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Autoantibodies to Thyroglobulin in Health and Disease

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Abstract

Thyroglobulin (Tg)—a heavily glycosylated, iodinated protein—is a major autoantigen in autoimmune thyroiditis. Tg also induces thyroiditis by immunization of experimental animals. Humans with chronic lymphocytic thyroiditis characteristically produce autoantibodies to thyroglobulin, but similar autoantibodies are also found in some clinically normal, euthyroid individuals. A comparison of the fine specificity of autoantibodies in humans and in experimentally immunized mice was carried out, based on their ability to inhibit a panel of monoclonal antibodies (MAbs). Patients with autoimmune thyroid disease, as well as normal individuals, produced autoantibodies mainly to the conserved, cross-reactive determinants of thyroglobulin. Patients developed additional autoantibodies to species-restricted epitopes. The determinants recognized by patients with Graves' disease differed in some respects from epitopes recognized by thyroiditis patients or patients with differentiated thyroid carcinoma. Similarly, mice that are genetically susceptible to thyroiditis produced autoantibodies that reacted with the mouse-specific antigenic determinants. Using an autoantibody that reacts with one of the epitopes associated with thyroiditis, a reactive 15-kDa fragment of human Tg—localized at the carboxy end of the molecule—was isolated and sequenced. Iodine plays an important role in the precise specificity of the disease-associated epitope, since T cells from patients with thyroiditis react with iodinated but not noniodinated human thyroglobulin. Addition of iodine to Tg generates new or cryptic epitopes. Use of a selected MAb as a surrogate for the T-cell receptor suggests that a specific iodine-containing epitope is sometimes involved in recognition. Finally, thyroglobulin-reactive autoantibodies exhibit proteolytic activity on thyroglobulin.

Index Entries: Thyroglobulin; iodine; thyroiditis; autoimmune disease; autoimmunity; autoantigen; thyroxine.

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Introduction

Among the autoimmune diseases with the highest prevalence in the United States are Graves' disease and chronic lymphocytic (Hashimoto's) thyroiditis *(1)*. Based on published studies, the incidence of thyroiditis is approximately 21.8/100,000, and of Graves' disease, 13.9/100,000. Both diseases occur predominantly in women in the fourth and fifth decades of life. Both are relatively insidious in onset and, therefore the diagnosis is frequently delayed or missed. The diseases represent a spectrum of clinical findings from the hyperthyroidism characteristic of Graves' disease to frank hypothyroidism, the usual long-range consequence of thyroiditis. Both diseases are characterized by the production of autoantibodies to antigens that are specific to the thyroid gland. In Graves' disease, the most prominent autoantibodies are directed to the thyrotropin receptor on the thyroid follicular cells, whereas in thyroiditis the major autoantibodies react with thyroperoxidase or thyroglobulin. Both of these antigens are present in the cytoplasm of the follicular cells, and thyroglobulin is also stored in large amounts in the lumen of the follicle. Of these three autoantigens, thyroglobulin is especially useful for experimental study, because it is capable of inducing thyroiditis by immunization of experimental animals *(2)*.

Epitope Mapping of Thyroglobulin

Thyroglobulin (Tg) is a high molecular weight glycosylated iodoprotein (660 kDa), which is the site of synthesis and storage of the thyroid hormone, thyroxine. It can be dissociated by reduction into two 330-kDa monomers, and comprises up to 75% of the total protein in the mammalian thyroid. Immunization of mice with murine Tg induces autoantibodies to Tg. In genetically susceptible strains bearing the H-2^k or H-2^s haplotypes, the animals also develop thyroiditis characterized by mononuclear infiltration and destruction of the thyroid gland.

In order to map the autoepitopes responsible for the induction of thyroid lesions, we prepared a large panel of MAbs to murine Tg *(3)*. Using competitive inhibition enzyme immunoassays, the fine specificity of these MAbs was determined. They were grouped into reactivity clusters based on their ability to cross-inhibit by other MAbs. Some of the clusters proved to be highly specific for mouse Tg, whereas others represented immunodeterminants that were widely shared by Tgs of other mammalian species. These findings are in accord with many previous studies which show that Tg is a highly conserved protein. Some of the MAbs to the conserved sites were also inhibited by the iodoamino acids, triiodothyronine (T3) and thyroxine (T4), which represent the actual thyroid hormones.

Our studies showed that the autoantibody response to thyroglobulin is very diverse. Using the panel of MAbs, at least seven major determinants were distinguished, some being species-restricted and others cross-

reactive with Tg of other species. In general, all of the strains of mice tested developed autoantibodies to the conserved antigenic determinants. The animals susceptible to the induction of thyroid lesions also produced autoantibodies to the species-restricted epitopes.

Parallel studies were carried out by Bresler et al. *(4,5)*, using human Tg. To map the antigenic determinants, a large panel of mouse MAbs was generated. Based on competitive inhibition-enzyme immunoassays, the MAbs were divided into those reactive with conserved or species-restricted determinants. A selected group of the MAbs was then tested for competitive inhibition by sera from patients with chronic lymphocytic thyroiditis as well as from individuals who were clinically euthyroid, but had autoantibodies to Tg. The determinants recognized by autoantibodies from seropositive, clinically unaffected individuals were primarily directed to the conserved epitopes. In most instances, these MAbs were inhibited by T3 and T4. Sera of patients with chronic lymphocytic thyroiditis recognized additional sites defined by MAbs that were specific for human Tg and were not involved in the hormonal function of the molecule. We suggest that these sites are the ones that have more recently evolved.

To determine whether thyroid autoantibodies associated with different clinical conditions recognize different epitopes on the Tg molecule, we compared patients with chronic lymphocytic thyroiditis, Graves' disease, and thyroid carcinoma. In addition, normal subjects who had autoantibodies to Tg were assessed. All of these patients were selected because they produced antibodies to thyroglobulin *(6)*. This study confirmed our previous observation that autoantibodies to Tg present in patients with thyroiditis recognize a limited number of epitopes on Tg. With our panel of MAbs, autoantibodies from thyroiditis patients recognized three major clusters with multiple epitopes. There was no difference between autoantibodies from the more common goiterous and the less frequent atrophic variants of thyroiditis. In contrast, autoantibodies from Graves' patients shared specificities with only two of these three clusters; there was no difference between the presence or absence of ophthalmopathy in the patients. A surprising finding in our investigation was that patients with differentiated thyroid carcinoma recognized—although weakly—the same clusters as the patients with thyroiditis. There is often a close association between thyroid cancer and thyroiditis, and this finding is in accordance with that association. Finally, antibodies from healthy subjects reacted primarily with the cross-reactive, conserved determinants of the Tg molecule.

Disease-Associated Epitopes

To localize the actual epitopes of Tg responsible for autoantibody reactivity, we incubated human Tg with trypsin for various times under nonreducing conditions to release the peptides in their native configuration. These peptides were then studied by Western immunoblot for their reactivity with the panel of MAbs *(6)*. Most of the reactive peptides were released

from intact Tg after relatively short incubation with trypsin, suggesting that they are located on relatively accessible parts of this large molecule. Most of the MAbs recognized conformational epitopes that were inactivated by reduction.

To localize the actual epitopes responsible for autoantibody formation, we incubated Tg with trypsin for different times under nonreducing conditions to release peptides in their natural conformation *(7)*. This procedure produced a number of small peptides that were reactive with the murine MAbs to human Tg. Some peptides were released after only 1 h of incubation with trypsin, suggesting that they are located on the more accessible portions of the Tg molecule. Most of the MAbs failed to react with peptides after reduction, indicating that they bound conformational epitopes.

The tryptic peptides of human Tg were then analyzed by Western immunoblot for their reactivity with circulating autoantibodies from patients with chronic thyroiditis, Graves' disease, and thyroid carcinoma, or from normal human controls *(8)*. The sera of the patients with autoimmune thyroid disease or thyroid carcinoma reacted predominantly with three molecular weight peptides of 25 kDa, 20 kDa, and 15 kDa, whereas the sera of normal individuals did not bind these fragments of Tg. Autoantibodies from all three groups of patients recognized the 15-kDa peptide with high frequency. Most of the sera from Graves'-disease patients also recognized the 25 kDa fragment, whereas sera from most of the thyroiditis patients and thyroid-cancer patients recognized the 20-kDa peptide.

A chance outcome of this study was our discovery that one of the MAbs—137C1—closely paralleled the reaction of the thyroiditis autoantibodies. We also found that most of the sera from thyroiditis patients completely inhibited the reactivity of MAb 137C1 with intact thyroglobulin. We were therefore able to use this MAb to isolate and characterize the 15-kDa peptide *(9)*. Using two-dimensional SDS-PAGE, we found that 137C1 and the thyroiditis sera produced similar patterns when separation was carried out on the basis of both isoelectric point and molecular weight.

In order to isolate the tryptic peptide reactive with MAb 137C1, we separated the tryptic digest by high-pressure liquid chromatography (HPLC). The digest resolved into 14 major peaks, which were then analyzed by SDS-PAGE followed by protein staining or immunoblotting with MAb 137C1 and sera of patients with thyroiditis. One of the HPLC peaks that contained reactive peptide was then submitted for amino-acid sequencing. By determining the first 15 amino acids at the amino-terminal end of the separated peptide, we could localize it to the carboxy end of human Tg, representing amino acids 2657–2748. Comparison of the amino-acid sequence with the published sequences of bovine and rat Tg indicated that there were both conserved and nonconserved stretches. We also proceeded to clone and sequence the C-terminal region of mouse Tg *(10)*. The derived amino-acid sequence was compared with the published rat and human sequences, and revealed amino-acid homology between rat and mouse of 96% and between human and mouse of 78%. Furthermore, the mouse-Tg

shared homology with two thyroiditic peptides described by other investigators, using rat and human Tg peptides. These findings may suggest that species-specific regions of Tg are involved in the induction of thyroiditis. In order to test this hypothesis, we have recently cloned and characterized the complete murine Tg cDNA *(11)*.

Role of Iodine in the Disease-Associated Epitopes

Thyroglobulin is the storage form of the hormonally active iodoamino acids T3 and T4. Several epidemiological and clinical reports suggest that the incidence of autoimmune thyroid disease has risen concomitantly with increased iodine consumption. Furthermore, experimental evidence in animal models showed that increased dietary iodine enhances the development of autoimmune thyroiditis in genetically susceptible chickens, rats, and mice. Finally, the studies of Champion et al. have shown that T-cell hybridomas respond to Tg in direct proportion to their iodine content *(12)*. The T4-containing peptides recognized by these hybridomas were able to induce experimental thyroiditis in mice *(13)*. On the other hand, Kong et al. *(14)* showed that not all Tg peptides were dependent on iodine to induce T-cell proliferation. In our studies, we found that T-cell recognition of human Tg was dependent upon the presence of iodine in the molecule. T cells that failed to respond to human Tg lacking detectable iodine responded vigorously to Tg that was iodinated 24 in vitro *(15)*.

The mechanism by which iodine promotes the autoantigenic properties of Tg is unknown. It may have multiple effects, including alteration of the stereochemical formation of the Tg molecule or the creation of novel iodine-containing binding sites. We have recently found evidence for both of these possibilities *(16,17)*. Using selected MAbs, we concluded that iodinization of Tg either through natural processes in vivo or artificially in vitro changed its conformation in such a way that some epitopes were lost and some novel or cryptic epitopes were expressed. The appearance of novel epitopes may be particularly important in the induction of autoimmune responses, leading to autoimmune disease. We took advantage of one MAb—42C3—that bound to iodinated but not noniodinated human Tg *(18)*. In this sense, MAb 42C3 mimicked the reactivity of the T-cell receptor from thyroiditis patients. It could be used to determine the fine specificity of the corresponding epitope. We found that the binding of MAb 42C3 to intact Tg was inhibited strongly by T4. T3, which differs from T4 only in its lack of one iodine substitution, inhibited the binding of MAb 42C3 to Tg 1,000-fold less. Reverse T3—which also contains three iodine substitutions but in positions different from T4—was only one-tenth as effective. Thyronine with two iodine substitutions was relatively ineffective, and thyronine with no iodine failed to inhibited this antibody in any concentration tested. These reactions were highly specific for Tg, since iodinated bovine serum albumin did not inhibit at any concentration. Based on these findings, we suggest that the binding site of MAb 42C3 has the

best fit with an epitope that contains four iodine atoms. If fewer iodines are present, the fit is less perfect, but binding will still occur. No binding occurs in the absence of iodine. Further studies are necessary to determine the specificity of recognition of iodinated human Tg by human T cells.

Catalytic Properties of Thyroglobulin Autoantibodies

In collaboration with Paul and his colleagues, the catalytic properties of autoantibodies from healthy subjects in patients with autoimmune thyroid disease were tested for their ability to cleave human Tg *(19)*. Cleavage was determined by eletrophoretic identification of peptide bands smaller than the 330-kDa Tg monomer. IgG preparations from human subjects without autoantibodies displayed little or no Tg cleavage activity. In contrast, reproducible cleavage of Tg by IgG preparations from 4 of 8 patients with autoimmune thyroiditis was evident. The importance of these findings in the induction of autoimmune thyroid disease is still unknown. Thirty years ago, however, we were able to show that proteolytically degraded thyroglobulin is more effective in inducing autoimmune thyroiditis than the intact molecule *(20)*. It is possible, therefore, that catalytic autoantibodies play an important role in the progression from a benign autoimmune response to pathogenic autoimmunity.

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Discussion

Sela: Do you know the differences between the T-cell characteristics of healthy individuals and the T-cell characteristics of the patients?

Rose: Quantitatively, the T cells from patients proliferate to thyroglobulin more vigorously than T cells from euthyroid individuals. The extent of proliferation of T cells with a self-antigen is unfortunately very low, and is very hard to dig out of the background. We really need to compare T-cell clones to get their precise specificity and to determine if—like autoantibody—T cells from patients recognize epitopes not seen by T cells from normals.

Shoenfeld: Noel, I was impressed about the link between disease and environmental factors, for example, factors that influence iodination of the thyroglobulin. Actually, we have similar data that the disease might be increased when thyroglobulin is enriched with iodine. The second point is: somehow you escape the notion that actually Hashimoto's thyroiditis is a cell-mediated autoimmune disease and the antithyroglobulin antibodies are actually an epiphenomenon.

Rose: So sayeth all the textbooks, including your book. I have studied thyroiditis for 40 years, and I still do not know how much of the damage is cell-mediated or antibody-mediated. We use the term antibody-disease-associated antibodies. The literature on the subject is quite mixed. Weigle showed that you can actually transfer the disease with serum in the rabbit if you take the serum from a thyroidectomized donor, and we reported that you can transfer the disease with serum in the OS chicken if there is already preexisting mild disease. On the other hand, in the mouse and the guinea pig, the disease is transferred most easily with cells. So in different experimental models of disease, there are divergent results. In the human, one can certainly demonstrate antibody and complement localized in the thyroid gland. On the other hand, newborns from affected mothers do not

develop thyroiditis in contrast to Grave's disease. So we do not know the exact contribution of the antibodies and T cells. In our own studies, we are not looking at antibody at the moment as the cause of the disease, but at the B cell as an important antigen-presenting cell. We think antigen presentation may determine, to a great extent, the course of the autoimmune response.

Capra: My question has to do with your first slide in which you showed some catalysis data. Could you speculate for us as to the meaning of circulating antibodies in a patient with thyroiditis that can cleave thyroglobulin?

Rose: I have thought about it during the last two-and-a-half days. The course of the synthesis and release of thyroglobulin is a very interesting one. Thyroglobulin is synthesized in the thyroid follicular cells—the cells that surround the sac-like follicles in the thyroid gland. Iodine is incorporated into thyroglobulin during its synthesis, and then it is secreted into the colloid. As the body requires it, thyroglobulin is reabsorbed into the cells, broken down, and the thyroxine is released into the bloodstream. So cleavage of the thyroglobulin molecule is an important step in producing the thyroid hormones. If the cleavage is abnormal, the possibility arises that the abnormal fragments are antigenic. There are reasons to think that is the case. We have tested the antigenicity of rabbit thyroglobulin cleaved by a number of different enzymes. It turned out that papain-digested thyroglobulin injected intravenously into rabbits not only produces thyroglobulin autoantibodies, but also provokes thyroid lesions. So it is possible that the pathogenic disease-associated antibodies may split the molecule in an abnormal manner, exposing cryptic epitopes and triggering an autoimmune response in genetically predisposed individuals.

Capra: But we don't know that the nondisease-associated antibodies do not initiate a catalytic response, do we?

Rose: If I had known then what I know now, that is the group of antibodies I would have sent to Dr. Paul to test.

Unidentified: Is there any disease association with IgG antibodies as opposed to IgM antibodies? I had the impression that when class switching to IgG occurs, this is a much more serious situation than having circulating IgM.

Rose: Of course, the natural antibodies are usually IgMs. In this case, however, there are plenty of IgGs found in normal euthyroid patients along with the IgMs. We looked at isotypes and subclasses to see if that is where the difference between patients and normal lies. It does not.

Kohler: Noel, your finding that the evolutionary-conserved epitopes are less autoimmunogenic seems to be a general phenomenon. Several years ago, we analyzed the data on the response to another autoantigen, and

we found that the most autoantigenic epitopes are found in regions with the greatest evolutionary variability.

Marchalonis: I work with sharks and I work with people, and often I can't tell the difference. Sharks have only IgM in their circulation, and about half of their blood protein can be IgM. They don't have a class shift because they don't have anything to shift to. They only have a primary immune response. Their antibody genes are organized in little cassettes that are not linked to one another. Each cassette contains one V gene, a couple of Ds, a J—and there are hundreds of thousands of those. We were interested in the background antibody reactivity of the system. We found, among other things, that happy, healthy, free-living sharks had antibodies to pig and cow thyroglobulin. Noel kindly gave us some human thyroglobulin, and the shark antibodies reacted with the human thyroglobulin. We made peptides and we found that the reactivity was essentially to the conserved thyroglobulin epitopes. We also found antibodies to DNA in the sharks. The healthy free-living shark has as much antibody to DNA in its blood as women who are very sick with lupus. Having autoantibodies doesn't necessarily make you sick. They may reflect only the background reactivity of the system.

Paul: I ask your consideration for an alternative mechanism whereby the proteolytic antibodies might be important, which is by way of altered antigen presentation. We have not spoken of this at all during this conference because no data are available. But based on what we know about autoantigen presentation to T cells, it is a reasonable hypothesis. Concerning evolution of thyroglobulin, why and how have we evolved the new epitopes if they are susceptible to negative autoimmune reactions? What are the advantages of these epitopes, if any?

Rose: I think I look at it the other way, and I say that we have not mutated those parts of the molecule that are physiologically important—the parts that are related to hormonal function. The rest of the molecule is free to diversify. There are no evolutionary constraints; therefore, that is where the variations occurred.

Paul: The implication is that the immune system is not an important selection force in evolution of thyroglobulin.

Rose: I think that is correct. Harmful autoimmunity to thyroglobulin is evolutionarily irrelevant. Hashimoto's thyroiditis is a disease that occurs most commonly in women beyond the child-bearing years. There would probably be little or no evolutionary pressure against harmful autoimmunity.

Shoenfeld: Just a final comment about the difference between autoimmunity and autoimmune disease. The fact is that you have many conditions in which you have high levels of autoantibodies. Very high levels are found in first-degree family members of patients without disease. In patients with

myeloma, sometimes you may have paraproteins with autoantibody activity and the myeloma patients do not have the disease. Some other factor may be needed to induce the disease. On many occasions, the autoantibodies may just be an epiphenomenon and may be nonpathogenic.

Rose: You are right. Of course, you and I worked together to determine whether some of your MAbs are directed to the determinants that are disease-associated. One of the explanations might be that the MAbs are directed to the nondisease-associated determinants.