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*J Immunol* 2006; 176:5084-5092; doi: 10.4049/jimmunol.176.8.5084

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Monoclonal Pathogenic Antibodies to the Thyroid-Stimulating Hormone Receptor in Graves’ Disease with Potent Thyroid-Stimulating Activity but Differential Blocking Activity Activate Multiple Signaling Pathways

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The thyroid target Ag for disease-inducing autoantibodies in Graves’ disease is the receptor for thyroid-stimulating hormone (TSH), but little is known about the molecular basis of this pathogenic Ab response. We describe the characteristics of two high-affinity mAbs developed from an experimental murine model of hyperthyroid Graves’ disease that exhibit potent thyroid-stimulating activity. Nanogram concentrations of the IgG mAbs KSA1 and KSA2 and their Fab induce full stimulation of the TSH receptor that is matched by the ligand TSH and, thus, act as full agonists for the receptor. However, KSA1 and KSA2 display differential activities in their ability to block TSH-mediated stimulation of the receptor, indicating subtle differences in their biological properties. In displacement studies, IgG and Fabs of KSA1 and KSA2 compete with Graves’ disease autoantibodies as well as thyroid-blocking Abs present in some hypothyroid patients, indicating a close relationship between these autoimmune determinants on the receptor. In passive transfer studies, single injections of microgram quantities of KSA1 or KSA2 IgG led to rapid elevation of serum thyroxine and a hyperthyroid state that was maintained for a number of days. The thyroid glands showed evidence of cell necrosis, but there was no accompanying mononuclear cell infiltrate. In studying their receptor activation pathways, both KSA1 and KSA2 provoked phosphorylation of the intracellular ERK1/2 pathway in primary thyrocytes, indicating that multiple signaling pathways may participate in the pathogenesis of Graves’ disease. In summary, our findings emphasize the similarities of the experimental mouse model in reproducing the human disorder and provide improved means for characterizing the molecular basis of this pathogenic response.

G raves’ disease is a common Ab-mediated disorder in which the primary target Ag has been identified as the thyroid follicular cell surface receptor (TSHR)3 for the thyroid-stimulating hormone (TSH) (1). Recent evidence also suggests involvement of a second autoantigen, the insulin-like growth factor-1 receptor (IGF-1R), in extrathyroidal complications of Graves’ disease such as thyroid-associated ophthalmopathy (2). The members of one group of anti-TSHR Abs behave as agonists, mimicking the action of the natural ligand TSH on the receptor, and are known as thyroid-stimulating Abs (TSAs) (3). The TSAbs hyperstimulate the thyroid follicular cells to secrete thyroxine, resulting in hyperthyroidism. Another group of anti-TSHR (blocking) Abs (TSBAbs) may act as antagonists of TSH binding to the receptor, a role that may occasionally lead to hypothyroidism (3). One recent model indicates potential cross-reactivity of anti-TSHR Abs with IGF-1R, resulting in the T cell-mediated induction of extracellular matrix components and leading to the manifestation of thyroid-associated ophthalmopathy and pretibial myxoedema (2, 4, 5). Neutral class Abs to the TSHR that have neither agonist nor antagonist activity have also been described, although their role in disease remains to be clarified.

The TSHR belongs to the family of G protein-coupled receptors with a large extracellular domain, a seven-transmembrane region, and a short cytoplasmic tail (6). The transmembrane region of G protein-coupled receptors is responsible for the transmission of the activating signal by regulating small secondary messengers such as cAMP, diacylglycerol, and inositol 1,4,5-triphosphate (7). It is likely that the mode of TSHR activation and the consequent intracellular regulatory cascade may ultimately be responsible for the variations observed in different patients with regard to toxic hyperplasia (gland enlargement and goiter), extrathyroidal complications, and response to treatment (8). The TSHR is unique among the large family of G protein-coupled receptors in undergoing complex posttranslational modifications such as cleavage into two disulfide-linked subunits known as the A subunit and the B subunit (9, 10). The A subunit of 53 kDa, corresponding to the ectodomain of TSHR, is of special interest because it preferentially binds TSAbs (11), and it has been proposed that the resulting cleaved fragment released into the bloodstream may be the primary stimulus for provoking autoimmunity in susceptible individuals (10, 11).

Received for publication July 7, 2005. Accepted for publication October 4, 2005.

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1 This work was supported by funds from the Department of Diabetes, Endocrinology, and Internal Medicine, King’s College School of Medicine, London, U.K. (J.P.B and A.M.M.) and by National Institutes of Health Grants EY008976, EY11708, DKD63121 (T.J.S), and K23 RR017304 (to A.G.G.).

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3 Abbreviations used in this paper: TSHR, thyroid-stimulating hormone receptor; TSH, thyroid-stimulating hormone; bTSH, bovine TSH; Ad, adenovirus; CHO, Chinese hamster ovary; IGF-1R, insulin-like growth factor-1 receptor; TBI, TSH binding inhibiting Ig; TSAb, thyroid-stimulating Ab; TSAB, anti-TSHR (blocking) Ab.
In addition to signaling through heterotrimeric G proteins, a number of reports have suggested that TSH can activate inositol 1,4,5-triphosphate kinase-related pathways. This activation leads to the phosphorylation of ERK1/2 MAPK pathway (12–15). It is suggested that these actions are related to the growth-promoting effects of TSH in thyroid stimulation. These pathways are prominently known as downstream targets for the IGF-1R. The relationship between TSHR and IGF-1R was appreciated nearly 20 years ago, when IGF-1R was found to substantially enhance the effects of TSH on the proliferation of cultured thyrocytes (16). Whether thyroid-stimulating Abs directed to the TSHR can, like TSH, initiate ERK phosphorylation has yet to be demonstrated.

The isolation of TSAsbs as mAbs has been a long sought goal but, until recently, has proved to be extraordinarily difficult to achieve (17). The establishment of experimental animal models of hyperthyroid Graves’ disease has led to the development of IgG mAbs with limited TSAb activity (18–20). At the same time a human IgG mAb to TSHR, developed from a patient with Graves’ disease, was described as having powerful thyroid activity in the nanogram range (21). The human mAb acted as a full agonist by activating the TSHR to maximal stimulation, equivalent to that achieved with subsaturating concentrations of TSH (22). More recently, a murine mAb with similar efficacy has also been described (23) that was developed from an experimental model and behaves as a full agonist for the TSHR. Moreover, this mAb was pathogenic in Ab transfer experiments in vivo and reportedly led to a lymphocytic infiltrate of the thyroid gland (23). The determinants on the receptor for the full agonist and other stimulatory and blocking murine mAbs are dependent on the conformational, three-dimensional folding of the ectodomain residing in a region rich in leucine repeats within the horseshoe structure (23). The paucity of full agonist autoantibodies to TSHR present in patients’ serum (24, 25) precluded a comparison of their properties, which may impact on the pathogenesis of Graves’ disease. In this report, we describe the properties of two mAbs with full agonist activity to the TSHR that also show subtle differences in their behavior in terms of the antagonism of TSH-mediated receptor stimulation and that activate multiple signaling pathways. Finally, we show that the mAbs are pathogenic in vivo when transferred into mice whereby a single injection of microgram quantities of IgG induces rapid hypersecretion of thyroxine, leading to sustained hyperthyroidism with considerable morphological changes but with minimal mononuclear cell infiltrate in the thyroid glands.

Materials and Methods

Measurement of TSHR Abs and thyroid function tests

Depending on the samples for assessment, Abs to TSHR were measured using two different types of TSH binding inhibition assays (porcine TRAK RIA and TRAK II DYNOTest human kits (Brahms Diagnostika) requiring 50 and 100 μl of neat serum, respectively, essentially according to the manufacturer’s instructions (26)). The results were expressed as the percentage of inhibition of 125I-labeled TSH binding. TSABs and TSBAbs activities were measured by bioassay using CHO cells stably transfected with a human TSHR gene, with the hybridomas being added to each well in triplicate and incubated for 48 h at 37°C. The cAMP released into the medium was measured by RIA (R&D Systems), and the results were expressed as picoeinstein per milliliter or stimulation over basal value obtained with medium as described (28). TSABs were detected similarly by adding a subsaturating dose of bTSH (40 μU/ml) to the test sample or control serum and measuring the reduction in TSH-mediated stimulation of cAMP, as described previously (28). In our laboratory the inter-assay and intra-assay coefficients of variation for TSABs have been measured as <16 and <14%, and for TSBAbs these coefficients were <24 and 11%, respectively (28). Total thyroid hormone (TT4) was determined by RIA with 25 μl of sera (DS Laboratories) from four normal BALB/c animals for determination of basal values.

Recombinant adenosviruses (AdS)

Recombinant Ad expressing the human TSHR holoreceptor (TSHR-Ad) was constructed using the AdEasy adenoviral vector system (Quantum Biotechnologies). Briefly, TSHR CDNA (26) was excised from pBluescript II SK by digestion with KpnI and NotI and ligated into the Ad transfer vector (pShuttleCMV; Quantum Biotechnologies). After linearization of the pShuttleCMV/HTSHR CMV vector with Pmel and treatment with alkali phosphate, the linear DNA was cotransformed by electroporation into electropocompetent Escherichia coli/B35183 together with a supercoiled plasmid, pAdEasy-1, containing viral DNA. Recombinants were selected in kanamycin, extracted, and digested with PciI to expose the inverted terminal repeats, and finally transfected into HEK293A cells to generate viral particles. An Ad containing the human TSHR-A subunit (aa 1–289) (referred to as A subunit-Ad) was generously provided by Drs. S. M. McLachlan and B. Rapoport (29, 30). An Ad expressing β-galactosidase was used as control and prepared using the AdEasy system. All virus constructs were propagated in HEK293 cells, purified twice with CsCl gradient centrifugation (29, 30), and dialyzed against PBS, and viral concentration was determined by optical absorbance at 280 nm. Purified Ads were aliquoted and stored at −80°C.

Immunization and selection of animals for hyperintegrines

All mice were obtained from Harlan. For TSHR-Ad injection, female BALB/c mice (n = 10, ages 7–8 wk) were injected i.m. with the recombinant virus (50 μl containing 10⁷ particles) in PBS three times at three weekly intervals (31). The animals were bled 1 wk after the second injection and subsequently sampled again 4 wk after the third injection and tested individually for TSAb activity.

For A subunit-Ad injections, a low-dose immunization protocol of 10⁶ particles was used (30). Female BALB/c mice (16 animals, ages 7–8 wk) were immunized as described, bled 1 and 3 wk after the second injection, and tested individually for TSAb activity. The animals with consistently elevated TSAb activity received a third injection of A subunit-Ad. One week later, this injection was followed by a booster i.p. injection of CHO CHO cells expressing the TSHR ectodomain via a GPI anchor (2 × 10⁹ cells in 500 μl of PBS) (32). The animals were sacrificed 3 days later, and the spleens were removed aseptically for hyperintegridection followed by the collection of blood by cardiac puncture for serum and the excision of thyroid glands for histological analysis. All animal experiments were performed with the approval of the Home Office Regulations (U.K.) and King’s College London under full veterinary welfare care.

Screening of hyperintegrides and cloning

Spleen cell suspensions were fused with X63-Ag8653 myeloma cells at a ratio of 5:1 using polyethylene glycol fusion medium for hybridoma production followed by selection in hypoxanthine, aminopterin, and thymidine (HAT medium; Invitrogen Life Technologies) and HT medium (Immune Systems). The supernatants (100 μl) from wells showing growth were tested for TSH binding inhibiting Ig by RIA with 25 μl of sera (DS Laboratories) from four normal BALB/c animals for determination of basal values. The mixture was mixed with 2.5 mg of IgG, 1 M cysteine (25 μl), 20 mM EDTA (25 μl), and 1 mg/ml papain in acetate buffer (5 μl) and incubated overnight at 37°C (33). Following the addition of 100 mM iodoacetate (16 animals, ages 7–8 wk) to terminate the reaction, the digest was mixed with protein A-Sepharose for 1 h at 4°C. After a brief microfuge centrifugation step, the supernatant was collected and dialyzed overnight against PBS. The purity of the Fabs was examined by SDS-PAGE, and quantified for protein by Bradford protein assay.

Preparation of Fabs

Fabs were prepared from IgG by digestion with papain (Sigma-Aldrich) using 2.5 mg of IgG, 1 M cysteine (25 μl), 20 mM EDTA (25 μl), and 1 mg/ml papain in acetate buffer (5 μl) and incubated overnight at 37°C (33). Following the addition of 100 mM iodoacetate (16 animals, ages 7–8 wk) to terminate the reaction, the digest was mixed with protein A-Sepharose for 1 h at 4°C. After a brief microfuge centrifugation step, the supernatant was collected and dialyzed overnight against PBS. The purity of the Fabs was examined by SDS-PAGE, and quantified for protein by Bradford protein assay.

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by SDS-PAGE, and TSHR reactivity was confirmed by TBII activity. 

Iodination of IgG and Fabs and displacement studies 

IgG (0.25 nM) or Fabs (0.25 nM) of KSAb1 or KSAb2 in 10 μl of PBS were labeled with 5 μl of Na125I using iodogen-coated tubes by incubating for 10 min at room temperature. Free iodine was removed by gel filtration in Sepharose G25 columns, and specific activity was calculated (34). Ab affinity was measured by saturation binding analysis. Briefly, different concentrations of 125I-labeled IgG of KSAb1 or KSAb2 were added in duplicate to human TSHR-coated tubes (from TRAK II DYNOtest human kit), resuspended in binding buffer from the kit in a 200-μl final volume, and incubated overnight in the cold room to reach equilibrium. After washing the plates three times with washing buffer from the kit, the bound 125I-labeled IgG was measured by counting the radioactivity in a gamma counter (DPC Laboratories). Nonspecific binding was subtracted, and the KD values were calculated at 50% saturation using Excel software. The affinity results were expressed as reciprocal KD values (liter per mole).

Competition studies were conducted similarly as those described above. Different concentrations of unlabeled IgG or Fabs were resuspended in binding buffer (from TRAK II DYNOtest human kit) in a final volume 200 μl and added to human TSHR-coated tubes from the kit. After 2 h of incubation with shaking at room temperature, the tubes were washed twice with washing buffer from the kit. A subsaturating concentration of 125I-labeled KSAb1 or KSAb2 IgG was added, incubation continued for 1 h, and the tubes were washed and counted in a gamma counter. For competition with sera from Graves’ patients, 100 μl of serum was added to the human TSHR-coated tubes as described above. The inhibition of binding of 125I-labeled KSAb1 or KSAb2 was determined and expressed as a percentage of inhibition.

Injection of KSAb1 and KSAb2 IgG for in vivo stimulation of thyroid gland

Injection of different doses of purified IgG of KSAb1 or KSAb2 was performed by the i.v. and i.p. routes. Female BALB/c mice (18 animals ages 7–8 wk) were treated with a single i.v. injection in the tail vein of KSAb1 or KSAb2 IgG in sterile PBS (50 μl) containing 10 or 100 μg Ab (three mice per group). Another group of mice were treated with a single i.p. injection of KSAb2 IgG in sterile PBS (100 μg). For controls, animals were injected i.v. with glutamic acid decarboxylase (mAb GAD-1), an isotype-matched, 100-μg IgG mAb specific for the islet cell Ag. All animals were bled at 7, 28, and 70 h after injection, and serum TT4 levels were determined. Mice were sacrificed at 70 h, and thyroid glands were excised for histological analysis.

TSHR signaling and phosphorylation of ERK

Thyroid tissue was obtained as surgical waste from patients undergoing thyroidectomy for the treatment of variety of conditions, including Graves’ disease, nodular disease, and focal well-differentiated malignancies. Tissue was processed as described previously (35). Liberated thyrocytes were pipetted into 25-mm flasks to allow the attachment of cells and the formation of monolayers. These were maintained in RPMI 1640 medium supplemented with 10% FCS and penicillin/streptomycin/Fungizone. They were disrupted with gentle trypsin treatment and used between the second and the sixth passages. Cells in 60-mm-diameter plates were shifted to medium containing 1% FCS for 16 h before the initiation of experimental treatments. To the confluent thyroid monolayers were added human TSH (1 mU/ml; Sigma-Aldrich), recombinant human IGF-1 (10 nM; R&D Systems). KSAb1 IgG (10 ng/ml), and KSAb2 IgG (10 ng/ml), and the monolayers were incubated for 15 min; anti-IGF-1-Rab (mAb 1H7) (5 μg/ml) (BD Pharmingen) was added to another plate for 45 min. PBS was added to the control plates. Following the appropriate incubation time, the plates were rinsed with PBS, and the cells were harvested in Tris-saline buffer containing 15 mM CHAPS (Sigma-Aldrich), 1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 5 μM/10% Nonidet P-40, and 0.1 μM/ml PMSF (100 mM). Lysates were solubilized in Laemmli buffer and subjected to SDS PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore). After overnight blocking in 1% BSA solution in Tris-saline, the membrane was incubated with anti-phosphorylated ERK Ab (Santa Cruz Biotechnology) overnight at 4°C, washed, and then incubated with peroxidase-labeled secondary Ab for 2 h at room temperature. The bound Ab was revealed using the ECL-Plus system (Amersham Bioscience), and the specific signals were captured on X-Omat film (Kodak) and densitometrically with a Bioimage quantified scanner.

Thyroid histology

Thyroid glands were fixed and processed in Formalin. Sections were stained with H&E for morphological analysis. Immunohistochemistry was performed for the detection of B and T cells on the fixed thyroid sections with anti-mouse CD20 followed by detection with the Immunocruz anti-goat (Santa Cruz Biotechnology), rat anti-mouse CD4 and CD8 mAbs followed by detection with biotinylated anti-rat Ab (Vector Laboratories), and a streptavidin-biotin peroxidase conjugate (Dako). Ag retrieval was performed before staining by pressure cooking at pH 6.0 for CD20 and CD8 Abs and at pH 9.0 for the CD4 Ab.

Results

BALB/c mice were immunized with a recombinant Ad expressing the TSHR holoreceptor (TSHR-Ad) or TSHR-A subunit (A subunit-Ad) to induce hyperthyroid Graves’ disease (29, 30). Initial assessment for anti-TSHR Abs was performed for TSAs in individual sample bleeds of all animals. In the TSHR-Ad (holoreceptor) group, 1 wk after the second immunization only one animal demonstrated a marked elevation in TSAb activity with a 90-fold increase over basal (not shown). Thus, in our laboratory the 10% incidence of induced Graves’ hyperthyroid disease following the full-length holoreceptor immunization protocol differs from those of other groups who have reported a higher disease incidence (30, 31).

For A subunit-Ad injections, we used the improved low dose immunization protocol that preferentially induces hyperthyroidism in a higher frequency of immunized animals (30). One week after the second injection nine animals were positive (56%) for TSAb activity, ranging from 3.1- to 92.6-fold increased activity over basal levels (Table I). Eleven animals (68%) showed significant elevation of serum TT4 levels and, hence, were hyperthyroid (Table I). These results are in complete agreement with those of Chen et al. (30). One animal from this group with the highest stable TSAb levels was selected for hybridoma production and boosted with a third injection of A subunit-Ad, followed 1 wk later by an i.p. injection of CHO cells expressing high levels of human TSHR ectodomain linked by the GPI anchor to the plasma membrane cell surface to expand the Ab-secreting splenic B cell population. Serum from the selected animal at sacrifice showed it to be hyperthyroid with elevated TT4 (134 μg/ml); TT4 for control BALB/c mice cAMP (pmol/ml) TT4 (μg/ml)

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a Eleven animals (68%) were hyperthyroid (shown in bold).
b Control BALB/c mice.
mice was 56.25 ± 8.26 μg/ml, as well as being highly positive for TBII activity with 87% inhibition of 125I-labeled TSH binding.

Monoclonal antibodies
Culture supernatants (100 μl) were collected from 70–80% confluent wells and tested neat for TBII activity using TRAK II DYNOtest human kits. A total of 250 wells were screened, resulting in three positive wells (well 9, 98%; well 17, 96%; and well 233, 80% inhibition). Upon expansion, the TBII activity of the well 233 primary cell line declined rapidly. The remaining two well 233, 80% inhibition). Upon expansion, the TBII activity of the well 233 primary cell line declined rapidly. The remaining two lines were cloned twice at 0.3 cells/well and renamed KSAb1 and KSAb2, which have been in continuous culture for >7 mo. The H and L chain subtypes for KSAb1 and KSAb2 were shown to be IgG2b/k and IgG2a/k, respectively.

Thyroid-stimulating activity of the mAbs
Both KSAb1 and KSAb2 IgG stimulated cAMP production in CHO cells stably transfected with human TSHR. Initial dose-response studies were conducted in NaCl-free, sucrose-containing medium routinely used for its increased sensitivity for detecting TSAb (36). As shown in Fig. 1A, in dose-response studies both KSAb1 and KSAb2 IgG showed typical sigmoid curves by stimulating TSHR to reach >98% of the response achieved with a subsaturating dose of bTSH. However, although both the mAbs show full agonist activity by achieving near maximal cAMP stimulatory responses, they showed differences in their cAMP stimulatory responses at lower doses of IgG. Thus, overall KSAb1 and KSAb2 showed maximal stimulation of 199- and 183-fold over basal value, with 3-fold stimulation obtained at 1.2 ng/ml and 2.2 ng/ml IgG, respectively (Fig. 1A). The EC_{50} values for KSAb1 and KSAb2 IgG were determined to be 9.4 and 93 ng/ml, respectively (Fig. 1A). Fabs of KSAb1 and KSAb2 also gave similar TSAb responses to the intact parental IgG (Fig. 1A) and therefore also behaved as full agonists for the TSHR.

We also performed dose-response studies under physiological salt concentrations, although these assay conditions demonstrate reduced sensitivity compared with the use of salt-free, sucrose-containing isotonic HBSS buffer (36). The dose-response for TSH induced cAMP production was not altered significantly in the NaCl-containing buffer, with 40μU/ml giving maximal stimulation (not shown). Importantly, both KSAb1 and KSAb2 IgG continued to show full agonist activity with maximal cAMP stimulatory responses reaching >98% of the response obtained with subsaturating dose of bTSH (Fig. 1B). Typical sigmoid dose-response curves were observed, which again at lower doses showed differences in the cAMP stimulatory activity of KSAb1 and KSAb2 IgG (Fig. 1B). Under the physiological salt conditions, KSAb1 and KSAb2 continued to be active at concentrations of <1 and 3 ng/ml respectively. The IgGs showed EC_{50} values in the nanomolar range of 16.5 and 100 ng/ml, respectively (Fig. 1B). Moreover, Fabs of KSAb1 and KSAb2 also showed similar efficacies of cAMP stimulation (Fig. 1B).

Blocking of TSH-mediated stimulation (TSAb) activity
The ability of KSAb1 and KSAb2 IgG to block TSH-mediated stimulation of cAMP in JPO9 cells was measured in a TSH-mediated stimulation blocking assay. Different concentrations of IgG were examined in the assay to ensure that the blocking activity was not dependent upon the Ab concentration. Whereas KSAb1 showed negligible TSAb activity, KSAb2 IgG showed a reproducible >20% TSAb activity in all Ab concentrations tested (≤30 ng/well) equivalent to 240 ng/ml, which was below the subsaturating concentration of agonist activity for KSAb2. Consequently, KSAb2 acted with weak antagonism to TSH-mediated stimulation of cAMP (Fig. 2). Interestingly, neither KSAb1 nor KSAb2 IgG demonstrated reactivity with any specific peptide in a complete set of synthetic peptides of the TSHR ectodomain by ELISA (28), giving compelling evidence about the recognition of conformational epitopes on the receptor (not shown).

FIGURE 1. Dose-response curves of TSAbs of KSAb1 and KSAb2 IgG and Fabs assessed by bio-assay in JPO9 cells. Varying concentrations of KSAb1 IgG ( ), and Fabs ( ) and KSAb2 IgG ( ) and Fabs ( ) (in ng/ml) were added to JPO9 cells in salt-free isotonic HBSS buffer containing sucrose and HEPES (A) or physiological isotonic HBSS buffer containing NaCl (B), and the stimulated cAMP was measured (in picomoles per milliliter) as described in Materials and Methods. The cAMP responses of a subsaturating dose of bTSH in the salt free and physiological isotonic medium were 164 and 157 pmol/ml, respectively. The results shown are representative of at least three independent experiments performed in triplicate.

FIGURE 2. TSAb activity of KSAb1 ( ) and KSAb2 ( ) IgG measured in JPO9 cells using a subsaturating concentration of 40μU of bTSH. The TSAb activity, expressed as the percentage of inhibition of TSH-induced cAMP, was calculated as described in Materials and Methods. Different concentrations of KSAb1 and KSAb2 IgG were examined to ensure that the blocking activity was not dependent upon the Ab concentration. The results shown are representative of at least two independent experiments performed in triplicate.
**TBI activity**

In TBI assays using TRAK II DYNOtest human kits, dose-response analysis of KSAb1 and KSAb2 IgG showing concentrations of 3.3 and 10 ng/ml were sufficient to give 50% inhibition of 125I-labeled TSH binding activity (Fig. 3). Moreover, for both of the mAbs 20% inhibition was achieved at concentrations of 0.7–4.4 ng/ml, whereas 100 ng/ml was sufficient to give 95% inhibition (Fig. 3). Fabs gave similar TBI activity to the intact, parental IgG (Fig. 3).

**Competition studies with KSAb1 and KSAb2**

By saturation binding analysis using human TSHR-coated tubes (from TRAK II DYNOtest human kits), both 125I-labeled KSAb1 and KSAb2 IgG bound the receptor with high affinity, with equilibrium dissociation constants of 4.5 × 10^{-10} liter/mol and 6.25 × 10^{-10} liter/mol, respectively (Fig. 4). The labeled IgG and Fabs of the mAbs were then used as tracers in competition studies to study the epitopes on the receptor. We investigated whether labeled KSAb1 and KSAb2 IgG displaced heterogeneous autoantibodies to TSHR from Graves’ disease patients. Sera from normal individuals with no family history of autoimmunity were used as controls. As shown in Fig. 5A, sera from Graves’ disease patients inhibited the binding of KSAb1 or KSAb2 to the immobilized receptor. Furthermore, although different sera competed to a similar degree with both labeled KSAb1 and KSAb2 IgG, the sera varied in their inhibitory activity, indicating the heterogeneous nature of anti-TSHR autoantibodies in sera from different patients (Fig. 5A). To investigate the autoimmune determinants on TSHR present in other conditions, we assessed sera with strong blocking activity from autoimmune hypothyroid patients that also competed in binding to the receptor with labeled KSAb1 or KSAb2 IgG (Fig. 5A). Finally, using sub saturating concentrations of 125I-labeled IgG on human TSHR-coated tubes, both KSAb1 and KSAb2 IgG competed with each other, showing that their epitopes overlapped on the TSHR (Fig. 5B). Moreover, Fabs of KSAb1 and KSAb2 also competed with each other, indicating the close association of their determinants on the TSHR (Fig. 5C).

Displacement studies using labeled KSAb1 and KSAb2 IgG as tracers were also performed with another panel of anti-TSHR IgG mAbs that are specific for linear determinants on the receptor and that show negligible thyroid stimulatory activity (37). Neither KSAb1 or KSAb2 IgG showed any competition with the mAbs A10, A9, and A7, which are specific for residues located in the amino-terminal, middle, and carboxyl-terminal regions of the receptor, respectively (37) (not shown). Thus, the stimulatory epitopes on the TSHR are different from the linear epitopes recognized by this panel of anti-TSHR mAbs.

**TSHR signaling and phosphorylation of ERK**

A number of reports have appeared recently suggesting that thyrocyte activation through TSHR may be mediated by signaling pathways other than those involving G protein-coupled cAMP generation (12–14). An as yet unanswered question relates to whether phosphorylation of ERK is dependent upon cAMP generation. We found that both KSAb1 and KSAb2 IgG could provoke the phosphorylation of ERK1/2 in primary thyrocytes (Fig. 6). The actions of the two mAbs were similar and occurred at comparably low concentrations. They mimicked the effects of human TSH (1 mU/ml) and IGF-1 (10 nM) (Fig. 6A). Because of the potential cross-talk between the TSHR and IGF-1 receptor (4, 5), we added the IGF-1R-blocking Ab 1H7 to cultures treated with these mAbs and found that 1H7 could partially attenuate ERK phosphorylation (Fig. 6B). The addition of 1H7 alone or irrelevant Abs failed to alter levels of ERK phosphorylation. Thus, it would appear that both KSAb1 and KSAb2 can initiate signaling in thyrocytes through activation of the ERK pathway and that the IGF-1R may be at least partially involved in this action (Fig. 6).
Passive transfer studies on KSAb1 and KSAb2 IgG

We assessed the effect of in vivo injection of KSAb1 and KSAb2 IgG into naive mice in terms of inducing hyperthyroidism. We anticipated KSAb1 and KSAb2 to cross-react with mouse TSHR, because the two mAbs were derived from a mouse that was significantly hyperthyroid. Two IgG doses of 10g and 100g of each mAb were injected i.v. into mice, and the induced hyperthyroxinemia was determined at different time points. As control, an isotype-matched mAb GAD1 to a pancreatic islet cell Ag was used. The results in Fig. 7 show that both KSAb1 and KSAb2 IgG are pathogenic with a dose of 10 or 100 μg of KSAb1 and 100 μg of KSAb2, resulting in a rapid thyroid stimulatory response characterized by hyperthyroxinemia within 7 h of administration. Serum thyroxine levels returned to baseline by 70 h. Injection of KSAb2 at a dose of 10 μg demonstrated a more delayed response in elevating TT4 levels, peaking at 28 h (Fig. 7). We also examined the effect of i.p. injection of KSAb2 IgG, which paralleled the stimulation mediated by i.v. delivery (Fig. 7). These results also confirm cross-reactivity of KSAb1 and KSAb2 to mouse TSHR.

Histological analysis of the thyroid glands from KSAb1- and KSAb2-treated mice showed both follicular and epithelial changes as compared with glands from the animals treated with the control mAb (Fig. 8, A–F). In contrast with the thyroid gland from the control GAD1 mAb-treated animals (Fig. 8 A), the thyroid follicles from KSAb1 and KSAb2 IgG-treated mice were of variable size and shape, with focal areas exhibiting the total loss of luminal colloid associated with the collapse of follicular lumina; in other areas, the colloid appears pale, thin, and also finely vacuolated (Fig. 8, B–E). Moreover, the follicles containing the pale colloid were lined with flattened and attenuated epithelial cells, whereas the follicles without colloid showed epithelial cell lining of columnar and cuboidal cells with multilayering (broken arrows in Fig. 8). Moreover, individual necrotic cells were found to be present within the luminal colloid and also within the follicular lining epithelium with pyknotic nuclei (shown in Fig. 8F). Finally,
histological analysis of the H&E sections revealed no mononuclear cell infiltrate in the glands of the KSAb1 and KSAb2 IgG-treated animals, irrespective of the dose or the route of administration (Fig. 8, B–F). This finding was further substantiated by immunohistochemical staining of the thyroid glands, whereby staining with Abs to mouse CD4, CD8, and CD20 failed to identify any B or T cell infiltrate (not shown).

Discussion
The generation of mAbs with agonist activity for the TSHR has been a long sought goal for a number of laboratories that has only recently been attained (17). Progress in this achievement has been dependent on the establishment of viable experimental models of Ab-induced hyperthyroidism. However, the one major exception has been the notable achievement of a human IgG mAb with all the essential properties of a bona fide pathogenic Ab (21). Among the several thyroid-stimulating mAbs derived from experimental models, only one showed full agonist activity and potencies that matched those present in the human disorder (23). The mAbs described in this study, KSAb1 and KSAb2, fall into this category of disease-associated Abs as they display the following: 1) full agonist activity for the human TSHR; 2) potency in nanogram quantities of IgG; 3) high affinity for human TSHR in the 10^10 liter/mol range; and 4) in vivo pathogenicity with the induction of hyperthyroidism.

Our results show that the epitopes of KSAb1 and KSAb2 on TSHR are likely to be conformational in nature and overlap with autoreactive determinants recognized by patients’ autoantibodies. Their epitopes on the TSHR are likely to be close and intimately associated with each other, because the smaller Fabs of the KSAb1 and KSAb2 also compete for binding to the receptor. Additionally, our finding that these epitopes also overlap with the determinants associated with strong antagonism for TSH binding to the TSHR is in agreement with previous studies on the close relationship of the receptor epitopes associated with receptor stimulation and TSH antagonism (23, 38).

The notable aspects of our studies with KSAb1 and KSAb2 are based on the derivation of two thyroid-stimulating mAbs with full agonist TSHR activity, allowing us to compare their behavior. Our striking finding that KSAb1 IgG and KSAb2 IgG show differences in cAMP-mediated signaling of TSHR raises the possibility that Abs to different epitopes of the receptor can influence thyroid function. Although TSH is known to stimulate both the adenyl cyclase...
and phospholipase C cascades in the human thyroid gland, the concentrations of TSH required for stimulation through the latter pathway (phospholipase C) are considerably higher (7). KSAb1 and KSAb2 have comparable affinities and exhibit differences in cAMP generation, particularly at very low doses of TSH. However the phosphorylation of ERK following treatment with the two mAbs is similar. This observation calls into question the assertion that phosphorylation of ERK (or p38) provoked by TSH is the trivial consequence of some unidentified contaminant (14, 39, 40). Thus, it can be surmised that the overlapping epitopes recognized by the two mAbs, as suggested by the competitive binding studies, may be related to the activities associated with ERK1/2 phosphorylation, whereas the cAMP activities may be related to the distinct aspects of the respective epitopes. Clearly, future studies using cells overexpressing IGF-1R may prove enlightening. Changes in the profile of anti-TSHR Abs over time (41–43) could account for the fluctuations in thyroid hormone status commonly observed in Graves’ disease patients (8, 41, 42). Moreover, the substantial variations seen in different patients with regard to gland enlargement and extrathyroidal manifestations suggest that other factors, perhaps involving another receptor, may play important roles in the pathogenesis of Graves’ disease (2, 4, 44). Whether either monoclonal mAb recognizes an epitope(s) on the IGF-1R or whether this receptor interacts, directly or indirectly, with TSHR is uncertain.

Abs with potent TSAb activity are pathogenic and directly responsible for the hyperthyroid status. Examination of serum thyroxine levels in individual mice showed a number of animals in which the hormone levels failed to correlate with serum TSAb activity. This was not surprising, because a lack of concordance is also recognized both in human patients and in various models of induced Graves’ disease (26, 29, 45, 46). We confirmed, in an acute study, by passive transfer of KSAb1 and KSAb2 IgG that these Abs led to rapid hyperthyroidism in the animals. Moreover, the i.p. route of injection of KSAb2 also led to elevated serum TT4 levels, the kinetics of which paralleled the i.v. route. The differences in potency between KSAb1 and KSAb2 observed at lower doses in the in vitro experiments were also apparent in the in vivo studies. Thus, i.v. injections of a lower dose of 10 μg of IgG of KSAb2 showed delayed kinetics of induced hyperthyroidism in the animals, in contrast with the induced hyperthyroidism with KSAb1 that showed a similar induction of elevated serum thyroxine at the 10- and 100-μg doses tested. However, once maximal serum thyroxine levels were achieved with either the 10- or the 100-μg doses of either KSAb1 or KSAb2 IgG, hormone levels declined with similar kinetics until reaching basal levels by 70 h after injection.

Interestingly, histological analysis of the thyroid glands at 70 h after injection showed morphological changes suggestive of both stimulatory and cytotoxic effects. The stimulatory effects were characterized by proliferative epithelial changes as evidenced by the foci of hyperplastic follicles with the loss of colloid formation associated with multilayering and luminal collapse. The cytotoxic effects were present as individual cell necrosis within the lining epithelium and as dying thyrocytes present within the colloid material. These cytotoxic effects are likely to be mediated as a result of the increased thyroid hormone production induced in the gland by the TSAbs, leading to a massive accumulation of hydrogen peroxide within the apical surface of the thyroid follicles (47, 48). There was no evidence of a mononuclear cell infiltrate such as that present in the glands of patients with Graves’ disease. It is possible that a prolonged chronic study of mAb administration may lead to inflammatory changes within the thyroid gland, but it is interesting that in a similar acute study with their potent TSAb mAb, the group of Costagliola et al. (23) reported a mononuclear cell infiltrate in the glands of their hyperthyroid mice. Because the time frame in their study following the single injection of the TSAb mAb was similar to that in the acute study in this report, this finding suggests that perhaps Abs to different epitopes on the TSHR may be linked to the inflammatory reaction in the thyroid gland (23). Another recent study from Davies and colleagues (18) used a hamster mAb to TSHR with weaker TSAb activity than that reported in this study to examine both the acute and chronic effects of the mAb administration to mice (49). Although it is difficult to draw correlates of the hamster mAb with the powerful stimulating mAbs reported herein, it is interesting that chronic stimulation failed to induce hyperthyroxinemia in the animals but did lead to desensitization of the receptor as well as to considerable morphological changes such as hypertrophy and colloid depletion in the thyroid glands (49). Nevertheless, there was no reported mononuclear cell infiltration into the thyroid glands of the animals following chronic stimulation with the hamster mAb (49).

The development of potent thyroid-stimulating mAbs such as KSAb1 and KSAb2 opens the way to a molecular dissection of the TSHR and the epitopes associated with autoimmune disease. Mapping of the conformational epitopes on the TSHR recognized by KSAb1 and KSAb2 may be performed by phage random peptide library screening in conjunction with mutagenesis (50). This will help in the characterization of the epitopes associated with thyroid stimulation on the TSHR and their modes of intracellular signaling. An understanding of these signaling events may also be relevant to complications of Graves’ disease such as thyroid-associated ophthalmopathy and pretibial myxoeida. Finally, the generation of anti-idiotypic Abs to KSAb1 and KSAb2 for the identification of individual clonotypes of anti-TSHR Ab specificities present in patients with Graves’ disease may allow studies in the future to correlate their response to treatment and hence tailor therapies for individual patients without risk of relapse.

Acknowledgments
We acknowledge our deep appreciation of Drs. Sandra McLachlan and Basil Rapoport for provision of the A subunit-Ad and, along with Dr. Chun-Rong Chen, for sharing their experimental protocol for scale-up and purification of the virus suitable for immunizations. We thank Dr. Monika Gora for help in the early stages of construction of the full-length human TSHR recombinant Ad and Nick Bailey for the immunohistochemical staining of the thyroid glands.

Disclosures
The authors have no financial conflict of interest.

References
THYROID-STIMULATING mAbs


is the culprit as well as the victim. Thyroid 5: 5092 THYROID-STIMULATING mAbs


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