Moreover, the conception of organ-specific autoimmunity was predominantly influenced either by animal models with mostly systemic immune defects or by in vitro or in vivo experimentally manipulated immune models. Both approaches produced a prevailing interest in genetic deficiencies or genetic predisposition in patients with organ-specific autoimmune disorders. Very often the theory collided with the clinical observations, the course of the disease or the results of treatment in man.

Recently, clinical and experimental evidence has accumulated which suggests antigenic homologies between plasmid-encoded release proteins from enteropathogenic *Yersinia enterocolitica* (YE-RPs) and thyroid antigens.

Most virulence properties of enteropathogenic YE, such as cell adherence, phagocytosis and serum resistance and cytotoxicity, are encoded on a 42- to 46-MDa plasmid. The plasmids of YE and other *Yersinia* strains are closely related on the basis of DNA homologies. Chromosomal genes, however, appear to contribute and/or to control plasmid-mediated pathogenicity of YE [1]. The plasmid of enteropathogenic YE codes for proteins of the outer membrane (YOP 1–5) [2], and also for proteins secreted in vitro in Ca^{2+}-deficient cultures of YE (RP 1–5) [3] (Fig. 1).

Evidence for antigenic homologies between antigens of thyroid epithelial cells (TECs) – i. e. the TSH receptor – and RPs was derived from experiments using ligand blotting with ^{125}I-TSH on RP blots, from the radioligand receptor assay with RP antibodies (RP-Ab) and solubilized TSH receptor protein or

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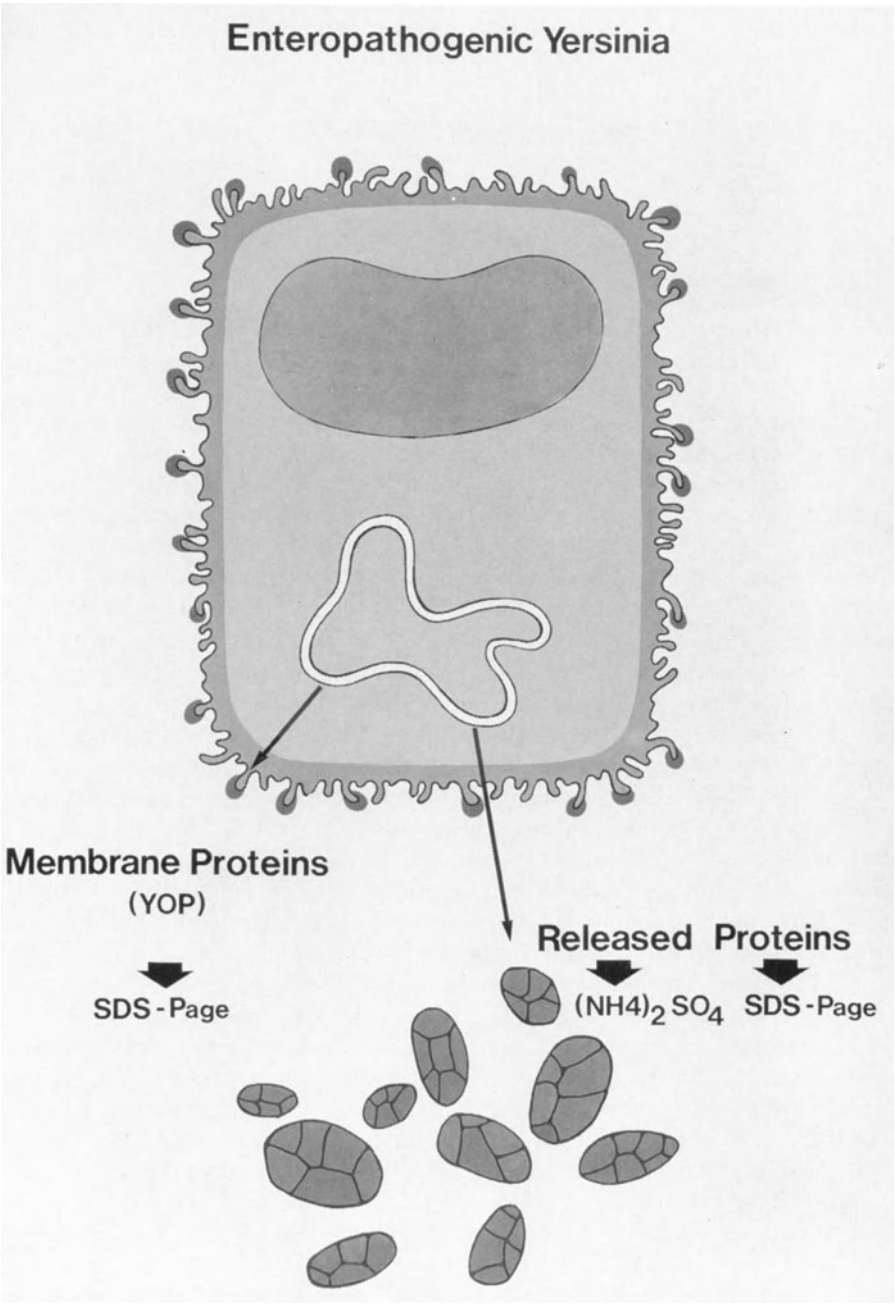

Fig. 1. Enteropathogenic *Yersinia enterocolitica* and expression of release proteins. These are secreted in vitro after Ca^{++} deprivation of nutrient medium

from applying RP-Ab in the indirect immunofluorescence test on TECs from patients with Graves' disease (GD) [4, 5].

YE-RP-Ab raised in rabbits showed one or two specific bands in Western blots with TEC homogenates from TSH-stimulated cell culture. Using purified thyroid peroxidase (TPO) or thyroglobulin (Tg) in Western blots, one band emerged which could be blocked by preincubation with the appropriate antigen (TPO or Tg) [6].

Western blotting with sera from patients with AITD on YE-RPs revealed a high prevalence of RP-Ab in patients with GD. This prevalence comprised RP-Ab of IgG class and IgA class antibodies. The latter indicated that those patients with AITD had had a recent infection with enteropathogenic YE [7]. No RP-Ab were found to develop in serum when patients who presented with overt GD hyperthyroidism for the first time and who had TSH receptor antibodies were followed up for 2–6 weeks after diagnosis. Thereafter, GD patients developed IgG and IgA RP-Ab sequentially against all RPs [4].

The RP-negative sera from the first 6 weeks after diagnosis of GD were reassessed on Western blots. This time RPs of enteropathogenic YE of all three commonly encountered serotypes (0:3, 0:8, 0:9) were used. In seven out of eight patients' sera, RP-Ab of IgM specificity showed one band in Western blots with YE serotype 0:9 but not with the other serotypes [6]. Serotype 0:9 of enteropathogenic YE is frequently found in Germany as a cause of yersiniosis.

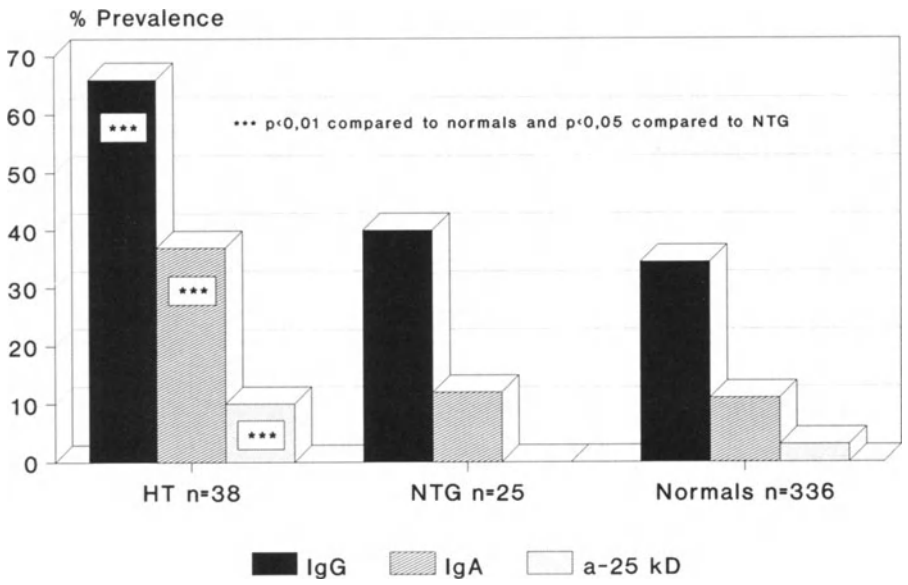

Fig. 2. Prevalence of antibodies to plasmid-encoded proteins of enteropathogenic *Yersinia enterocolica* in sera of patients with Hashimoto's thyroiditis (HT), nontoxic goiter (NTG) and normals

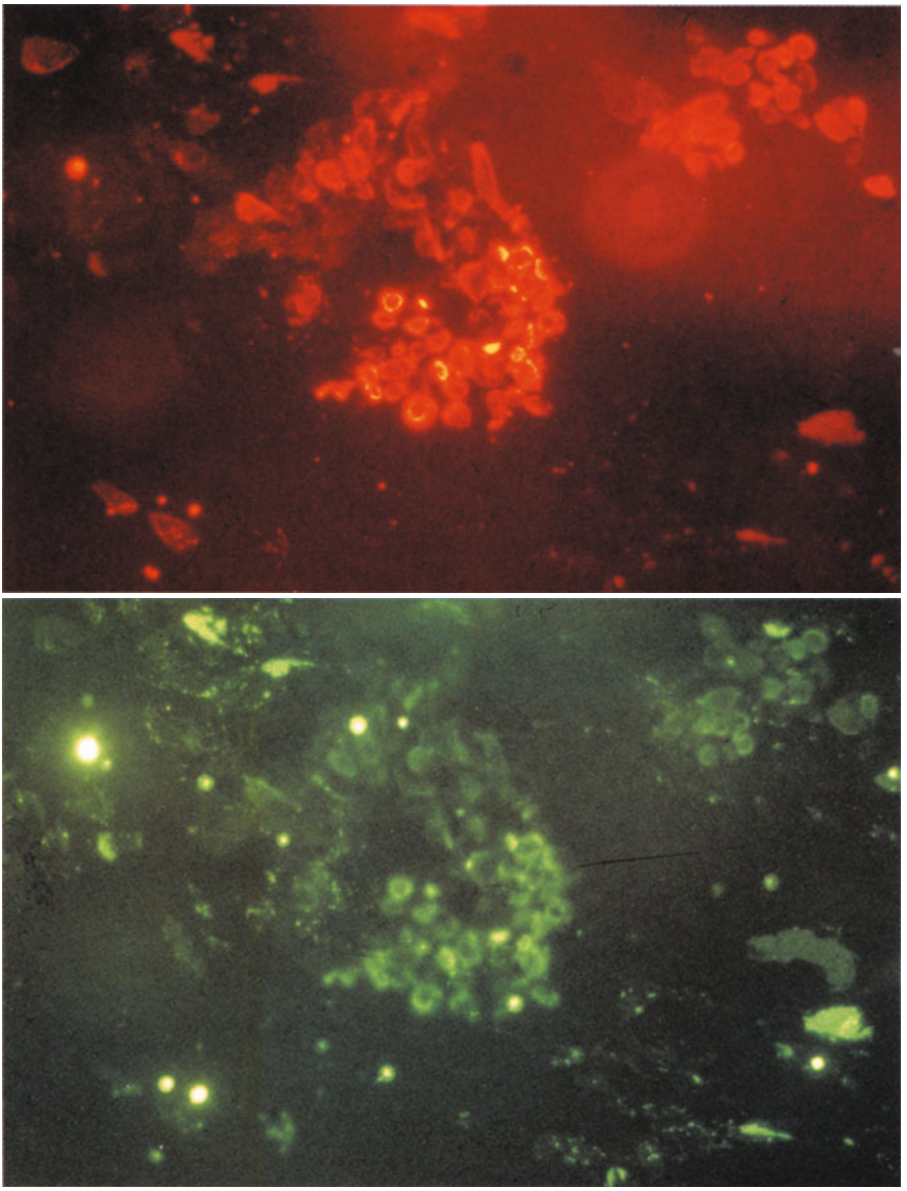

Fig. 3 a, b. Indirect immunofluorescence of thyroid epithelial cells derived from thin-needle aspiration biopsies of HT goiters. **a** Anti-YE-RP raised in rabbits; **b** anti-microsomal/TPO from an HT patient

These findings may indicate that even on the plasmid level, serotype-specific genes could have a role in triggering autoimmunity.

We investigated sera from patients with Hashimoto's thyroiditis (HT) for antibodies against YE-RPs using the Western blotting method. The prevalence of YE-RP antibodies in HT was compared to the antibody frequencies in patients with non-autoimmune euthyroid goiter and in a representative group of blood donors (Fig. 2). The prevalence of YE-RP antibodies of IgG class (68%, $n = 38$) and of IgA class (38%) in HT patients was found to be significantly higher than in the non-autoimmune groups.

Since HT patients develop antibodies against YE-RP, we were curious to discover whether the TECs of HT goiters might express YE-RP antigenic epitopes. Antisera against YE-RP were raised in rabbits, absorbed and applied in the indirect immunofluorescence staining of tissue obtained by thin-needle aspiration biopsies of HT goiters (Fig. 3). Clear immunofluorescence was displayed by all cells which also stained with antimicrosomal TPO sera.

Our findings in HT indicate that, as with GD, enteropathogenic YE may play a role in triggering the autoimmune disease.

An alternative scenario might involve the heat shock proteins (HSP) [8]. These proteins are expressed in cells exposed to calcium deficiency or low oxygen pressure. Bacteria and human cells have HSP epitopes in common [9]. A recent study links thyroid autoantigen epitopes of TPO and Tg to sequences of *E. coli* starvation protein [10], which recalls the earlier findings of TSH binding sites in YE and *E. coli* [11]. An antigenic epitope sequence of the autoantibodies to TPO and Tg also has a high degree of homology with a peptide sequence of the plasmid-encoded YOP 1 of enteropathogenic YE (personal communications).

Although normal individuals also have T lymphocytes reactive with HSP epitopes [9, 12], the increased expression of HSP epitopes following exposure of epithelial cells to stress and MHC class II expression of epithelial cells may lead to immunity against "self" [13].

There are indications that RPs from YE share epitopes with HSP 70 [6]. This would link the autoimmune reaction to thyroid epithelial cells with bacterial infections. Investigation of this hypothesis are under way in our laboratory.

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Discussion

Sundick:
It should be interesting in these BB/W rats to actually test for microsomal antibody.

Wenzel:
We would love to do so. To my knowledge, however, there is only one person who is able to do this assay. We couldn't convince Dr. Taurock to measure anti-rat TPO antibodies. As I learned, the TPO is species specific, and the antibody assays do not work with human TPO.

Bottazzo:
As far as I am aware, BB rats do produce thyroid microsomal antibodies. Do you expect these animals to produce these specificities if one follows them long enough?

Wenzel:
Well, this is a discussion with the Worcester group. I don't know. They think they can develop it; I don't know.

Bottazzo:
Going back to antibody titers: now, you have 50% of normal population in Germany . . .

Wenzel:
No, 35%.

Bottazzo:
I looked at the slide: it's 50%.

Wenzel:
Well, then the slide is wrong, it's 35%.

Bottazzo:
I take your point. But, in any case a prevalence of 35% is quite high, don't you agree? Do you expect that these individuals will ultimately develop Graves' disease? My prediction is that they wouldn't, despite their being exposed to the

agent that you postulate as that potentially responsible for causing Graves' disease.

Wenzel:
OK, first of all, Germans as well as Belgians eat a lot of ground meat, and you do find a lot of *Yersinia* in these populations, which is a reason for a high incidence of antibodies also against plasmid-encoded release proteins. Originally, I thought that infection with *Yersinia* might be a trigger for autoimmunity, only restricted by a genetic predisposition. I am not that sure about it any more. We were able to test serum samples of Graves' patients from a country and a geographic region where yersinosis triggered through the food chain should not occur. We also found in a considerable number, however, not statistically significant, a high incidence of IgG antibodies. The spectrum of bacterial involvement in autoimmune disease is not restricted to the thyroid but exists also in the liver; you may also recall the discussion in rheumatic arthritis. Maybe it just reflects a cross-reactivity of something which the bacteria have in common with these organs. The link between the bacterial virulence genes and the eukaryotic genes might be the chloroblast which developed into the mitochondria. Moreover, there is an exciting discussion at present about the involvement of inducible heat shock sequences in autoimmunity. The hypothesis hinges on the assumption that you have highly preserved genes or gene sequences which are encoded in the eukaryotic cells. These genes are normally not immunogenic at all because they are highly preserved. Under certain circumstances, for example, if a retrovirus occurs, these genes become activated, and then we will observe antigenic mimicry or whatever.

Bottazzo:
What you just said reminds me of the Coxsackie virus story in type I diabetes. Until a few years ago, Coxsackie B was fashionable, and these viruses were proposed as the possible causative agent of type I diabetes. However, as Coxsackie are widespread viruses in the community, why do only a few individuals develop the disease? By analogy, the same concept seems now to apply to *Yersinia* and Graves' disease. Can you comment on this and further elaborate on the pathogenetic mechanism possibly driven by molecular mimicry.

Wenzel:
I can give you an information about diabetes. We checked diabetes, of course, and this obviously has nothing to do with my story with virulence sequences. We don't find that.

Khoury:
A technical point: It is quite common that hyperimmunized rabbits produce autoantibodies, mainly against nucleoproteins and actin, and I think that this is a possibility for the reactivity you showed us on the thyroid monolayer. Have you tried to absorb out that rabbit antiserum with, let us say, liver powder?

Wenzel:
No. Our sera, however, are quite well defined by Western blotting, where we get a full pattern of specific bands for these release proteins. What I showed in the immunofluorescence was a whole serum, and we can block this immunofluorescence by antigen blocking. We did not do what you asked for using actin, for example. But we feel quite confident; because of the blocking experiments we also can perform in Western blotting with the actual immunogen.

Yersinia enterocolitica and Thyroid Disease

K. BECH

The observation that the concordance rate of Graves' disease in identical twins is 30%–60% points to the influence of environmental factors, and infections have been incriminated in the pathogenesis [1–3]. Over 15 years ago we demonstrated an increased frequency of antibodies to *Yersinia enterocolitica* serotype 3 [4], a finding which was later confirmed by others [5–6]. The incidence was especially high in newly diagnosed Graves' disease and in recurrent Graves' disease [7], whereas the prevalence in the general population was considerable lower. These findings suggested a link between *Yersinia* and thyroid disease.

During infection with *Yersinia* an endotoxin of lipopolysaccharide (LPS) is released. LPS is known as a B cell mitogen able to substitute for T cells in the induction of the primary immune response and to interfere with T cell dependent delayed hypersensitivity as well as having a direct adjuvant effect on T cell function [8]. We have been able to demonstrate cell-mediated immunity against *Yersinia* in peripheral leukocytes of patients with newly diagnosed Graves' disease [9], and the degree of immunity was related to titer of antibodies, in contrast to infected patients without thyroid diseases, who display normal leukocyte migration. On the other hand, we were not able to demonstrate lymphocyte activation by lymphocyte transformation test after stimulation with whole heat-killed *Yersinia* bacteria [10].

A high incidence has also been reported in the United States, despite a very low prevalence of antibodies against *Y. enterocolitica* in the population [5]. One characteristic feature is that none of the thyroid patients showed clinical symptoms compatible with *Yersinia* infection, which suggests that the relationship between *Yersinia* and thyroid disease may be due to a cross-reaction between some thyroid antigens and those of *Yersinia.* Further evidence for this suggestion is that patients infected with *Yersinia* showed, by immunofluorescence, antibodies directed against the membrane of thyroid epithelial cells [11].

Recently we have demonstrated antibodies present in sera of patients infected with *Yersinia,* which not only bound to human thyroid plasma membrane but also elicited effects resembling thyroid-stimulating immunoglobulins present in patients with Graves' disease [12]. Western blotting revealed similarity of the molecular characterization between *Yersinia* antibodies and thyroid-stimulating immunoglobulins [12]. Most recently BYFIELD et al. [13] have supported this finding by chromatographic separation showing that the same

molecules reacted with the bacteria-binding protein and the human TSH receptor.

Thus, accumulating evidence has led to the suggestion that antibodies against a bacterial protein such as *Y. enterocolitica* cross-reacts with the TSH receptor of the human thyroid cell. The potent immune stimulation of LPS during the infection may result in autoimmunity by activation of the autoreactive B cells to production of antibodies against TSH receptor, leading to thyroid disease. These findings are consistent with the hypothesis that thyroid autoimmunity may be triggered by bacterial infection via cross-reaction at the level of the TSH receptor.

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Discussion

Wenzel:
Let me add something, although it is not my favorite hypothesis. It could well be that infection plays a role in the different reaction of these individuals, the autoimmune patient and the so-called normals. We have had evidence, and this evidence comes also from the arthritis field, that the product ot the virulent *Yersinia* modulates macrophage function and probably also interleukin-6 secretion. This means that there is a difference between autoimmune patients; they have a suppressed IL-6 secretion. So it could well be connected to your hypothesis.

Bech:
We are going to report some of our results in the next session today about the influence of endotoxin developed during infection with *Yersinia enterocolitica* as endotoxin has a potent effect on the immune reaction. However, the endotoxins do not influence the thyroid cells. On the other hand, it is important to see if expecially the LPS from *Yersinia* infection is able of influencing either the macrophages to produce IL-1 or the thyrocytes to produce IL-6 following an infection with *Yersinia.*

Gaitan:
It is a very important hypothesis that *Yersinia* or bacteria could start a process of Graves' disease or autoimmunity. However, there are several answers that must be given before accepting this concept. First, if one looks at the slides, normal IgG per se exhibits a significant, dose-dependent inhibition of TSH binding to thyroid membranes. Second, why thyroid-stimulating immunoglobulin or TSI, which should be more important in Graves' disease than TBII, shows such minimal activity in sera of *Yersinia* patients when compared with TSH. Therefore, I think it is extremely important to determine how TBII and TSI activities of patients with *Yersinia enterocolitica* compare quantitatively with those activities of Graves' disease patients. If the percentage activity of *Yersinia* patients is very low as compared to Graves patients, then the specificity of the IgG fraction of *Yersinia* patients to react with thyroid membranes comes into question. A comparison of results with IgG fractions of patients recovering from other febrile illnessess would then be required to clarify this question. I believe these studies would give a better perspective as to the potential role of *Yersinia* infection in the pathogenesis of Graves' disease.

Bech:
First of all, it is well known that normal IgGs are able to inhibit or displace TSH from the TSH receptor to some degree, while normal IgGs do not influence thyroid adenylate cyclase activity. In this case the IgGs purified by protein A Sepharose are able to displace the TSH more effectively than normal IgGs and stimulate adenylate cyclase activity. Furthermore, others have found a specific TSH binding site on *Yersinia* bacteria with a relatively high affinity. These studies just show that the *Yersinia* bacteria share antigens with the TSH receptor on the human thyroid membrane. If you get an infection with *Yersinia,* and you are predisposed to develop thyroid disorders, *Yersinia* antibodies are able to bind to the THS receptor and maybe initiate an autoimmune reaction.

Furmaniak:
Just a word of caution in interpreting your Western blot results. The fact that there are different bands precipitated of similar molecular weight from patients with *Yersinia* infections and from patients with Graves' disease does not indicate that you can see TSH receptor, because there hasn't been any serious evidence for visualization of receptor by this means. The second thing is: incidence of antibodies to *Yersinia.* You showed that they are more frequent in patients at 0–6 months of duration of disease, and yet Dr. Wenzel showed that in the first 4 weeks of clinical symptoms he couldn't find any antibody.

Wenzel:
IgM.

Furmaniak:
Sorry, but could you provide any antibody levels after a shorter duration of clinical symptoms in the first 4 weeks for example?

Bech:
One characteristic feature is that the thyroid patients in general do not have symptoms compatible with *Yersinia* infection, but the incidence and levels of antibodies are higher in newly diagnosed Graves' disease and recurrent Graves' disease. Concerning the TSH receptor and *Yersinia* bacteria, I think that Western blotting supported the fact that there is some homology between the IgGs of Graves patients and the antibodies developed in patients with *Yersinia* infections. Studies by others have shown binding sites on the bacteria with relatively high affinity for TSH where Graves IgGs can displace TSH from these binding sites, indicating some homology.

Bottazzo:
When we were in Helsinki at the exophthalmos meeting, one hypothesis that was put forward was that fibroblasts have TSH receptors. This might explain why there is proliferation of collagen, collagen products in the eyes. Now, you are saying that *Yersinia* has TSH receptors, and this explains why they have Graves' disease.

Bech:
We have to explain why patients with thyroid diseases have agglutinating antibodies against *Yersinia enterocolitica* serotype 3 in sera. It has been shown that patients infected with *Yersinia* without thyroid disease display antibody reaction with thyroid cell membrane by immunofluorescence, and that *Yersinia* antibodies acted similarly as Graves' IgGs on the TSH receptor. Furthermore, it has been shown that also the *Yersinia* bacteria has binding sites, which bind TSH with a relatively high affinity. Therefore, we conclude that *Yersinia* and the TSH receptor share some antigens, which may be important in some cases for development of Graves' disease.

In Vitro and In Vivo Effects of Cytokines

Regulation of ICAM-1 Expression on Human Thyroid Follicular Cells

E. TOLOSA, M. MARTI, C. ROURA, A. LUCAS, and R. PUJOL-BORRELL

Introduction

The finding that thyroid follicular cells affected by autoimmune disorders, i. e., Graves' disease and Hashimoto's thyroiditis show increased levels of HLA class I and de novo expression of class II [1, 2] suggested that the target cells can play a role in the initiation and/or perpetuation of the autoimmune responses [3]. Central to this line of thinking was the model of antigen presentation in which HLA class II molecules present peptides to the helper T cells. In recent years advances in the understanding of the recognition of antigens by T cells have shown that this process is more complex. Among the newly recognized interactions between the T-lymphocytes and the target and/or antigen-presenting cell, the pair of ligands lymphocyte function antigen-1/intercellular adhesion molecule 1 (ICAM-1) seemed to play a decisive role in the initial adhesion process that enables the T cell receptor to interact with its antigen. Because of its possible involvement in the interaction between the autoreactive T cells and the thyroid follicular cells, we have studied the modulation of ICAM-1 expression in primary cultures of nonautoimmune thyroid glands.

Material and Methods

Thyroid tissue was obtained from four patients who underwent surgery for multinodular goiter. All glands were from adult women. Routine thyroid function tests showed normal values. Thyroid autoantibodies (T and M Thymune Test, Wellcome, Beckenham, UK).

Glands were processed as previously described [1]. Briefly, thyroid tissue disrupted by incubation with trypsin 2.5 mg/ml and collagenase 0.2 mg/ml) at 37° C for 2 h under continuous agitation. Supernatants were collected every 30 min and were kept on ice after the addition of FCS (10%) to stop the digestion, while fresh enzymatic solution was added to the tissue. Pooled supernatants were passed through a 500 μm mesh and washed three times by centrifugation at 1200 rpm (300 *g*) for 8 min. Red blood cells in the pellet were lysed by osmotic shock with ammonium chloride, and after another washing step cell viability and number were assessed by ethidium bromide/acridine orange staining in a hemocytometer under a UV microscope. Cells were then cryopreserved in complete

TCM plus 10% DMSO. For each experiment freshly thawed thyrocytes from a single thyroid (viability > 80%) were plated in complete TCM at a density of 10^5/ml on 13 mm round coverslips placed in a 24-well plate and kept in a 5% $C0_2$, 95% humidified incubator. After overnight culture an incipient monolayer was already visible under the inverted microscope. Cultures were washed, medium was replaced, and supplements were added and kept for a further 48 h until staining. Parallel cultures without supplements were used as controls.

Recombinant human interferon gamma (IFN-γ, specific activity 2×10^7 U/mg, endotoxin contamination less than 0.1 EU/mg) and tumor necrosis factor alpha (TNF-α, specific activity 6×10^7 U/mg, endotoxin contamination less than 0.125 EU/ml; kindly provided by Dr. G.R. Adolf, Boehringer Institute, Vienna, Austria) were added to cultures in doses ranging from 1 to 1000 U/ml final concentration.

Monolayer cultures were stained on the coverslips while viable, as previously described [1]. For the identification of thyroid follicular cells the cultures were incubated first with serum from a patient containing high titter of microsomal antibodies followed by a FITC labeled goat anti-human IgG serum (Southern Biotechnology, Alabama, USA). For the detection of ICAM-1 molecules the monoclonal antibody (MoAb) RR1/1 (kindly provided by T. Springer) followed by a FITC-labeled goat anti-mouse IgG serum (Southern Biotechnology) were used. After staining, cultures were fixed with ethanol/acetic acid (95/5 v/v), mounted, and observed under a Zeiss Axioplan UV microscope using a × 63 oil immersion objective. Morphology of the cells was assessed by simultaneous observation under phase contrast. A minimum of 200 epithelial cells per culture were evaluated, and results are expressed as an index of relative positivity.

Results

Preliminary staining showed that at 48 h more than 90% of the cells present in the monolayer cultures were microsomal positive and were therefore identified as thyrocytes. At 48 h, more than 30% of the epithelial cells in the culture were positive for ICAM-1. As shown in Fig. 1, the addition of TNF-α and IFN-γ both resulted in a dose-dependent increase of ICAM-1 expression in the thyroid follicular cells. A clear synergism between TNF-α and IFN-γ was demonstrated (Fig. 2). The induction of ICAM-1 expression was parallel to the induction of HLA class I and class II induced in parallel cultures by IFN-γ (data not shown).

Discussion

The capability of normal thyroid cells to express ICAM-1 under stimulation with IFN-γ and TNF-α is of interest because it has been shown that these cytokines are produced in autoimmune thyroid tissue [4]. Is therefore highly prob-

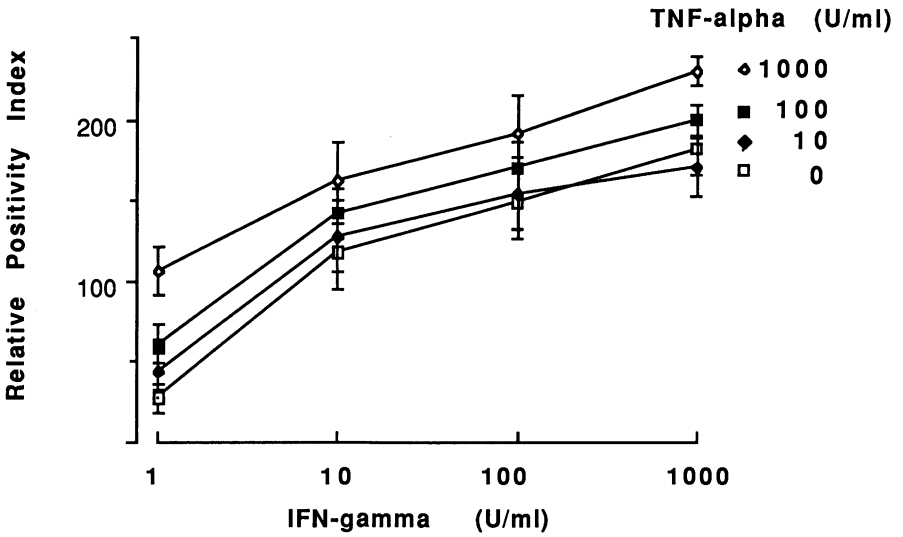

Fig. 1. Monolayers of thyroid follicular cells were incubated for 2 days with culture medium alone or in the presence of 1, 10, 100, and 1000 U/ml IFN-γ (□) or TNF-α (◆) and were stained for ICAM-1 expression

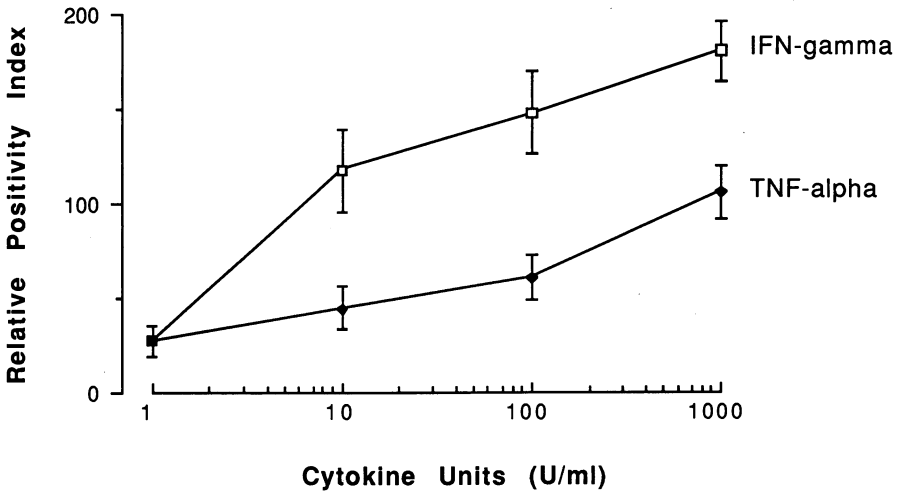

Fig. 2. Synergistic effect of different concentrations of IFN-γ and TNF-α. Thyroid follicular cells were simultaneously supplemented with 1–1000 U/ml IFN-γ and/or TNF-α and stained 2 days later for ICAM-1

able that thyrocytes in thyroid autoimmune disease do express elevated levels of ICAM-1 expression as a result of local cytokine production. In a preliminary communication Weetman et al. [5] have found increased levels of ICAM-1 in four out of five glands from Graves' disease patients. The implications of increased ICAM-1 expression by the target cells of thyroid autoimmunity will

have to be established in a functional way. The results presented here provide the basis for an in vitro model in which to test the role of adhesion molecules in the presentation of autoantigens to T cells by thyrocytes.

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Discussion

Scherbaum:
Is the ICAM-1 expression the cause or the result of the inflammation in the thyroid gland?

Pujol-Borrell:
I think we cannot answer that with the present information. I can only say that it is one of the related phenomena.

Hüfner:
This induction of ICAM seems not to be very specific. We have tried similar experiments in an ovarian cancer cell line, and we found that one can also induce ICAM with interferon and TNF.

Pujol-Borrell:
I am not saying that this is specific. Most cells have ICAM-1. Normally macrophages and antigen-presenting cells have a higher basal level, which is regulated by IFN-gamma and TNF, and this is a little bit different from HLA class II regulation. Probably what is important is that the basal expression in normal thyroid cells is rather low. When the whole process starts, this expression may play a role in perpetuating the response or merely favoring the contact between T cells and the thyroid cell.

Bottazzo:
Can you be more precise. Do normal thyroid cells express ICAM-1?

Pujol-Borrell:
Very low, yes; about 20% of them.

Bottazzo:
Do Graves' thyrocytes have an enhanced expression of ICAM-1? What is the effect of IFN-gamma?

Pujol-Borrell:
Yes, they have.

Bottazzo:
Is there any difference in the behavior of Graves thyrocytes and that of the ovarian cancer cell line, in relation to the paramaters you have studied?

Pujol-Borrell:
You can heavily induce or hyperinduce ICAM expression by these two components. They constitutively express it, but it is very much increased by IFN and TNF.

Bottazzo:
So it is more or less the same.

Todd:
Have you on the thyroid cell line HT 93, Ricardo, looked at the time course of the induction by gamma-IFN?

Pujol-Borrell:
Yes, I have done that. The time course is very fast. In 6–8 h you have a very high level, which suggests that you are increasing the transcription of a gene that is already switched on. So, while with class II you have a bit of a gap, you need a bit more time, suggesting that it is a gene that is originally off, and then you have to switch it on, but this is more clear. I just wanted to give the message. All this work has also been done on primary cultures with normal thyroid cells where these differences are even more evident.

Mariotti:
Dr. M. Bagnasco of Genua and Dr. C. Betterle of Padua have recently reported very similar results on spontaneous expression of ICAM-1 in Graves' disease, but they found very poor expression in Hashimoto's thyroiditis. Do you have any data on Hashimoto's thyroiditis?

Pujol-Borrell:
No, we never operate on Hashmotos's thyroiditis, but this is an interesting observation.

Davies:
You are claiming 20% positivity of normal thyrocytes?

Pujol-Borrell:
Expressing ICAM-1.

Davies:
Yes.

Pujol-Borrell:
It depends where exactly you put your gate, but basically around that.

Davies:
This is the crude thyroid preparation?

Pujol-Borrell:
These are primary cultures. You wash the day after plating the nonadherent cells, and if you do a thyroid microsomal staining, about 90% are positive.

Davies:
So 90% are positive for microsomal antigen, and 20% are positive for ICAM? Would you say that the normal thyroid cell expresses ICAM-1?

Pujol-Borrell:
I would say that probably it is a matter of threshold, and probably all thyroid cells express, but by cytofluorometry we have a sensitivity of around 3000 molecules per cell with the system we are using, and it may well be that the normal cells have less than that. But the fact is that they can be induced very quickly to express more, thus suggesting that the genes are already transcribed.

Davies:
We haven't been able to see it in normal thyroid, only in diseased thyroid or by inducing ICAM-1 expression.

Pujol-Borrell:
Well, we can discuss the technical details: what we are using is the MoAb RR1, and we are using it at about 5 μg/ml, and we use cytofluorometry.

Davies:
Probably more important than just fluorescing cells, now, is whether we can show a functional result of ICAM-1 expression, in other words, does it have any biological function that you could test? Have you looked for any?

Pujol-Borrell:
I can show you another slide. We are moving now to a functional assay, and I will be very surprised if you do not get adhesion, because it has been shown in many systems. The function of ICAM-1 has been very clearly demonstrated by the experiments of Nancy Hogg and John Trowsdale in which, if you take class II positive L cells plus a peptide antigen, and try to do presentation experiments to T cells, it doesn't work very well. If you then transfect again for ICAM-1, you get a very efficient presentation. So, I think that the general principle has been established. Now, if that is true in the thyroid cells, I gather it will be; but we will test it.

Davies:
I am familiar with those recombinant experiments, but they are restricted to recombinant products. As far as I know, demonstrated enhanced adhesion to ICAM-1 expressing cells has been very difficult.

Pujol-Borrell:
But you can block it with monoclonals to ICAM-1 in the tissue.

Davies:
It has been very difficult in normal epithelial cells to show T cell binding.

Pujol-Borrell:
With endothelial cells you can show it.

Scherbaum:
Did you do double-labeling studies to see whether your 10% or 20% were not thyrocytes but, for example, already constitutively expressing class II positive dendritic cells which were positive for ICAM?

Pujol-Borrell:
Yes, I have done this on monolayers and double-labeling, and there is no doubt that they are ICAM-positive thyroid cells.

Interleukin 6: A Pathogenic Factor in Autoimmune Thyroid Diseases?*

Å. KROGH RASMUSSEN, L. KAYSER, K. BECH, U. FELDT-RASMUSSEN, H. PERRILD, and K. BENDTZEN

Introduction

Interleukin 6 (IL-6) is a biologically active cytokine produced primarily by activated monocytes. IL-6 has many properties in common with interleukin 1 (IL-1), and it may be directly involved in infectious and autoimmune diseases [1, 2]. We have previously demonstrated an IL-1 induced inhibition of the function of cultured human thyroid cells, evaluated as the decreased production of cAMP and thyroglobulin (Tg) [3]. The influence of recombinant IL-6 (rIL-6) on the function and growth of human thyrocytes was examined in the present study.

Material and Methods

Human thyroid cells were obtained from para-adenomatous tissue during strumectomies and prepared as previously described [3]. The cells were grown in Ham's F12 medium as modified by Coon and supplemented with 5% fetal calf serum (Flow Labs, Irvine, UK) and bovine thyrotropin, human insulin, somatostatin, human transferrin, hydrocortisone, and glycyl-histidyl-lysine acetate (6H). After 1 week the cells were passaged and seeded into 24-chamber culture plates. The cells were grown for 3 days with 6H and 4 days without thyrotropin. The cells were then exposed for additional 3 days to human rIL-6 [(0.01 fg/ml–1 ng/ml (10^{-5}–10^{3} U/ml); kindly provided by Dr. T. Hirano and T. Kishimoto, Osaka, Japan] in the presence of TSH.

The release of extracellular cAMP and Tg into the supernatants was measured – by a competitive protein binding assay and a double antibody radioimmunoassay, respectively. Growth was expressed as the content of DNA in culture chambers.

All results were carried out in triplicate and performed at least twice. A confidence limit of 95% was used as the significance level. Friedman's test was used.

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Table 1. The influence of IL-6 on the production of Tg and cAMP in human thyrocytes

IL-6 (U/ml)	Tg (ng/μg DNA; $n = 6–9$)	cAMP (pmol/μg DNA; $n = 6–9$)
0	367.2 (108.0–1156.3)	11.9 (9.3–40.4)
10^{-5}	497.7 (78.2–1316.7)	19.8 (7.0–34.1)
10^{-3}	743.3 (94.7–1369.0)	22.6 (9.3–36.6)
10^{-1}	441.6 (63.8–1257.6)	9.6 (7.7–31.7)
10	450.0 (87.9–1130.3)	11.1 (9.0–29.8)
50	395.6 (57.8–1198.6)	10.6 (6.9–28.9)

Results

In secondary cultures of human thyrocytes rIL-6 did not influence the production of Tg (Table 1) or the DNA content of the cells; however it inhibited the production of cAMP (Table 1) to a small but significant degree. The maximal inhibitory effect of cAMP was exerted at 50 U/ml (16.4% inhibition; $n = 9$, $p < 0.02$).

Discussion

The effects of IL-6 do not correspond to those previously found with rIL-1α and rIL-1β. IL-6 may play a role in the communication of immune and inflammatory responses, but the different effects of IL-1α/β and IL-6 indicate that IL-6 is not a direct mediator of IL-1α/β in this system.

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Discussion

McLachlan:
Can you tell us how variable these responses are? When you take thyroid cells from different patients, do you consistently see that very low concentrations of IL-1 stimulate thyroglobulin secretion and higher levels inhibit secretion? Is it completely reproducible, or do you see variation between different individuals?

Krogh Rasmussen:
I have repeated it several times and found the same results, and it corresponds to what you find when you are researching the insulin release and insulin content of beta cells. In the same manner you have this biphasic dose response influence of recombinant IL alpha or beta.

McLachlan:
Similarly with the IL-6 data, you do not see any variation between one individual and another?

Krogh Rasmussen:
Also with the IL-6 data the relative changes compared to the control can be repeated from one individual to another, but we found a high interculture variation.

Scherbaum:
May I ask you if there is a difference according to the duration of cell culturing, if you test them in the beginning or after a long period of culturing in their response to interleukins?

Krogh Rasmussen:
Yes, I have not reported all the details. Of course, it is a relevant question. It is after 3 days influencing of the cytokine, and before that we have a period without TSH because then we have a more sensitive response from the thyroid cells.

Bottazzo:
You may recall that when Ulrich Deuss was working in our laboratory he showed that IL-1 had quite opposite effects when the cytokine was incubated with normal or thyrotoxic thyrocytes. In normal thyroid cells, in fact, IL-1 sup-

pressed both TSH-stimilated cAMP and proliferative responses; but in thyrotoxic cells, regardless of whether they were obtained from Graves or toxic nodular glands, exactly the opposite effects were observed. So, my question is: In your experiments did you employ normal or thyrotoxic thyrocytes?

Krogh Rasmussen:
Yes, I remember those discrepancies, and of course, it is another fact that you have to know: this is para-adenomatous tissue, so it is normal thyroid tissue, as normal as we can have it.

Bottazzo:
It would be interesting to try on thyrotoxic cells.

Krogh Rasmussen:
Yes, that's right, but in Denmark it is very difficult. There are very few strumectomies on patients with Graves' or Hashimoto's disease, so it is very difficult to obtain these tissues.

Endotoxins Do not Mimick the Effect of Interleukin-1 in Secondary Cultures of Human Thyroid Cells*

L. KAYSER, Å. KROGH RASMUSSEN, K. BECH, U. FELDT-RASMUSSEN, H. PERRILD, and K. BENDTZEN

Introduction

Interleukin (IL) 1β has previously been shown to inhibit production of cAMP and thyroglobulin (Tg) in TSH-stimulated primary and secondary cultures of human thyrocytes in vitro [1]. Endotoxins (LPS) are known to affect many cell systems and also to stimulate production of both IL-1 and tumor necrosis factor [2]. The effect of endotoxins upon thyroid cells is unknown. A preliminary study [3] has indicated that thyroid cells may produce IL-1α, and it is thus conceivable that endotoxins might inhibit the function of thyroid cells due to an autocrine IL-1 production. In the present study we evaluted whether endotoxins from two different species of bacteria affect the cAMP or Tg production in secondary cultures of human thyroid cells.

Material and Methods

Human thyroid cells were obtained from para-adenomatous tissue during strumectomies and prepared as previously described [1]. The cells were grown in Ham's F12 medium as modified by Coon supplemented with 5% fetal calf serum and 6H. After 1 week the cells were passaged and seeded into 24-chamber culture plates.

LPS from *Salmonella abortus equi* or *Yersinia enterocolitica* was a kind gift from Dr. A. Fomsgaard, State University Hospital, Copenhagen.

The human thyroid cells (1×10^5/ml) were plated in 24-well (175 mm^2/well) culture plates (Greiner) and grown for 3 days with 6H and 4 days without thyrotropin (5H). The cells were then exposed for an additional 3 days to the endotoxins (10^{-3}–10 µg/ml) in the presence of TSH (6H). For the measurement of cAMP 3-isobutyl-L-methyl-xanthine (0.5 mmol/l) was added. The function of the cells was evaluated by production of cAMP (extracellular, competitive pro-

* This study was supported by grants from the Danish Cancer Society, the Danish Hospital Foundation for Medical Research – Region of Copenhagen, the Faroe Islands, and Greenland, the Danish Medical Research Council, the Lykfeldt Foundation, and the Danish Biotechnology Program.

tein binding assay [4]) and Tg (a double antibody radioimmunoassay [5]). Growth inhibition was expressed as the percentage change in total content of DNA in culture chambers, measured by a modified diphenylamine method [6].

All experiments were carried out in triplicate and performed at least twice. Results were expressed as medians and ranges. They were analyzed nonparametrically using Friedman's test comparing the response at different concentrations of the same cytokine (balanced design). A confidence limit of 95% was used as the significance level.

Results

The cAMP production was marginally reduced when exposing the cells to a very high concentration of LPS (10 μg/ml) (*S. abortus equi*, $p = 0.10$; *Y. enterocolitica*, $p = 0.14$; Table 1). Also, the Tg concentration was marginally decreased when exposed to the same high concentrations of LPS (*S. abortus equi*, $p = 0.40$; *Y. enterocolitica*, $p = 0.38$; Table 1).

There was no change in the DNA contents (Table 1). Thus, the endotoxins failed to affect the growth of thyroid cells in vitro.

Table 1. The influence of endotoxin from *Salmonella abortus equi* and *Yersenia enterocolitica* on cAMP and thyroglobulin production and on DNA content in secondary cultures of human thyroid cells

Concentration of endotoxin	cAMP (pmol/μg DNA)	Tg (ng/μg DNA)	DNA (μg)
S. abortus equi			
0 μg/ml	159 (114–200)	322 (285–462)	2.2 (1.7–2.7)
10^{-3} μg/ml	164 (107–187)	331 (280–369)	2.0 (1.6–2.3)
10^{-1} μg/ml	157 (149–191)	341 (297–459)	2.0 (1.5–2.7)
10 μg/ml	140 (108–154)	269 (238–372)	2.2 (1.7–3.6)
Y. enterocolitica			
0 μg/ml	157 (98–212)	345 (309–404)	2.1 (1.7–2.6)
10^{-3} μg/ml	160 (110–214)	320 (273–426)	2.0 (1.8–2.2)
10^{-1} μg/ml	153 (131–171)	338 (279–398)	2.1 (1.8–2.6)
10 μg/ml	116 (97–157)	313 (223–358)	2.2 (1.8–2.6)

Discussion

We have previously reported that rIL-1β causes a 70%–80% inhibition of cAMP production and a 50% inhibition of Tg production in secondary cultures of human thyroid cells [1]. The two different endotoxins marginally inhibited cAMP and Tg production but only when given at a very high concentration. Thus, it is unlikely that the previously reported inhibitory effect of IL-1β upon cAMP and Tg production could be ascribed to endotoxin contamination. The present study also, though indirectly, shows that secondary cultures of human thyroid cells do not synthesize biologically active IL-1 after endotoxin stimulation, since such a production would also have caused inhibition of the cAMP and Tg production.

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Blocking the Induction of HLA Class II Expression by Thyroid Epithelial Cells In Vitro*

I. TODD, V. GUERIN, and G. F. BOTTAZZO

Introduction

Cells expressing class II molecules of the major histocompatibility complex (MHC) have the potential, in the presence of antigen, to interact with CD4 + T lymphocytes which are mainly implicated in the induction of immune responses. This may help to explain the limited tissue distribution of HLA class II since it restricts the possibilities for immunological interactions to defined, controllable proportions. Thus, most tissue cells are normally HLA class II negative, and this includes thyroid epithelial cells (thyrocytes). By contrast, thyrocytes of patients with autoimmune thyroid diseases frequently express HLA class II [1–3]. These cells, which are themselves the target of the autoimmune attack, may then interact with CD4 + T cells and thereby influence the autoimmune process. It has been hypothesised that the activation of helper T cells by such an interaction might stimulate the pathogenesis [4]. This is supported by the observations that thyroid-specific, autoreactive T cells can be stimulated in vitro by autologous [5–7] or syngeneic [8] thyrocytes bearing MHC class II. However, another possibility is raised by recent findings that the interaction of T cells with antigen plus MHC in the absence of "accessory signals" can induce a state of anergy in the T cells (referenced in [9]). Thus it is feasible that MHC class II positive thyrocytes might in some circumstances down-regulate rather than stimulate the activity of thyroid autoreactive T cells [10] if the required accessory signals are not provided. In any case, the potential of MHC class II bearing thyrocytes to modulate T cells reactivity, whether positively or negatively, makes it important to understand the regulation of this class II expression.

Interferon-γ (IFN-γ) induces MHC class II expression by thyrocytes in vitro [11, 12], and this induction can be enhanced by thyroid-stimulating hormone (TSH) [13, 14] and tumour necrosis factor (TNF) [15, 16]. It is feasible that all of these mediators contribute to the thyrocyte class II seen in thyroid autoimmunity in vivo, but it is equally important to investigate the nature of potential inhibitors of this expression, since the actual level of class II expression may well depend on the balance between stimulatory and inhibitory fac-

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tors. Here we consider investigations into two types of substance which we have found to inhibit HLA class II induction in cultured monolayers of human thyrocytes.

Effects of Epidermal Growth Factor

Epidermal growth factor (EGF) has been shown to have a number of effects on thyrocytes in culture; not only does it stimulate growth of thyroid cells, but it also suppresses certain differentiated characteristics of these cells (e.g. iodide metabolism) [17]. We therefore investigated the effects of EGF, and the related mediator transforming growth factor-α (TGF-α), on the induction of HLA class II expression by thyrocytes [18]. The culture and assay procedures employed have been described in detail elsewhere [11, 13, 19]. Briefly, monolayer cultures of human thyrocytes from non-autoimmune patients were established and incubated for 5–7 days with the various mediators under investigation. These were then stained for surface expression of HLA class II molecules by indirect immunofluorescence, and assessed by UV microscopy.

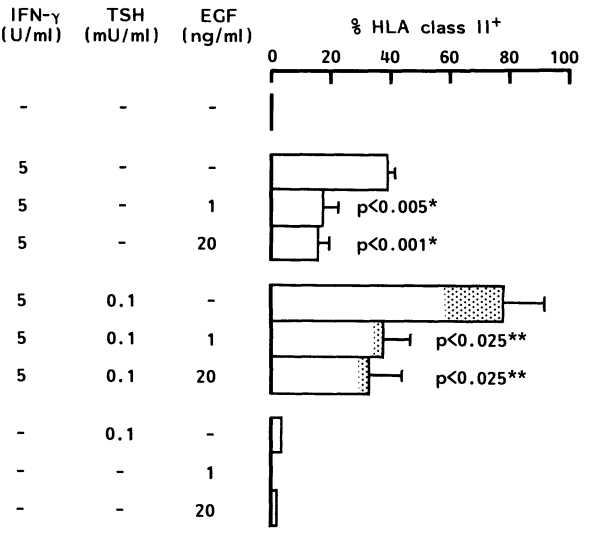

Fig. 1. Modulation of cell surface HLA class II expression in thyrocyte monolayers treated with IFN-γ, TSH and EGF for 6 days. HLA class II expression was detected by indirect immunofluorescence employing a mouse monoclonal anti-HLA class II antibody (MID-3) followed by fluoresceinated rabbit anti-mouse immunoglobulins. The proportion of cells expressing HLA class II was determined by differential counts by phase and fluorescence microscopy on the same fields of cells. The fluorescence intensity was estimated of each cell scored as positive: □ lower intensity; ▨ higher intensity. The p values show the level of statistical difference between the indicated responses and the responses to: *IFN-γ alone, **IFN-γ plus TSH

EGF was found to inhibit, usually by about 50%, the induction of HLA class II in thyrocytes by IFN-γ (Fig. 1). This occurred when the expression of class II was stimulated by IFN-γ only or by IFN-γ plus TSH (Fig. 1). Furthermore, EGF also suppressed the low level of HLA class II expression stimulated by TSH alone. This suggests that the effect of EGF is at the level of metabolic processes intrinsic to the thyrocytes rather than simply inhibiting the binding of, or triggering by, IFNγ per se. Further support for this interpretation was provided by experiments in which thyrocytes were cultured with EGF alone for 3–4 days either before or after treatment with IFN-γ alone for a similar length of time. EGF suppressed HLA class II induction to a similar extent when used to treat the thyrocytes before or after IFN-γ, although a somewhat greater degree of inhibition was observed when IFN-γ and EGF were present simultaneously throughout the culture period. However, there appears to be no strict requirement for EGF to act on thyrocytes at precisely the same time as IFN-γ in order to exert its effect.

We found TGF-α to behave similarly to EGF in inhibiting HLA class II induction in thyrocytes by IFN-γ (Fig. 2). This is not surprising in view of the fact that TGF-α shows substantial amino acid sequence homology with EGF and binds to the same cell surface receptor [20]. It is also worth noting that TGF-α is produced not only by neoplastic and early foetal cells, but also by certain normal adult cell types, as demonstrated in keratinocytes [21].

EGF is present in normal human serum at about 0.2 ng/ml [22]. In most of our experiments EGF was employed at concentrations of 1–20 ng/ml, but concentrations in culture at least as low as 0.1 ng/ml were found partially to suppress HLA class II induction in thyrocytes by a low dose of IFN-γ (e.g. 1 U/ml). Furthermore, the serum concentration of EGF appears to be affected by thy-

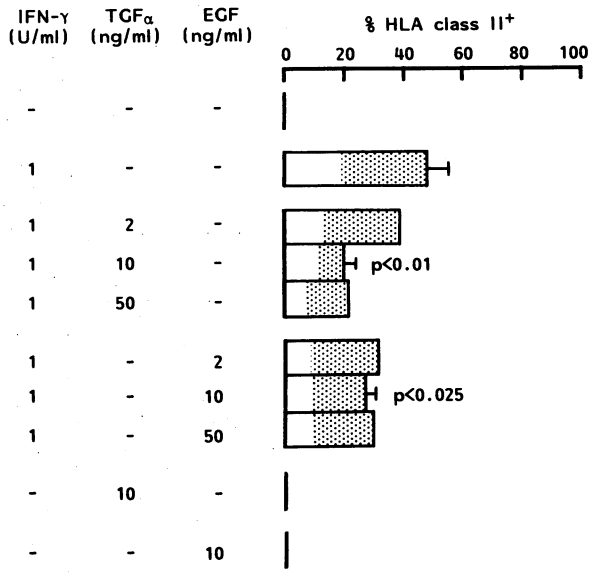

Fig. 2. Modulation of cell surface HLA class II expression in thyrocyte monolayers treated with IFN-γ and TGF-α or EGF for 6 days. The staining and scoring were performed as detailed in the legend to Fig. 1. The p values show the level of statistical difference between the indicated responses and the response to IFN-γ alone

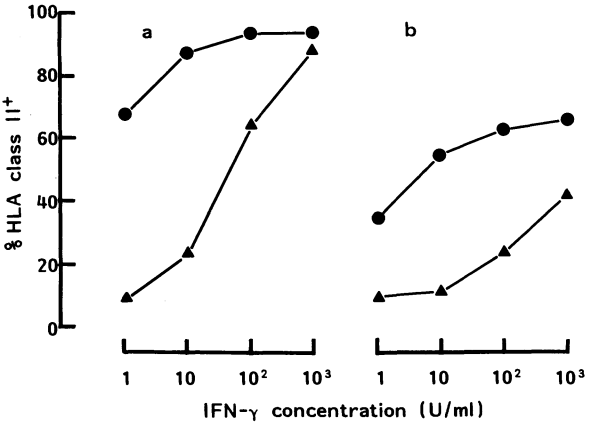

Fig. 3a, b. Modulation of cell surface HLA class II expression in thyrocyte monolayers treated with various doses of IFN-γ with (▲—▲) or without (●—●) EGF (10 ng/ml) for 7 days. HLA class II was detected as outlined in the legend to Fig. 1. The results of two independent experiments with the same thyroid specimen are shown

roid function and has been reported to be raised in patients with hyperthyroidism and, to a lesser extent, in hypothyroid individuals [22]. However, one must also bear in mind that during active autoimmune thyroiditis high concentrations of class II inducing mediators may be present in the thyroid. In this context, we found that the in vitro inhibitory effect of EGF at 10 ng/ml on class II induction in thyrocytes could be at least partially overcome by increasing the concentration of IFN-γ in the cultures (Fig. 3). This raises the possibility that although EGF or substances with similar activity may restrict HLA class II expression by thyrocytes under normal circumstances, they may be unable to prevent the induction and perpetuation of such expression brought about by high concentrations of IFN-γ and synergising factors produced in situations of severe autoimmune activation.

Effects of IFN-α

Both IFN-γ and type I interferons (i.e. IFN-α and IFN-β) are able to enhance HLA class I expression by thyrocytes [11]. However, unlike IFN-γ, type I interferons do not induce HLA class II in thyrocytes [11, 12]. Our more recent studies [33] have further indicated that IFN-α inhibits HLA class II induction in thyrocytes in vitro by IFN-γ (Fig. 4). This activity was noted on thyrocytes from both non-autoimmune individuals and patients with Graves' disease. Lymphoblastoid IFN-α derived from the Namalva cell line was more potent than recombinant IFN-α_1 (Fig. 4); this may be because the former preparation contains at least ten subspecies of IFN-α, some of which may be more potent in this activity than the IFN-α_1 subspecies per se.

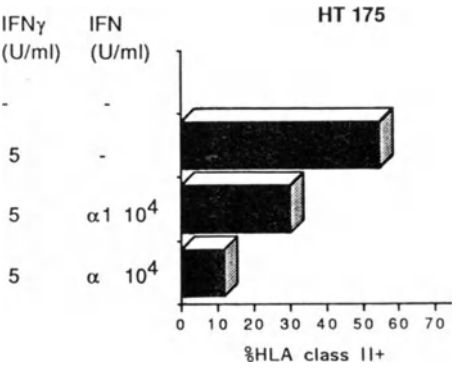

Fig. 4. Inhibition by IFN-α_1 and lymphoblastoid (Namalva-derived) IFN-α of IFN-γinduced cell surface HLA class II expression on monolayers of thyrocytes from a non-autoimmune donor (HT-175). HLA class II was detected as outlined in the legend to Fig. 1

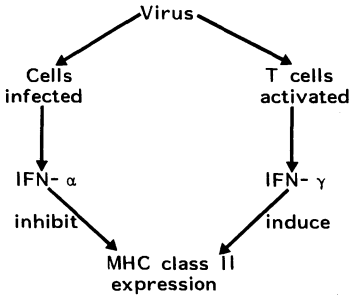

Fig. 5. Hypothetical scheme for the modulatory influences on MHC class II expression resulting from viral infection. (From Morris and Tomkins [23])

The inhibition of class II induction in thyrocytes by IFN-α_1 and lymphoblastoid IFN-$\alpha$was dose dependent, with the degree of inhibition achieved increasing up to the highest concentrations of IFN-αemployed (i.e. 10^4 U/ml). However, it is conceivable that high concentrations of IFN-α may occur in pathological situations in vivo: for example, levels of type I interferons up to 1000 U/ml have been detected in brain tissue of mice infected with neurotropic Semliki Forest virus [23]. Furthermore, as suggested by Morris and Tomkins [23], the possibility of IFN-α mediated inhibition of MHC class II induction could be relevant in viral infections where T cells responding to the virus are activated to secrete IFN-γ, but cells infected by the virus are stimulated to produce IFN-α(Fig. 5).

Different Effects of MHC Class II Modulators on Different Cells Types

We have so far discussed evidence that EGF (and TGF-α) and IFN-$\alpha$are inhibitors of HLA class II expression by thyrocytes in culture. However, when looking at the effect on a cell of its interaction with a cytokine, one must consider not only the nature of the cytokine but also the type of cell on which it is acting. This is because different cell types may respond in different ways to the same cytokine. To

Table 1. Differential effects of mediators on HLA class II expression by different cell types

Mediators	HLA class II expression	
	Decreased	Increased
EGF	Thyrocytes [18]	T-depleted PBMC [26]
IFN-α_1	Thyrocytes [33]	Monocytes [32]

give just one example, TNF inhibits the proliferation of various cells, including thyrocytes [24], but stimulates the proliferation of fibroblasts [25]. The findings of different groups summarised in Table 1 indicate that such considerations may apply to some of the mediators that we have discussed here. Thus, in contrast to our finding that EGF suppresses HLA class II induction in thyrocytes, Acres et al. reported that EGF stimulates HLA class II expression by blood mononuclear cells (other than T cells) and this is paralleled by an increase in their ability to induce antigen-dependent T cell proliferation [26]. With regard to IFN-α, in accord with the results described here, a number of reports examining various cell types indicate that IFN-α and IFN-β inhibit IFN-γ mediated enhancement of MHC class II expression [23, 27–29], although in some cases weak induction of class II by IFN-α has been reported [23, 30, 31]. Of particular relevance here is the finding by Rhodes et al. that recombinant IFN-α_1 enhanced HLA class II expression by monocytes (although it actually suppressed the antigen-presenting function of these cells) [32]. This again contrasts with the class II suppressive effect of this subspecies of IFN-α on thyrocytes (Table 1). In general, these various studies raise the possibility that certain mediators might inhibit "inappropriate" MHC class II expression in some cell types (e.g. thyrocytes) without simultaneously suppressing class II expression by cells which normally function in antigen presentation. This may help to maintain the physiological distribution of MHC class II expression, which is presumably tuned to a level which allows adequate activation of the immune system when necessary. By contrast, as suggested in the introduction, an inappropriate level or distribution of MHC class II expression might, depending on the circumstances, either permit excessive immune activation or unnecessarily limit the T cell repertoire through over-zealous induction of anergy.

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Discussion

McLachlan:
I have a problem which perhaps you and Ricardo would clear up for me: I understood from Ricardo Pujol-Borrell's talk that in some cases where there is a tumor, and presumably proliferating cells, you observe increased expression of class II and yet EGF is one of the factors with the most potent ability to decrease expression of class II. So how do you explain class II positivity in these experiments?

Todd:
Well, one can only speculate. There are many ways that one can get malignancy other than by the effects of EGF-like factors. There are other instances where people have shown MHC class II expression by tumor cells or by virally infected cells, where the MHC class II induction appears to be independent of cytokine action. So, I would speculate that in the case of some thyroid tumors it must be one of the other mechanisms of tumor induction involving oncogenes inrelated to EGF or its receptor that leads to the neoplasia.

McLachlan:
And not proliferation itself?

Todd:
No.

Ludgate:
I was interested in the effect of TSH on the stimulation of expression of class II. Did you look to see whether thyroid-stimulating antibodies would have the same effect or whether analogues of cyclic AMP would have the same effect, or perhaps phorbol esters?

Todd:
We have certainly looked at dibutyryl cyclic AMP, and it has similar effects to TSH. In the experiments we did, its effect was additive rather than synergistic with IFN-gamma, but it certainly enhanced the expression. We haven't ourselves looked at the effects of thyroid-stimulating antibodies on thyrocyte MHC class II expression, but other people have. I think Björn Wenzel has, and he found these antibodies to enhance MHC class II expression.

Analysis of Intrathyroidal Cytokine Production in Thyroid Disease

B. GRUBECK-LOEBENSTEIN and M. FELDMANN

Introduction

In spite of the generally accepted importance of cytokines in autoimmune disease, little is known about their production at the site of autoimmune processes. Recent work from our laboratory has demonstrated the increased production of interleukin (IL) 1α and 1β, tumour necrosis factor (TNF) α and β, as well as of IL-2 and interferon (IFN) γ in rheumatoid joints [1, 2].

This and other work in this field has, however, been hampered by the lack of appropriate control tissues, and it is not yet clear whether the phenomena described are specifically linked to autoimmune disorders or are to be regarded as a correlate of cell infiltration by immune cells in tissue inflammation in general. Non-toxic goitre, a benign thyroid enlargement of non-autoimmune origin, is frequently accompanied by a lymphocytic infiltration of the thyroid gland [3] and thus represents a good control for classical thyroid autoimmune disorders such as Graves' disease.

It was the aim of the present study to compare cytokine production in autoimmune and non-autoimmune thyroid tissue. Special emphasis was given to determining whether the production of one or a certain spectrum of cytokines was disease-specifically impaired in one of the cell types involved in autoimmune processes.

Materials and Methods

Most of the methods have been described in detail in recent publications [3–6] and are thus discussed only briefly here.

Thyroid tissue was obtained at surgery. Tissue from nine patients with Graves' disease, as a typical thyroid autoimmune disease, was compared with thyroid tissue from seven patients with multinodular non-toxic goitre, as a non-autoimmune control group. Small amounts of normal thyroid tissue were obtained from patients undergoing parathyroidectomy. Hyperplastic parathyroid tissue was in some experiments used for control purposes.

Tissue was digested with collagenase and purified into infiltrating mononuclear cells and thyroid follicular cells as described [3–6]. Purified cells were either used directly for mRNA analysis or further cultured: thyroid follicular

cells were established in monolayers and cultured for 3 days in the presence or absence of stimulatory factors such as lipopolysaccharide, thyroid-stimulating hormone or iodide. The conditioned supernatants were then analysed for the presence of IL-1, IL-6 and transforming growth factor-β (TGF-β) bioactivity as described [1, 4, 5]. Infiltrating mononuclear cells were stimulated with IL-2 (25 ng/ml) only for 1 week and then with a combination of OKT3 (30 ng/ml) and IL-2 (25 ng/ml). T-cell clones were established from the stimulated cells as described [6]. Cytokine mRNA was analysed in unstimulated clones as well as after stimulation with OKT3, bound to plastic and IL-2.

Total cytoplasmatic RNA was extracted from cells with the NP40 lysis method, dried, redissolved, denatured and blotted onto nitrocellulose as described elsewhere [1, 2, 4–6]. The filters where then baked, prehybridized and hybridized with cDNA probes specific for the respective cytokines. The sources and structures of the various probes have also been mentioned in previous publications [1, 2, 4–6]. The filters were exposed to films and the resulting autoradiographs scanned on a densitometer. The integral values from the linear range of scan (scanning units = abundance of mRNA) were used to define the amount of lymphokine per sample. The values were adjusted for the amount of mRNA per sample detected by the probe 7B6.

Student's *t* tests or, when data were not normally distributed, Willcoxon's rank-sum tests were used.

Results

All cytokines analysed could be detected in mononuclear cells purified from Graves' disease as well as from non-toxic goitre thyroids (Fig. 1). Cytokine mRNA production varied considerably within the disease groups. Although values tended to be higher in Graves' disease than in non-toxic goitre (mean values were higher in each cytokine), non-producers were found in each group, and the differences between the groups were not statistically significant.

Most of the T-cell clones from Graves' disease as well as from non-toxic goitre tissue were $CD4^+$. No cytokine mRNA expression could be detected in resting $CD4^+$ T-cell clones. However, after stimulation with OKT3 and IL-2 most of the clones produced all cytokines simultaneously. The respective mRNA concentrations varied among clones and among different patients but not between clones from autoimmune and non-autoimmune thyroid tissue.

No mRNA for IFN-γ and for TNF-α or -β was found in purified thyroid follicular cells. IL-1β and platetet-derived growth factor A mRNA signals were, although detectable in a few samples, negligible in their intensity. In contrast, relatively high concentrations of IL-1α and of IL-6 message were found in 4/8 Graves' disease samples and in 1/6 non-toxic goitre samples, suggesting that these cytokines were produced by thyroid follicular cells. In accordance with this suggestion, IL-1 and IL-6 bioactivity could be detected in supernatants conditioned by thyroid follicular cells in monolayer (Fig. 2). IL-1 but not IL-6

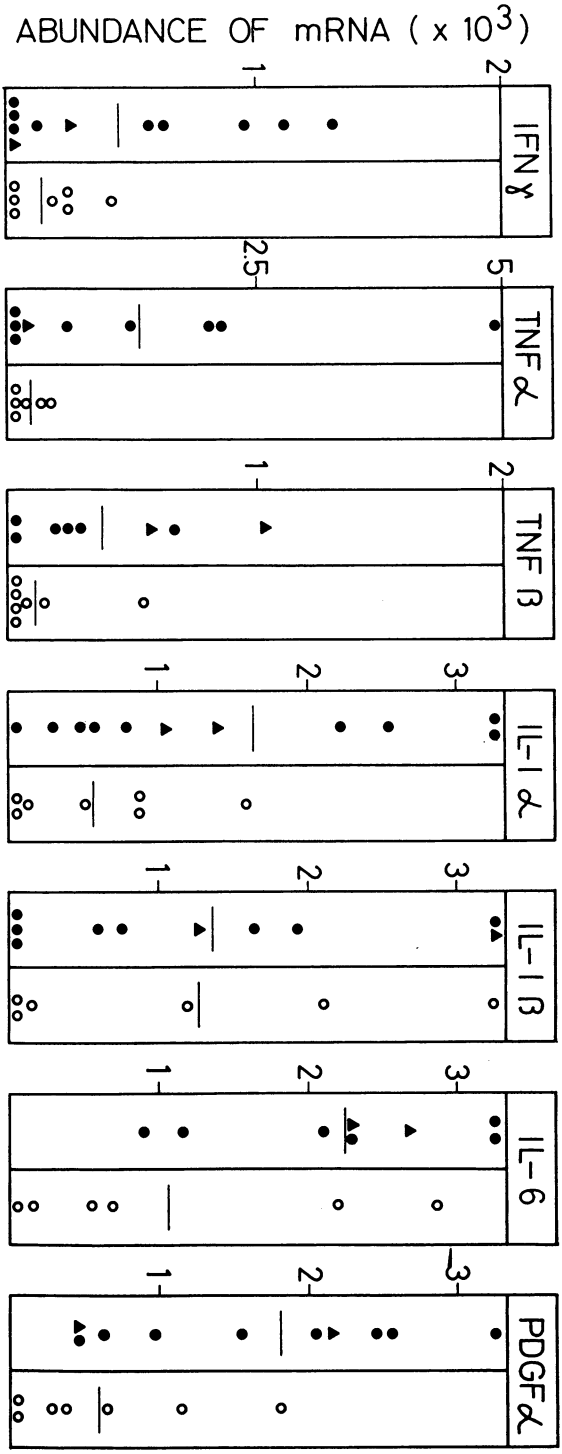


Fig. 1. Slot blot analysis of cytokine mRNA in thyroid-infiltrating mononuclear cells from patients with autoimmune disease *(left columns)* and from non-toxic goitre *(right columns)*. Values correspond to densitometer scanning units (= abundance of mRNA). (From Grubeck-Loebenstein et al. [5])

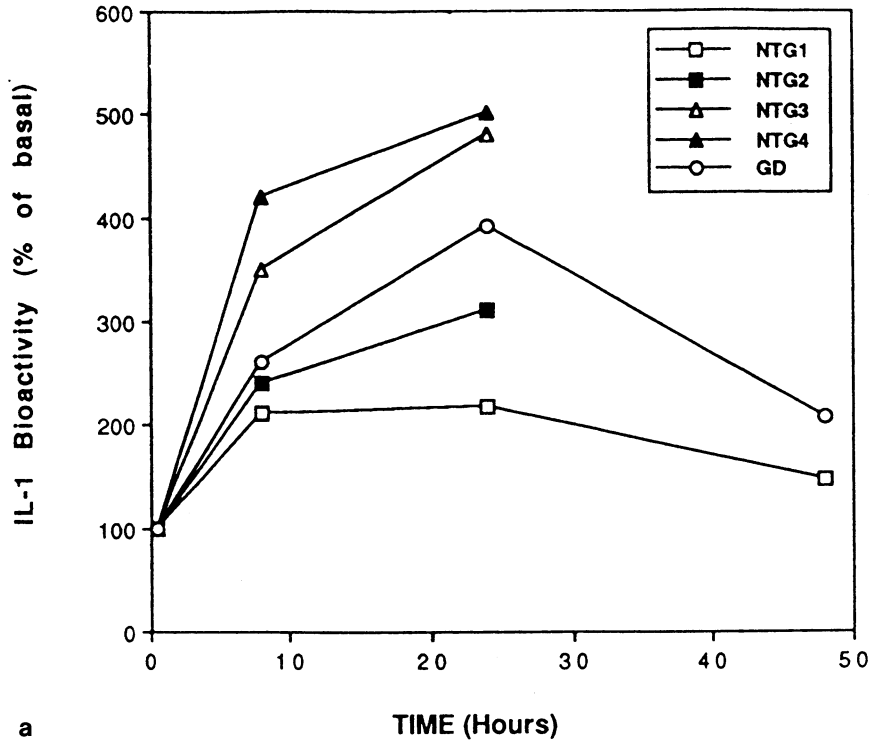
bioactivity secretion could be increased by stimulation of the cells with lipopolysaccharide (Fig. 2). Thyroid follicular cells from non-toxic goitre and from Graves' disease secreted similar amounts of IL-1 and IL-6 bioactivity.

Intrathyroidal production and growth regulatory effects of TGF-β have been discussed in detail elsewhere [4] and one thus only briefly mentioned here. Although TGF-β message was also found in thyroid-infiltrating mononuclear cells, thyroid follicular cells were the major source of TGF-β in the thyroid gland. The respective concentrations were, however, lower in non-toxic goitre than in Graves' disease ($p < 0.0.1$), lower than in normal control thyroids ($p < 0.05$) and lower than in a control group of hyperplastic parathyroid tissue samples ($p < 0.01$). Culutred thyroid follicular cells from non-toxic goitre also secreted less TGF-β bioactivity than thyroid-follicular cells from Graves' disease specimens or from normal control thyroids. The secretion of TGF-β by thyroid follicular cells could be increased by addition of micromolar concentrations of sodium iodide to the culture medium. Recombinant TGF-β inhibited, at a concentration of 10 ng/ml, the growth-stimulatory influence of insulin-like growth factor (IGF I), epidermal growth factor, and TGF-α on cultured thyroid follicular cells. This inhibition was weaker in non-toxic goitre than in Graves' disease ($p < 0.05$).

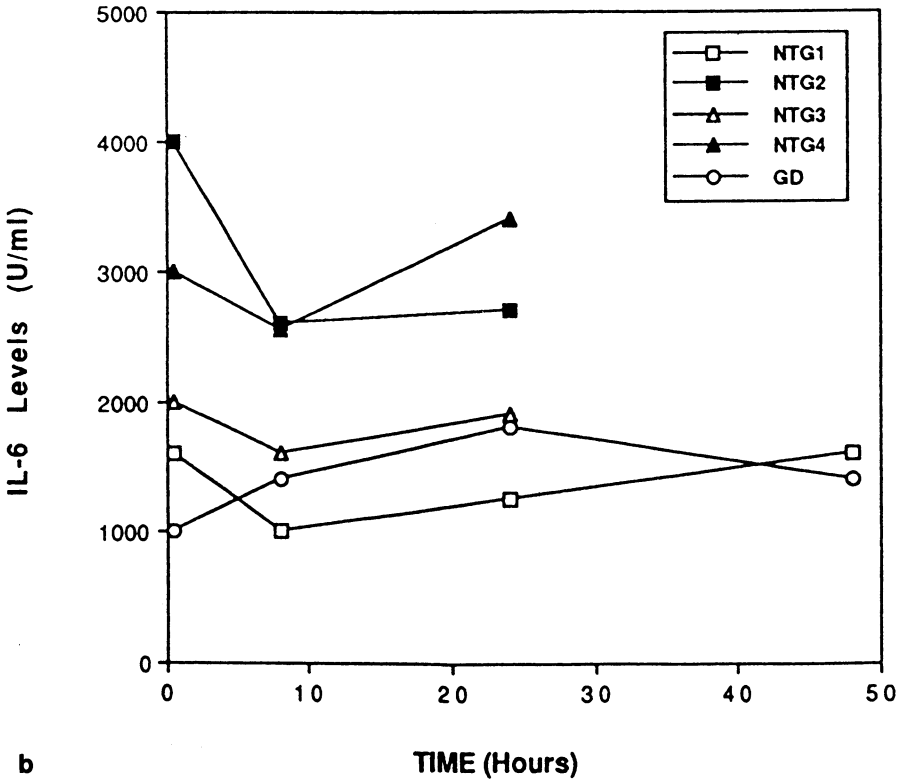
Summary and Conclusions

These results demonstrate that intrathyroidal cytokine production is not a specific feature of thyroid autoimmune disease but can also be found in non-autoimmune thyroid disorders. They show additionally that intrathyroidal cytokine production is not necessarily restricted to thyroid-infiltrating mononuclear cells but may also be found in thyroid follicular cells. It is of special interest that TGF-β, which acted as autocrine inhibitor on thyroid cell growth, was specifically decreased in non-toxic goitre. This, as well as the decreased responsiveness of non-toxic goitre cells to the inhibitory influence of TGF-β may be important for the pathogenesis of non-toxic goitre.

Fig. 2. IL-1 (**a**) and IL-6 (**b**) bioactivity in supernatants conditioned by lipopolysaccharide-stimulated thyroid follicular cells from patients with non-toxic goitre or Graves' disease. From Grubeck-Loebenstein et al. [5])


IL-1 Bioactivity (% of basal)
600
500
400
300
200
100
0
0
10
20
30
40
50
TIME (Hours)
NTG1
NTG2
NTG3
NTG4
GD

a


IL-6 Levels (U/ml)
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
TIME (Hours)
NTG1
NTG2
NTG3
NTG4
GD

b

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Discussion

Bottazzo:
Just for clarification: the IL-6 is produced only by autoimmune thyrocytes, as well as nontoxic nodular goiters, but you do not find any in normals?

Grubeck-Loebenstein:
We didn't look at normals for IL-6. It was a different series that we used for TGF beta. We had very few normals and not very much tissue.

Bottazzo:
As you know, Ian Campbell – who started all this work and published it a couple of months ago, by working on RIN cells – has found that they spontaneously produced IL-6. So, there is the great chance that IL-6 is normally produced by epithelial cells like thyroid cells. Do you go along with this idea?

Grubeck-Loebenstein:
Yes, I do. I think the data are pretty clear as far as IL-6 production is concerned.

Bottazzo: .
So, by taking all these lymphokines that have been studied thus far, produced by epithelial cells, can we say that the only one which is found in an autoimmune situation is IFN alpha in diabetic islets? You have not got anything in autoimmune thyroids which has something specific for autoimmune thyroiditis?

Grubeck-Loebenstein:
No, according to our results, there is nothing specific to thyroid autoimmune disease. Normal as well as Graves' disease thyroid epithelial cells, as well as infiltrates can produce cytokines. Probably there are different combinations of stimulators and inhibitors in autoimmunity so that finally a different pattern is created, but I think principally there is no specific defect as far as the production is concerned, at least not in our hands.

Kayser:
I would like to make a comment and also put a question: recently our group has published preliminary data showing that cultures of human thyroid epithelial cells produce IL-6, and also that the production is enhanced by IL-1 beta

[Bendtzen et al. (1989) Lymphokine Res 8: 335–340]. In preliminary cytochemical studies not yet published, we have also localized the production of IL-6 to the epithelial cells, whereas we have not been able to demonstrate any immunoreactive IL-1 alpha or IL-1 beta in the epithelial cells or in supernatants from the cultures. I would therefore like to ask you: do you think these differences could be due to differences between your and our culture systems, or could the differences be due to endothelial cells in your cultures? Have you tried to localize the IL-1 alpha production to the thyrocytes by immunocythochemical technique?

Grubeck-Loebenstein:
Well, of course, we were also interested in this question. We were trying to stain thyroid sections, and these studies are being continued at Charing Cross in Marc Feldmann's laboratory right now, but I am not quite sure what sort of results they have got. About the supernatants and about the bioactivity, I think that our studies pretty well agree with what's been published by Dr. Nagataki. He gets identical results as far as bioactivity is concerned. What finally convinced us that it was really IL-1 alpha were the experiments with neutralizing antibodies, because they demonstrated so nicely that bioactivity analyzed in the thymocyte proliferation assay was to 50% inhibited by anti-IL-1 alpha and to 50% by anti-IL-6 but not by anti-IL-1 beta.

Scherbaum:
Do you have any evidence proving that your culture system actually was devoid of endothelial cells? Using a method to deplete a system from endothelial cells is no guarantee of a complete depletion.

Grubeck-Loebenstein:
We depleted them as much as we could. We definitely reduced the contaminating cell population by 4% and that didn't change our results, so if it had been the contaminating cells which produced the cytokines, one would have expected at least some change, some reduction which we did not see.

Bottazzo:
Going back to the Nagataki story: perhaps Ulrich Deuss can help us, because he was in contact with him recently. My impression was that he did not find IL-1 in Graves' disease, when he presented his poster in Copenhagen.

Grubeck-Loebenstein:
He found the bioactivity.

Bottazzo:
Just a second, this is very important: he was finding the activity because he was not working with pure thyrocytes like you. I can't remember exactly the details of the experiment, but the conclusion was: thyrocytes are not making IL-1.

Grubeck-Loebenstein:
Well, I think, we did our best to get these results well documented: we did find the message, we found the bioactivity, and we could neutralize the bioactivity with the respective antibodies. Additional depletion of contaminating cells did not reduce the activity.

Bottazzo:
Don't you think it would be important to take FRCP cells and do an IL-6 assay there?

Grubeck-Loebenstein:
Yes of course, that would be nice, one could certainly do that, but if lines don't produce it, that would not be evidence that fresh human thyrocytes don't produce it either.

Bottazzo:
No, because that goes back to what Terry Davies was saying this morning, that work has to progress more and more, working with epithelial cell lines to make sure that there is no contamination.

Grubeck-Loebenstein:
I would not go along with your statement that Dr. Nagataki does not find IL-1, because it is always a question how you interpret the data.

McLachlan:
That was spontaneous secretion you looked at? Could you change the pattern of cytokines produced by thyroid cells if you gave TSH?

Grubeck-Loebenstein:
If you stimulate with LPS, you could increase stimulatory activity in the mouse thymocyte proliferation assay, but not in the B9 hybridoma assay. LPS-stimulated thyrocytes produce more IL-1 alpha.

McLachlan:
But was that with the highly depleted thyroid cell monolayer?

Grubeck-Loebenstein:
Yes, but we never tried TSH in this system.

Lymphokine-Mediated Induction of HLA-DR Antigen Expression on Thyroid Follicular Cells In Vitro by Autologous Mononuclear Leukocytes and by Cytomegalovirus Infection*

E. L. KHOURY

Introduction

A characteristic immunopathologic feature of human autoimmune thyroid disease (ATD) is the ectopic expression of HLA class II antigens by a proportion of the thyroid follicular cells (TFC) in the affected gland [1]. This finding prompted the notion that this expression might be the primary aberration which would enable TFC to present specific self constituents to potentially autoreactive T lymphocytes in the "appropriate" context, and thus initiate the autoimmune response leading to chronic inflammation and parenchymal damage [2]. In support of their putative role as antigen-presenting cells, HLA-DR positive TFC cultured from ATD tissues have been shown to present virally-encoded [3] and autologous thyroid [4] antigens to specific T cell clones in vitro.

A role for viral agents in the etiology of thyroid autoimmune disorders has long been postulated, and there is evidence that viruses are involved in the development of autoimmune thyroiditis in some experimental animal models [5]. Cytomegalovirus (CMV) infection is very prevalent in the general human population and can cause severe disease in immunocompromised individuals, with the frequent involvement of endocrine tissues [6, 7]. Several autoantibodies have been described after CMV infection in humans [8] and experimental animals [9]. Moreover, a high prevalence of persistent CMV infection has been recently reported in newly diagnosed cases of type I diabetes mellitus with islet-cell autoantibodies [10].

Although the role of ectopic HLA class II expression by TFC as a sufficient or even a necessary stimulus for the initiation of ATD remains speculative, a proposed mechanism whereby a viral infection might be able to trigger autoimmunity would operate through the induction of that ectopic expression [2]. Such an induction can be envisioned as the result of either (a) a direct effect of the virus on the host cell or (b) the local release of lymphokines (i.e., interferon-γ) by sensitized T lymphocytes after their encounter with viral antigens. We have previously shown that, in glands affected by ATD, ectopic HLA class II expression on TFC appears to be entirely dependent upon the local mononuclear cell (MNC) infiltration and not the result of a primary aberration

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in the TFC [11, 12]. The present communication focuses on the de novo induction of HLA class II antigens on TFC cultured from normal or nodular tissues, devoid of detectable MNC infiltrates, by autologous peripheral blood MNC (PBMNC) and by CMV infection in vitro.

De Novo Induction of HLA Class II Antigens on TFC Cultured from Normal Tissues by Autologous PBMNC

HLA class II negative TFC, obtained from normal thyroid tissue of patients undergoing thyroidectomies for medullary carcinoma and with no humoral markers of thyroid autoimmunity, were used in these experiments [12]. TFC were cocultured with autologous PBMNC, or with their E rosette positive (T cell enriched) subset, at an approximate MNC/TFC ratio of 25 to 1, for up to 4 weeks. In some instances TFC and MNC were cocultured using Millicell (0.45 μm pore size) filters (Millipore, Bedford, MA, USA) to prevent physical contact between the two cell populations. Parallel thyroid monolayers were treated with phytohemagglutinin (PHA, 10 μg/ml), known to induce HLA class II expression on TFC in primary cultures [13], to obtain positive controls. Cell-surface HLA class II antigen expression in the viable monolayers was detected by indirect immunofluorescence (IF) with monoclonal antibodies (MAb) against HLA-DR (L243) and HLA-DQ (Leu 10) determinants (Becton-Dickinson Monoclonals, Mountain View, CA, USA).

As shown in Fig. 1, coculture of TFC with total PBMNC resulted, after a latency of 3–5 days, in a pronounced induction of HLA-DR antigens on the TFC, which reached its peak (> 80% HLA-DR positive TFC) after 8–12 days in coculture. Under these conditions, TFC were also induced to express cell-surface HLA-DQ antigens although the degree of expression was noticeably lower. TFC in monolayers which were cocultured with MNC separated by Millicell filters showed a comparable degree of induction, although in mixed cultures where both populations are in physical contact there is also evidence for a direct transfer of HLA-DR molecules from MNC to TFC [12]. In contrast, purified T lymphocytes, separated from the same PBMNC suspensions by E rosetting, were unable to induce de novo HLA class II antigens on autologous TFC in the mixed cultures (Fig. 2). Moreover, the T cells themselves also remained negative for HLA-DR expression during the entire period of coculture, presumably reflecting their lack of activation.

Taken together, these and our previous results [12] indicate that TFC cultured from either normal or autoimmune thyroid tissues possess a similar susceptibility for being induced to express HLA class II antigens by autologous MNC in vitro, and that a preexistent thyroid autosensitization of the MNC donors is not required for the induction to occur. On the other hand, purified T lymphocytes appear to be unable by themselves to induce de novo HLA-DR antigens on autologous TFC, although they can maintain a preexisting TFC expression under similar conditions [12, 14]. The induction observed in these

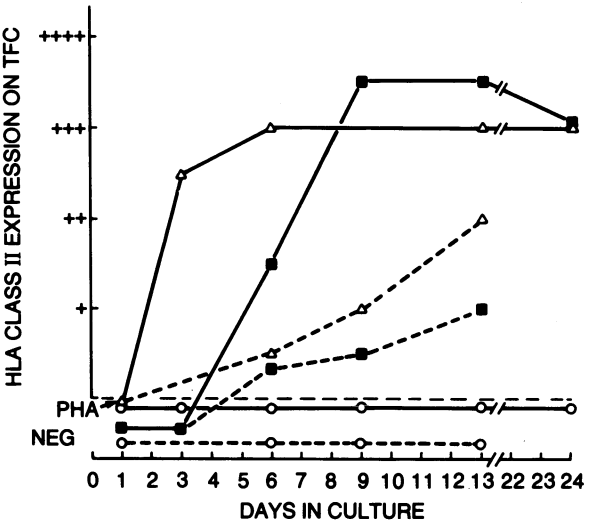


Fig. 1. Time course of cell-surface HLA class II antigen expression (—, HLA-DR detected by MAb L243; - - -, HLA-DQ detected by MAb Leu 10), induced in vitro on TFC cultured from normal thyroid tissue: ○, control cultures; △, PHA (10 μg/ml) treated cultures; ■, mixed (with 10^6 autologous PBMNC/4 × 10^4 TFC) cultures. HLA class II antigen expression was graded according to the proportion of TFC with surface IF in the monolayer, as + (20%–30%), + + (45%–55%), + + + (70%–80%), and + + + + (>95%). *NEG*, negative

mixed autologous TFC/MNC cultures is largely mediated by a soluble factor which has been identified as interferon-γ[14], but a synergistic effect of macrophage-released tumor necrosis factor α [15] might also contribute to its ocurrence.

Interferon-γ Associated Induction of HLA-DR Antigen Expression on TFC in Primary Monolayers Infected In Vitro with CMV

To explore the possibility of a de novo induction of HLA class II antigen expression on TFC triggered by CMV, we employed [16] primary thyroid monolayers cultured from nodular and normal (paranodular) tissues, devoid of detectable lymphoid infiltrates, of four patients who underwent thyroidectomies for large nodular goiters. Two of these individuals were CMV seropositive and two CMV seronegative. Four to 8 days after plating freshly dispersed cells, the monolayers were infected with CMV (strain AD169) at a mutiplicity of infection of approximately 1. As a positive control for induction, parallel uninfected monolayers were treated with PHA as described above. Four MAb (clones L243, 2.06, O3-D7, and 1800 D2.4) directed against nonpolymorphic HLA-DR determinants, and two MAb (clones CH16-1 and CH167-5) against CMV-encoded proteins were used, in single- and double-label IF stainings, to detect

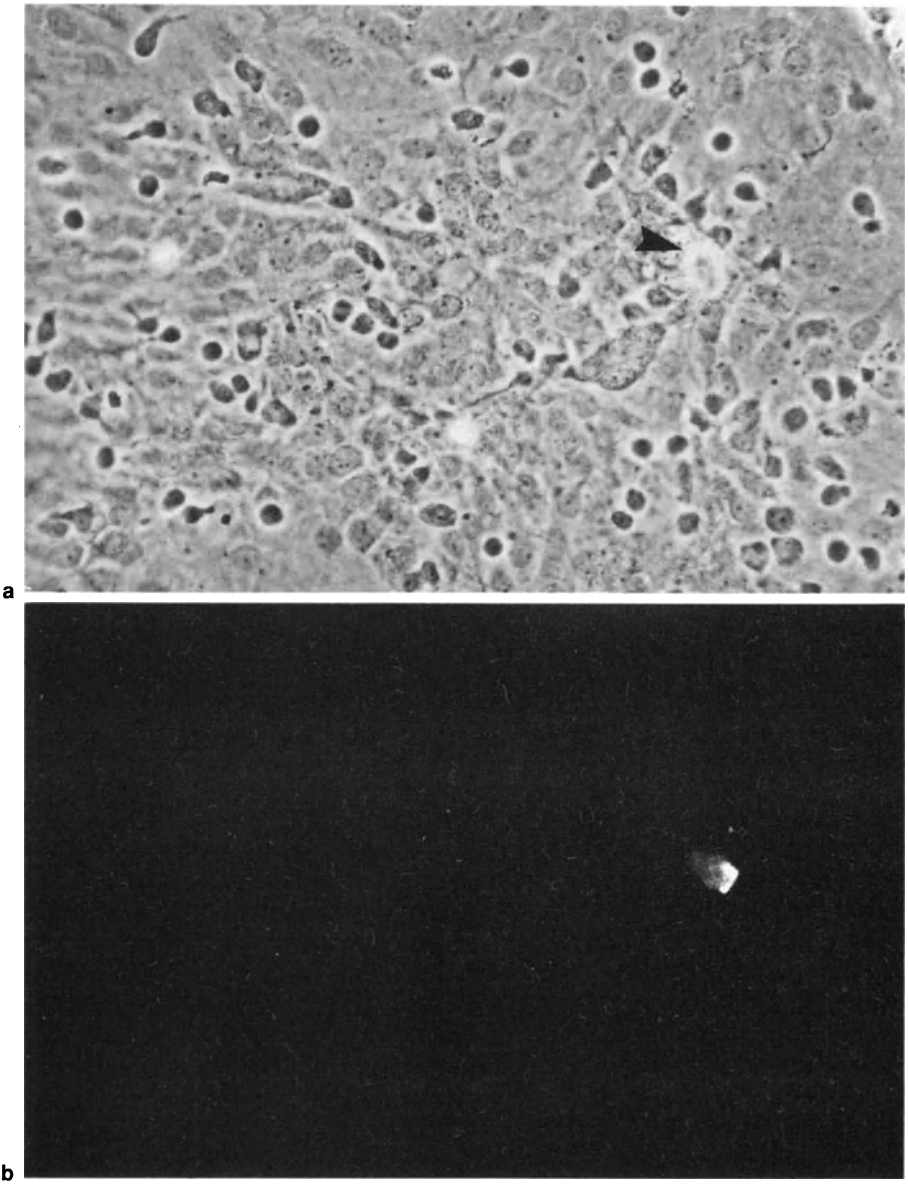


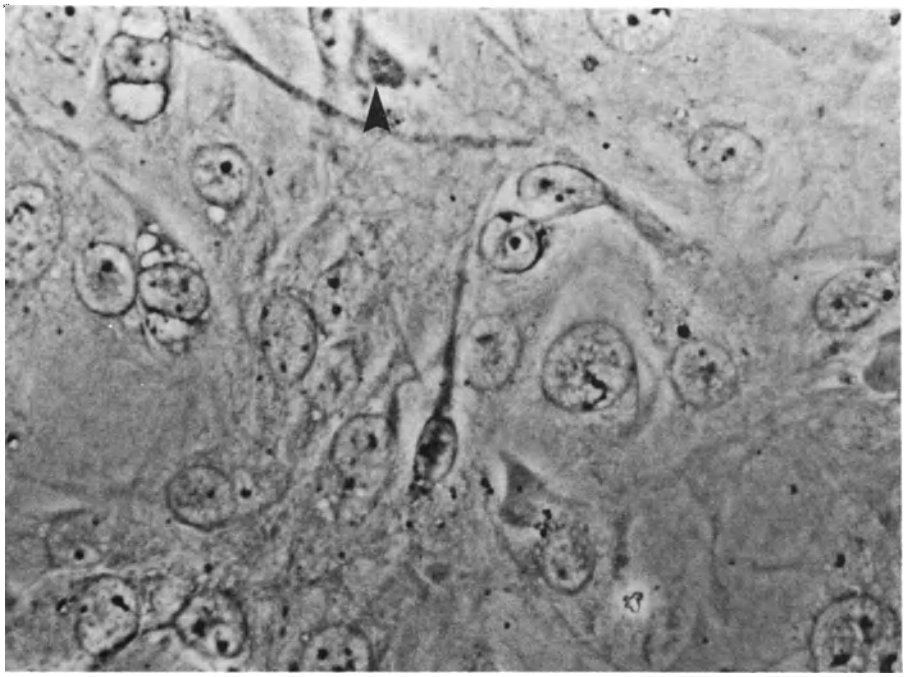
Fig. 2 a, b. Indirect IF staining with anti-HLA-DR MAb (clone L243, 20 μg/ml) on a viable 18-day-old mixed (with 10^6 autologous E^+ PBMNC) culture established from normal thyroid tissue (original magnification ×300). **a** Phase-contrast microscopy, showing a confluent monolayer of TFC, numerous attached lymphocytes, and a partially spread and refringent macrophage *(arrowhead)*. **b** IF staining on the same field; neither TFC nor T lymphocytes express cell-surface HLA-DR antigens, and only the macrophage displays positive IF staining in the entire field. In contrast, about 80% of the TFC cocultured with equal numbers of unseparated PBMNC for a similar period were positive for cell-surface HLA-DR expression (see Fig. 1).

cell-surface HLA-DR expression and individual CMV-infected cells (before and after permeabilizing the monolayers, respectively). The polypeptide profile of the induced HLA class II antigens was determined by SDS-PAGE analysis of ^{125}I-labeled cell-surface glycoproteins after immunoprecipitation with anti-HLA-DR MAb. Interferon-γ levels in conditioned culture supernatants were measured with a solid-phase ELISA kit (Holland Biotechnology, The Netherlands).

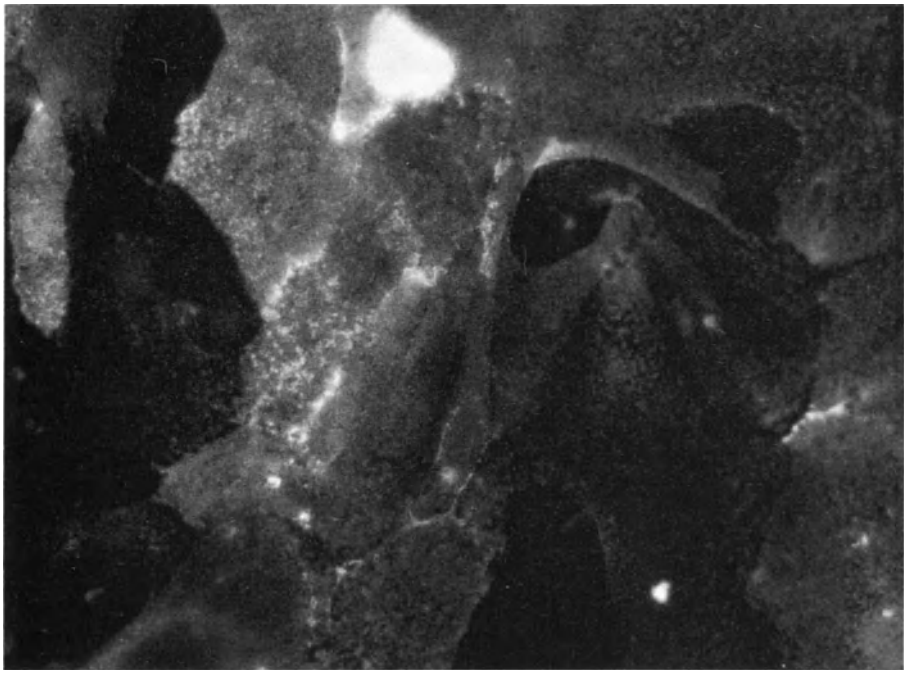
CMV-infected monolayers cultured from the normal (paranodular) tissues of the two CMV-seropositive thyroid donors showed induction of HLA-DR antigen expression on 30%–50% of the TFC. This expression was initially detected 3–4 days after infection and was fully manifested 4 days later. On the other hand, CMV-infected cultures from the nodular tissues of the same donors, constituted by an extremely homogeneous population of TFC, showed only marginal or no induction. All four anti-HLA-DR MAb recognized the induced cell-surface molecules, which displayed the characteristic polypeptide profile of conventional HLA class II antigens upon immunoprecipitation and SDS-PAGE analysis [16]. The large majority of TFC showing this early and widespread induction, however, lacked morphologic and antigenic evidence of individual CMV infection (Fig. 3), suggesting the mediation of a diffusible factor. This possibility was supported by the detection of interferon-γ in the conditioned supernatants from CMV-infected and PHA-treated but not from control cultures [16]. Moreover, in all cases the interferon-γ levels in the supernatants correlated with the proportion of HLA-DR positive cells seen in the corresponding monolayers. In contrast, this induction did not occur in CMV-infected monolayers from either normal or nodular tissues of the two CMV-seronegative donors, although parallel cultures showed the expected HLA-DR expression in response to treatment with PHA.

A second, direct mode of induction was detected later on in all CMV-infected cultures, irrespective of their nodular or paranodular origin and of the immune status of the tissue donor toward CMV. Starting 6–8 days after infection, a proportion (up to 50%) of the individually infected TFC having large, CMV antigen positive, intranuclear inclusions also displayed cell-surface HLA-DR related determinants recognized by one (clone 1800 D2.4) of the MAb employed. This induction, which was fully manifested 10–18 days after infection, was restricted to TFC, and CMV-infected fibroblastoid cells present in the primary monolayers remained negative even at advanced stages of their individual infection [16]. The mechanism(s) underlying this direct induction and the identity of the molecule induced are presently unknown.

These results suggest the intriguing possibility that primary monolayers cultured from histologically normal thyroid tissue, devoid of detectable lymphoid infiltrates, of CMV-seropositive individuals may contain sufficient numbers of CMV-sensitized T lymphocytes to mount an in vitro immune response, with local release of interferon-γ, upon encounter with CMV. The interferon-γ-induced HLA class II antigen expression on neighboring uninfected cells which would result from a similar local immune response in vivo might confer upon TFC the capability to participate directly in this response, presenting viral anti-



a



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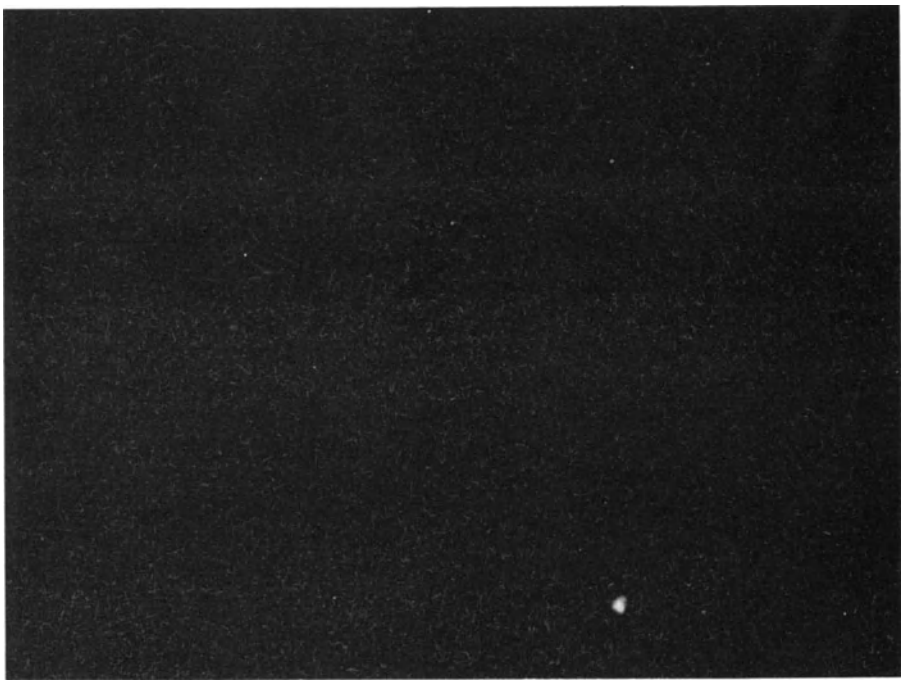

c

Fig. 3 a–c. Double-label IF staining on the same microscopic field of a 12-day-old monolayer, cultured from normal (paranodular) thyroid tissue of a CMV-seropositive donor, 8 days after CMV infection in vitro (original magnification × 500). **a** Phase contrast, showing several spread-out TFC and one partially spread mononuclear leukocyte *(arrowhead)*. None of the cells in this particular field show the characteristic cytopathic effect (intranuclear inclusions) of CMV infection. **b** IF staining given (on viable cells, before fixation of the monolayer) by anti-HLA-DR MAb (clone 1800 D2.4), followed by FITC-labeled goat $F(ab')_2$ fragments anti-mouse IgM. Most of the cells display a dense surface staining, particularly strong on the mononuclear leukocyte. **c** IF staining given (on permeabilized cells, after acetone-fixation of the monolayer) by a MAb directed against CMV-encoded ICP 36 (clone CH16-1), followed by rhodamine-labeled goat $F(ab')_2$ fragments anti-mouse IgG. None of the cells in the field show the characteristic intranuclear binding of this MAb on CMV-infected cells [16], as further indication that they are uninfected.

gens, should they become infected by CMV. Furthermore, HLA-DR expression might enable infected TFC to become more efficient targets for anti-CMV T cell cytotoxic responses, since T cell mediated cytotoxicity against some CMV-infected human cells is HLA-DR restricted [17]. In another context, CMV-induced HLA-DR expression on other human epithelial cells, i. e., those in kidney tubules, might play a significant role in allograft rejection associated with CMV infection [18].

Regarding the possible relevance of these findings in the pathogenesis of ATD, we [12] and others [14, 19] have obtained evidence indicating that ectopic HLA class II expression in thyroid tissues affected by autoimmune conditions is not an intrinsic abnormality of the TFC but a result of their response to the

local lymphoid infiltration and lymphokine release. This would argue against a direct HLA class II induction on TFC due to their productive or latent viral infection, as the initial step in the development of ATD. Nevertheless, the possibility still remains of an initial interferon-γ mediated (indirect) induction triggered by a local antiviral immune response, induction which might then be maintained by the autoreactive lymphoid infiltration.

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Discussion

Bottazzo:
The egg and chicken story that characterized our discussion in the last few years: Are we going back now to common ground?

Khoury:
Well, I would say that whether or not these results with cytomegolovirus have a direct relevance for the pathogenesis of thyroid autoimmune disease has yet to be established. There are two situations, however, in which the relevance of these phenomena is more straightforward: one is the immune response against a local infection by cytomegalovirus. Because of their interferon mediated induction, the newly infected thyroid cells might directly participate in the immune response, presenting viral antigen. Not only that, but in the case of T-cytotoxic responses against CMV-infected human cells, in some circumstances the T-cytotoxic cell is HLA class II restricted, i. e., is a CD4-positive T-cytotoxic cell restricted by HLA-DR, not by class I; so the fact that the cell is expressing class II together with the putative CMV-encoded antigens on the membrane may allow the cytotoxic T cell to recognize a CMV-infected cell. The second situation is organ (e. g., kidney) graft rejection associated with CMV infection.

Seif:
Nuclear and cytoplasmic inclusion bodies of the CMV infection are aggregates, as I understand, of viruses?

Khoury:
Yes, but they are not really viral particles.

Seif:
My question would by, in these cells, where you cannot see the inclusion bodies, and where you do not have an immune reaction with a specific antibody, how can you exclude that the virus is distributed diffusely in these cells?

Khoury:
This is a very good point, especially in the case of CMV which, like all the herpes viruses, may be latent. However, the fact that most of these cells later on showed a full-blown infection with inclusion bodies etc. makes me think that there was no latent infection, but just lower susceptibility with the very low dose

of virus that we were using here. We were using one plaque-forming unit per cell, which is quite a low dose, especially for thyroid cells which appear to be much less susceptible to CMV infection in vitro than fibroblasts.

Todd:
About 3 years ago, with Will Irving, when he was in Oxford, we infected thyroid cells with various viruses. I remember that CMV was one of the viruses we tried, but we could detect no MHC class II expression. I was interested that you said that some anti-MHC class II monoclonal antibodies stain the CMV-infected thyrocytes, but others did not.

Khoury:
Well, that was in the case of the direct induction. In the case of the gamma interferon mediated induction, all the monoclonals reacted, and the molecule induced, as shown after immunoprecipitation and SDS gel electrophoresis, was certainly class II. Probably the donors you used were CMV seronegative. So there was no previous sensitization, there were no primed T cells in the primary cultures, there was no local immune response, no release of interferon gamma, no induction.

Scherbaum:
Yes, but you obtained a positive induction with the seronegative thyroid.

Khoury:
Only for the direct induction. The direct induction works on cultures from both seronegative and seropositive donors. The interferon gamma induction, the first one I showed . . .

Bottazzo:
We are talking about the direct now?

Todd:
Yes, I am referring to the direct induction in virally infected thyrocytes. We had infected cultures looking like the ones shown by Dr. Khoury with the cytopathic effects in the virally infected cells.

Khoury:
I am saying that if the donors you use happen to be seronegative for CMV, you will miss, for sure, one of the types of induction, the indirect type. The second one, the direct type, was picked up only by one monoclonal antibody, and not by three others.

Todd:
There are other possible explanations of the apparently direct induction of MHC class II. That is why I was particularly asking about the fact that some monoclonal antibodies to MHC class II pick it up and others do not. The fact,

on the one hand, may be that we just used monoclonals that didn't react for some reason. On the other hand, there are some viruses that have reactivities that cross-react with MHC molecules.

Khoury:
Yes, and for CMV there is some cross-reactivity between an immediate early (IE) 2 gene product and a nonpolymorphic portion of the beta chain of DR, described recently by Fujinami et al. (J Virol 62: 100–105, 1988), but this is not the case.

Todd:
Why?

Khoury:
That particular mechanism is not the one underlying the direct induction that we saw. Of course, there may be some other cross-reactivity.

Bottazzo:
Emilio, what he is asking you is: is there the possibility that your antiserum is picking up cross-reactivity products from the virus? Did you check for that?

Khoury:
Yes, I did for the already mentioned cross-reactivity. I checked for that, and it is not the cause of the induction since CMV-infected fibroblasts, where the cross-reactivity was described, were negative for the direct induction that we found. Of course, there may be another type of cross-reactivity. So, the molecule induced by the direct mechanism may not be a real HLA-DR molecule. On the other hand, there might be reasons for having an induced molecule that is not a complete molecule, e. g., some of the CMV genes induce the activation of a DNA-binding factor like the NF-kappa B factor which would act, perhaps, on the transcription of one HLA-DR chain and not on the other.

Bottazzo:
You will have to prove that experimentally.

Thyroid Dysfunction After High-Dose Interleukin-2 Therapy: An Update*

M. B. ATKINS, M. M. KAPLAN, P. A. DEMCHAK, N. J. ROBERT, S. REICHLIN, and J. W. MIER

Introduction

Interleukin-2 (IL-2) is a secretory product of activated T-lymphocytes that has profound effects on the immune response. In particular, IL-2 has been shown to enhance the *in vitro* tumoricidal activity of peripheral-blood mononuclear cells [1] and to induce the formation of lymphokine-activated killer (LAK) cells which are able to lyse a broad spectrum of malignant cells *in vitro* [2]. Clinical trials using high doses of recombinant human IL-2, with or without autologous LAK cells, have yielded promising results, particularly in patients with melanoma or renal cell carcinoma [3–8]. This therapy has also been accompanied by multiple acute but generally reversible toxic effects, including fever, chills, lethargy, diarrhea, anemia, thrombocytopenia, eosinophilia, confusion, diffuse erythroderma, hepatic dysfunction, myocarditis and myocardial infarction, defects in neutrophil function, bacterial sepsis, and a capillary leak syndrome leading to hypotension, fluid retention, and renal insufficiency [3, 5, 8–13].

The appearance of a large goiter (Fig. 1 A) and hypothyroidism in a 28-year-old man 3 months after IL-2/LAK cell treatment for metastatic melanoma prompted us to investigate thyroid function in all of our patients. In June 1988 we reported that hypothyroidism occurred in 7 of the first 34 (21%) patients with advanced neoplasms treated with IL-2 and autologous LAK cells at the New England Medical Center [14], Hypothyroidism typically developed 4–11 weeks after treatment, and appeared at least in some patients to be self-limited, resolving within 10–11 months. IL-2 induced hypothyroidism was frequently associated with the presence of humoral immunity against thyroid antigens, suggesting an autoimmune mechanism, appeared to require the use of LAK cells in the treatment regimen, and was associated with tumor regression.

As of August 1989 a total of 140 patients with advanced malignancies had received various forms of high-dose IL-2 therapy at the New England Medical

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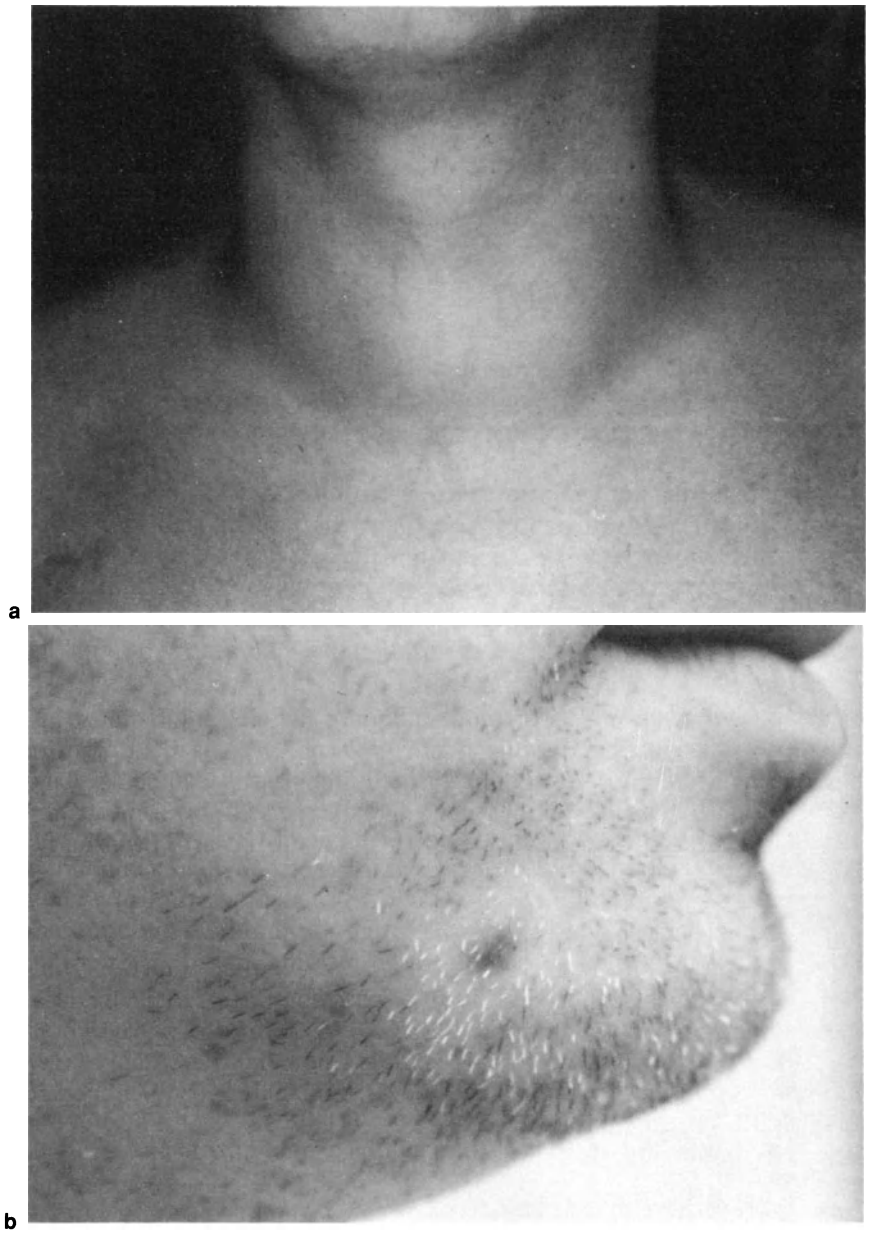

Fig. 1a, b. Physical findings in the index case of IL-2 and LAK cell related hypothyroidism 22 weeks after the patient began his first course of IL-2 and LAK cell therapy. **a** Diffuse goiter. **b** Halo nevus with hypopigmentation of surrounding skin and facial hair

Center, and 115 were evaluable for thyroid function at least 4 weeks after treatment. We detected thyroid dysfunction in an additional 10 patients (17 overall). We now report our evaluation of this expanded series of patients and update our discussion of the possible mechanisms and clinical and basic scientific relevance of this consequence of IL-2 therapy.

Methods

Patients were treated in the Clinical Study Unit of the New England Medical Center either as part of a larger, six-center clinical trial conducted by the National Cancer Institute Extramural IL-2 Working Group or institutional trials including high-dose IL-2. All treatment protocols were designed in cooperation with the National Cancer Institute and approved by the Food and Drug Administration and the Human Investigation Review Committee at the New England Medical Center. Written informed consent was obtained from each patient. All patients had measureable metastatic cancer, normal organ function, and a performance status of 0 or 1 as defined by the Eastern Cooperative Oncology Group.

Patients were treated according to six distinct schedules involving maximum tolerated doses of IL-2 (Table 1). IL-2 was administered either by intermittent bolus injection, continuous infusion or a combination of the two and either alone or in combination with interferon alpha (IFN-α), LAK cells, or both. Thirty-five patients were treated with IL-2 and LAK cells according to a protocol essentially identical to that originally described by Rosenberg et al. [3] (B-IL2/LAK). Nineteen patients were treated according to a modified protocol in which IL-2 (Cetus, Emeryville CA, USA) was administered by continuous intravenous infusion during both the initial and LAK cell phases of treatment (CI-IL2/LAK). Seventeen patients were treated according to a second modified protocol (Hybrid IL2/LAK) in which IL-2 was administered by bolus intravenous injection before leukapheresis (days 1–3) and continuous infusion during the LAK cell phase (days 9–15). Eight patients received IL-2 (Hoffmann La Roche, Nutley NJ, USA) together with IFN-α (Hoffmann La Roche) intravenously every 8 h on days 1–5 and days 11–15, with leukapheresis procedures performed on days 7–10 and LAK cells administered on days 11, 12, and 14 (IL2/IFN/LAK). Twenty-eight additional patients received bolus injections of IL-2 without LAK cells on a schedule that was otherwise identical to the original IL-2/LAK cell protocol except that in 12 patients the beginning of the second course of IL-2 was delayed until day 15 (B-IL2 alone). Finally, three patients received IL-2 (Cetus) and IFN-α (Schering-Plough, Kenilworth NJ, USA) by bolus intravenous injections every 8 h on days 1–5 and 15–19 (B-IL2/IFN).

Each patient was evaluated for side effects and tumor response in the Oncology Clinic at the New England Medical Center at 4-week intervals after the completion of therapy. Patients whose tumors had progressed were withdrawn

from the study, while those whose tumors had regressed were offered retreatment at 3-month intervals.

Thyroid function and thyrotropin (TSH) assays were performed before treatment and on all follow-up clinic visits while the patients remained under study. Although some patients were unavailable for follow-up due to treatment-related toxicity and/or rapid early disease progression, no patient was excluded from analysis *apriori*. Serum thyroxine (T_4) and TSH were measured by radioimmunoassays and the free thyroxine index (FTI) was calculated as previously described [14]. Hypothyroidism was considered to be present if the FTI was low and serum TSH concentration elevated, while hyperthyroidism was considered to be present if the reverse were true.

Serum samples were obtained before treatment and at follow-up clinic visits and were stored frozen at $-20°C$. In all patients with thyroid dysfunction and the initial 27 who remained euthyroid, serial serum samples were assayed for titers of antithyroglobulin and antithyroid microsomal antibodies as previously described [14]. Thyrotropin-receptor antibodies were assayed, in seven patients with thyroid dysfunction, including those patients with hyperthyroidism, at Nichols Institute Diagnostics (San Juan Capistrano CA, USA) according to standard procedures. ^{123}I labeled thyroid scans were performed on three patients, including one patient while hyperthyroid in the Nuclear Medicine Department at New England Medical Center using standard techniques.

Fisher's exact test was used to test the hypothesis of a relationship between thyroid status and treatment protocol, tumor type, sex, thyroid autoantibody titers, and tumor regression.

Results

A total of 140 patients were treated with high-dose IL-2 regimens at the New England Medical Center between April 1986 and August 1989, and 115 were evaluable for thyroid function at least 4 weeks after treatment. Specific data on these patients are summarized in Table 1. Of these patients, 79 received LAK cells, and 36 did not; 58 had melanoma, 40 had renal cell carcinoma, and 17 had any of a variety of other malignancies; 75 were men and 40 were women. Thyroid abnormalities were detected in 17 patients (15%) after IL-2 based treatment, including: 14 of 79 (18%) patients treated with LAK cells and 3 of 36 (8%) of those treated without LAK cells; 13%, 20%, and 12% of patients with melanoma, renal cell carcinoma, or other tumors, respectively; and 12 of 40 (30%) women, but only 5 of 75 (7%) men.

Specific information on the 17 patients with thyroid dysfunction is shown in Table 2. Four patients (numbers 4, 7, 9, and 14) had elevated pretreatment TSH concentrations which increased still further with the decline in T_4 level after treatment. One patient (number 16) had an abnormally low TSH pretreatment. Twelve patients developed straightforward hypothyroidism, and two de-

Table 1. Incidence of thyroid abnormalities following IL-2 therapy

	Total	Evaluable	Thyroid Dysfunction	
Protocol				
IL-2/LAK				
B-IL2/LAK	39	35	7	(20%)
Hybrid IL2/LAK	18	17	2	(12%)
CI-IL2/LAK	25	19	4	(21%)
IL2/IFN/LAK	9	8	1	(12%)
Total LAK	91	79	14	(18%)
IL-2 alone	46	33	2	(6%)
B-IL2 alone				
B-IL2/IFN	3	3	1	(33%)
Total IL-2 alone	49	36	3	(8%)
Disease				
Melanoma	71	58	7	(13%)
Renal Cell CA	47	40	8	(20%)
Other	22	17	2	(12%)
Gender				
Male	91	75	5	(7%)
Female	49	40	12	(30%)
Overall total	140	115	17	(15%)

veloped clear-cut hyperthyroidism with TSH levels falling to 0.1 mU/l or lower and FTI values rising to 328 and 287 units, respectively, after IL-2 therapy without subsequent hypothyroidism. Three patients (numbers 6, 11, and 13) had a biphasic pattern characterized by a transient period of hyperthyroidism before becoming hypothyroid. Peak thyroid dysfunction occurred between 6 and 11 weeks after beginning treatment in 13 of 14 patients with either straightforward hypo- or hyperthyroidism, but hypothyroidism was delayed in the three patients with a biphasic pattern occurring at 16, 19, and 22 weeks, respectively. While follow-up was limited in many patients, four of five patients followed for at least 10 months after treatment showed normalization of thyroid function in the absence of thyroid hormone replacement (data not shown). An ^{123}I-labeled thyroid uptake scan performed at the time of the peak T_4 level in one hyperthyroid patient (number 16) showed heterogenous uptake of only 3%; however, as this scan was performed only 2 weeks after a radiographic contrast computed tomography, this result is difficult to interpret. TSH receptor antibodies were not detected in any of seven patients tested, including the two patients with clear-cut hyperthyroidism.

Several clinical and laboratory characteristics of the patients exhibiting thyroid dysfunction and those remaining euthyroid are compared in Table 3. Although a higher precentage of patients receiving a LAK cell containing regimen developed thyroid dysfunction, this difference was not statistically significant ($p = 0.3$). There was no significant correlation between thyroid dysfunction and tumor type, but a significantly greater proportion of women developed thyroid abnormalities ($p < 0.002$).

Table 2. Thyroid function, thyroid antibodies, and tumor response

Patient number	Age/sex/ tumor	Protocol sample time[a]	T_4 (mmol/l)	FTI (index units)	TSH (mU/l)	Antibody titer		Best tumor response
						ATA	AMA	
1	36/F/ melanoma	B-IL2/LAK						
		Pre	57	52	0.4	Neg	Neg	
		9 Weeks	52	26	30	Neg	Neg	None
2	38/M/ melanoma	B-IL2/LAK						
		Pre	63	52	1.6	Neg	Neg	
		10 Weeks	35	32	7.2	Neg	Neg	None
3	28/M/ melanoma[b]	B-IL2/LAK						
		Pre	67	81	1.1	Neg	1:100	
		11 Weeks	8	23	95	Neg	1:1600	Complete
4	54/F/ renal cell	B-IL2/LAK						
		Pre	58	52	22	Neg	1:102,400	
		6 Weeks	6	5	72	1:1600	1:102,400	Minor
5	55/F/ melanoma	B-IL2/LAK						
		Pre	95	111	2.4	Neg	1:12800	
		6 Weeks	6	8	143	1:6400	1:102,400	Partial
6	55/M/ melanoma[c]	Hybrid IL2/LAK						
		Pre	74	70	4.1	Neg	1:100	
		7 Weeks	118	118	0.2	Neg	1:100	
		22 Weeks	12	14	60	Neg	1:100	Partial
7	29/F/ melanoma	Hybrid IL2/LAK						
		Pre	84	70	8.6	Neg	1:1600	
		8 Weeks	12	17	166	Neg	>1:102,400	Minor
8	48/F/ renal cell	CI-IL2/ LAK						
		Pre	81	68.2	4.8	Neg	Neg	
		7 Weeks	<1	ND	154.7	1:100	1:100	None
9	53/F/ ovarian	B-IL2/LAK						
		Pre	115	102	5.7	Neg	1:400	
		7 Weeks	13	10	72.6	1:100	1:1600	None
10	52/F/ renal cell	CI-IL2/ LAK						
		Pre	62	19.3	2.5	Neg	1:400	
		7 Weeks	21	22	20.3	Neg	1:6400	Partial
11	53/M/ renal cell[c]	CI-IL2/ LAK						
		Pre	102	90	2.1	Neg	Neg	
		7 Weeks	61	97	0.5	Neg	Neg	
		19 Weeks	18	17	23.9	Neg	Neg	Complete
12	66/F/ renal cell[c]	CI-IL2/ LAK						
		Pre	94	85	1.3	Neg	Neg	
		19 Weeks	6.6	59	6.2	Neg	Neg	Partial

Table 2. Continued

Patient number	Age/sex/ tumor	Protocol sample time[a]	T_4 (mmol/l)	FTI (index units)	TSH (mU/l)	Antibody titer		Best tumor response
						ATA	AMA	
13	45/F/ renal[c]	B-IL2/LAK						
		Pre	98	91	1.4	Neg	Neg	
		7 Weeks	123	192	0.1	Neg	Neg	
		16 Weeks	44	18	71.7	Neg	1:100	Complete
14	58/F/ renal	B-IL2/ INF/LAK						
		Pre	72	71	6.5	Neg	Neg	
		11 Weeks	44	18	37.7	Neg	1:100	Complete
15	55/F/ ovarian	B-IL2 alone						
		Pre	126	115	0.8	Neg	1:100	
		7 Weeks	175	328	0.1	Neg	1:1600	Partial
16	66/M/ renal	B-IL2/IFN						
		Pre	102	98	< 0.1	1:1600	1:400	
		7 Weeks	219	287	< 0.1	1:1600	1:25,600	Partial
17	44/F/ melanoma	B-IL2 Alone						
		Pre	124	116	2.7	Neg	Neg	
		7 Weeks	69	64	5.4	Neg	Neg	Partial
Normal range			65–148	54–155	0.5–5.5	< 1:100	< 1:100	

Neg, negative; Pre, pretreatment; ATA, antithyroglobulin antibodies; AMA, antimicrosomal antibodies; TSH, thyrotropine; T_4, thyroxine; FTI, free thyroxine index; ND, not determined.
[a] Posttreatment values are from the time of thyrotropin peak in hypothyroid patients, nadir in hyperthyroid patients, or both in biphasic patients.
[b] Index patient.
[c] Received a second course of treatment at 12–20 weeks.

Forty-four patients, including all 17 with thyroid dysfunction, were evaluated of the presence of serum antimicrosomal (AMA) and antithyroglobulin antibodies (ATA). ATA were detected in 5 patients (3 pretreatment) and AMA were detected in 12 patients (9 pretreatment) with thyroid dysfunction (Table 2). Five patients remained seronegative even while hypothyroid. Antibody titers generally peaked at 6–11 weeks after treatment, similar to the timing of the TSH peaks, and tended to return to baseline thereafter (data not shown). Only one of 27 patients tested who remained euthyroid developed even a marginally elevated AMA titer, and no euthyroid patients had elevated ATA. The correlations between AMA and ATA elevation and thyroid dysfunction were significant at $p < 0.00001$ and $p < 0.01$, respectively (Table 3).

The index patient, in addition to developing goiter and hypothyroidism, developed hypopigmented halos or vitiligo around each of his subcutaneous melanoma deposits (Fig. 1B) folowed by a complete regression of his melanoma, which has persisted for over followed years. This correlation between thyroid dysfunction and tumor regression was more than coincidental. In fact, 13 of 17 (77%) patients with thyroid dysfunction showed tumor regression (4 complete,

Table 3. Comparison of the study groups

	Euthyroid ($n = 98$)	Thyroid dysfunction ($n = 17$)	p
Regimen			
LAK	65 (66%)	14 (82%)	0.3
Non-LAK	33 (34%)	3 (18%)	
Tumor			
Melanoma	51 (52%)	7 (41%)	NS
Renal	32 (33%)	8 (47%)	
Other	15 (15%)	2 (12%)	
Gender			
Male	70 (7%)	5 (29%)	< 0.002
Female	28 (29%)	12 (71%)	
Antibodies			
ATA	0/27 (0%)	5/17 (29%)	< 0.01
AMA	1/27 (4%)	12/17 (71%)	< 0.00001
Tumor regression	26 (27%)	13 (75%)	< 0.0002

Table 4. Predictive value of preexisting thyroid antibodies for tumor regression

	Thyroid antibodies[a]	Tumor Regression	p
Yes	9	8 (89%)	< 0.002
No	35	10 (29%)	

[a] Only 44 patients were studied before treatment.

6 partial, 3 minor; Table 2) as compared with 26 of 98 (27%) who remained euthyroid (6 complete, 15 partial, 5 minor; $p < 0.0002$; Table 3). Furthermore, 8 of 9 (89%) patients with detectable AMA pretreatement, including all 8 with either renal cell carcinoma or melanoma, had tumor shrinkage, as compared with only 10 of 35 (29%) without AMA ($p < 0.002$); Table 4).

Discussion

This update largely confirms our earlier report on IL-2 related hypothyroidism [14]. In particular, the approximately 20% incidence of thyroid dysfunction and its strong association with serologic evidence of thyroid autoimmunity and tumor regression support our earlier conclusions. However, in contrast to our previous report, this expanded series: (a) fails to confirm the requirement for the inclusion of LAK cells in the treatment regimen; (b) more clearly shows the female predisposition of this entity; and (c) identifies three different patterns of

thyroid dysfunction: hypothyroidism, hyperthyroidism, and a biphasic pattern in which a period of enhanced thyroid hormone release precedes hypothyroidism.

Several potential mechanisms for IL-2 related thyroid dysfunction were postulated in our prior report [14]: (a) the exposure to inorganic iodine in the form of antiseptics and/or radiographic contrast agents; (b) inhibition of thyroglobulin production by secondary cytokines such as IL-1 or tumor necrosis factor (TNF); (c) a direct toxic effect of LAK cells on thyrocytes; (d) a direct or indirect stimulation of humoral immunity to thyroid autoantigens with or without the contribution of antibody-dependent cellular cytotoxicity; or (e) reactivation of preexisting T cell clones directed against thyroid autoantigens resulting in autoimmune thyroiditis. Although scientific rationales exist to support each of these possible mechanisms [14], the weight of the evidence strongly favors a cellular autoimmune etiology. The evidence favoring an autoimmune etiology includes: (a) the strong association with elevated titers of antithyroid antibodies (although 5 of 17 patients with thyroid dysfunction remained seronegative for AMA while hypothyroid, it is conceivable that if a more sensitive assay were used [15], antibodies might be detected in these patients, further strengthening this correlation); (b) the strong female predisposition; (c) the appearance in plasma of IL-2 recipients of cytokines such as IFN-γ and TNF capable of inducing HLA antigen expression on thyrocytes [16, 17] – cells which have previously been shown to possess the ability to present antigens [18]; (d) the association of IL-2 therapy with other potentially autoimmune phenomenon such as vitiligo (Fig. 1B), anti-insulin antibodies, pemphigus, and vasculitides (unpublished observations).

Furthermore, a similar incidence and pattern of thyroid dysfunction has been reported in patients receiving IFN-α therapy [19–21]. While we did not perform thyroid biopsies in our patients at the time of peak thyroid dysfunction, biopsies performed by Karlsson and coworkers in several of their patients with IFN-related thyroid dysfunction reportedly showed an intense lymphocytic infiltrate – a pattern highly suggestive of autoimmune thyroiditis (F. A. Karlsson, personal communication 1989).

Taken together, these factors imply that the most likely mechanism for IL-2 mediated thyroid dysfunction involves the IL-2 induced synthesis of secondary cytokines which lead directly to enhanced expression of HLA antigens on thyrocytes, rendering them competent to present thyroid-specific antigens to IL-2 activated, specific, and autoreactive T cells, thereby initiating an immune response. As such, this side effect is likely to be a consequence of any biological therapy that directly or indirectly enhances T-lymphocyte activity and target cell immunogenicity. In that many of these patients developed profound and symptomatic thyroid dysfunction, awareness and close monitoring for thyroid abnormalities, as well as other potentially autoimmune phenomena, is recommended in all patients receiving IL-2, IFN, or other such cytokine therapy.

The strong association of thyroid dysfunction with tumor regression previously reported [14] appears to persist in this larger series of patients. Of 17 pa-

tients with thyroid dysfunction, 13 had evidence of significant tumor regression following therapy, including four patients who have had durable complete responses. This association strongly suggests that a common mechanism exists for tumor regression and thyroid dysfunction, that is, that IL-2 related tumor regression is mediated through activation of specific autoreactive T cells capable of recognizing target cells expressing increased amounts of antigen in conjunction with HLA molecules. Such a hypothesis is supported by the observed correlation of tumor regression with HLA DR expression by tumors [22] and the enhanced cytolytic capabilities of IL-2 activated and expanded, antigen-restricted, tumor-infiltrating lymphocytes [23, 24]. Such a mechanism implies that the benefit of such therapy may be limited to patients with relatively immunogenic tumors (i.e., melanoma and renal cell carcinoma) who also possess an inherent propensity toward autoimmunity. The observation that tumor regression was seen in eight of nine patients with pretreatment seropositivity for AMA, including all eight with either melanoma or renal cell carcinoma, suggests that this test may have some utility in screening potential candidates for IL-2 therapy.

The further evaluation of IL-2 associated thyroid dysfunction should serve not only as a useful model for understanding the pathophysiology of autoimmune thyroiditis but also for clarifying the interaction of the immune system and certain malignancies and for unraveling the mechanisms by which IL-2 mediates its antineoplastic effect.

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Discussion

Scherbaum:
The majority of your patients had thyroid antibodies to begin with, before treatment?

Atkins:
Of the 17 patients who developed thyroid abnormalities nine had antimicrosomal antibodies prior to treatment. The titer usually increased after treatment, and in three patients antibodies were detectable only after treatment.

Scherbaum:
Have you done biopsies?

Atkins:
We did one biopsy of our index patient about 3 months after his second course of therapy, and there was no lymphocytic infiltration. We didn't stain the cells for antigen expression. We have not done subsequent biopsies on these patients.

Höfer:
It is very interesting that we found exactly the same numbers in patients treated, with IFN alpha. It was exactly 20% that developed very high antibody titers. What could be the reason for that? You suggested the preexisting immunity or preexisting T-cell abnormality. We have found something that speaks more or less against that, because all our patients were HLA-typed, and there was no finding as to sort out the patients at risk, none at all. So, this would be an argument against the preexisting abnormality.

Atkins:
We performed HLA-DR typing in about four or five of our early hypothyroid patients. When no correlation was found, we decided that it wasn't worth the expense to test everyone. So I agree with the finding that there is no DR correlation, but it could be either that we are looking at the wrong class II antigen, or that other antigens maybe important.

Höfer:
These tests were done by Dr. Mayr; they were on only seven patients. I agree, for any HLA association seven patients is too few.

Becker:
I was wondering about the strong correlation you found between tumor regression and development of autoimmunity. Or, to put the question the other way round, have you gone through the trouble to determine trough levels in these patients? Both could be a hint in the same direction that in those where you have a tumor regression, you have a sufficient level, perhaps able to induce autoimmunity?

Atkins:
What you mean by trough levels?

Becker:
The levels of the medication you have been delivering, in this case, IL-2, in the patient, because every patient, as you know, reacts differently.

Atkins:
I don't believe that trough levels would be relevant considering the high doses of IL-2 that we use. Furthermore, there appears to be no relationship between IL-2 related thyroid dysfunction and either other IL-2 side effects, which are known to be dose dependent, or various protocols which utilize different IL-2 doses.

Bretzel:
Did you check the peripheral lymphocyte subsets in these patients?

Atkins:
Yes, particular subsets do not appear to correlate with any clinical parameter including thyroid dysfunction.

Human Recombinant Interleukin-1 beta Decreases Plasma Thyroid Hormone and TSH Levels in the Rat

A. G. BURGER

Introduction

While physiopathological studies with human recombinant interleukin 1 (hrIL-1) have focused mainly on its role as a mediator between macrophages and monocytes and the immune system, effects on endorine functions have also been seen. The first reports concerned the cytotoxic effects of hrIL-1 on islet beta cell function [1]. Shortly there-after studies in thyroid cell cultures were reported [2]. These studies both found that very low doses of hrIL-1 (10^{-14}–10^{-16} *M*) stimulated the secretion of insulin and potentiate the effect of TSH on the secretion of thyroid hormones. Many other metabolic effects have now also been reported. hrIL-1 affects the regulation of glucocorticoids as it increases ACTH in stress, yet a direct effect on adrenal function remains to be established [3]. Fluid balance is also affected as hrIL-1 decreases renal sodium absorption [4].

In severe illness and stress, thyroid function undergoes major changes. The hormonal changes are a decreased hepatic and renal conversion of thyroxine (T_4) to triiodothyronine (T_3), with a concomitant increase in serum rT_3 levels and a transient inhibition of TSH secretion. In these situations hrIL-1 concentrations can increase in at least two different ways: stimulated monocyte-macrophages and many other cell types may secrete hrIL-1 into the surrounding biological fluids, stimulating, for instance, the phagocytotic activity of leukocytes: With very pronounced stimulations this may lead to increased circulating levels of hrIL-1. The second way is a local increase of hrIL-1 in the central nervous system, which in turn stimulates the hypothalalmic production of prostaglandin E_2.

The aim of this study was to evaluate thyroid function, in particular its hypophyseal control and the metabolism of thyroid hormones in vivo, after administration of pharmacological doses of hrIL-1 [5]. In view of the known effect of hrIL-1 on the phagocytotic activity, we also studied a minor pathway of thyroid hormone metabolism which has been specifically reported to occur in phagocytosing leukocytes: the ether link cleavage of T_4.

Materials and Methods

rhIL-1β (specific activity 4×10^7 U/mg) was kindly supplied by Prof. J. M. Dayer. Inner-ring labeled T_4 ([^{125}I-3,5]T_4, specific activity 50 μCi(μg) was synthetized from [^{125}I] diiodotyrosine, as previously described [6]. Outer-ring labeled [^{125}I] T_4 and T_3 were obtained from Amersham (UK). The free T_4 and free T_3 fractions were measured by equilibrium dialysis. The activity of the 5'deiodinase was determined according to the method of Leonard and Rosenberg [8], the hepatic activity (5'deiodinase type I) in presence of 0.1 μM rT_3 and 1 mM dithiothreitol, the activity in brain cortex (5'deiodinase type II) in presence of 1 nM rT_3 and 10 mM dithiothreitol.

A single intraperitoneal dose of hrIL-1 was administered to each animal, ranging from 0.125 to 12.5 μg. As in preliminary experiments, we noticed that the food intake was markedly reduced for approximately 12 h. The control rats, receiving 0.9% NaCl, were starved over the first 12 h. In most instances the 6- to 10-week-old rats were euthyroid; some experiments were also performed in hypothyoid rats.

The metabolism of thyroid hormones was studied in two types of experiments. Rats were either (a) infused continuously over 14 days intraperitoneally by implanting [^{125}I] T_4-filled minimumps (Alzet 2002) for the study of serum [^{125}I] T_4 levels or (b) given a single intravenous injection of inner-ring labeled [^{125}I] T_4 and outer-ring labeled [^{131}I] T_4. The latter experiments were intended to study the ether link cleavage of T_4, the metabolic product of which is diiodotyrosine. Only the inner-ring labeled T_4 yields a radioactive diiodotyrosine. This metabolite is, however, very rapidly cleared, and the experiments were therefore performed in rats which had received a potent anti-diiodotyrosine antiserum shortly before the hrIL-1 injection. In preliminary experiments this antiserum proved able to slow markedly the disappearance of [^{125}I] diiodotyrosine from serum.

The following statistical analysis were used: two-way analysis of variance, Wilcoxon's test, and for representing TSH changes in percentage a one-way analysis of variance followed by a test a posteriori according to Scheffee's procedure. The results are expressed as means $\pm$ SEM.

Results

After an injection of 12.5 μg hrIL-1 the rats tended to isolate themselves for 4–8 h and to reduce their food intake. Later their behavior was restored to normal.

Five hours after injection of 12.5 μg hrIL-1 serum TSH levels had decreased by $77 \pm 3\%$ ($p < 0.01$). A similar decrease was obtained with 250 ng T_3/100 g body wt (1.9 ± 0.2 versus 3.0 ± 0.5 μU/ml; $p < 0.05$). A significant (but less marked) decrease in serum TSH by starvation required that food be withheld for at least 12 h.

The effect of 12.5 μg hrIL-1 on serum TSH levels over a period of 6 days is shown in Fig. 1. There was not only a marked decrease in serum TSH but 3 days later a rebound of serum TSH levels above the control levels. Not shown is the concomitant fall in total serum T_4 and T_3 levels which were significantly reduced for more than 24 h (25 μg hrIL-1, from 43 ± 4 to 6 ± 2 pmol/ml; 12.5 μg hrIL-1, to 24 ± 3 pmol/ml). However, the most dramatic effect was observed on the free T_4 and free T_3 fractions (Fig. 2); the free T_4 fraction increased by 150% above baseline. This resulted in significant increases in free T_4 concentrations while free T_3 concentrations tended to be slightly lower than in the control groups. The percentage changes in free fatty acids (FFA) is also shown in Fig. 2. As FFA are known to interfere with the binding of T_4 and T_3 to their serum transport proteins, we fed hrIL-1 treated rats by tube treatment with 2.5 ml 2.2 *M* sucrose at the moment of the hrIL-1 injection and 6 h later. This treatment markedly diminished the effect of hrIL-1 on the free T_4 fraction; the changes in total T_4 were, however, the same (data not shown).

Despite the marked changes in serum T_4 levels the plasma clearance rate of [^{125}I]T_4 did not change (1.09 ± 00.9 versus 1.03 ± 0.05 ml/100 g per hour). The results on ether link cleavage were also negative. Although immunosequestration of diiodotyrosine clearly confirmed the occurrence of ether link cleavage

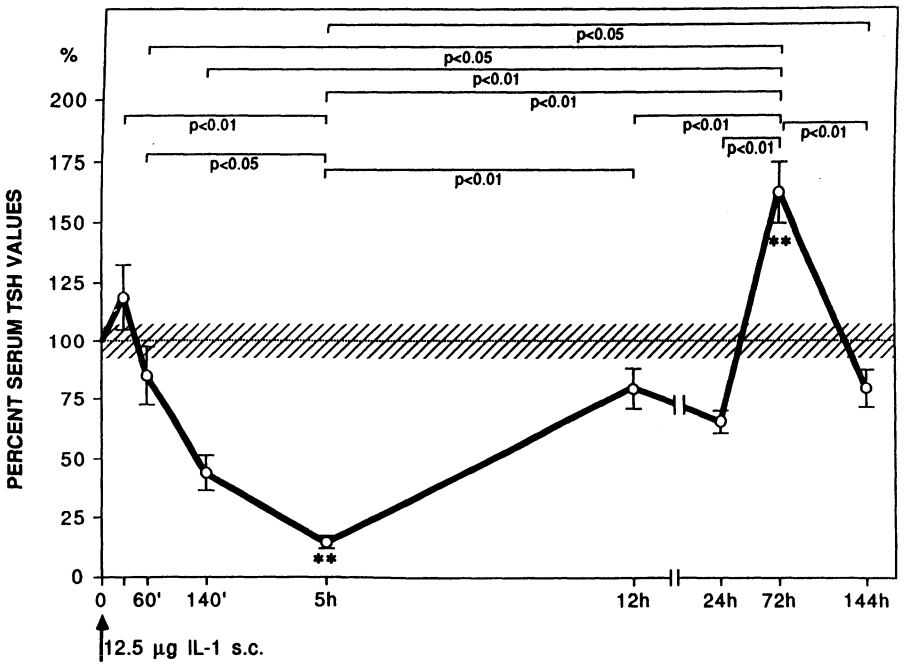

Fig. 1. Changes in serum TSH values over 6 days after a 12.5 μg hrIL-1 injection ($n = 20$). *Hatched area* mean serum TSH values of the NaCl injected rats

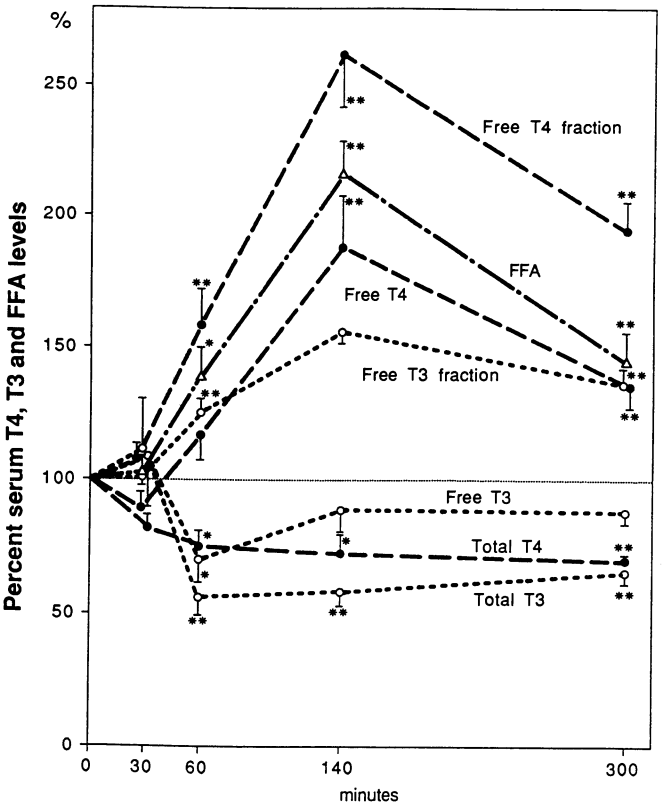

Fig. 2. Percentage changes in serum thyroid hormones after a single injection of 12.5 μg hrIR-1

in rats, the amount of diiodotyrosine production was minimal, and hrIL-1 was unable to increase this pathway of T_4 metabolism.

In brain cortex, 5′deiodinase activity increased, probably a reflection of the decreased serum and tissue T_4 levels. Hepatic 5′deiodinase activity was unaltered.

Discussion

The present data show unmistakably that hrIL-1, at least when given in pharmacological doses, can induce a marked and rapid fall in serum TSH levels. This fall can be seen in hypothyroid (data not shown) as well as in euthyroid animals and therefore reflects a thyroid hormone-independent mechanism of TSH control. One may question whether these changes are of any physiological significance, yet at 100 times less than the standard dose, 0.125 μg hrIL-1 per

animal still reduced serum TSH levels markedly while not affecting serum thyroid hormone levels. While we lack direct evidence on how hrIL-1 acts on TSH secretion, we postulate that under physiopathological conditions the increased concentration of hrIL-1 in brain could result in increased prostaglandin E_2 production which in turn affects TRH or TSH secretion. However, in vitro hrIL-1 has been shown to stimulate thyrotroph cells. A predominant direct effect of hrIL-1 on TSH secretion therefore seems unlikely.

With the rapid decrease in serum T_4 levels, thyroidal secretion was rapidly inhibited, leading to the postulate of a direct inhibition of thyroidal secretion. This has since been confirmed by Fuji' et al. [7], who found a reduced sensitivity of thyroid hormone secretion in intact rats to standard doses of bovine TSH. Our data certainly support these findings when pharmacological doses are used. One may, however, wonder why in our hands smaller doses had no effect on serum thyroid hormone levels despite a marked inhibition of TSH secretion. This could possibly be due to by the sluggish reponse of thyroid function and to the long half-life of serum T_4, in contrast to the very rapid half-life of serum TSH. The results may also indicate that under pathophysiological conditions hrIL-1 is more likely to exert its effects on TSH secretion than on thyroidal secretion.

Peripheral metabolism is apparently little affected by hrIL-1. At first glance one might consider the marked increase in free T_4 and T_3 fractions as a major effect, yet these effects are clearly not directly related to hrIL-1 as we were to a large extent able to prevent the increase in the free hormone fractions by abolishing the increase in the FFA. Concerning the ether link cleavage, the experiments are one of many examples showing that even marked in vitro effects need in vivo confirmation as their physiological relevance is otherwise void of meaning. We postulate therefore that in the "euthyroid sick syndrome" hrIL-1 could influence thyroid function at the hypothalamic-pituitary level and possibly affect thyroid secretion.

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Discussion

Orgiazzi:
What about corticosteroids?

Burger:
They go up. However, you can use 100 times less IL-1, and you can get the same inhibition of TSH without any change in serum T_4 and T_3. That is why I believe that IL-1 is a potent inhibitor of TSH secretion.

Scherbaum:
You talked about secretion. Does your effect work at the mRNA level?

Burger:
Unfortunately we did not test mRNA levels, but we should do so.

Thyroid Autoimmunity Induced by Interferon Therapy*

F. A. KARLSSON, P. BURMAN, K. ÖBERG, A. KARLSSON-PARRA

Introduction

We have previously reported [1] that patients with carcinoid tumors on long-term therapy with human leukocyte-derived (huLe) interferon (IFN) may develop signs of thyroid autoimmunity: among 49 patients hyperthyroidism occurred in two and hypothyroidism in five. Antibodies against thyroid microsomal antigen and/or thyroglobulin were found in 13 patients, and in 7 of these the antibodies appeared after the start of therapy. In the present report, we have extended these observations in a larger patient series and include data on patients given long-term treatment with recombinant (r) IFNα. Moreover, the occurrence of simple epithelial cell autoantibodies, frequently seen in carcinoid patients receiving huLe-IFNα [2], and the correlation of these autoantibodies to thyroid dysfunction and/or thyroid-specific antibodies, was examined in sera from patients given huLe-IFNα or r-IFNα.

Materials and Methods

IFN was administred to three groups of patients with carcinoid tumors [3]. In group 1, 88 patients (52 women, 36 men) received long-term therapy with huLe-IFNα ($3–6 \times 10^6$ U per day); in group 2, 34 patients (19 women, 15 men) were given r-IFNα (Introna, Schering; $3–6 \times 10^6$ U per day); and in group 3, 13 patients (8 women, 5 men) received r-IFNα (Roferon Roche; $3–6 \times 10^6$ U per day). Thyroid function was assessed in each patient every 3rd month by measurement of total thyroxine (T_4), total triiodothyronine (T_3), and TSH in serum by radioimmunoassays in routine use at the hospital. Antibodies against thyroid microsomal antigen and thyroglobulin were measured with hemagglutination kits (Wellcome, Beckenham, UK). TSH receptor antibodies were measured with a radioreceptor kit (TRAK, Henning Berlin, West-Berlin). Antibodies against simple epithelial cells were examined by indirect immunofluorescence using bile duct epithelium in cryostate sections of rat liver tissue as substrate [2].

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Results

Among the 135 patients on long-term therapy with IFNα, thyroid dysfunction was observed in 18. Hyperthyroidism (defined as serum TSH < 0.1 mU/l, $T_4 > 160$ nmol/l and/or $T_3 > 2.8$ nmol/l) was registred in 8 and hypothyroidism (defined as TSH > 7 mU/l and subnormal or low levels of T_3 and/or T_4) in 15, 5 of whom had biphasic thyroid dysfunctions. Of these 18 individuals 13 were women. Antibodies against thyroid microsomal antigen and/or thyroglobulin were found in 27 patients, of whom 22 were women. In 24 of these, 14 of whom developed hypothyroidism, the thyroid antibodies appeared, or antibody titers were significantly increased, after start of IFNα therapy. Altogether, development of thyroid dysfunction and/or thyroid autoantibodies were seen in 19 of 88 patients (22%) given huLe-IFNα and in 9 of 47 patients (20%) given r-IFNα preparations.

Hyperthyroidism developed in eight patients at a median of 4.8 months, range 3–12 months. In one of the patients the thyrotoxicosis was associated with elevated levels of TSH receptor antibodies, as typical of Graves' disease. In four cases, the hyperthyroidism was transient and was followed by hypothyroidism. Thyroid fine-needle biopsy revealed lymphocytic infiltration in three cases examined.

Hypothyroidism appeared in 15 of the 18 patients after start of therapy at a median of 8.5 months, range 2–48 months. Fine-needle biopsies were performed in nine patients and showed a picture of lymphocytic thyroiditis. Thyroxine supplementation was instituted in 11 cases. In two out of three patients, thyroid function was eventually normalized following cessation of huLe-IFNα therapy. In one case, hypothyroidism was followed by hyperthyroidism.

When examined, antibodies against simple epithelial cells were found to develop in sera from 9 out of 12 patients given huLe-IFNα treatment but in sera from none of the 9 patients given r-IFNα. No apparent correlation between the occurrence of epithelial cell antibodies and thyroid dysfunction and/or thyroid autoantibodies was observed (Table 1).

Discussion

This study corroborates and extends our previous report [1] of an increased risk of thyroid autoimmunity among patients receiving treatment with huLe-IFNα. This finding has been confirmed by FENTIMAN et al. [4] who noted that five of ten patients with breast cancer given huLe-IFNα developed de novo thyroid autoantibodies; three of these patients became hypothyroid. In the present study, we also describe that patients treated with r-IFNα preparations present thyroid autoreactivity. Abstracts with data in support of these findings have recently been published [5, 6]. It seems possible that the capacity of IFNα to increase the expression of proteins of the major histocompatibility complex [7] is of relevance in this respect, since these proteins serve as recognition structures for

Table 1. Thyroid dysfunction, thyroid antibodies, and simple epithelial cell antibodies in 12 patients given huLe-IFNα therapy

Patient no.	Hyper-thyroidism	Hypo-thyroidism	Microsomal antibodies	Thyroglobulin antibodies	Epithelial cell antibodies
1	–	Yes	1600	80	0
2	–	Yes	100	10	0
3	–	–	0	0	0
4	Yes	–	400	0	160
5	Yes	–	0	0	20
6	–	Yes	25600	163480	40
7	–	Yes	6400	2560	160
8	–	–	6400	40	80
9	–	–	6400	0	40
10	–	–	400	160	320
11	–	–	0	0	160
12	–	–	0	0	80

cytotoxic T cells [8]. Development of epithelial cell antibodies was confined to the group given huLe-IFNα, suggesting that this preparation contains factors in addition to those of IFNα that contribute to inducing this type of autoimmunity.

A state of tolerance may be altered and autoimmune disease induced by changes in the target cells or by changes within the immune system itself. Thus, a fall in thyroid autoantibody titers seen in patients with Hashimoto's thyroiditis when put upon T_4 medication [9] may be due to reduced thyroid cellular activity brought about by lowered TSH levels. On the other hand, the occurrence of autoimmune thyroid disease in the postpartum period [10, 11] is likely to be due to alterations of the immune system rather than the target cells. The present report of enhanced thyroid-specific autoreactivity in patients given IFNα therapy may represent an example of changes in both the target cells and the immune cells. IFNα increases the expression of MHC class I molecules on cells [12, 13] as well as directly enhancing the activity of cytotoxic T cells [14]. The development of hypothyroidism after treatment with interleukin-2 and lymphokine-activated killer cells [15] may be another example of changes at the level of the immune cells as well as the target cells.

The clinical picture of hyperthyroidism in most cases was not that of Graves' disease but rather that of silent thyroiditis, i.e., a thyrotoxic phase of autoimmune thyroiditis. Most of the patients with thyroid disease had thyroid autoantibodies, and when fine-needle biopsies were performed, lymphocytic infiltration was uniformly present. These findings as well as the female preponderance among the patients with thyroid dysfunction suggest that the disturbancies seen were due to induction or activation of autoimmune thyroiditis. In studies with experimental animals, IFNα has been found to promote the progression of autoimmune disease in NZB mice [16] and to increase the frequency and severity of diabetes in ICR Swiss mice [17]. Clearly, patients receiving IFNα are at increased risk of developing thyroid disease and should be followed regularly with respect to thyroid function.

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Discussion

Todd:

After your early studies using the leukocyte-derived interferon alpha, you suggested that the effects might have been due to contaminating IFN gamma, but presumably you do not feel that now you have made similar findings with your recombinant material.

Karlsson:
Yes, that's right. There is some gamma IFN in the human leukocyte-derived preparation that you can detect, and as we reported in that paper, you can see an effect on thyroid epithelial DR expression, due to the gamma IFN. Clearly now, recombinant IFN, the pure preparation in itself, is sufficient to produce autoreactivity in patients.

Todd:
Your present results are in accord with our own findings, made in collaboration with the ICRF group (Fentiman, Balkwill, and collaborators) who also found that leukocyte-derived IFN alpha induced some thyroid autoimmunity and dysfunction. We could not find IFN gamma activity in this IFN alpha preparation in terms of ability to induce MHC class II expression in thyrocytes. We therefore concluded that the in vivo effects on thyroid of the leukocyte-derived IFN alpha could not be explained in terms of contaminating IFN gamma. I think that your finding or similar effects with recombinant IFN alpha is in agreement with this conclusion.

Karlsson:
Yes.

Bottazzo:
And your patients, like the previous ones, are doing better when they develop thyroid disease?

Karlsson:
That we couldn't say. I think that the disease is heterogeneous, and it is difficult to evaluate. It runs for a very long time. We haven't seen a clear correlation between response in terms of tumor regression and development of autoimmune thyroid disease.

Bottazzo:
Have you done biopsies?

Karlsson:
Yes, we have done, as I indicated. In nine patients we did biopsies, and in all of them lymphocytic infiltrates like Hashimoto's were seen. In one patient we also stained with monoclonal antibodies for various subtypes of lymphocytes, and the picture was that of Hashimoto's thyroiditis.

Scherbaum:
You didn't do HLA typing?

Karlsson:
We haven't had the money to do it.

Effects of Interferon-γ and Interleukin-2 on Xenotransplanted Human Thyroid Tissue*

P.-M. SCHUMM-DRAEGER, B. O. BOEHM, H. J. C. WENISCH, and K. SCHÖFFLING

Introduction

On the basis of in vitro data it has been suggested that the inappropriate class II expression on thyroid follicular cells may play an important role in the initiation and/or perpetuation of thyroid autoimmune disease (BOTTAZZO et al. 1983; PUJOL-BORELL 1983; JANSSON et al. 1985). However, the ongoing discussion concerning the pathogenesis of human thyroid autoimmune disease demonstrates that there are still many open questions. In particular, the mechanisms which induce inappropriate HLA class II expression on thyroid cells in vivo are unknown at present (LUCAS-MARTIN et al. 1988).

Previous studies by our group and others have shown that it is possible to xenotransplant human thyroid tissue from patients with various thyroid diseases and to study the pathophysiology as well as the effect of therapeutic measures under in vivo conditions (SCHUMM-DRAEGER et al. 1987, 1989). As hyperfunction of thyroid tissue of Graves' disease depends upon extrinsic thyroid stimulators, it loses all signs of thyrotoxicosis as well as the characteristics of autoimmune thyroid disease 4–8 weeks after transplantation to athymic nude mice (SCHUMM et al. 1982, 1984). Thus the xenotransplantation model seems to be well suited to investigate factors originating from the patients' immune system which may be important for the initiation of thyroid directed autoimmune processes.

The aim of the present study was to examine the in vivo-effects of interferon-γ (IFN-γ) and interleukin-2 (IL-2) on xenotransplanted human thyroid tissue from patients with Graves' disease.

Material and Methods

Immediately following surgery, human thyroid tissue from two patients with Graves' disease was transplanted to 60 athymic nude mice (NMRI; initial body weight 28–30 g; 5–6 weeks of age; FORTMEYER 1981). Each recipient

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animal received two grafts subcutaneously. Four weeks after transplantation groups of ten animals each received the treatment summarized in Table 1 up to week 6. Fifty recipient animals (groups II–VI) were subjected to IFN-γ (200 IU per day; human, recombinant, specific activity 2×10^7 U/mg; Boehringer, Mannheim FRG), administered as continuous infusion by means of intraperitoneally implanted minipumps (Alzet, model 2002) for 2 weeks. Animals of group II received no additional treatment. The following groups were additionally treated with daily intraperitoneal injections of either bovine TSH (Thyreostimulin, 0.1 IU/ml; group III), IL-2 (100 IU per day, human, recombinant, specific activity 2×10^6 U/mg, Boehringer Mannheim; group IV), a combination of IL-2 (100 IU per day) and bovine TSH (0.1 IU/ml; group V), or a combination of IL-2 (100 IU per day) and methimazole (Favistan, 0.3 mg per day; group VI) from week 4 to week 6. Controls received saline only (group I).

Histological and Immunohistochemical Examination. At the end of the treatment period one thyroid transplant was removed and stored deep-frozen at $-70\,^{\circ}C$ for histological and immunohistochemical examination. Series of 10-µm sections per transplant were either stained according to HOPA (TONUTTI et al. 1960) for histological evaluation or by means of monoclonal antibodies (Dianova/Immunotech, HLA-DR/IOT 2a) to determine HLA class II expression of thyroid cells immunohistochemically. The percentage of HLA class II positive thyroid cells was determined by examination of five sections (microscopical magnification 10×16) from different regions of each sample, comprising at least 100 follicular cells each.

^{131}I Scintigraphy. In addition to the morphological evaluation of the transplants, iodine kinetics were examined in the animals at the end of the trial by means of a single injection of ^{131}I (30 µCi, 0.3 ml per mouse, i.v.) and subsequent scintiscans 2, 24, 48, 72, and 96 h later. The scintiscans were carried out as described previously (SCHUMM-DRAEGER et al. 1987).

Data are given as means ± SEM. For statistical evaluation a model of hierarchic analysis of variance was used according to SCHEFFE (1959; level of significance, $p < 0.01$). Treated groups were compared with controls bearing transplants of the same tissue.

Results

Histological and Immunohistochemical Examination. Histological evaluation of the thyroid transplants at the end of the trial period revealed that continuous infusion of IFN-γ had induced numerous atypies and destruction of thyroid cells (Table 1). Simultaneously, HLA class II expression (HLA-DR) on thyroid follicular cells was detected immunohistochemically after the IFN-γ infu-

Table 1. Transplanted thyroid tissue (Graves' disease) in athymic nude mice

Group	Treatment (weeks 4–6; ten animals per group)	Results	
I	Saline		
II	IFN-γ, 200 IU/day (continuous infusion)	Cell damage	+
		HLA-DR expression	+
		^{131}I retention	+
III	IFN-γ + bovine TSH (0.1 IU/daily)	Cell damage	+ +
		HLA-DR expression	+ +
		^{131}I retention	+ +
IV	IFN-γ + IL-2 (100 IU/weekly)	Cell damage	(+)
		HLA-DR expression	(+)
		^{131}I retention	(n)
V	IFN-γ + IL-2 + bovine TSH	Cell damage	+
		HLA-DR expression	+
		^{131}I retention	+
VI	IFN-γ + IL-2 + methimazole (0.3 mg/daily)	Cell damage	(+)
		HLA-DR expression	(0)
		^{131}I retention	↓

(+), Slightly increased; +, increased; + +, strongly increased; (n), normal; (0), not detectable; ↓ decreased.

sion (50% HLA class positive cells; group II) in contrast to cells of transplanted thyroid tissues of the control group which did not express HLA class II at all. Both histological changes and HLA class II expression were found to be even more pronounced by additional administration of bovine TSH to IFN-γ treatment (70% HLA class II positive cells; group III) but were reduced when IL-2 was added (20% HLA class II positive cells; group IV). In group V the enhancing effects of bovine TSH were compensated by the effects of IL-2, so the results are similar to those of group II which received IFN-γ alone (50% HLA class II positive cells; group V). After the combined treatment with IL-2 and methimazole the histological changes, including numerous cell destructions, were less obvious. However, no evidence of IFN-γ induced HLA-antigen expression was found in this group (group VI).

^{131}I Scintigraphy. After continuous infusion of IFN-γ (group II) there was a significant increase in ^{131}I retention in the tissue of thyroid transplants 2 h after ^{131}I injection as well as a significant increase in ^{131}I turnover rate (as indicated by significant lower values of ^{131}I retention of thyroid transplants 24–96 h after ^{131}I injection) as compared to controls (group I; Tables 1, 2). These effects were more pronounced after addition of bovine TSH (group III). ^{131}I retention and turnover rates were still significantly increased after a combined treatment with IFN-γ, IL-2, and bovine TSH (group V) but decreased to normal (as found in controls, group I) after treatment with IL-2 (group IV). After the combined treatment with IFN-γ, IL-2, and methimazole (group VI) ^{131}I retention was significantly reduced in the thyroid transplants.

Table 2. ^{131}I retention of human thyroid transplants of Graves' disease (ten recipients per group)

Group	Treatment	Time after ^{131}I injection (hours)				
		2	24	48	72	96
I	Saline	4.9 ± 0.8	3.9 ± 0.5	2.7 ± 0.6	2.3 ± 0.3	2.2 ± 0.4
II	IFN-γ	9.8 ± 0.7[a]	2.5 ± 0.6[a]	1.5 ± 0.5[a]	1.3 ± 0.5[a]	1.2 ± 0.4[a]
III	IFN-γ + IL-2 + bovine TSH	11.8 ± 0.9[a]	2.0 ± 0.8[a]	1.0 ± 0.5[a]	0.8 ± 0.7[a]	0.6 ± 0.5[a]
IV	IFN-γ + IL-2	5.0 ± 0.4	3.8 ± 0.6	2.8 ± 0.6	2.3 ± 0.5	2.3 ± 0.4
V	IFN-γ + IL-2 + bovine TSH	9.6 ± 0.6[a]	2.6 ± 0.6[a]	1.4 ± 0.4[a]	1.2 ± 0.6[a]	1.1 ± 0.5[a]
VI	IFN-γ + IL-2 + methimazole	1.0 ± 0.3[a]	0.5 ± 0.2[a]	0.3 ± 0.2[a]	0.2 ± 0.1[a]	0.2 ± 0.1[a]

[a] $p < 0.01$

Discussion

Our data demonstrate, for the first time under in vivo conditions, that recombinant human IFN-γ is able to induce cell damage and, more importantly, expression of HLA class II antigens on thyroid follicular cells of transplanted human Graves' disease tissue. These findings are in good agreement with in vitro data of other authors (PUJOL-BORELL et al. 1983; HANAFUSA et al. 1983; MATSUNAGA et al. 1986; CONE et al. 1988). Furthermore, our results show that in response to IFN-γ treatment the ^{131}I retention and ^{131}I turnover rates increase significantly. The signs of thyroid hyperfunction – which were not found in the control group – may be due to the numerous cell destructions in the transplanted thyroid tissue and thus increased thyroid hormon release after IFN-γ treatment.

The described effects of IFN-γ treatment were seen only after continuous infusion of IFN-γ. Single daily injections of the cytokine affected neither HLA class II expression nor thyroid hyperfunction in the transplanted Graves' disease tissues. Our findings clearly indicate that the effects of IFN-γ treatment on transplanted thyroid tissue were more pronounced after additional administration of bovine TSH. Similar data were found by TODD et al. (1987) in vitro.

In our study additional treatment with IL-2 reduced the IFN-γ induced cell damage and HLA class II expression on thyroid follicular cells and normalized the ^{131}I turnover rate and thus the function of thyroid transplants. This again demonstrates an interaction of different cytokines, which most probably plays an important role in the modulation of immune reactions in man.

No HLA-DR antigen on follicular cells of human thyroid transplants was observed when IL-2 and methimazole were added to IFN-γ. This indicates an immunosuppressive effect of the antithyroid compound methimazole, which was also postulated by others referring to in vitro data (MCGREGOR et al. 1984; WEETMAN et al. 1986; MCLACHLAN et al. 1986; KARLSSON and TÖTTERMAN 1986; TÖTTERMAN et al. 1987).

As we studied human thyroid tissue under in vivo conditions, our results should represent the situation in the patient quite well, giving a good idea of the

pathomechanisms of the human disease. Further studies must clarify the role of other cytokines and immunomodulatory compounds in thyroid autoimmune disease as well as the effect of antithyroid drugs. Ongoing studies in our laboratory will elaborate on the role of mononuclear cells which can be identified in grafts of Graves' disease tissue up to 8 weeks after transplantation.

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Discussion

Bottazzo:
How preliminary are these results? Are you sure that these are solid data?

Schumm-Draeger:
In the ongoing studies we do examine other cytokines, and more importantly we try to characterize lymphocytic populations in thyroid transplants and want to distinguish between lymphocyte subsets in thyroid transplants and the residual autoimmune reactivity of the nude mouse which has NK cells and B cells, and we are working on it to make it more clear.

Bottazzo:
Which lymphocytes do you want to look for in the nude mice?

Schumm-Draeger:
Not in the nude mice, in the thyroid transplants. We try to distinguish if there are influences from the model, and I hope to present clearer data.

Scherbaum:
How much imformation do you have thus far on the lymphocytes which remain in the graft, and how big were the grafts?

Schumm-Draeger:
The grafts are very small, about 0.5 cm or less in diameter. The lymphocyte population that we have to characterize I cannot comment on now, but I can say that a longer time after transplantation, up to 8 weeks, you do not find mononuclear cells in thyroid transplants of Graves' disease, in contrast to the material you get at surgery. We did not characterize it at an earlier time point or in combination with these therapy forms.

Karlsson:
I saw an article in the *Journal of Experimental Medicine* recently, using an experimental model of mice which were made diabetic. The authors transplanted islets, both species-identical islets and islets with separate H2 antigens. What one could find then was that this mixed graft, the islets which were not histocompatible, were destroyed whereas the other ones survived completely without any problems. This puts some interesting aspects on cytokines and the ef-

fects of cytokines on the endocrine cell being transplanted in these situations. I would like to hear your comment. What is the relevance of cytokines influencing different cells in this situation?

Schumm-Draeger:
This is a difficult question, but I think ongoing studies may eludicate this issue.

Closing Remarks

G. F. BOTTAZZO

Research specialist in thyroid autoimmunity meet several times during the year. When we set out to plan this Workshop, we were not sure whether we were organising "yet another one". Our initial doubts and scepticisms proved to be totally wrong. The Workshop was lively, punchy and very much to the point. We discussed several "hot" topics which needed further clarification and certainly proffered new ideas for future exploration in autoimmune thyroid research in particular and endocrine autoimmunity in general.

The HLA genetic contribution is becoming increasingly clear in type I diabetes, but in thyroid autoimmunity we still face difficulties in grasping its real impact in the pathogenesis. Has the time come when we need to move to other chromosomes to better clarify the picture? Some indications in this direction came from the meeting. Whether this is the right approach, only time will tell.

Cytokines are definitely fashionable. My personal prejudice is that too much weight has been put on them. They certainly must contribute to pathogenesis but their precise role in the autoimmune process remains difficult to frame in the appropriate context. However, it is true to say that cancer patients treated with certain cytokines develop de novo or exacerbated thyroid autoimmunity. Was I too hasty in my initial negative comment about cytokines?

Since thyroid peroxidase was cloned and sequenced and found to be the major autoantigen of the thyroid microsomal function, it has become a topic in its own right in any thyroid workshop or symposium. The availability of the cloned enzyme will enable much new clinical and basic work to be carried out. This is certainly the key molecule for understanding autoantibody production but some of its sequences could also be essential for T cell recognition and interaction. If this latter possibility proves to be correct, we will face another discrepancy with type I diabetes, where it appears that autoreactive T cells recognise epitopes in molecules distinct from those recognized by circulating autoantibodies, i.e., islet cell antibodies. Differences cannot be discarded a priori but, if critically analysed, they can provide relevant information for understanding heterogeneities in the autoimmune attack to distinct endocrine cells.

Environmental factors are constantly invoked as possible causative agents of human autoimmunity but their final identification and characterization still remains extremely elusive. Will it be finally a bacterium (i.e. yersinia), or a retrovirus, or iodine itself, or a completely unsuspected culprit? I hope that

more interest will be taken in this aspect of thyroid autoimmunity in the 1990s. It could pay a lot of dividends and important information could emerge.

At the Workshop, several questions were asked about the long awaited final characterization of the structure of the TSH receptor. Researchers working in certain key laboratories kept their answers vague when challenged on this specific point: they did not want to disclose secrets, but it was obvious that something important was afoot. A few months later news of the TSH receptor cloning exploded like a bombshell. If the topic was important before, now it will be the focus of even more attention. What a pity that this Workshop just missed the chance to become the arena for discussion on such a relevant and topical aspect of autoimmune thyroid investigation.

Nevertheless, this Workshop on thyroid autoimmunity proved to be very enjoyable. We learnt a great deal and we fought and criticized each other a lot, but all in good humour. What all the participants were interested in was to get to the crunch of the matter, not just to scratch the surface, so that novel ideas could emerge and important venues be opened. After all, this is what counts in all areas of medical research.