In vivo effects of a human thyroid-stimulating monoclonal autoantibody (M22) and a human thyroid-blocking autoantibody (K1-70)

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Abstract

Purpose To study in vivo effects of the human monoclonal TSH receptor (TSHR) autoantibodies M22 (stimulating type) and K1-70 (blocking type) on thyroid hormone levels in rats.

Methods Serum levels of total T4, free T4, M22 and K1-70 were measured following intramuscular injection of M22 IgG (2–4 μg/animal), K1-70 IgG (10–200 μg/animal) or both into rats. Thyroid pathology was assessed in M22-injected rats.

Results Serum levels of total T4 and free T4 increased in a dose-dependent manner following injection of M22 IgG. Thyroid follicular cell hypertrophy was dependent on the dose of M22 IgG. K1-70 IgG caused a dose dependent decrease of total T4 and free T4 levels in rats receiving K1-70 only. The stimulating effects of M22 IgG on T4 levels in rats were completely inhibited by K1-70 IgG.

Conclusion M22 is a potent stimulator of thyroid hormone secretion in vivo. In contrast, K1-70 inhibits thyroid hormone secretion in vivo. Furthermore, K1-70 has the ability to inhibit the stimulating activity of M22 in vivo and as such has potential as a new drug to block TSHR stimulation by autoantibodies in Graves’ disease.

Keywords Thyroid · Graves’ disease · TSH receptor · Thyroid-stimulating antibodies · Thyroid-blocking antibodies · Autoimmunity

Introduction

The TSH Receptor (TSHR) is a major thyroid autoantigen and is a key protein in the control of thyroid function [1–5]. The hyperthyroidism of Graves’ disease is caused by TSHR autoantibodies (TRAbs), with stimulating (agonist) activity while the rare blocking (antagonist) type TRAbs are responsible for hypothyroidism in some patients [1–5]. TRAbs with thyroid-stimulating activity mimic the actions of TSH and initiate the first step of the TSHR signalling cascade leading to increased synthesis of thyroid hormones in vivo. In contrast, TRAbs with blocking activity inhibit TSHR stimulation by TSH or stimulating antibodies leading to a decrease of thyroid hormone synthesis.

Recently, we have produced a panel of human monoclonal TRAb with different functional activities [6–10]. One TRAb M22, which was isolated from a hyperthyroid patient with Graves’ disease [6, 7], has potent thyroid-stimulating activity, while another TRAb K1-70 [9, 10] isolated from a patient with autoimmune hypothyroidism has strong antagonist (blocking) activity. The crystal structures of both TRAbs alone and in complex with the TSHR extracellular fragment amino acids (aa) 22–260 (TSHR260) have been solved and analysed [11, 12]. TSHR260 comprises almost the entire (except for the last 21 C-terminal aa) leucine-rich domain (LRD) of the receptor. M22 and K1-70 bind to the concave surface of the TSHR LRD and both form networks of strong interactions with the receptor [11, 12]. The binding sites of the two TRAbs on the TSHR overlap extensively, however, the interactions of the blocking TRAb K1-70 involve regions located more towards the N-terminus of the LRD than the stimulating TRAb M22. In particular, K1-70 interacts with TSHR leucine rich repeats 1–8, while M22 binds to repeats 1–10 of the TSHR LRD [11–13].
M22 is a potent (at ng/mL concentrations) stimulator of cyclic AMP in CHO cells expressing the TSHR [6, 7]. K1-70 has the ability to completely inhibit the stimulation of cyclic AMP production in CHO cells expressing the TSHR by TSH, by the human thyroid-stimulating TRAb (M22) and by patient sera containing TRAb with stimulating activity [9, 10]. The potent biological activities of human TRAbs have potential important in vivo applications. For example, M22 has great potential as an alternative to recombinant human TSH in the management of patients with thyroid cancer [5, 9, 14, 15]. K1-70 has the potential to be used as a specific inhibitor of thyroid stimulation by TRAbs in patients with Graves’ disease and to control Graves’ ophthalmopathy caused by stimulating TRAbs.

We now describe the in vivo effects of the thyroid-stimulating TRAb M22 and the blocking TRAb K1-70 in rats. In particular, rat serum total T4 and free T4 levels were analysed after intra muscular injection of M22 IgG or K1-70 IgG alone or in different combinations with each other. Furthermore, thyroid pathology was assessed in rats injected with M22.

Materials and methods

Human monoclonal autoantibodies

The human monoclonal TRAbs (M22 and K1-70) [6, 7, 9, 10] and the human monoclonal thyroid peroxidise (TPO) autoantibody (2G4, control) [16] were purified from heterohybridoma culture supernatants as described previously [7].

Measurement of TSHR-blocking (antagonist) activity

The ability of K1-70 IgG to inhibit the stimulating activity of M22 was assessed in low salt buffer using CHO cells expressing the human TSHR (5 × 10^4 receptors per cell) as described previously [17]. The effect of M22 (3 ng/mL) on stimulation of cyclic AMP in CHO-TSHR cells was measured in the absence or in the presence of K1-70 IgG (0.001–100 μg/mL).

In vivo studies in rats

Female Han Wistar rats (Harlan Laboratories, Hillcrest, UK), approximately 5 weeks old at the beginning of the study, were used to exclude any effects of the estrus cycle or sex hormones. In some experiments, the high levels of endogenous TSH in rats were suppressed by allowing access to T3 in the drinking water (3 μg/mL) for 4 days before administration of test IgG (or buffer control) and thereafter. In all experiments, 15 animals were used per each dose group with 5 animals having blood samples taken at 0 and 48 h, 5 animals at 4 and 96 h and 5 at 24 and 120 h to incorporate all time points. The 120-h time point was not evaluated in the in vivo experiment using different doses of K1-70 in rats not receiving T3.

Antibodies (M22 or K1-70 IgG) were diluted to the appropriate concentration in phosphate-buffered saline (PBS; 137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 8.1 mmol/L disodium hydrogen phosphate, 1.5 mmol/L potassium dihydrogen phosphate pH 7.4) with 3% mannitol and 1% Tween-80 on the day of injection (0.1 mL intramuscularly).

The experiments with M22 IgG (2, 4 and 10 μg per animal) were carried out in T3-suppressed rats whereas K1-70 IgG was tested at 10, 50, 100 and 200 μg per animal in non-suppressed rats. Experiments involving injection of both K1-70 IgG (200 μg) and M22 IgG (4 μg) were carried out in T3-suppressed rats. In this series of experiments, K1-70 IgG was injected 3 h before administration of M22 IgG, or M22 IgG was injected 3 h before administration of K1-70 IgG or K1-70 and M22 IgGs were injected at the same time.

The thyroids were removed from the rats injected with different doses of M22 at the time of terminal blood sampling (48, 96 or 120 h), weighed, preserved and both a pathological and histological examinations carried out.

Measurement of total T4 and free T4 in rat serum samples

Free T4 in rat serum samples was assayed using the Beckman Coulter Access 2 analyser (Beckman Coulter, High Wycombe, UK) and total T4 was measured using the Siemens Immulite analyser (Siemens, Frimley, UK) according to the manufacturer’s instructions.

Measurement of M22 and K1-70 in rat serum samples

A TSHR autoantibody ELISA based on binding of M22 (labelled with peroxidase) to TSHR-coated ELISA wells [18] was used. In the assay, 100 μL of test sample (diluted in 50 mmol/L NaCl, 20 mM Tris–HCL pH 7.8, 1% Triton X-100 and 1 mg/mL BSA; assay buffer) was added to the plate wells and incubated for 1 h at room temperature with shaking (500 shakes/min). After washing 100 μL of M22-peroxidase (RSR Ltd, Cardiff, UK) was added and incubation continued for 25 min without shaking. The wells were washed three times, substrate added, followed by stop solution and absorbance read at 450 nm.

The concentration of M22 IgG or K1-70 IgG in the rat serum samples was calculated using an M22 IgG or K1-70 IgG calibration curves, respectively.
Effect of M22 IgG on thyroid hormone levels in T3-suppressed rats

M22 IgG levels increased after injection in a dose-dependent manner over time (Fig. 1a) being detectable 4 h after injection and reaching a peak after 24 h of $62.3 \pm 7.3$, $149.3 \pm 16.0$ and $264.0 \pm 59.3$ ng/mL (mean $\pm$ SD; $n=5$) after injection with 2, 4 and 10 $\mu$g of M22 IgG, respectively. Then M22 IgG levels decreased steadily over time to $4 \pm 4$, $26 \pm 4$ and $43 \pm 15$ ng/mL (mean $\pm$ SD; $n=5$) at 120 h in animals receiving 2, 4 and 10 $\mu$g M22, respectively (Fig. 1a). M22 IgG was not detectable in sera from control animals not receiving M22.

The serum concentration of total T4 in the T3-suppressed rats injected with buffer only were below the detection limit of the assay ($<26$ nmol/L). Measurement of total serum T4 in the T3-suppressed rats after injection of M22 IgG showed a dose dependent increase compared to buffer only control (Fig. 1b). The total T4 levels were highest at 24 h; with the highest level of $72 \pm 17$ nmol/L (mean $\pm$ SD; $n=5$) observed in animals receiving 10 $\mu$g M22. The levels of total T4 then decreased slowly over time and fell to control levels after 48, 96 and 120 h in rats receiving 2, 4 and 10 $\mu$g M22, respectively.

The serum concentrations of free T4 in all T3-suppressed rats injected with buffer only were below the detection limit of the assay ($<6.4$ pmol/mL). Measurement of free serum T4 in the T3-suppressed rats after injection of M22 IgG showed a dose dependent increase compared to buffer only control (Fig. 1c). The free T4 levels peaked at 24 h with the highest levels of $20 \pm 6$ and $19 \pm 5$ pmol/L (mean $\pm$ SD; $n=5$) observed in animals receiving 5 and 10 $\mu$g M22, respectively. The levels of free T4 then decreased slowly over time and were reduced to control group free T4 levels after 96 h for rats receiving 2 and 5 $\mu$g of M22 and after 120 h for animals receiving 10 $\mu$g M22.

Thyroid pathology and histology

Examination of the thyroid glands from M22 IgG-injected rats showed no significant differences in thyroid weight. Furthermore, the thyroids were macroscopically unremarkable and the findings seen were consistent with the usual pattern of findings in rats of the same age and strain.

However, thyroid histology showed increases in the incidence and severity of follicular cell hypertrophy in M22-treated animals, which were more marked when higher amounts of M22 were injected. Follicular cell hypertrophy was characterised by a diffuse change in which the follicular epithelium showed increased height with scant colloid. In the control group all 15 rats had normal thyroid morphology (Fig. 2a), while 2/15, 5/15 and 10/15 rats showed follicular cell hypertrophy in animals receiving 2, 5 and 10 $\mu$g M22, respectively (Fig. 2b).
Effect of K1-70 on M22 stimulated cyclic AMP production in CHO cells expressing the TSHR

Experiments carried out using CHO cells expressing the TSHR showed that K1-70 IgG inhibited the cyclic AMP-stimulating activity of M22 IgG (3 ng/mL) in a dose-dependent manner with near complete inhibition at 0.1 μg/mL and complete inhibition at 0.5 μg/mL. From this in vitro data, the relative concentration of K1-70 IgG required to prevent stimulation of the rat thyroid by 4 μg of M22 IgG in vivo was estimated to be between 100 and 500 μg. Consequently, doses of K1-70 IgG of 10, 50, 100 and 200 μg per animal were selected for in vivo studies.

Effect of in vivo administration of K1-70 IgG on thyroid hormone levels in non-suppressed rats

Rats were injected intramuscularly with buffer only (control), 10, 50, 100 and 200 μg K1-70 IgG per animal and blood samples taken at 0, 4, 24, 48 and 96 h.

After injection, K1-70 IgG concentrations in the rat serum showed a dose-dependent increase over time while K1-70 IgG concentrations in animals not receiving K1-70 were undetectable (Fig. 3a). K1-70 IgG was detectable after 4 h and maximum levels observed after 24 h of 0.47 ± 0.07, 4.18 ± 0.47, 6.06 ± 0.24 and 14.0 ± 4.18 μg/mL (mean ± SD; n = 5) after injection with 10, 50, 100 and 200 μg of K1-70 IgG, respectively. K1-70 IgG levels then decreased steadily over time but were still detectable after 96 h (Fig. 3a).

The serum total T4 levels in pre-treated animals not suppressed with T3 were in the range of 60–79 nmol/L. Injection of 100 or 200 μg K1-70 IgG caused a dose-dependent decrease in total T4 (Fig. 3b) to levels similar to those seen in T3-suppressed rats <26 nmol/L (mean ± SD; n = 5) at 24 h. Lower doses of K1-70 (10 and 50 μg per animal) were less effective. The levels of total T4 were still low (<26 nmol/L; mean ± SD; n = 5) at 96 h in animals receiving 200 μg K1-70 while in rats receiving 10 μg K1-70 the total T4 levels at 96 h (64 ± 9 nmol/L; mean ± SD; n = 5) were similar to the control group (67 ± 18 nmol/L; mean ± SD; n = 5) (Fig. 3b).

Prior to K1-70 treatment the serum free T4 levels in rats not suppressed with T3 were in the range 12.1–17.2 pmol/L. Injection of 50–200 μg K1-70 IgG caused a decrease in free T4 whereas 10 μg had no effect (Fig. 3c). At 24 h, the levels of free T4 decreased to <7.5 and <7.0 pmol/L in animals receiving 100 and 200 μg K1-70, respectively. The levels of free T4 were still low (<6.6 pmol/L) at 96 h in rats receiving 200 μg K1-70 but showed a slight increase to 10.5 ± 2.3 pmol/L (mean ± SD; n = 5) and to approximately 9 pmol/L, respectively, in animals receiving 50 and 100 μg.

Effect of K1-70 before or after M22 IgG on thyroid hormone levels in T3-suppressed rats

T3-suppressed rats were injected intramuscularly with buffer only, or 4 μg M22 IgG, or 200 μg K1-70 IgG at time 0 h. Other groups of animals received 200 μg K1-70 IgG at time 0 h followed by 4 μg M22 IgG at 3 h, or 4 μg M22 IgG at 0 h followed by 200 μg K1-70 IgG at 3 h or a mixture of 200 μg K1-70 IgG and 4 μg M22 IgG. Blood samples were taken at 0, 4, 24, 48, 96 and 120 h. After

![Fig. 2 Histology of rat thyroid sections from a control rat injected with buffer only showing normal thyroid histology and b a test rat at 96-h post-injection with 10 μg of M22 showing follicular cell hypertrophy characterised by a diffuse change in which the follicular epithelium showed increased height with reduced colloid](image-url)
injection of K1-70 IgG, M22 IgG or a mixture of K1-70 IgG and M22 IgG the concentration of IgG in the rat serum showed a dose-dependent increase over time and maximum levels were observed at 24 h similar to that seen in the experiments with 200 µg of K1-70 IgG and 4 µg M22 IgG described above.

Injection of K1-70 IgG alone had no detectable effect on total T4 levels in the T3-suppressed animals (as the basal total T4 levels were already low in these rats). Injection of 4 µg of M22 IgG per animal alone had a similar effect on total serum T4 as observed in the earlier experiments with a peak concentration of 69 ± 10 nmol/L (mean ± SD; n = 5) at 24 h compared to <26 ± 0 nmol/L (mean ± SD; n = 5) in the control group (Fig. 4a).

When M22 IgG was injected together with K1-70 IgG in different combinations an increase in total T4 levels was no longer evident (Fig. 4a).
Injection of K1-70 IgG alone had no detectable effect on free T4 levels in the T3-suppressed rats. Serum free T4 in T3-suppressed rats after injection of M22 IgG alone increased to a maximum level of 17.2 ± 1.7 pmol/L (mean ± SD; n = 5) at 24 h compared to <6.4 ± 0 pmol/L in the control group (injected with buffer only) and decreased over time reaching the levels seen in the control group after 96 h (Fig. 4b). However, when K1-70 IgG was administered 3 h before injection of M22 IgG a rise in free T4 levels was no longer observed (Fig. 4b). When K1-70 IgG was administered 3 h after M22 IgG or injected together with M22 IgG-free T4 concentrations also remained at low levels except for a slight increase at 4 h (Fig. 4b).

**Discussion**

The thyroid-stimulating human monoclonal TRAb M22 has the characteristics of patient serum-stimulating TRAbs [19]. It has high affinity for the TSHR (5 × 10¹⁰ L/mol) and is a potent stimulator of cAMP production in CHO cells expressing the TSHR [6, 7]. The blocking type human monoclonal TRAb K1-70 also has high affinity for the TSHR (4 × 10¹⁰ L/mol); however, it is a potent inhibitor in vitro of the stimulating activities of TSH, M22 and patient sera with stimulating TRAb [9, 10]. We now show that K1-70 is also effective in vivo.

The high endogenous levels of TSH and hence the high levels of total and free T4 in rat serum were suppressed by adding T3 to the drinking water for a few days prior to various experiments with M22. After intramuscular injection of different amounts of M22 IgG the total and free T4 concentrations in rats increased in a dose-dependent manner. The maximum concentrations of total and free T4 in the rat sera were detected at 24-h post-injection and coincided with the peak in the levels of M22 IgG measured in the same rat serum samples. These experiments indicate that rat thyroid hormone levels increase in response to intramuscular administration of M22 IgG and the dose responses were similar to those we observed in our preliminary experiments when rats were injected with M22 IgG subcutaneously [9]. Consequently, M22 is an effective stimulator in vivo irrespective of the method of injection used.

Histology of the thyroids from rats injected intramuscularly with M22 showed an increased incidence of thyroid cell hypertrophy with increasing doses of M22. However, there were no visible signs of lymphocytic infiltration or enlargement of the thyroid gland at 48, 96 or 120 h post-injection. In earlier reports, thyroid-stimulating mouse MAbs IRI-SAb2 (1–10 μg injected intravenously [20]), KSAb1 and KSAb2 (100 and 10 μg; administered intravenously and intraperitoneally [21]) and a hamster MAb MS-1 (10 and 5 μg; administered intraperitoneal y [22]) into mice caused hyperstimulation of the thyroid with increased T4 levels peaking approximately at 24-h post-injection similar to the effects observed after injection of M22. Thyroids from the mice in these earlier studies showed evidence of follicular and epithelial changes and thyroid cell hypertrophy [20–22] as observed after injection of M22. However, lymphocytic infiltration of the thyroid gland was reported only in one study [20].

Intramuscular injection of different concentrations of K1-70 IgG into rats caused a dose dependent decrease of total and free T4 levels in vivo (these experiments were carried out without suppressing total and free T4 with T3). The total and free T4 concentrations decreased to their lowest levels at 24-h post-injection and this coincided with the maximum levels of K1-70 IgG measured in the same samples. Doses of 100 or 200 μg per animal of K1-70 IgG caused a decrease of the total and free T4 to the very low serum levels observed in the T3-suppressed rats. Furthermore, administration of K1-70 IgG at 200 μg per animal caused complete inhibition of the stimulation of the rat thyroid in vivo by M22 IgG (4 μg per animal) when injected 3 h before the M22 or 3 h after the M22 or at the same time as the M22. Thus our study shows for the first time that a blocking type monoclonal TRAb can act in vivo as an effective inhibitor of thyroid stimulation by TSH and TRAb.

Overall our studies indicate that the biological activities of M22 and K1-70 observed in vivo are similar to those reported previously in vitro [6, 7, 9, 10]. The ability of K1-70 to inhibit autoantibody stimulation of the thyroid emphasizes its potential as a new type of anti thyroid drug.

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**Conflict of interest** All authors are employees of RSR Ltd. RSR Ltd is a developer of medical diagnostics including kits for measuring TSH receptor antibodies.

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