Synthesis and characterization of anti-idiotypic anti-T4 antibodies*

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We injected rabbits with purified monoclonal murine immunoglobulin (IgG1) or polyclonal antithyroxine antibodies (anti-T4) and polyclonal anti-triiodothyroacetic acid (anti-Triac) antibodies to stimulate the production of anti-idiotypic antibodies. Purified immunoglobulins from all five rabbits immunized with monoclonal primary antibodies were able to inhibit the interaction between [125I]T4 and the primary antibody. The preimmune sera were inactive. This effect was not due to endogenous T4 contamination or contamination with the injected primary antibody. Half-maximal inhibition of binding of primary antibody was at 1.6 and 30 μg of total immunoglobulins. Addition of normal mouse IgG1 did not alter the inhibitory effect of the anti-idiotypic antibody, suggesting that this effect is specific. These anti-idiotypic antibodies reacted differently with different polyclonal antibodies, reflecting the heterogeneous nature of polyclonal antibody populations. Polyclonal antibodies were less effective in stimulating anti-idiotypic antibody production. One polyclonal anti-T4 and one anti-Triac antibody produced weak anti-idiotypic antibody that had to be used at a concentration of >600 μg of total immunoglobulins to be inhibitory. Both inhibited the binding of T4 to the monoclonal anti-T4 antibody. However, they were ineffective in inhibiting the function of their own antigen, the polyclonal anti-T4 or anti-Triac antibody. We tested the most potent anti-idiotypic antibodies for their ability to compete with T4 for other T4-binding proteins. Specific inhibition of T4 binding to thyroid-binding globulin was observed with half-maximal effect at approximately 450 μg of total IgG. The antibody was negative when tested against Transthyretin, rat liver deiodinase type I, triiodothyronine cell uptake and liver cytoplasmic triiodothyronine binding. In conclusion, the technique described herein allows production of anti-idiotypic anti-T4, which can be useful in the characterization of the range of iodothyronine-binding sites involved in thyroid hormone action.

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Thyroid hormones are thought to have many specific binding sites (1, 2); in human serum both thyroxin (T4) and triiodothyronine (T3) are bound to at least three different proteins (3). Within cells and in addition to nuclear receptors, binding sites have been identified at the cell surface (4), nuclear membrane (5) and also in the cytoplasm (6). Most tissues, principally brain, liver and kidney, also possess deiodinating enzymes (7), which contain an active site for iodothyronines. Despite remarkable advances in our knowledge of protein structure and the molecular biology of T3 synthesis and action, a definitive role for many of these sites has yet to be determined.

Over the last decade one approach to the investigation of hormone-binding sites has been the use of anti-idiotypic antibodies (8). According to the network theory of immunity (9), the production of an antibody is regulated by a dynamic equilibrium between an antigen, an antibody and its anti-idiotypic antibody. The structure of anti-idiotypic antibodies may reflect the internal image of the original antigen and their production can be stimulated by injecting animals with purified preparations of the primary antibody (9). Anti-idiotypic antibodies have been described for a range of hormones, including insulin, thyroid-stimulating hormone and human chorionic gonadotrophin (8). Anti-idiotypic antibodies against smaller molecules such as alpenolol, glucocorticoids and aldosterone have also been described (10, 11). In each instance the anti-idiotypic antibody showed characteristics of the primary antigen and could be used as a probe for the structure of ligand-binding sites for that antigen.

We used antibodies elicited by two types of antigen. Thyroxine as one antigen was coupled to albumin by a peptide bond, triiodothyroacetic acid (Triac) as another antigen was coupled to albumin by the diazotized benzidine reaction. In the first case of a peptide bond, the phenolic ring of the T4 nucleus is antigenically exposed: in the second case it is the side-chain. Anti-Triac antibodies were used because antibodies to the side-chain of iodothyronines are difficult to elicit. The anti-

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Triac antibodies may be used as convenient surrogates for the T₃ idiotype. Some of the anti-idiotypic antibodies inhibited binding of T₄ to the primary antibody and also competed for binding at the T₄-binding site on thyroid-binding globulin (TBG). Such antibodies may have utility in defining the range of structures of T₄- and T₃-binding sites found in mammalian tissues.

Methods

Antigen preparation

As antigens, mouse monoclonal anti-T₄ antibodies of the IgG1 subclass (generously provided by Oris, France) were used. In addition, rabbit anti-T₄ antibodies and rabbit anti-Triac antibodies were purified by extracting the IgG fractions on DEAE-Sepharose columns. In order to obtain specific antibodies to thyroid hormones, the IgG fractions were purified by chromatography on Affigel 10 (Bio-Rad Laboratories, München, Germany) linked to T₄ or T₃ and eluted with an excess of hormone. The eluted specific anti-T₄ antibody (anti-T₄-pab) and anti-Triac antibody (anti-Triac-pab) and the monoclonal anti-T₄ antibody (anti-T₄-mab) were then coupled to Affigel 10 at a concentration of 10 g/l gel in 0.1 mol/l HEPES (pH 7.5) according to the supplier’s instructions. The amount of coupled immunoglobulin (IgG) was calculated by the difference between the initial and final protein concentration in the supernatant. Thyroid hormones or the antibodies bound to the gel were eluted by washing the gel with 40% acetonitrile in 0.2 mol/l acetic acid. The gel then was re-equilibrated rapidly in phosphate-buffered saline containing 0.02% sodium azide. In control experiments, less than 1% of [¹²⁵I]T₄ (> 3000 µCi/µg. Amersham International, Amersham, UK) was retained.

Immunization protocol

In order to avoid saturating the binding sites of the injected antibody with circulating thyroid hormones, thirteen rabbits were rendered hypothyroid with 0.02% methimazole, 1% perchlorate in their drinking water and then substituted twice weekly with 1 µg of 3'-isopropyl-3,5-dioiodothyronine/kg intramuscularly. This compound is a potent iodothyronine analogue that is not bound to anti-T₄ and anti-Triac antibodies. Five rabbits were immunized with an injection of anti-T₄-mab Affigel (40 µg of bound IgG) in complete Freund’s adjuvant (Calbiochem-Behring Diagnostic, La Jolla, CA, USA), followed 3 weeks later by the same dose in incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). Boosting was repeated every 10 days and blood collected 7 days after the injections. Four other rabbits received anti-T₄-pab Affigel and four received anti-Triac-pab Affigel. Weekly injections were given using 5 mg of polyadenylic-polyuridylic acid (Sigma Chemical Co., St Louis, MO, USA) as adjuvant and blood was collected 5 days after each injection.

Inhibition of T₄ binding to its antibody

The IgGs from the serum of preimmunized or immunized rabbits were isolated by 45% ammonium sulphate precipitation and DEAE-Sephadex (Pharmacia, Uppsala, Sweden) chromatography (12) and tested for their capacity to inhibit binding of [¹²⁵I]T₄ (specific activity > 1200 µCi/µg) to either the monoclonal or polyclonal anti-T₄ antibody or binding of [¹²³I]Triac to polyclonal anti-Triac antibodies. Isolated IgG preparations contained less than 0.01% endogenous T₄. Rabbit IgG was preincubated with mouse anti-T₄-mab (6 ng/tube) in 100 µl of 20 mmol/l NaH₂PO₄ (pH 7) overnight at 4°C. Then, 5000 cpm of [¹²⁵I]T₄ or [¹²³I]Triac (obtained by the iodination of 3.5-diodothyroacetic acid, Henning Berlin, Germany) was added and after a further 4-h incubation the bound hormone was precipitated by adding 50 µg of bovine gammaglobulins and PEG 6000 at a final concentration of 16%. Tubes were centrifuged at 2000 g for 10 min and the pellet was counted in a gamma counter. These antisera inhibiting [¹²⁵I]T₄ binding were preabsorbed with an excess of mouse IgG1, 87.5 µg (kindly provided by Dr H Jacot, Hoffman-La Roche, Basel).

Recognition of TBG or Transthyretin (TTR) by anti-idiotypic antibodies

The IgGs from immunized rabbits or untreated controls were extracted as described above and then coupled to Affigel 10 at a concentration of 3 g/l gel. In addition, T₄ was coupled to a separate Affigel column according to the manufacturer’s instructions. Columns, 0.5-ml bed volume, were prepared in 0.02 mol/l phosphate buffer (pH 7). Either 20 ng of TBG or 20–100 ng of TTR (UCB Bioproducts, Brussels, Belgium) in 300 µl was applied to these columns. The columns were closed and then left at 4°C overnight. The nonspecifically bound proteins were eluted by adding 10 ml of phosphate buffer. The specifically bound proteins were eluted with the same buffer containing 20 mg/l T₄. The concentrations of TBG or TTR in each effluent fraction were measured by radioimmunoassay.

The ability of anti-idiotypic antibodies to inhibit binding of T₄ to TBG or TTR was determined also using dilute normal human serum and a dextran-coated charcoal separation system as described previously (13).

Effects of anti-idiotypic antibodies on deiodinase type I activity

We tested the effect of the antibodies (up to 1 mg per incubation) on deiodinase type I activity in rat hepatic microsomes, as described previously (14). We searched also for immunostaining of proteins migrating to 27 kD
on polyacrylamide gel electrophoresis. The enzyme was localized at this position by labelling with bromoacetyl \(^{[125]}\)T\(_4\). After electrophoresis the proteins were transferred to nitrocellulose membranes and exposed to the anti-idiotypic antibody solution. \(^{[125]}\)T\(_4\)-labelled donkey anti-rabbit antibody was used for revealing bound antibodies.

**Other assays**

Competition by the antibodies for \(^{[125]}\)T\(_4\)-uptake sites was tested using rat H4 liver cells as described previously (4). Inhibition of \(^{[125]}\)T\(_4\) binding was examined also in cytosolic extracts prepared from m. fascicularis using methods described previously (6).

**Results**

**Anti-idiotypic anti-T\(_4\)-mab**

All five rabbits immunized with monoclonal antibody anti-T\(_4\) (mab) developed antibodies that were able to inhibit \(^{[125]}\)T\(_4\) binding to anti-T\(_4\)-mab in a dose-dependent manner (Fig. 1). Antibody 331 was the most potent inhibitor, with a half-maximal effect at 1.6 \(\mu\)g of total IgG. Preimmune sera of these rabbits were without effects.

To determine the specificity of this effect, preabsorption experiments were performed with normal mouse IgG. For this purpose, the anti-idiotypic antibodies were used at a submaximal dilution. We selected two concentrations of each rabbit IgG to give approximately half-maximal inhibition and preincubated them with 87.5 \(\mu\)g of mouse IgG1. Addition of non-immune mouse IgG1 had minimal effect on the inhibition of \(^{[125]}\)T\(_4\) binding to the anti-T\(_4\)-mab (Fig. 2). The excess of normal mouse IgG1 over that in the antibody preparations can be estimated only to be at least 3.5 times (antibody 333) to more than 100 times (antibody 331) greater. Even a 100-fold excess of mouse normal IgG1 had minimal effect on the binding inhibition of the most potent antibody. With antibody 331 we performed a dilution curve of inhibition of T\(_4\) binding with and without a constant amount of mouse IgG1 (Fig. 3). Preincubation with normal mouse IgG1 did not alter the inhibiting effect over the entire range of antiserum concentrations. Similar results were obtained with antibody 334 (not shown). From these results we concluded that anti-idiotypic antibodies were present in all five sera and that the highest titre was present in antibodies 331 and 334.

These antisera were tested also for their ability to

![Fig. 1. Inhibition of \(^{[125]}\)T\(_4\) binding to anti-T\(_4\) monoclonal antibody by anti-idiotypic antibodies. Different concentrations of anti-idiotypic IgGs were incubated with anti-T\(_4\) monoclonal antibody overnight at 4\(^\circ\)C. 5000 cpm of \(^{[125]}\)T\(_4\) was added and 4 h later bound and free \(^{[125]}\)T\(_4\) were separated by the addition of bovine gamma globulin and PEG 6000 and centrifugation. The IgGs from preimmune sera were not inhibitory.](image)

![Fig. 2. Effects of preabsorption with IgG1 on inhibition of \(^{[125]}\)T\(_4\) binding by anti-idiotypic antibodies. Here, B control (100% value) is the binding of \(^{[125]}\)T\(_4\) to its monoclonal antibody in the presence of preimmune IgGs. Two concentrations of purified anti-idiotypic antibodies were incubated in the presence (■) and absence (○) of 87.5 \(\mu\)g of normal mouse IgG1, which served to absorb non-specific anti-mouse IgGs. After overnight incubation and addition of \(^{[125]}\)T\(_4\), bound and free \(^{[125]}\)T\(_4\) were separated as described in Fig. 1. It can be seen clearly that the binding could not be restored to 100% by absorption.](image)
inhibit $[^{125}]$T4 binding to two different polyclonal antibodies. When tested for inhibition of $[^{125}]$T4 binding against one rabbit anti-T4 polyclonal antibody (pab), none of the anti-idiotypic antibodies showed any inhibitory activity. Antibodies 331 and 334 also were tested against a second polyclonal antibody by measuring their activity in the routine T4 RIA described by Stockigt et al. (15). Ninety-two and 140 µg of IgG of antibodies 331 and 334 resulted in apparent T4 values of 3350 and 2900 nmol/l, respectively. Contamination with T4 was excluded because after ethanol extraction the corresponding T4 values were 40 and 58 nmol/l. Precipitation of $[^{125}]$T4 with PEG 6000 was not significantly different from the control in these two sera.

**Anti-idiotypic anti-T4-pab**

Four antisera were raised against rabbit anti-T4 and were tested in an RIA using the antigen as ligand for $[^{125}]$T4. No inhibitory activity of these anti-idiotypic anti-T4-pabs on the binding of T4 to its pab was observed. Only one of them (antibody 335) inhibited T4 binding to anti-T4-mab. However, the concentrations required were high, because 600 µg was needed to observe an inhibition of T4 binding of only 30%.

**Anti-idiotypic anti-Triac-pab**

None of the antisera raised against anti-Triac-pab were able to inhibit $[^{125}]$Triac binding to rabbit anti-Triac-pabs. Again, one of them, antibody 342, weakly inhibited T4 binding to anti-T4-mab.

**Interaction of anti-idiotypic antibodies with serum binding proteins**

Antibodies 331 and 334 were tested for their ability to bind to the T4-binding sites on TBG and TTR. With the antibodies linked to Affigel 10, 24.4% of the applied TBG was retained by the column with antibody 331 and 17.5% with antibody 334. More importantly, TBG could be eluted with excess T4. In the control experiment with a column of non-immune IgGs, only 6% of TBG was retained. In contrast, an Affigel T4 column retained 84% TBG. Identical experiments with TTR showed that this protein could not be retained on columns with antibody 331 or 334.

The interaction between TBG and antibodies 331 and 334 was tested further using dilute normal serum, low concentrations of $[^{125}]$T4 and a dextran–charcoal separation system. In these experiments antibodies 331 and 334 were able to displace radiolabelled T4 from the high-affinity binding site (Fig. 4). Assuming that all of the purified antibody was directed towards the T4-binding site, the affinity of the interaction between TBG and antibody 331 was approximately 10 µmol/l.

**The effect of anti-idiotypic antibodies in other iodothyronine sites**

We tested antibody 331 for its ability to inhibit deiodinase activity. There was no difference in enzyme activity

![Fig. 3. Effect of preabsorption with IgG1 on the inhibitory activity of antibody 331. Purified IgG (0.2–12 µg) was incubated with anti-T4 monoclonal antibody in the presence (○) and absence (●) of 87.5 µg of IgG1. After addition of $[^{125}]$T4 and polyethylene glycol, bound and free $[^{125}]$T4 were separated as described in Fig. 1.](image)

![Fig. 4. Effect of anti-idiotypic antibodies on binding of $[^{125}]$T4 to thyroid binding globulin. Normal human serum (1:10 000 final dilution) was incubated overnight at 4°C with $[^{125}]$T4 and unlabelled T4 (●) and purified rabbit IgGs 331 (○) and 334 (△) in a volume of 200 µl. Bound and free hormone was separated by the addition of 0.5 ml of dextran (0.4%) and charcoal (1%) and centrifugation. Purified normal rabbit IgG showed minimal displacement of $[^{125}]$T4 up to 250 µg per tube. Results are means ± SEM (N = 3).](image)
in the presence of immune or non-immune rabbit IgGs. In addition, we were unable to demonstrate any immunostaining of the 27,000-D protein band with antibody 331.

We also tested this antibody in a T3 cell uptake assay. At the highest concentration it was weakly inhibitory but the level of inhibition was not significant within the limits of the assay. No inhibition was observed in a T3 binding assay using cytoplasmic extract prepared from liver as a source of binding protein.

Discussion

In earlier studies we have tried, on several occasions, to produce anti-idiotypic anti-T4 antibodies by immunizing rabbits and mice with soluble polyclonal antibodies specific to thyroid hormones or their antigen-binding fragments as well as with intact monoclonal antibodies. The results were negative and further studies with antibodies stripped free of T4 on affinity columns also were unsuccessful. In the latter case the antibodies had lost their binding capacity following dissociation from the affinity column. In other experiments, we found that the same antibodies immobilized on Affigel were very stable and they could be stripped with 2 mol/l acetic acid, 40% acetonitrile or even with 1 mol/l NH4OH without loss of binding (data not shown). These findings were the basis of the present study. The positive results obtained with these antigens suggest a critical role for an intact binding site even though the experiments were not designed to investigate this question.

A second important point in these experiments was the use of a monoclonal antibody to look for inhibition of binding. Polyclonal antibodies are usually the antibody of choice because they have a much higher affinity then their monoclonal counterpart. This was not the case for our monoclonal antibody, which had a high affinity constant (2.6 × 1091/mol). Competition for binding sites of a single configuration is more likely to be observed than with a polyclonal antibody, which has a mixed population of sites, even though they may be of similar affinity. Interestingly, antibodies 331 and 334, while being inactive against one polyclonal antibody, were active in an RIA that uses another polyclonal antibody. Presumably this variability reflects the diversity of antibody response to a particular antigen (8).

There are two alternative explanations for our initial results. The inhibition of binding that we observed could have been due to contamination with endogenous T4. This is unlikely to be the case because during purification less than 0.01% of the initially added [125I]T4 could be recovered. Based on the total T4 concentration of the antisera, the T4 concentration in the purified antibody was less than 2 pmol/l. A second possibility is that the anti-idiotypic antibody preparations contained excess anti-T4 antibodies, which could prevent the binding of T4 to the monoclonal antibody. Again, this is unlikely because precipitation of [125I]T4 with PEG 6000 in the antisera 331 and 334 was not significantly different from that in the preimmune serum.

Monoclonal anti-T4 antibody binding was inhibited not only by anti-idiotypic anti-T4 antibodies but also weakly by anti-idiotypic antibodies against Triac antibodies. The Triac antibodies were raised using an antigen in which Triac was coupled to albumin by diazotized benzidine (16). With this method the side-chain of Triac (acetic acid) was the exposed antigenic site. The T4 antibodies were raised with T4 coupled by a peptide bond to albumin (16) such that the phenolic ring was exposed. The fact that the anti-idiotypic antibodies could inhibit the binding of both types of antibodies favours the view that the anti-idiotypic antibodies present a large idiotype that includes both the phenolic ring and the side-chain.

The most interesting finding is the binding of antibodies 331 and 334 to the T4-binding site of TBG, which could be dissociated with an excess of T4. This reaction was proved by two methods. The affinity column method having the advantage over the competition assay with TBG and charcoal separation that contamination of TBG or of the antibodies with T4 would even reduce the interaction of anti-idiotypic antibodies with idiotype. In contrast, identical experiments with TTR suggested that the antibodies did not bind to this protein. The binding site on TTR is a narrow deep cleft that accommodates the entire length of the T4 molecule (17). On the basis of homology with serine protease, the structure of the TBG-binding site is probably a wide hydrophobic pocket that may be able to accommodate T4 in a different orientation (18). An anti-idiotypic antibody with an active site directed towards both the phenolic ring and the side-chain of T4 is consistent with this view.

Clearly, we would like to know if the antibodies could interfere with other iodothyronine-binding sites. Our antibodies did not bind to the type I deiodinase, to cell uptake sites or to cytoplasmic binding sites for T3. Even so, the method described has been successful, for the first time, in producing anti-idiotypic antibodies to T4 with activity at a T4-binding site. This method can be used to produce anti-idiotypic antibodies of higher affinity, which may have a wide range of applications in thyroid hormone biology.

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References

4. Topliss DJ, Kolliniatis E, Barlow JW, Lim C-F, Stockigt JR. Uptake of 3,5,3′-triodothyronine by cultured rat hepatoma cells is inhibited by non bile acid cholephils, diphenhydantoin, and nonsteroidal antiinflammatory drugs. Endocrinology 1989;124:980–6
18. Flink JL, Bailey TJ, Gustafson TA, Markham BE, Morkin E. Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to the serine antiproteases. Proc Natl Acad Sci USA 1986;83:7708–12

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