

A Human Monoclonal Autoantibody to the Thyrotropin Receptor with Thyroid-Stimulating Blocking Activity

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Background: Human monoclonal autoantibodies (MAbs) are valuable tools to study autoimmune responses. To date only one human MAb to the thyrotropin (TSH) receptor (TSHR) with stimulating activity has been available. We now describe the detailed characterization of a blocking type human MAb to the TSHR.

Methods: A single heterohybridoma cell line was isolated from the peripheral blood lymphocytes of a patient with severe hypothyroidism (TSH 278 mU/L) using standard techniques. The line stably expresses a TSHR autoantibody (5C9; IgG1/kappa). Ability of 5C9 to bind and compete with ¹²⁵I-TSH or TSHR antibodies binding to the TSHR was tested using tubes coated with solubilized TSHR. Furthermore, the blocking effects of 5C9 on stimulation of cyclic AMP production was assessed using Chinese hamster ovary (CHO) cells expressing the wild-type human TSHR or TSHRs with amino acid mutations.

Main outcome: 5C9 IgG bound to the TSHR with high affinity (4×10^{10} L/mol) and inhibited binding of TSH and a thyroid-stimulating human monoclonal autoantibody (M22) to the receptor. 5C9 IgG preparations inhibited the cyclic AMP-stimulating activities of TSH, M22, serum TSHR autoantibodies and thyroid-stimulating mouse monoclonal antibodies. Furthermore 5C9 reduced the constitutive activity of wild-type TSHR and TSHR with some activating mutations. The effect of different amino acid mutations in the TSHR on 5C9 biological activity was studied and TSHR Lys129Ala or Asp203Ala completely abolished the ability of 5C9 to block TSH-mediated stimulation of cyclic AMP production.

Conclusions: The availability of 5C9 provides new opportunities to investigate the binding and biological activity of TSHR blocking type autoantibodies including studies at the molecular level. Furthermore, monoclonal antibodies such as 5C9 may well provide the basis of new drugs to control TSHR activity including applications in thyroid cancer and Graves' ophthalmopathy.

Introduction

THE THYROTROPIN RECEPTOR (TSHR) is a key protein in the control of thyroid function and is a major autoantigen in Graves' disease (1–4). Thyroid over-activity in Graves' disease is due to autoantibodies to the TSHR which activate the receptor in a similar way to the hormone thyrotropin (TSH) (1–4). These autoantibodies are usually described as thyroid-stimulating autoantibodies (TSAb) or TSHR autoantibodies with stimulating or TSHR agonist activity (1–4).

In a few patients with various autoimmune thyroid diseases however, TSHR autoantibodies are found which act as TSHR antagonists, blocking the stimulating effects of TSH and of thyroid-stimulating autoantibodies (1–4). In some cases,

these blocking type TSHR autoantibodies cause hypothyroidism, particularly when transferred from mother to fetus (5).

Human monoclonal autoantibodies (MAbs) are very useful tools to study the autoantigen–autoantibody interactions at the molecular level (1–4). A human thyroid-stimulating monoclonal autoantibody to the TSHR, which has the characteristics of patient serum autoantibodies with stimulating activity has been isolated and characterized in detail (4,6–10). Extensive studies of the interaction of M22 with the native or mutated TSHR allowed us to obtain a valuable insight into M22 binding sites on the TSHR (4,8). In addition, M22 crystal structure guided mutation of amino acids on the surface of M22 antigen binding site provided further information as to which TSHR and M22 residues interact with each other

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(4,10). These studies culminated in solving the crystal structure of the TSHR leucine-rich domain in complex with M22 Fab (4,9) thus providing details of binding arrangements and molecular interactions between the TSHR and M22 (4,9).

Experimental evidence indicates that the TSHR binding sites for autoantibodies with TSHR antagonist activity are closely related to regions of the TSHR that bind TSH and TSHR-stimulating antibodies (animal and human) (1–4). Until very recently however (4), no human MABs with the characteristics of patient serum blocking type TSHR autoantibodies have been available. We now describe detailed characteristics of the first such human MAB (5C9) (4) isolated from peripheral blood lymphocytes of a 27-year-old patient with postpartum hypothyroidism and high levels of TSHR autoantibodies (260 units/L). 5C9 binding to the TSHR, its effect on constitutive and stimulated cyclic AMP activity, V region sequences and interaction with the TSHR have been studied.

Material and Methods

5C9 heterohybridoma cell line

The heterohybridoma cell line secreting 5C9 monoclonal autoantibody (IgG1/kappa) was established as described before (4) from peripheral blood lymphocytes of a 27-year-old patient with postpartum hypothyroidism and high levels of TSHR autoantibodies (260 units/L) as measured by inhibition of TSH binding to TSHR coated tubes (4,6,11). Local Ethical Committee approval for the study was obtained. The patient developed severe hypothyroidism (TSH levels 278 mU/L 6 months postpartum) after a normal pregnancy and delivery of a healthy child. She had no signs of ophthalmopathy or pre-tibial myxoedema and was started on L-thyroxine. The patient's serum showed strong inhibition (79% at 1/10 dilution of serum) of TSH-stimulated cyclic AMP production in TSHR-transfected Chinese hamster ovary (CHO) cells (4,12). In addition, the patient's serum (1/10 dilution) on its own showed TSHR-stimulating activity (530% of basal activity; >180% is positive). Serum thyroglobulin antibodies were detectable (780 U/mL of NIBSC reference preparation 65/093) and thyroid peroxidase antibody levels were undetectable (both measured in their respective direct radioimmunoassays (13) from RSR Ltd. Tests for other autoantibodies (to glutamic acid decarboxylase 65, IA-2, insulin, and steroid 21-hydroxylase) were negative. IgG was purified from the patient's serum using protein A affinity chromatography on MabSelect™ (GE Healthcare, Chalfont St. Giles, UK) as described before (9). The 5C9 heterohybridoma has been maintained in cell culture for over 28 months and stably expresses approximately 15 mg/L of 5C9 IgG.

Purification of 5C9 IgG, labelling, and fragment preparations

5C9 IgG was purified from culture supernatants using protein A affinity chromatography on MabSelect™ (GE Healthcare) and purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) (14). 5C9 IgG was labelled with ¹²⁵I as described in detail previously (11).

5C9 F(ab')₂ preparations were obtained from 5C9 IgG (10 mg/mL in 20 mmol/L sodium acetate pH 4.5) incubated with immobilized pepsin prepared according to the manu-

facturer's instructions (Perbio Science UK Ltd., Cramlington, UK) for 4.5 hours at room temperature with shaking. Thereafter, immobilized pepsin was removed by centrifugation (1000×g, 5 minutes at room temperature) and the supernatant dialyzed against 300 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.5 overnight at 4°C. The dialyzed mixture containing 5C9 F(ab')₂ and small amounts of intact IgG was separated using a Sephacryl S-300 High Resolution Matrix (GE Healthcare). The 5C9 F(ab')₂ preparations purified in this way did not contain intact IgG as judged by SDS-PAGE and HPLC gel filtration (TSK gel G3000SW column; Tosoh Bioscience; Anachem Ltd., Luton, UK).

In addition, to obtain Fab preparations, 5C9 IgG was treated with mercuripapain (Sigma, Poole, UK) at an enzyme/protein ratio of 1:100, dialyzed into 50 mmol/L NaCl, Tris-HCl pH 9.0 and passed through an anion exchange Sepharose column (Q-Sepharose Fast flow from GE Healthcare) to separate intact IgG and Fc from the Fab preparation. Analysis by SDS-PAGE and gel filtration (Sephacryl S-300) indicated that intact IgG was undetectable in the Fab preparation.

Serum samples and preparations of MABs other than 5C9

Preparations of the human MAB M22 with powerful thyroid-stimulating activity were obtained as described before (6,7). RSR-B2, a mouse MAB which blocks the thyroid-stimulating activities of TSH and TSHR autoantibodies, was prepared as described before (12). A panel of mouse monoclonal TSHR antibodies with stimulating activity (mTSMABs) was produced as previously described (15). Negative control monoclonal antibodies used were a human MAB to thyroid peroxidase 2G4 (16) and a mouse MAB to thyroglobulin 2G2 (15).

Sera containing TSHR autoantibodies with thyroid-stimulating or -blocking activity were obtained from patients who had given informed consent for the study. Healthy blood donor sera (HBD; Golden West Biologicals, Vista, CA) with no detectable TSHR autoantibodies were used as controls.

Inhibition of ¹²⁵I-TSH, ¹²⁵I-M22, or ¹²⁵I-5C9 binding to the TSHR

Binding inhibition assays were carried out using TSHR coated tubes as described before (7,11,12). In the assay, 100 μL of test sample (MAB preparation, patient serum or unlabelled TSH) and 50 μL of start buffer (RSR Ltd.) were incubated in TSHR-coated tubes for 2 hours at room temperature with gentle shaking (duplicate determination). After aspiration, the tubes were washed, 100 μL of ¹²⁵I-labelled protein (5×10⁴ cpm) added and incubated for 1 hour at room temperature with shaking. The tubes were then aspirated, washed again, and counted in a gamma counter. Inhibition of labelled protein binding was calculated as

$$100 \times \left(1 - \frac{\text{cpm bound in the presence of test material}}{\text{cpm bound in the presence of control material}} \right)$$

Control material was a pool of healthy blood donor sera or individual healthy blood donor sera or other materials as indicated in the results of various experiments.

Analysis of 5C9 IgG binding to the TSHR

For Scatchard analysis, unlabelled 5C9 IgG in 50 μ L of assay buffer (50 mmol/L NaCl, 10 mmol/L Tris pH 7.8, and 1% Triton X-100) and 50 μ L of 125 I-labelled 5C9 IgG (30,000 cpm in assay buffer) were incubated in TSHR-coated tubes for 2 hours at room temperature with shaking (maximum binding occurred under these conditions), aspirated, washed twice with 1 mL of assay buffer and counted in a gamma counter. A plot of the concentration of IgG bound against bound/free was used to calculate the affinity of binding to the TSHR (17).

Analysis of stimulation of cyclic AMP production

The ability of 5C9 IgG and other preparations to stimulate production of cyclic AMP in CHO cells expressing the human TSHR was tested as described previously (7,18). CHO cells expressing approximately 5×10^4 TSHRs per cell were seeded into 96-well plates at 3×10^4 cells per well, adapted into DMEM (Invitrogen Ltd., Paisley, UK) without fetal calf serum and then test samples (TSH, IgG or patient serum) added (100 μ L diluted in cyclic AMP assay buffer, i.e., NaCl free Hank's Buffered Salts solution containing 1 g/L glucose, 20 mmol/L HEPES, 222 mmol/L sucrose, 15 g/L bovine serum albumin, and 0.5 mmol/L 3 isobutyl-1-methylxanthine pH 7.4) and incubated for 1 hour at 37°C. After removal of test solutions, cells were lysed and cyclic AMP concentration in the lysates assayed using a Correlate-EIATM Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs; Cambridge Bioscience, Cambridge, UK). For analysis of the effect of 5C9 on the constitutive activity of wild-type TSHR a CHO-K1 cell line expressing approximately 5×10^5 TSHRs per cell was used.

Measurement of blocking (TSHR antagonist) activity

The ability of 5C9 IgG and other preparations to inhibit the stimulating activity of porcine (p) TSH (RSR), recombinant human (rh) TSH (National Institute for Biological Standards and Control [NIBSC] 94/674) and native human (h) TSH (NIBSC 81/565), thyroid-stimulating human MAb M22, mTSMABs (15), and patient serum TSHR autoantibodies was assessed using CHO cells expressing the TSHR (12). These studies were performed by comparing the stimulatory effect of TSH, M22, mTSMABs, or sera, in the absence or in the presence of 5C9 IgG (or other preparations being tested). The assays were performed using the procedure for measuring stimulation of cyclic AMP levels, except 50 μ L of 5C9 (or other preparations being tested) diluted in cyclic AMP assay buffer was added to the cell wells prior to addition of 50 μ L of TSH or M22 or patient serum (diluted as appropriate in cyclic AMP buffer). Other MABs and sera from patients with blocking type TSHR autoantibodies were tested in this assay in addition to 5C9.

Variable region gene analysis

Variable region genes of 5C9 heavy chain (HC) and light chain (LC) were determined as described previously (7) using total RNA prepared from 1×10^7 5C9 secreting cells to produce mRNA for reverse transcriptase polymerase chain reaction (RT-PCR). Specific IgG1 HC and kappa LC sense and antisense strand oligonucleotide primers were designed

using the Medical Research Council's V-base (<http://vbase.mrc-cpe.cam.ac.uk/>) and synthesized by Sigma Genosys (Poole, UK). The RT reaction was carried out at 50°C for 15 minutes followed by 40 cycles of PCR at 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. DNA products were cloned into pUC18 and sequenced by the Sanger-Coulson method (19). V region sequences were compared with available sequences of human Ig genes using Ig blast (<http://www.ncbi.nlm.nih.gov/igblast/>).

Analysis of the effects of amino acid mutations in the human TSHR sequence on 5C9 activity

The methods used, to introduce specific mutations into the TSHR sequence and transfection of mutated TSHR constructs into CHO cells using the Flp-In system, have been described in detail previously (8). In addition to these, amino acid mutations that have been reported to cause an increase in TSHR basal cyclic AMP activity (Ser281Ile in the extracellular domain [20], Ile568Thr in extracellular loop 2 of the transmembrane domain [21] and Ala623Ile in intracellular loop 3 of the transmembrane domain [22]) were also produced.

Flp-In-CHO cells expressing either wild-type or mutated TSHRs were seeded into 96-well plates and used to test the ability of 5C9 preparations to block the stimulating activity of TSH, M22, or patient serum TSHR autoantibodies as described above.

TSHR preparations

Full-length human TSHR was expressed in CHO-K1 cells and extracted in 1% Triton-X-100 (23). The extracts of solubilized TSHR were run on 9% SDS-PAGE gels, blotted onto nitrocellulose, and reacted with MABs as described previously (23).

Epitope analysis using TSHR peptides

Twenty-six peptides each 25 amino acids long covering the whole of the extracellular domain of the human TSHR were kindly provided by Dr. J. Morris (24). A TSHR peptide (amino acids 367–386) that binds a mouse TSHR MAB 8E3 (25) (10 μ g/mL) was used as a positive control. The peptide enzyme-linked immunoabsorbant assay was carried out as described previously (23).

Immunoprecipitation of 35 S-labelled TSHR

Immunoprecipitation assays using 35 S-labelled TSHR produced in the *in vitro* transcription/translation system (Promega, Southampton, UK) were carried out as described before (26).

Results

TSHR-blocking activities of 5C9 IgG

The ability of 5C9 to block stimulation of cyclic AMP production in CHO cells expressing the TSHR by hTSH, rhTSH, pTSH, and the thyroid-stimulating MAB M22 is shown in Fig. 1. Both rhTSH and hTSH stimulated cyclic AMP production in CHO cells expressing the TSHR well, giving 3340 ± 580 fmol/cell well (mean \pm SD) with 100 ng of rhTSH and 8155 ± 1380 fmol/cell well with hTSH at the same concentration. Partial inhibition of both rhTSH- and

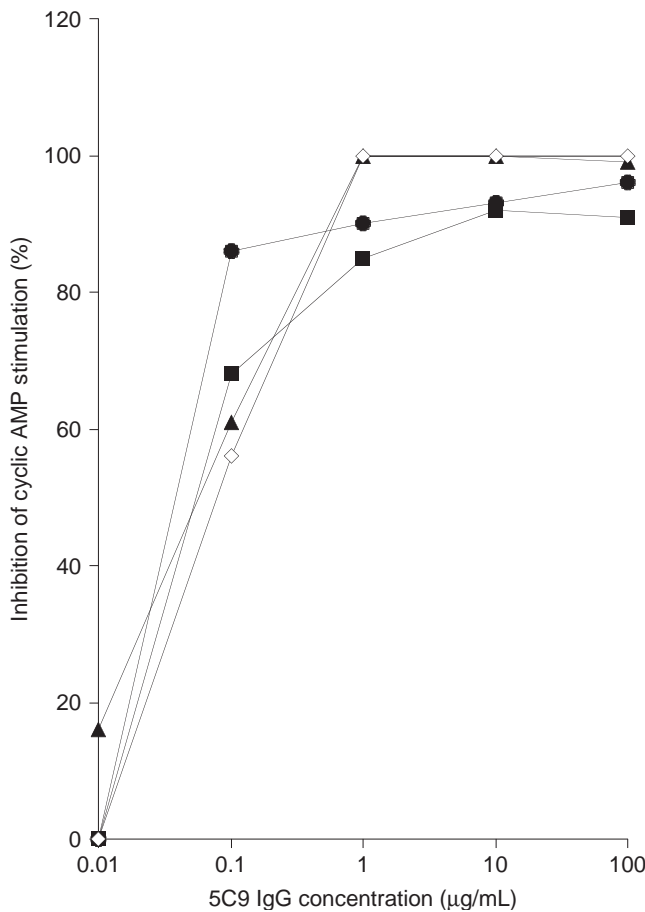


FIG. 1. 5C9 immunoglobulin (IgG) inhibition of porcine thyrotropin (TSH) (■-■), native human TSH (▲-▲), recombinant human TSH (◇-◇), and M22 Fab (●-●) stimulation of cyclic adenosine monophosphate (cyclic AMP) production in thyrotropin receptor (TSHR)-transfected Chinese hamster ovary (CHO) cells. Porcine TSH was used at 3 ng/mL, human TSH preparations at 100 ng/mL and M22 Fab at 3 ng/mL.

hTSH-mediated stimulation of cyclic AMP production was observed with 100 ng/mL 5C9 IgG and complete inhibition with 1 µg/mL (Fig. 1). In the case of pTSH, 3 ng/mL stimulated cyclic AMP production strongly ($12,270 \pm 980$ fmol/cell well). In the presence of 100 ng/mL of 5C9 IgG the stimulating activity of pTSH was reduced to 3920 ± 464 fmol/cell well (68% inhibition) and the inhibiting effect was dose dependent with 85% inhibition of cyclic AMP production at 1 µg/mL of 5C9 IgG. Furthermore, 5C9 F(ab')₂ and 5C9 Fab were effective inhibitors of TSH stimulation (data not shown). The lymphocyte donor serum also had a strong inhibiting effect on TSH-mediated stimulation of cyclic AMP production in CHO cells expressing the TSHR. A 1/10 dilution of donor serum (1.43 mg/mL total serum IgG at this dilution) decreased cyclic AMP stimulation by pTSH from $19,020 \pm 2154$ fmol/cell well to 5931 ± 350 fmol/cell well (68% inhibition). This corresponded to the same inhibition seen with approximately 370 ng/mL of purified 5C9 IgG (calculated from a dilution curve of the effect of different concentrations of 5C9 on stimulation of cyclic AMP by pTSH in the

same assay). This indicates that purified 5C9 IgG is approximately 3900 times more active than the donor serum IgG in terms of its ability to block pTSH-stimulated cyclic AMP production.

M22 Fab (3 ng/mL) is a potent stimulator of cyclic AMP production (9432 ± 822 fmol/cell well) and in the presence of 5C9 IgG the stimulating effect of M22 was inhibited in a dose-dependent manner, with cyclic AMP levels reduced to 1298 ± 134 fmol/cell well in the presence of 100 ng/mL of 5C9 IgG (86% inhibition; Fig. 1).

Effect of 5C9 IgG on the stimulating activity of patient sera TSHR autoantibodies

Preliminary studies indicated that in the presence of 5C9 IgG (100 µg/mL final concentration), the stimulating activity of 16 different patient sera was markedly reduced and in 15/16 the reduction was to near-unstimulated (i.e., basal) cyclic AMP levels (4) while in the case of a negative control TPO MAb 2G4 (100 µg/mL) no significant effect on stimulation by any of the 16 sera was observed (4; Table 1). However, in the case of one serum (T3; Table 1) stimulating activity was only reduced by approximately 50% by 5C9 IgG. This serum (T3) and two other sera (T1 and T16) were analyzed further (Table 1). In the case of sera T1 and T16 both 5C9 and RSR-B2 were able to inhibit stimulation of cyclic AMP in a dose-dependent manner giving 95% and 94% inhibition of T1 stimulation and 94% and 97% inhibition of T16 stimulation at 10 µg/mL of IgG respectively (Table 1). In contrast, with serum T3, 89% inhibition of stimulation was observed with 100 µg/mL of RSR-B2 but only 56% inhibition of stimulation was observed with 5C9 IgG at the same concentration (Table 1).

Inhibition of ¹²⁵I-5C9 IgG binding to the TSHR by serum TRAbs

Binding of ¹²⁵I-5C9 IgG to TSHR-coated tubes was not markedly inhibited by sera from 10 different healthy blood donors (3.4–18.9%). Sera from 40 patients with Graves' disease, all positive for TSHR autoantibodies as assessed by ¹²⁵I-TSH and ¹²⁵I-M22 inhibition assays, inhibited ¹²⁵I-5C9 binding to TSHR-coated tubes (inhibition range 22.0–85.2%) to a greater extent than sera from healthy blood donors. Inhibition of ¹²⁵I-5C9 binding to TSHR-coated tubes correlated well with both inhibition of ¹²⁵I-TSH binding to TSHR-coated tubes ($r=0.95$) and with inhibition of ¹²⁵I-M22 binding to coated tubes ($r=0.95$). Labelled 5C9 binding was inhibited in a dose-dependent manner by the donor serum, and patient serum TSHR autoantibodies with either stimulating or blocking (TSH antagonist) activity (Fig. 2A).

Furthermore, mTSMAbs 1–7 (7,15) and our mouse blocking MAb (RSR-B2; [12]) effectively inhibited 5C9 binding to the TSHR compared to a negative control mouse MAb 2G2 specific for thyroglobulin (Fig. 2B). Furthermore, stimulation of cyclic AMP production by the mouse mTSMAbs 1–7 was completely inhibited by 100 µg/mL of 5C9 IgG (data not shown).

5C9 IgG showed no reactivity with full-length TSHR (produced in CHO cells and detergent solubilized) on Western blots (data not shown). In addition, 5C9 was unable to immunoprecipitate ³⁵S-labelled full length TSHR (1.7% of total cpm immunoprecipitated with 10 µg/mL 5C9 IgG

TABLE 1. BLOCKING EFFECTS OF HUMAN MAb 5C9 AND MOUSE MAb RSR-B2 ON STIMULATION OF CYCLIC AMP PRODUCTION BY SERA FROM THREE DIFFERENT PATIENTS WITH GRAVES' DISEASE^a

Test sample ^b	Cyclic AMP level ^c (fmol/cell well \pm SD; n = 3) with 5C9 IgG	Cyclic AMP level ^c (fmol/cell well \pm SD; n = 3) with RSR-B2 IgG	Cyclic AMP level ^c (fmol/cell well \pm SD; n = 3) with control 2G4 IgG ^d
Experiment 1			
Buffer only	707 \pm 147	707 \pm 147	707 \pm 147
Buffer + 100 μ g/ml MAb ^e	301 \pm 38	721 \pm 183	1207 \pm 123
Serum T1 only	20,336 \pm 1539	20,336 \pm 1539	20,336 \pm 1539
Serum T1 + MAb 0.001 μ g/ml	16,868 \pm 912	21,648 \pm 502	18,134 \pm 2126
Serum T1 + MAb 0.01 μ g/ml	20,314 \pm 279	19,943 \pm 1834	20,876 \pm 1887
Serum T1 + MAb 0.1 μ g/ml	16,152 \pm 3577	17,867 \pm 2220	16,659 \pm 1031
Serum T1 + MAb 1 μ g/ml	2428 \pm 594	4990 \pm 1670	19,025 \pm 1450
Serum T1 + MAb 10 μ g/ml	1119 \pm 348	1061 \pm 104	18,868 \pm 1806
Serum T1 + MAb 100 μ g/ml	724 \pm 28	721 \pm 183	22,078 \pm 2546
Experiment 2			
Buffer only	622 \pm 79	622 \pm 79	622 \pm 79
Buffer + 100 μ g/ml MAb	212 \pm 40	616 \pm 111	745 \pm 136
Serum T3 only	14,023 \pm 2487	14,023 \pm 2487	14,023 \pm 2487
Serum T3 + MAb 0.001 μ g/ml	11,641 \pm 2168	12,159 \pm 2970	13,650 \pm 1679
Serum T3 + MAb 0.01 μ g/ml	13,743 \pm 1687	12,178 \pm 1676	11,939 \pm 131
Serum T3 + MAb 0.1 μ g/ml	10,414 \pm 1094	11,960 \pm 1390	13,853 \pm 1589
Serum T3 + MAb 1 μ g/ml	8075 \pm 610	7385 \pm 554	12,931 \pm 891
Serum T3 + MAb 10 μ g/ml	7806 \pm 793	4262 \pm 367	12,862 \pm 250
Serum T3 + MAb 100 μ g/ml	6136 \pm 558	1597 \pm 323	12,086 \pm 2613
Experiment 3			
Buffer only	466 \pm 65	466 \pm 65	466 \pm 65
Buffer + 100 μ g/ml MAb	253 \pm 25	534 \pm 89	999 \pm 55
Serum T16 only	9781 \pm 1672	9781 \pm 1672	9781 \pm 1672
Serum T16 + MAb 0.001 μ g/ml	11,281 \pm 911	10,229 \pm 714	10,500 \pm 162
Serum T16 + MAb 0.01 μ g/ml	10,441 \pm 122	10,772 \pm 799	13,014 \pm 855
Serum T16 + MAb 0.1 μ g/ml	7483 \pm 415	8719 \pm 389	12,215 \pm 793
Serum T16 + MAb 1 μ g/ml	418 \pm 65	1383 \pm 66	10,831 \pm 140
Serum T16 + MAb 10 μ g/ml	303 \pm 107	605 \pm 254	10,355 \pm 469
Serum T16 + MAb 100 μ g/ml	210 \pm 60	442 \pm 32	10,848 \pm 373

^aMAb, monoclonal antibody.

^bSera diluted 1:10 (final concentration) in cyclic AMP assay buffer (Buffer).

^cIn Chinese hamster ovary cells expressing wild-type thyrotropin receptor.

^d2G4 is a human MAb to thyroid peroxidase (16).

^eMonoclonal antibody IgG concentrations shown are final concentrations.

compared to 15% with a 1/10 dilution of a positive control TSHR polyclonal rabbit serum reactive with the TSHR extracellular domain) (26,27). Furthermore, 5C9 IgG did not bind to a series of overlapping peptides representing the entire extracellular domain of the TSHR (maximum OD₄₅₀ signal generated = 0.03 with 10 μ g/mL 5C9 compared to a typical OD₄₅₀ values of 3.0 or greater with 1 μ g/mL of a positive control MAb).

Effect of 5C9 IgG on stimulation of cyclic AMP production in CHO cells expressing mutated TSHR

The effects of single amino acid mutations in the TSHR on the ability of 5C9 to block cyclic AMP-stimulating activity of pTSH in TSHR-transfected CHO cells are shown in Table 2. In particular, the following amino acids were mutated to alanine: Asp43, Lys58, Ile60, Glu61, Arg80, Tyr82, Thr104, His105, Glu107, Arg109, Lys129, Phe130, Phe134, Asp151, Glu178, Lys183, Tyr185, Asp203, Tyr206, Lys209, Asp232, Gln235, Lys250, Glu251, Arg255, Thr257, Trp258, Arg274, Asp276, and Ser281. In addition, the effects of change of charge

mutations Arg80Asp, Asp151Arg, Lys183Asp, Arg255Asp was studied. Previous experiments have shown that the change of charge mutation of TSHR Asp160Lys causes a loss of responsiveness to TSH while the response to M22 is not affected (8). Consequently, the effect of TSHR Asp160Lys mutation on 5C9 blocking activity was studied using M22 as a stimulator instead of TSH (Table 2).

Out of all the TSHR mutations investigated (Table 2), mutation of Lys129 and Asp 203 to alanine resulted in a complete loss of the ability of 5C9 IgG to block TSH stimulation of cyclic AMP production. In contrast TSHR mutation Lys183Ala caused a partial reduction of 5C9 IgG blocking activity (28% inhibition of TSH stimulation was observed compared to 84% inhibition with wild type TSHR at 1 μ g/mL 5C9 IgG). Even at 100 μ g/mL of 5C9 IgG, only partial inhibition of TSH stimulation (43%) was observed with TSHR Lys183Ala compared to 93% with wild-type TSHR. When positively charged Lys183 was mutated to negatively charged aspartic acid, the effect on 5C9 blocking activity was similar to that observed with the Lys183Ala mutation. Furthermore mutation of Glu178 and Glu251 to alanine (Table 2) showed

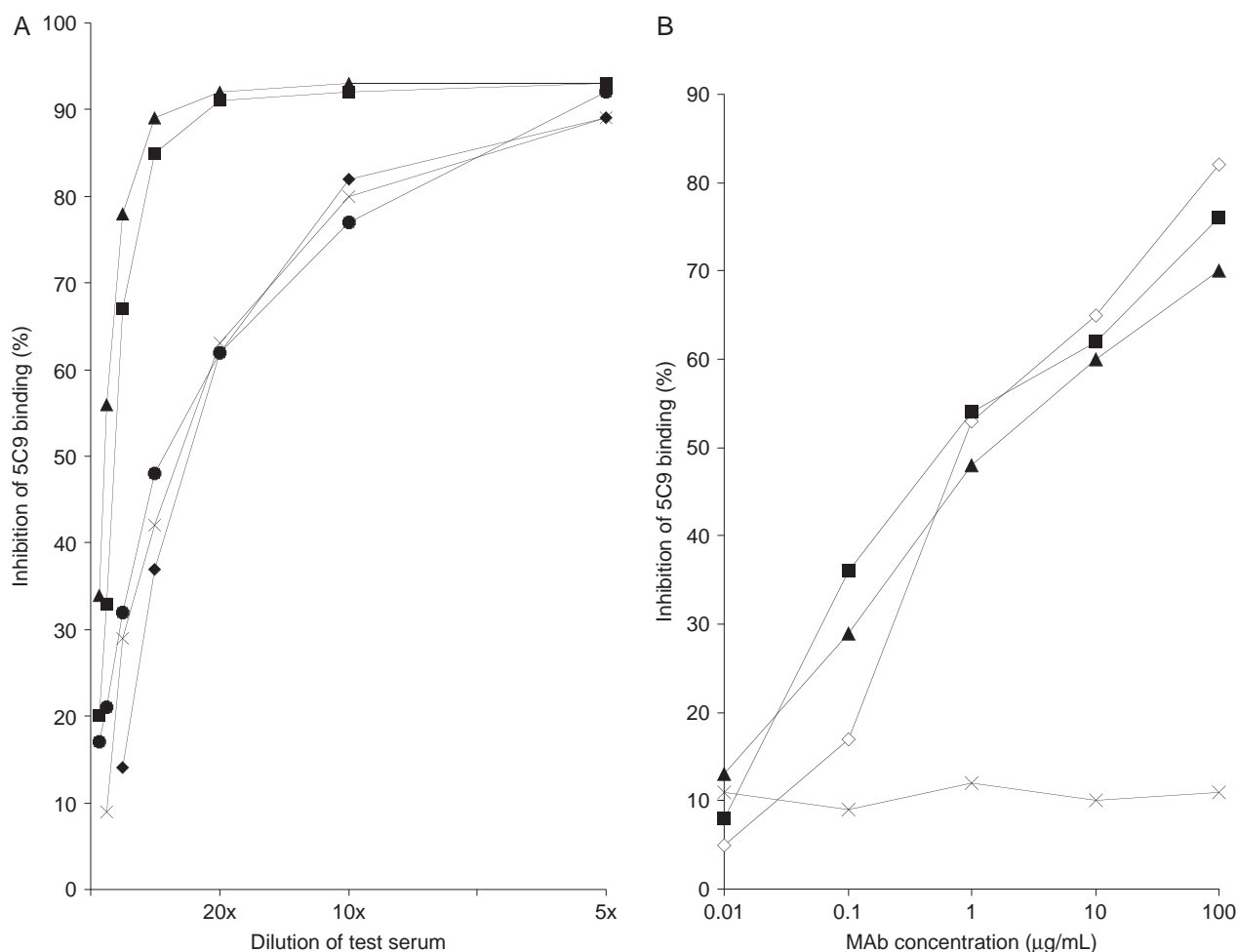


FIG. 2. (A) Effect of patient sera with stimulating activity (X-X and ●-●), patient sera with blocking (antagonist) activity (▲-▲ and ■-■), and the lymphocyte donor serum (◆-◆) on ^{125}I -5C9 IgG binding to thyrotropin receptor (TSHR)-coated tubes. Test sera were diluted in healthy blood donor (HBD) serum pool and labelled 5C9 binding to TSHR coated tubes in the presence of HBD pool only was approximately 17% of total radioactivity added. (B) Effects of unlabelled mouse thyroid stimulating monoclonal antibodies (mTSMABs), ◇-◇ = mTSMAB 4, ■-■ = mTSMAB 5 and a mouse blocking MAb on labelled 5C9 IgG binding to TSHR-coated tubes (▲-▲ = RSR-B2). X-X = negative control MAB 2G2 (mouse MAb to thyroglobulin [Tg]). In addition to mTSMABs 4 and 5 other mTSMABs were able to inhibit 5C9 binding (data not shown in Fig. 2B).

significantly reduced 5C9 blocking activity (40–60% and 60–80% of wild-type activity respectively).

Effect of patient sera with TSH blocking activity on cyclic AMP production in CHO cells transfected with mutated TSHRs

Two TSHR mutations (Lys129Ala and Asp203Ala) that abolished the ability of 5C9 to block TSH stimulation of cyclic AMP production were analyzed further. In particular sera from four patients with blocking type TSHR autoantibodies were studied. The effects of two sera (BS1 and BS3) were unaffected by the TSHR Lys129Ala mutation (Table 3) while two other sera BS2 and BS4 showed a marked decrease in blocking activity compared to their effect with wild-type TSHR. Furthermore in the case of TSHR mutation Asp203Ala (Table 3) two sera BS2 and BS4 showed some decrease in blocking activity relative to wild type while the blocking activities of BS1 and BS3 were affected slightly or not at all by the mutation.

Effect of 5C9 on constitutive activity of wild-type TSHR and TSHRs with activating mutations

Preliminary studies revealed a surprising feature of 5C9 IgG in that it was able to reduce the constitutive activity of wild-type TSHR and the constitutive activity of three TSHR activating mutations (S281I, I568T, and A623I) (4). Consequently, effects of 5C9 and the mouse MAb RSR-B2 on TSHR constitutive activity were compared in detail.

TSHR S281I constitutive activity (1978 ± 302 fmol/cell well) was inhibited 60% and 4% by $1 \mu\text{g/mL}$ of 5C9 or RSR-B2 respectively (Fig. 3A). A similar response was seen using TSHR I568T, the constitutive activity of 4277 ± 1060 fmol/cell well, was inhibited 74% by $1 \mu\text{g/mL}$ of 5C9 and was unaffected (0% inhibition) by $1 \mu\text{g/mL}$ of RSR-B2 (Fig. 3B). In the case of TSHR A623I the constitutive activity of 8380 ± 1738 fmol/cell well was reduced by 43% by addition of $1 \mu\text{g/mL}$ of 5C9 and was unaffected by RSR-B2 (0% inhibition) (Fig. 3C). Increasing the concentration of 5C9 IgG further (up

TABLE 2. EFFECTS OF 5C9 ON THYROTROPIN (TSH) STIMULATION OF CYCLIC AMP PRODUCTION IN CHINESE HAMSTER OVARY (CHO) CELLS TRANSFECTED WITH THE TSH RECEPTOR (TSHR) CONTAINING VARIOUS MUTATIONS

TSHR mutation	Stimulation of cyclic AMP production by TSH ^a	Blocking of TSH stimulation of cyclic AMP ^a by	
		5C9 IgG	RSR-B2 IgG
Wild type	+++++	+++++	+++++
Asp 43 Ala	+++	+++++	+++++
Lys 58 Ala	+++++	+++++	0
Ile 60 Ala	+++++	+++++	+++++
Glu 61 Ala	++++	+++++	+++++
Arg 80 Ala	+++++	+++++	0
Tyr 82 Ala	+++++	+++++	0
Thr 104 Ala	+++++	+++++	NT
His 105 Ala	+++++	+++++	NT
Glu 107 Ala	+++	+++++	+++++
Arg 109 Ala	+++++	+++++	0
Lys 129 Ala	+++++	0	0
Phe 130 Ala	+++++	+++++	+++++
Phe 134 Ala	+++++	+++++	++
Asp 151 Ala	+++++	+++++	NT
Glu 178 Ala	++++	++	++++
Lys 183 Ala	+++++	+	+++++
Tyr 185 Ala	++++	+++++	+++++
Asp 203 Ala	++++	0	+++++
Tyr 206 Ala	++++	+++++	+++++
Lys 209 Ala	++++	+++++	+++++
Asp 232 Ala	+++	+++++	+++++
Glu 235 Ala	+++++	+++++	+++++
Lys 250 Ala	+++++	+++++	++++
Glu 251 Ala	+++++	+++	+++++
Arg 255 Ala	+++++	+++++	+++++
Thr 257 Ala	+++++	+++++	+++++
Trp 258 Ala	+++++	+++++	+++++
Arg 274 Ala	+++++	+++++	+++++
Asp 276 Ala	+++++	+++++	+++++
Ser 281 Ala	++++	+++++	++++
Arg 80 Asp	++++	+++++	0
Asp 151 Arg	+++++	+++++	NT
Lys 183 Asp	+++	+	+++++
Arg 255 Asp	+++++	+++++	+++++
Asp 160 Lys	0	+++++ ^b	NT

^aResults shown are effects with mutated TSHR relative to wild-type TSHR expressed as a percentage; +++++=100% wild-type activity; ++++= <100–80% of wild-type activity; +++= <80–60% of wild-type activity; ++= <60–40% of wild-type activity; += <40–20% of wild-type activity; 0=<20% of wild-type activity, and increased activity relative to wild type: >100%=+++++. NT = not tested.

^bBlocking of stimulation for this mutation was carried out using stimulation by M22 as the mutant did not respond to TSH stimulation (see text for details).

to 100 µg/mL) showed no increase in the inhibition of cAMP production in the three activating mutations (data not shown).

In the case of the constitutive activity of the wild-type TSHR (11,600 ± 3040 fmol/cell well) this was reduced by 71% by addition of 1 µg/mL of 5C9 but was unaffected by RSR-B2 (0% inhibition) (Fig. 3D).

TABLE 3. EFFECT OF PATIENT SERUM BLOCKING TYPE THYROTROPIN RECEPTOR (TSHR) AUTOANTIBODIES ON TSH STIMULATION OF CYCLIC AMP PRODUCTION IN CHINESE HAMSTER OVARY (CHO) CELLS TRANSFECTED WITH MUTATED TSHRS

Test serum	Inhibition of TSH-mediated stimulation of cyclic AMP (%) with		
	Wild-type TSHR	TSHR Lys129Ala	TSHR Asp203Ala
BS1	97	94	80
BS2	67	52	30
BS3	98	94	98
BS4	74	35	59

Effect of patient sera with TSH-blocking activity on basal cyclic AMP levels in TSHR-transfected CHO cells

Previous studies showed that the effects of four sera with blocking type activity (BS1–BS4) on the basal activity of the wild-type TSHR and the TSHR with different activating mutations were different (4).

Two of the patient sera studied (BS2 and the 5C9 lymphocyte donor serum BS4), which were positive for blocking type TSHR autoantibodies, also showed some stimulating activity with the wild-type TSHR (4). To investigate this observation further, we analyzed the ability of the blocking monoclonal antibodies 5C9 and RSR-B2 to inhibit the stimulating activity of sera BS2 and BS4. As shown in Fig. 4 5C9 IgG (10 µg/mL) was able to inhibit the basal activity of the wild-type TSHR in the presence of a pool of healthy blood donor sera while the presence of RSR-B2 or a control IgG (5B3) at the same concentration had no effect. Incubation of CHO-TSHR cells with serum BS2 and serum BS4 caused an increase of cyclic AMP levels to 20,260 ± 2500 fmol/cell well and 23,680 ± 3180 fmol/cell well, respectively compared to the levels of 10,920 ± 1860 fmol/cell well in the presence of healthy blood donor sera (Fig. 4). In the case of serum BS2 the cyclic AMP-stimulating activity in the presence of 5C9 was reduced slightly to 13,760 ± 1400 fmol/cell well (*p* = 0.017) compared to cyclic AMP activity in the presence of buffer only and was unaffected by the presence of RSR-B2 or the control IgG (Fig. 4). In contrast, the cyclic AMP-stimulating activity of serum BS4 was reduced markedly to 5800 ± 660 fmol/cell well in the presence of 5C9 IgG (10 µg/mL) (*p* = 0.0007 compared to buffer only), and to 11,820 ± 1300 fmol/cell well in the presence of RSR-B2 IgG (*p* = 0.004 compared to buffer only) with no effect being observed in the presence of the negative control 5B3 IgG at the same concentration (Fig. 4).

Variable region sequences of 5C9

Sequence analysis of the genes coding for 5C9 IgG showed that the HC V region was from the VH3-53 germline gene, the D region was from the D2-2 gene and the J region was derived from the JH4 gene. There are somatic mutations in the 5C9 HC gene sequence compared to the germline gene sequences; in particular one silent mutation in CDR3 and one silent mutation in FWR4. In addition the 5C9 HC V region

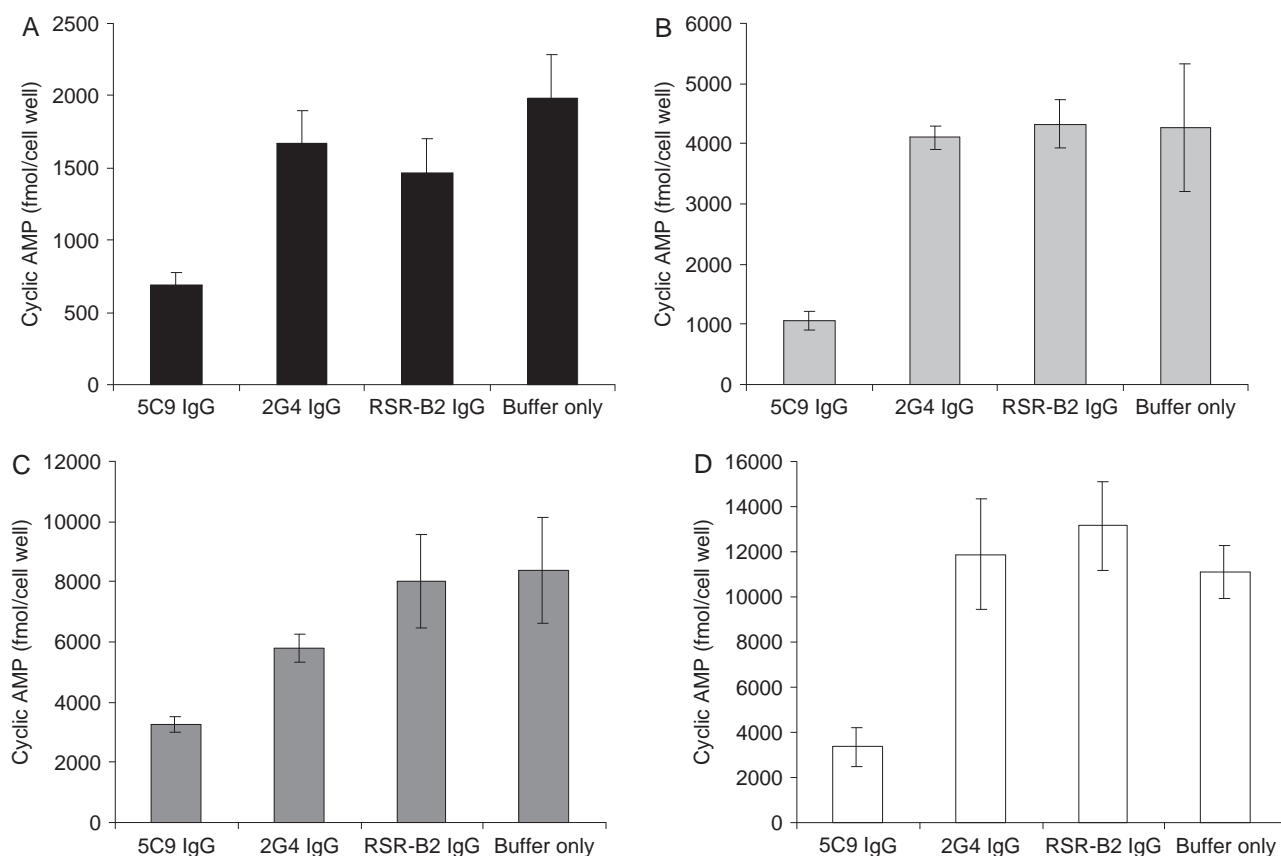


FIG. 3. Effect of 1 $\mu\text{g}/\text{mL}$ of 5C9 IgG or RSR-B2 IgG on constitutive (i.e., basal) activity of wild-type TSHR and TSHRs containing activating mutations: **A** = S281I (extracellular domain) ■; **B** = I568T (extracellular loop 2) □; **C** = A623I (intracellular loop 3) ■; **D** = wild type □. Results shown are means of triplicate determinations \pm SD. 2G4 is a negative control human autoantibody to thyroid peroxidase (TPO). The Chinese hamster ovary (CHO) cells transfected with wild type TSHR in this experiment expressed 500,000 receptors per cell (i.e., about 10 times more than cells usually employed in cyclic AMP stimulation assays).

sequence is characterized by two insertions; one 6 bp long between the V and D genes and one insertion of 15 bp long between the D and J genes. Consequently, the 5C9 HC CDR1 is 5 amino acids long, CDR2 is 16 amino acids long, and CDR3 is 18 amino acids long. The 5C9 LC V region is derived from the O12 germline gene and the J region from the JK2 gene. In the LC sequence there are one silent mutation in FWR1, one replacement mutation in CDR1, one replacement mutation in CDR3, and a 6 bp long insertion between the V and J genes. The 5C9 LC CDR1 is made up of 11 amino acids, CDR2 of 7 amino acids, and CDR3 of 10 amino acids.

Discussion

5C9 is the first human monoclonal TSHR autoantibody with the characteristics of patient serum blocking type autoantibodies to become available (4). It has a high affinity for the TSHR (4×10^{10} L/mol) in the range (3×10^{10} to 7×10^{10} L/mol) observed for patient serum blocking type TSHR autoantibodies (4,28) and is similar to that of the thyroid-stimulating human MAb M22 (5×10^{10} L/mol) we isolated using the same procedure (6,7).

5C9 is IgG1 subclass with a kappa light chain (M22 is IgG1 with a lambda light chain) and analysis of RNA from the 5C9 clone confirmed that the antibody was monoclonal. The V region genes are derived from VH3-53 (VH3 family), D2-2,

JH4, O12, and JK2 germlines and have undergone antigen-driven maturation by insertion (i.e., addition of nucleotides at the V-J and V-D-J junctions). In comparison, M22 V region genes are derived from VH32 (VH5 family), D6-13, JH5, VL1-11, and JL3b germlines (7) and also underwent antigen-driven maturation (29).

We tested the effects of 5C9 on several different thyroid stimulators including TSH (human and porcine), the human MAb M22, several mouse TSMABs, and patient sera with thyroid-stimulating activity. 5C9 was an effective inhibitor of the stimulating activity of all these TSHR agonists (4).

5C9 IgG itself showed no stimulating activity over a wide concentration range and consequently only acted as a TSHR antagonist. Similarly, the thyroid-stimulating human MAb M22 acts as a pure TSHR agonist (6,7) and consequently, neither human MAb has the mixed (or partial agonist activities) reported for some thyroid-stimulating MABs derived from TSHR immunized animals (30,31).

In most cases, 5C9 showed equal or greater potency than the mouse monoclonal blocking type TSHR antibody RSR-B2 (which has the characteristics of patient serum blocking type TSHR autoantibodies). In the case of one patient serum (T3 in Table 1) however RSR-B2 was more potent than 5C9. This suggests that although stimulating autoantibodies in different patient sera interact with the same region of the TSHR there

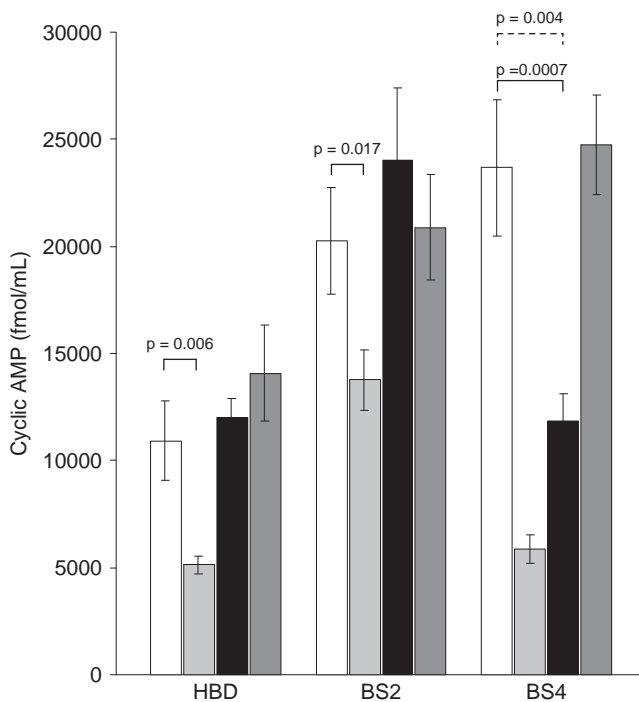


FIG. 4. Effect of the blocking MAbs 5C9 and RSR-B2 on thyrotropin receptor (TSHR) stimulation by sera that had both stimulating and blocking activities. □ = cyclic AMP buffer; ■ = +10 µg/mL 5C9 IgG; ■ = +10 µg/mL RSR-B2 IgG; ■ = +10 µg/mL 5B3 IgG (negative control IgG). Results shown are means of triplicate determinations ± SD. Serum BS4 was from the donor of the lymphocytes used for 5C9 preparation. 5B3 is a negative control human monoclonal autoantibody to glutamic acid decarboxylase. Antibodies were used at 10 µg/mL final concentration and serum samples were at 1/10 final dilution. HBD is a pool of healthy blood donor sera. The Chinese hamster ovary (CHO) cells transfected with the wild-type TSHR in this experiment expressed 500,000 receptors per cell (i.e. about 10 times more than cells usually employed in cyclic AMP stimulation assays). *p* values were calculated using Student's *t* test; *p* ≤ 0.05 was considered to indicate statistically significant difference.

are differences in the actual contact amino acids involved (8). Furthermore, this observation indicates that there are differences in the epitopes recognized by 5C9 and RSR-B2 even though RSR-B2 inhibits binding of 5C9 to the TSHR effectively.

5C9 IgG was 3900 times more potent than the donor serum IgG in blocking TSH stimulation. A similar ratio between the potency of the MAb and donor serum IgG was observed with our thyroid-stimulating human MAb M22 (3000 times) (6,7). These potency figures contrast markedly with those in previous reports of human MAbs reactive with the TSHR. For example, the blocking and the stimulating MAbs described by Valente *et al.* (32) and Kohn *et al.* (33) were of similar potency to the donor serum IgG. In addition some of these MAbs reacted with gangliosides. Yoshida *et al.* (34) have also reported the production of human monoclonal blocking and stimulating TSHR MAbs from patient lymphocytes. However the MAbs they describe are very different from 5C9 and M22 being far less potent and being able to recognize reduced and denatured TSHR on Western blots.

In addition, production of recombinant human MAbs to the TSHR with thyroid-stimulating activity has been reported (35,36) but these have affinities for the TSHR of about three orders of magnitude lower than 5C9 or M22. Furthermore, these recombinant TSHR MAbs did not inhibit labelled TSH binding to the TSHR. Also, blocking MAbs isolated from peripheral blood lymphocytes (37) and expressed as recombinant IgGs have been described (38). However they appeared to have low affinity for the TSHR, inhibited TSH binding weakly or not at all and were weak blockers of TSH-mediated cyclic AMP production (e.g., 100 µg/mL IgG giving 67% inhibition [38]). Another laboratory has reported the isolation of B cell lines producing IgGs with weak TSH-blocking activity but these IgGs were unable to inhibit TSH binding to the receptor (39).

Consequently, 5C9 has the properties of patient serum TSHR autoantibodies with blocking activity whereas human MAbs reported previously to have blocking activity do not (40). In particular, the earlier MAbs do not show the high affinity, high potency, and binding site features of 5C9 or patient serum blocking autoantibodies. As well as inhibiting cyclic AMP production stimulated by various TSHR ligands, 5C9 inhibited cyclic AMP production in TSHR-transfected CHO cells when no stimulating ligand was present. This effect on basal cyclic AMP production was observed with CHO cells transfected with wild type TSHR or TSHR with different activating mutations (4). Consequently, 5C9 has at least two mechanisms of action: (a) preventing binding of activating ligand to the TSHR and (b) an effect on TSHR activation not dependent on activating ligand binding. The mechanism by which inhibition of TSHR basal activity occurs is not clear at present and the effect is not seen with the blocking type mouse MAb RSR-B2 (Fig. 3). Recently however, a mouse MAb (CS-17) to the TSHR has been described (41) which has the ability to block both basal and TSH-stimulated cyclic AMP production in COS-7 cells expressing wild-type TSHR and TSHRs with activating mutations. Both 5C9 and CS-17 were able to inhibit the constitutive activity of TSHR S281I, TSHR I568T, and TSHR A623I at 1 µg/mL, however, 5C9 appeared to be more effective than CS-17 at low IgG concentrations (4,41). These differences in activity, may be due to differences in binding sites on the TSHR and/or differences in binding affinity of the two antibodies for the TSHR (4,41).

Analysis of the effects on basal TSHR activity of four patient sera with blocking type TSHR autoantibodies (4) indicated that one serum (BS3) has similar effects to 5C9 in CHO cells expressing wild-type TSHR and TSHR I568T. The other three sera studied caused an increase (relative to a pool of healthy blood donor sera) in basal cyclic AMP levels in CHO cells expressing wild-type TSHR suggesting that they contained a mixture of blocking-type and stimulating-type TSHR autoantibodies (4). Further analysis of two of these sera (BS2 and the 5C9 lymphocyte donor serum BS4, Fig. 4) showed that 5C9 inhibited the stimulating activity of both sera to different degrees and RSR-B2 only had an effect in the case of BS4. These observations emphasize the complexity of the overall biological effects of sera containing TSHR autoantibodies with both blocking and stimulating activity and the difficulty in interpreting the effects seen in various experiments. These observations emphasize the importance of producing more human monoclonal TSHR autoantibodies

to help investigate the intricate interactions between autoantibodies and the receptor and to analyze the mechanisms involved in receptor activation and inactivation.

The binding of ^{125}I -labelled 5C9 IgG to TSHR-coated tubes was inhibited by all 40 TSHR autoantibody positive patient sera studied. Furthermore, inhibition of 5C9 binding correlated well with inhibition of labelled TSH binding ($r=0.95$) and inhibition of labelled M22 binding ($r=0.95$). This emphasizes the close relationship between the binding sites on the TSHR for 5C9, M22, TSH, and the TSHR autoantibodies in the 40 different patient sera. Also the lack of reactivity of 5C9 IgG with (a) ^{35}S -TSHR (produced *in vitro* in rabbit reticulocyte lysates), (b) TSHR peptides, or (c) full-length TSHR on Western blots suggests that the human MAb 5C9 recognizes a conformational epitope on the receptor as do almost all patient serum TSHR autoantibodies (23,25,26,42).

Analysis of the effects of different TSHR mutations on the ability of 5C9 to block TSH stimulation enabled us to identify five residues important for 5C9 action, i.e., TSHR Lys129, Glu178, Lys183, Asp203, and Glu251 (Table 2, Fig. 5). Only one of these amino acids (Lys129) is important for the blocking actions of RSR-B2 (Fig. 5), while two amino acids (Lys129 and Lys183) are also important for the thyroid-stimulating effects of M22 (Fig. 5).

In summary, therefore, the main features of 5C9 are (a) a powerful antagonist of both TSH and thyroid-stimulating

antibodies, (b) an inhibitor of basal (i.e., constitutive) activity of both wild-type TSHR and TSHRs containing activating mutations, (c) high affinity for the TSHR, (d) conformational epitope, and (e) 5C9 binding to the TSHR is inhibited by patient serum autoantibodies with either stimulating or blocking activity as well as by thyroid-stimulating or -blocking MAbs. The ability of 5C9 to act as a powerful antagonist of thyroid-stimulating autoantibodies (and TSH) suggests potentially important *in vivo* applications. In particular, 5C9 preparations (or molecules derived from them) could be used as specific inhibitors of (a) thyroid stimulation by TSHR autoantibodies in patients with Graves' disease, (b) TSHR autoantibody induced Graves' ophthalmopathy (43), and (c) hyperthyroidism due to TSHR activating mutations (20–22). Also the ability of 5C9 to suppress TSHR constitutive activity could be useful in suppressing this activity in remnants of thyroid cancer tissue or in thyroid cancer metastases because, even in patients with complete TSH suppression, low constitutive TSHR activity may be undesirable.

The availability now of two types of human monoclonal autoantibodies to the TSHR with distinct biological activities (stimulating or blocking) provides new opportunities to study the autoantibody–TSHR interaction and the relationship between binding and biological activity of blocking autoantibodies can now be studied without the bias associated with use of polyclonal patient serum preparations.

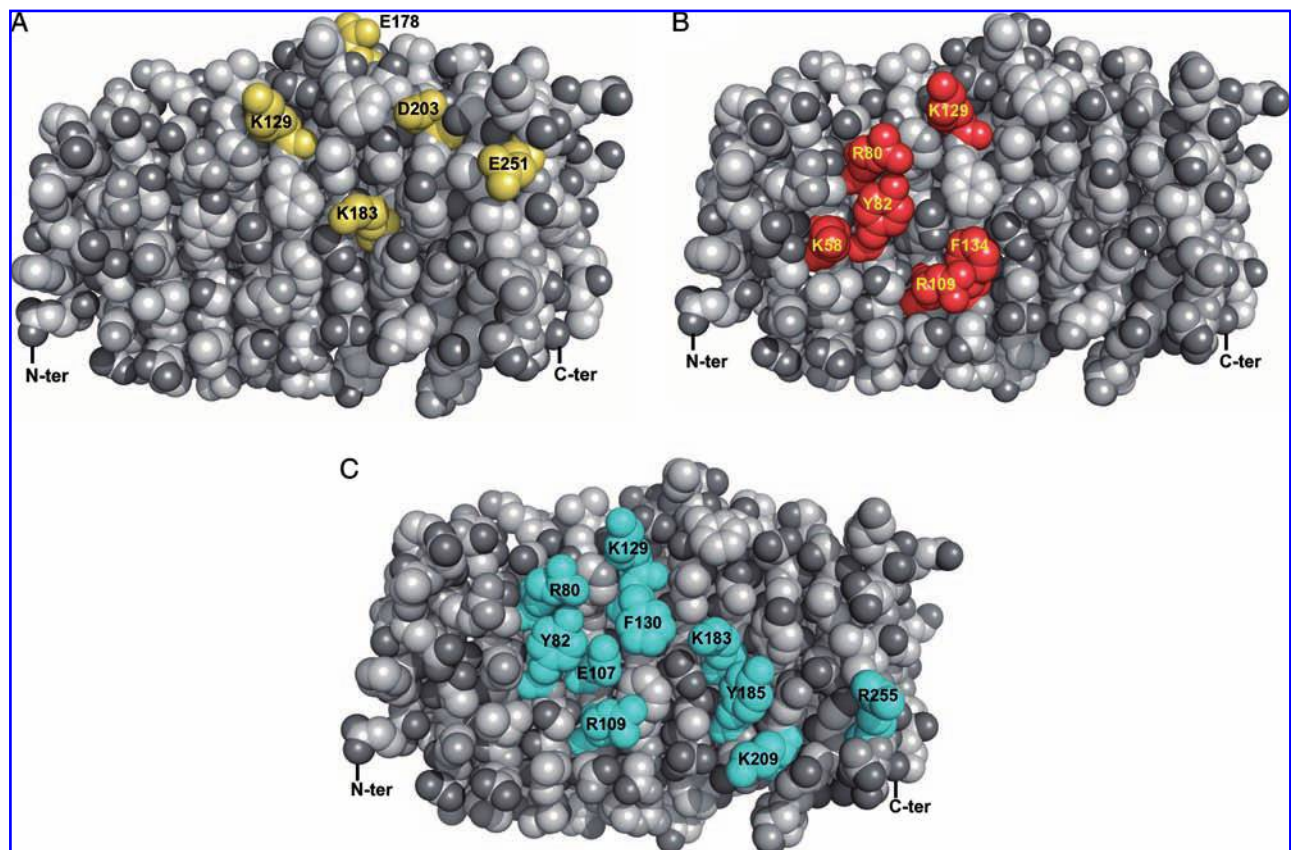


FIG. 5. Single amino acid mutations in the thyrotropin receptor (TSHR) leucine rich repeat domain (LRD) which (A) affect 5C9 inhibition of thyrotropin (TSH)-mediated stimulation of cyclic AMP production in TSHR-transfected Chinese hamster ovary (CHO) cells (shown in yellow), (B) affect RSR-B2 inhibition of TSH-mediated stimulation of cyclic AMP production in TSHR-transfected CHO cells (shown in red) and (C) affect M22 stimulation of cyclic AMP production in TSHR-transfected CHO cells (shown in blue). TSHR LRD structure shown is from the crystal structure of the TSHR in complex with M22 (9).

Acknowledgements

Carol James prepared the manuscript. Ricardo Núñez Miguel prepared Fig. 5A–C showing positions of TSHR amino acids important for 5C9, RSR-B2, and M22 activity in the TSHR leucine rich repeat domain (LRD) structure. RSR Ltd. is a developer of medical diagnostics including kits for measuring thyroid autoantibodies.

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