

A Thyroxine-containing Peptide Can Induce Murine Experimental Autoimmune Thyroiditis

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Summary

A synthetic peptide based on a sequence containing thyroxine at position 2553 in thyroglobulin (Tg), and already shown to be recognized by two clonotypically distinct murine Tg autoreactive T cell hybridomas, can trigger primed lymph node cells to transfer thyroiditis to naive recipients. Donor lymph node cells could be prepared from mice immunized either with intact mouse Tg or with this peptide itself. After a second exposure to the priming antigen *in vitro*, both these populations induced 100% thyroiditis in recipient animals. The importance of the T4 residue in the development of disease was demonstrated by the failure of Tg tryptic peptides depleted of T4 to stimulate pathogenic effectors *in vitro*, even when the lymph node cells had been taken from mice primed with whole Tg. We conclude that this T4-containing 12mer sequence is a major thyroiditogenic epitope in CBA/J mice although we cannot exclude the possibility that there are other pathogenic epitopes present in the whole Tg molecule.

Experimental autoimmune thyroiditis (EAT) is characterized by an inflammatory infiltrate of the thyroid gland. It has been shown that the cells comprising this infiltrate include macrophages and T cells, and in animal models of induced thyroiditis, the disease has been shown to be T cell dependent (1).

Thyroglobulin (Tg) a 660-kD glycoprotein, is synthesized in the thyroid by follicular epithelial cells, and tyrosine residues within the molecule are iodinated by thyroid peroxidase to produce the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4). In human Tg, T3 and T4 residues may be formed from tyrosines at positions 5, 2553, 2567, and 2746.

There is evidence from animal models that the iodine content of Tg may influence its autoantigenicity (2, 3), and EAT induced in mice is much less severe if poorly iodinated Tg is used for immunization (4). Similarly, human studies have shown that raised dietary iodine correlates with an increase in the incidence and severity of autoimmune thyroid lesions (5).

In previous studies (6) using synthetic T4-containing peptides, we showed that two clonotypically distinct murine Tg autoreactive T cell hybridomas recognized an epitope containing T4 at position 2553 in human Tg. Sets of overlapping 5mer to 12mer peptides around this T4 showed that

the most potent peptide was a 9mer beginning at Asp 2551, and replacement of the T4 with any of the 20 naturally occurring amino acids led to loss of antigenicity. Since the parent T cell lines of the hybridomas have been shown to induce thyroiditis on adoptive transfer, the T4-containing sequence appeared to be implicated as a pathogenic epitope in murine EAT. To confirm the pathogenic potential of this sequence, we have used the T4-containing 12mer to induce thyroiditis *in vivo*, and the results of these studies are the subject of this communication.

Materials and Methods

Preparation of Antigens, Mice, and Immunization Protocol. Mouse thyroglobulin (MTg) was prepared by extraction from pooled homogenized thyroids (Parkes outbred strain reared in our own laboratory) as previously described (7). CBA/J mice, 6–8 wk old, of either sex (NIMR, London, UK), were immunized either in the base of the tail with 50 µg MTg emulsified in CFA (MTg/CFA; H37Ra; Difco Laboratories, Detroit, MI) or with 50 µg MTg in PBS intravenously followed 3 h later by an intravenous injection of 20 µg LPS (MTg/LPS: *Salmonella enteritidis*; Sigma Chemical Co., St. Louis, MO). Mice were boosted 7 d later with 25 µg MTg/CFA in both hind footpads or 50 µg MTg/LPS as previously.

Preparation of Tg Peptides. Peptides lacking thyroxine (T4⁻)

were prepared as described previously (6, 8, 9), and the synthetic peptide Ac-STDD(T4)ASFSRAL-NH₂, hereafter referred to as the T4(2553) peptide, was produced on a synthesizer (9050; Milligen/Bioscience, Burlington, MA) also as described previously (10).

Transfer of Thyroiditis. The popliteal lymph nodes from MTg/CFA-immunized mice or spleens from MTg/LPS-immunized mice were taken on day 16 and cultured for 72 h in petri dishes or flasks at 5×10^6 /ml in RPMI 1640 (Gibco, Paisley, Scotland) with 5% FCS and other supplements, as described previously (6), together with MTg (40 μ g/ml) or peptide (2.5 or 5 μ g/ml) to activate and expand effector T cells. After washing, 2×10^7 viable lymph node cells or $3-5 \times 10^7$ spleen cells were injected intravenously into normal syngeneic recipients and their thyroids examined 14 d later.

Evaluation of Thyroid Infiltration. Thyroids were fixed in 4% phosphate-buffered formalin and sections stained with H and E. For each mouse, there are 12 possible scores (two lobes at six levels). 0, no infiltration; 1, any definite infiltration up to 20%; 2, between 20 and 50% infiltration; 3, between 50 and 75% infiltration; 4, gland totally infiltrated but follicles still discernible. The score for each mouse is the total divided by the number of observations (as occasionally, one lobe may be missing on some sections). The average thyroiditis for each group is the mean \pm SE of all of the mice in the group.

Proliferation Assay. Mice were immunized once in both hind footpads with 50 μ g MTg/CFA. The popliteal lymph nodes were excised 8 d later and cultured for 72 h in 96-well plates in supplemented RPMI 1640 at 5×10^5 /well with the antigens indicated. Proliferative responses were assessed by the overnight incor-

poration of ¹²⁵I-deoxyuridine and results expressed as the mean cpm \pm SEM of triplicate cultures.

Results and Discussion

Thyroiditis may be induced in high responder strains of mice by injecting Tg with a suitable adjuvant, usually CFA (11) or LPS (12), and in some studies, *Bordetella pertussis* has been used to augment the response (13). In our hands, thyroid lesions could be demonstrated in CBA/J mice on day 28 after the injection of 50 μ g MTg/CFA in the base of the tail on day 0 and into the hind footpads on day 7. Equally effective was the injection of 50 μ g MTg followed 3 h later by 20 μ g LPS both administered intravenously and repeated on day 7.

In many reports, thyroiditis can be induced by adoptive transfer of spleen or draining lymph nodes from mice immunized with Tg and adjuvants (14-17), and we found that adoptive transfer produced better thyroiditis than in situ immunization. Draining lymph nodes or spleens taken 16 d after the first priming dose from animals immunized with MTg/CFA or MTg/LPS, respectively, were cultured in vitro for 3 d with antigen as described in Materials and Methods, before transfer to normal syngeneic recipients. Thyroids were examined 14 d later when lesions were nearly always observed as shown in Table 1, Exps. 3, 4, and 6.

We confirmed the observations of other groups (14, 17)

Table 1. Transfer of Thyroiditis by T4 (2553) Peptide or MTg-primed Lymphocytes Reactivated In Vitro

Exp.	Immunization		In vitro Stimulation		Cells injected			Highest score (day 14)					Average thyroiditis \pm SEM						
	Antigen	Amount	Antigen	Amount	Cell	Amount	No. of mice	0	1	2	3	4		Incidence					
1	MTg/CFA	50	pep	2.5	Lymph node	2×10^7	4			2*	2			4/4	1.69 \pm 0.27				
2	pep/CFA	5	pep	2.5			3			3				3/3	0.59 \pm 0.17				
3	MTg/CFA	50	MTg	40	Lymph node	2×10^7	3	1	2	3			2/3	0.63 \pm 0.31					
			pep	5											3	3	3/3	2.3 \pm 0.1	
			MTg	40															2
pep/CFA	20	pep	5	3	3	3/3	2.73 \pm 0.13												
4	MTg/CFA	50	MTg	40	Lymph node	2×10^7	3		2	1	3	3/3	1.8 \pm 0.61						
			pep/CFA	20										MTg	40	2	2	0/2	0
			pep	5										3	1				
5	pep/LPS	20	MTg	40	Spleen	4×10^7	3	1	2	2/3	0.98 \pm 0.5								
			pep	5								3	3	3/3	3.25 \pm 0.4				
			MTg	40												4	4	0/4	0.09 \pm 0.02
6	MTg/CFA	50	PPD	25	Lymph node	2×10^7	4	4	4/4	1.7 \pm 0.075									
			T4-free tryptic peptide	2.5							3	3	0/3	0.02 \pm 0.03					

* Number of animals with that grade of thyroiditis in one or more sections.

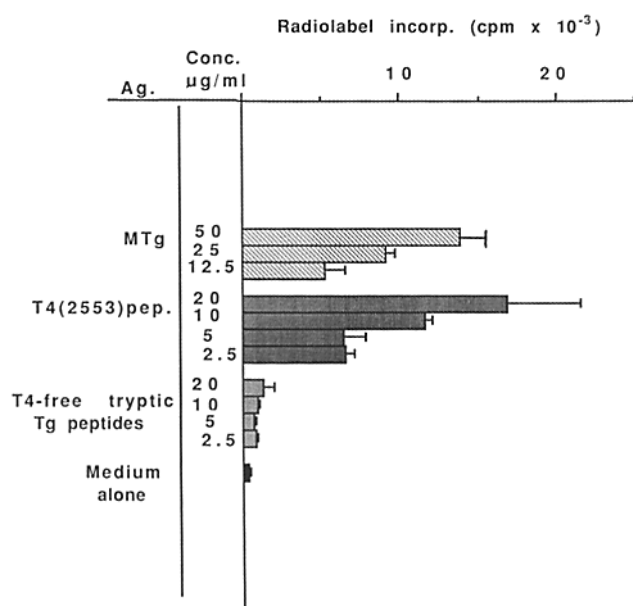


Figure 1. Proliferative response of MTg-primed cells to T4(2553) peptide in vitro. Popliteal lymph node cells from mice immunized in the footpad 8 d previously with MTg/CFA were stimulated for 72 h in vitro with MTg or peptide. Proliferative responses were assessed by the overnight incorporation of ¹²⁵I-deoxyuridine radiolabel and results expressed as mean cpm ± SE.

that in vitro activation with Tg antigens is essential for the transfer of thyroiditis to naive recipients (see Table 1, Exp. 6). Lymph node cells prepared from mice immunized with MTg/CFA 8 d earlier could be induced to proliferate by both MTg and the T4 (2553) peptide (Ac-STDD[T4]ASFSRAL-NH₂) (Fig. 1). This clearly shows that T cells recognizing the T4(2553) epitope, previously defined only by clonal T cell populations (6), are well represented in MTg-primed lymph node populations. To explore whether these T cells could elicit thyroiditis, the T4(2553) peptide was used to activate lymph node cells before transfer. MTg-primed lymph node cells cultured with T4(2553) peptide induced unequivocal thyroid lesions in all recipients examined (Table 1, Exp. 1).

In further experiments, mice were immunized with 5–20 µg T4(2553) peptide and either CFA or LPS as adjuvant. Lymph nodes or spleen were taken as before and activated for 72 h with peptide (2.5 or 5 µg/ml) before transfer. Every recipient of these cells had developed thyroid lesions by day 14 (Table 1, Exps. 2–5). These experiments show conclusively

that the T4(2553) peptide itself can act as a thyroiditogenic epitope, since neither donor nor recipient was primed or challenged with whole Tg in an immunogenic form. Furthermore, the ability of the peptide to stimulate T but not relevant B cells (as judged by the absence of anti-MTg antibodies in the recipient) provides added confirmation of the importance of T cells as pathogenic mediators of the thyroiditis lesion.

In spite of repeated attempts to induce thyroiditis in situ with T4(2553) peptide and CFA or LPS at doses ranging from 1 to 25 µg, no convincing thyroid lesions have been seen. Furthermore, although draining lymph nodes from T4(2553) peptide/CFA-immunized animals could be stimulated in vitro with peptide to transfer excellent thyroiditis (as shown already in Table 1), stimulation of these same cells in vitro with MTg failed to produce any lesions in recipient animals (Table 1, Exps. 3 and 4). However, in one experiment, spleen cells from mice immunized with peptide and LPS, rather than CFA as adjuvant, did transfer thyroiditis (although there were fewer lesions) after activation in vitro with MTg (Table 1, Exp. 5). The reasons for these data are not clear at present. Experiments are in progress to investigate whether the choice of adjuvant affects the T cell subsets stimulated and whether LPS augments antigen presentation by triggered B cells, since it is quite possible that the concentration in vitro of the relevant peptide after processing of MTg by cells from T4(2553)/CFA-treated animals may not reach the threshold for effector activation (David Wraith, personal communication). Although it has been shown that the presence of B cells is not obligatory for the development of thyroiditis (18), they may well augment responses to certain determinants by more efficient presentation (19).

The ability of the T4(2553) peptide to induce thyroiditis under appropriate experimental conditions accords well with our previous studies on the importance of iodination for the activity of Tg in this system. However, our inability to date to induce thyroiditis directly by immunization with T4(2553) peptide/CFA in situ (under circumstances where MTg itself is effective) raises the possibility that other thyroxine-containing epitopes may be of pathogenic importance. The failure of Tg tryptic peptides depleted of T4 to boost MTg immunized cells in vitro (Table 1, Exp. 6) provides a further argument against the view that noniodinated epitopes play a role in the disease model although in fairness, it should be noted that the peptides were derived from human Tg and one cannot exclude the possibility that the noniodinated epitopes do not crossreact adequately with those derived from the mouse protein.

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