

Demonstration of Immunoglobulin G, A, and E Autoantibodies to the Human Thyrotropin Receptor Using Flow Cytometry

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Human TSH receptor (TSHR) autoantibodies with biological activity result in thyroid dysfunction, but antibodies that simply bind do not. We have applied flow cytometry to the measurements of IgG, IgA, and IgE immunoreactivity to the TSHR in patients with Graves' disease (GD) and thyroid eye disease (TED) and in normal controls.

CHO cells stably expressing the extracellular domain of the TSHR with a glycosylphosphatidylinositol anchor were produced and found to express approximately 4 times as many receptors, but of similar affinity, as JP09 in TSH binding studies. Substantial increases in median fluorescence and peak channel fluorescence were obtained by flow cytometry using TSHR monoclonal antibodies on the glycosylphosphatidylinositol cells.

IgG autoantibodies were demonstrated in 55 of 65 un-

treated GD patients, 3 of 25 normal subjects, and 4 of 8 atypical TED sera (negative for TSHR autoantibodies with biological activity) by flow cytometry and correlated poorly with thyroid-stimulating antibodies. IgA antibodies were present in 1 of 12 normal, 1 of 7 treated GD with TED, and 3 of 8 atypical TED sera. IgE binding was observed in 1 of 12 normal, 2 of 8 treated GD without TED, 1 of 6 treated GD with TED, and 0 of 8 atypical TED sera.

In conclusion, we have demonstrated autoantibodies that bind directly to the TSHR in the majority of GD patients and in 50% of patients with atypical TED and a small number of normal controls lacking TSHR antibodies that affect function. Although predominantly IgG λ , TSHR autoantibodies of the IgA and IgE isotypes are also detectable. (*J Clin Endocrinol Metab* 87: 1754–1761, 2002)

AUTOANTIBODIES to the TSH receptor (TSHR) are found in patients with autoimmune thyroid disorders, particularly Graves' disease (GD). They are classified according to their biological activity. Thyroid-stimulating antibodies (TSAB) mimic the action of TSH and result in hyperthyroidism. Thyroid-blocking antibodies (TBAB) inhibit TSH induced stimulation and may be the cause of hypothyroidism in idiopathic myxedema. Both TSAB and TBAB may also be TSH binding-inhibiting Igs (TBII), which prevent hormone/receptor interaction (1).

Assays in current diagnostic use are not able to detect antibodies that simply bind to the receptor without any effect on the TSHR or TSH binding. The other major autoantigens in autoimmune thyroid disease are Tg and thyroperoxidase (TPO); both can be detected by direct binding, *e.g.* in ELISA (2). The incidence of TPO antibodies would be grossly underestimated if the only detection methods relied on a biological effect such as inhibition of enzyme activity. Direct binding has also revealed that 10–20% of normal euthyroid individuals, especially women, have circulating Tg and TPO autoantibodies, but little has been reported regarding TSHR antibodies in such a control population (3).

Abbreviations: ECD, Extracellular domain; FITC, fluorescein isothiocyanate; GD, Graves' disease; GPI, glycosylphosphatidylinositol; TBAB, thyroid-blocking antibodies; TBII, TSH binding-inhibiting Igs; TED, thyroid eye disease; TPO, thyroperoxidase; TSAB, thyroid-stimulating antibodies; TSHR, TSH receptor.

Previous studies investigating direct binding of autoantibodies to the TSHR have concentrated on sera from known TSAB- and/or TBII-positive GD patients. A variety of methods have been used, including ELISA, *in vitro* transcription/translation, Western blotting of bacterially produced TSHR, and flow cytometric analysis of cells expressing the full-length receptor or the extracellular domain (ECD) anchored via a glycosylphosphatidylinositol (GPI) link (3–10). Most studies report TSHR-binding autoantibodies in a proportion of sera containing TSAB and/or TBII and demonstrate the heterogeneity of TSHR autoantibodies, *e.g.* a GD patient with monoclonal gammopathy, having antibodies that bound to the TSHR in a Western blot, but were TBII and TSAB negative (11).

In the case of the TSHR, antibodies without biological effect may be of particular relevance in the extrathyroidal manifestations of GD, thyroid eye disease (TED), and pretibial myxedema. Pretibial myxedema is rare, but TED is frequently associated with GD, and several lines of evidence indicate that the TSHR may have a role in both disorders (12). These include the demonstration of TSHR transcripts and protein in the ocular tissues, especially in the adipose compartment (13–15), and the development of animal models of TED induced by transfer of TSHR-primed T cells (16) or TSHR genetic immunization (17). A strong argument against the TSHR being involved in TED is the fact that not all patients with TED have TSAB or TBII. This heterogeneous

group of individuals with severe symptoms of TED in the absence of TSHR autoantibodies in conventional assays, which we have termed atypical TED, may have no evidence of thyroid dysfunction or may have been thyrotoxic previously.

TSAB are predominantly of the IgG1 isotype, and there is some evidence for light chain restriction. Other isotypes have been implicated in the extrathyroidal manifestations of GD (18), and increases in the level of IgA secretion in tears have also been reported in 25% of patients (19). Furthermore, in the receptor-induced animal models of TED, mast cells were an early indicator of orbital pathology, and elevated IgE levels have been found in GD patients (20), both of which are suggestive of IgE immunoreactivity to the TSHR.

The aim of the present study was to investigate antibodies binding to the TSHR in individuals negative for TSAB and TBII, either normal controls or patients with atypical TED, and to determine whether TSHR autoantibodies are exclusively of the IgG isotype and/or light chain restricted in these and a panel of GD sera.

Subjects and Methods

Subjects studied

Serum samples from a total of 110 individuals were tested. This included 65 untreated patients with GD, defined by thyrotoxicosis, a diffuse goiter, and the presence of Tg or TPO antibodies or an appropriate family history or exophthalmos, and 12 patients with treated GD. Of the latter group, 6 also had TED, *i.e.* exophthalmos measured by exophthalmometry. All of these patients had TSHR autoantibodies detected as TSAB or TBII. We also selected 8 atypical TED patients who were negative for TSAB and TBII in conventional assays: 3 with no evidence of thyroid dysfunction, 1 with newly diagnosed GD, and 4 who were previously treated for GD. All 8 patients had exophthalmos of at least grade II; their details are shown in Table 1. We also studied 25 biochemically euthyroid normal blood donors (16 females and 9 males) selected at random from the local blood transfusion service. All samples were obtained with informed consent and with the approval of the local ethics committee in accordance with the Declaration of Helsinki.

Measurements of TSAB, TBAB, and TBII

TSAB and TBII were measured on CHO cell lines stably expressing the human TSHR, *lulu* or NA-4 for TSAB (21, 22) and JP09 for TBII (23), as previously described in detail.

Construction and characterization of GPI cell line

Construction of GPI-anchored TSHR ECD. A synthetic linker encoding a thrombin cleavage site and an artificial GPI attachment site based on a region of domain 3 of rat CD4 (7) was created by annealing of the following seven oligonucleotides and cloned into the *Xho*I and *Xba*I sites of pcDNA3.1 (Invitrogen, San Diego, CA), 5'-TCGAGCTGGTGCCAA-GAGGCTCTATCGAGGGCAGA-3', 5'-CGTGATGGATGTGCTCT-GCCCTCGATAGAGCCTCTTGGCACCAGC-3', 5'-GGCACATCCAT-CACGGCCTATAAGAGTGAG-3', 5'-CTCCGCTGACTCCCCCTCAC-TCTTATAGGC-3', 5'-GGGGAGTCAGCGGAGTCTTCTTCTCTACTC-3', 5'-CTAGCTAGACGAGCAGCAGCAGGAGCAGGAAGGATGAGT-AG GAAGAAGAA-3', 5'-ATCCTTCTGCTCCTGCTCGTCTCGTC-TAG-3', 5'-GGCTCGAGTATGTCTTACACGGGTT-GAAGTC-3', and 5'-GCCGGATCCATGAGGCCGGCGGA-CTTGCTG-3'.

The region coding the extracellular domain of human TSHR [amino acids 1 (methionine) to 412 (isoleucine)] was amplified using the High Fidelity *Taq* polymerase (Roche, Indianapolis, IN) and the following oligonucleotide primers: sense, 5'-GCCGGATCCATGAGGCCGGCG-GACTTGCTG-3'; antisense, 5'-GGCTCGAGTATGTCTTACACGGG-TTGAAGTC-3', and was cloned into the *Bam*HI and *Xho*I sites upstream of the GPI anchor sequence to give the plasmid pGPI-TSHR. The final construct was verified by sequencing using an ABI 310 system (PE Applied Biosystems, Foster City, CA). Plasmid pGPI-TSHR was transfected into CHO-K1 cells using Tfx-50 (Promega Corp., Madison, WI) according to the manufacturer's instructions, and stable lines were selected using G418 at a concentration of 400 μ g/ml. Clonal lines were obtained and screened by FACS using TSHR monoclonal antibody 2C11 (Serotec, Oxford, UK).

The expression of TSHR ECD on the GPI cell surface was demonstrated by flow cytometry using monoclonal antibodies A10 (1:50 dilution) (24), BA8 (1:10 dilution) (25), and 3G4 (1:50 dilution) (25), used as culture medium, and 2 μ g/tube purified 2C11 (Serotec) to the human TSHR, followed by an antimouse IgG-fluorescein isothiocyanate (Ig-FITC) conjugate (1:32; DAKO Corp., Carpinteria, CA), as described in detail below.

TSH binding studies were performed on 1.5×10^5 cells in 12-well plates to estimate the numbers of receptors expressed at the surface of the GPI cells and were compared with JP09 (and the control JP02, CHO

TABLE 1. 'Atypical' TED patient details: summary of flow cytometry results

No./sex/age			TSAB/TBII/TPO			Treatment/diagnosis	IgG	IgA	IgE
Euthyroid TED									
P 1	F	53	-ve	-ve	+ve	TED confirmed in all 3 cases by C.T. and/or M.R.I. scan	-ve	-ve	-ve
P 2	M	62	-ve	-ve	-ve		-ve	-ve	-ve
P 3	F	67	-ve	-ve	-ve		+ve	+ve	-ve
GD with TED TSAB and TBII negative									
P 4	F	37	-ve	-ve	-ve	Previous MMI	+ve	-ve	-ve
P 5	F	46	-ve	-ve	+ve	Previous PTU	+ve	+ve	-ve
P 6	F	60	-ve	-ve	-ve	Previous MMI	-ve	+ve	-ve
P 7	F	47	-ve	-ve	-ve	Radio-iodine	-ve	-ve	-ve
P 8	F	32	-ve	-ve	+ve	Untreated	+ve	+ve	-ve
GD with TED TSAB positive									
GD 1	F	60	+ve	+ve	+ve	Previous MMI	+ve	+ve	+ve
GD 14	F	62	+ve	-ve	+ve	Untreated	-ve	-ve	-ve
GD 15	F	49	+ve	-ve	+ve	Untreated	-ve	-ve	-ve
Normal subject									
N6	F	52	-ve	-ve	N.D.		+ve	+ve	N.D.

C.T., Computerized tomographic; M.R.I., magnetic resonance imaging; MMI, methimazole; PTU, propylthiouracil; N.D., not determined. TSAB and TBII were measured on CHO cell lines stably expressing the human TSHR, *lulu*, or NA-4 for TSAB (21, 22) and JP09 for TBII (23), as described previously, and TPO antibodies using a Roche Immunodiagnosics enzyme test, values greater than 32 kU/liter being positive.

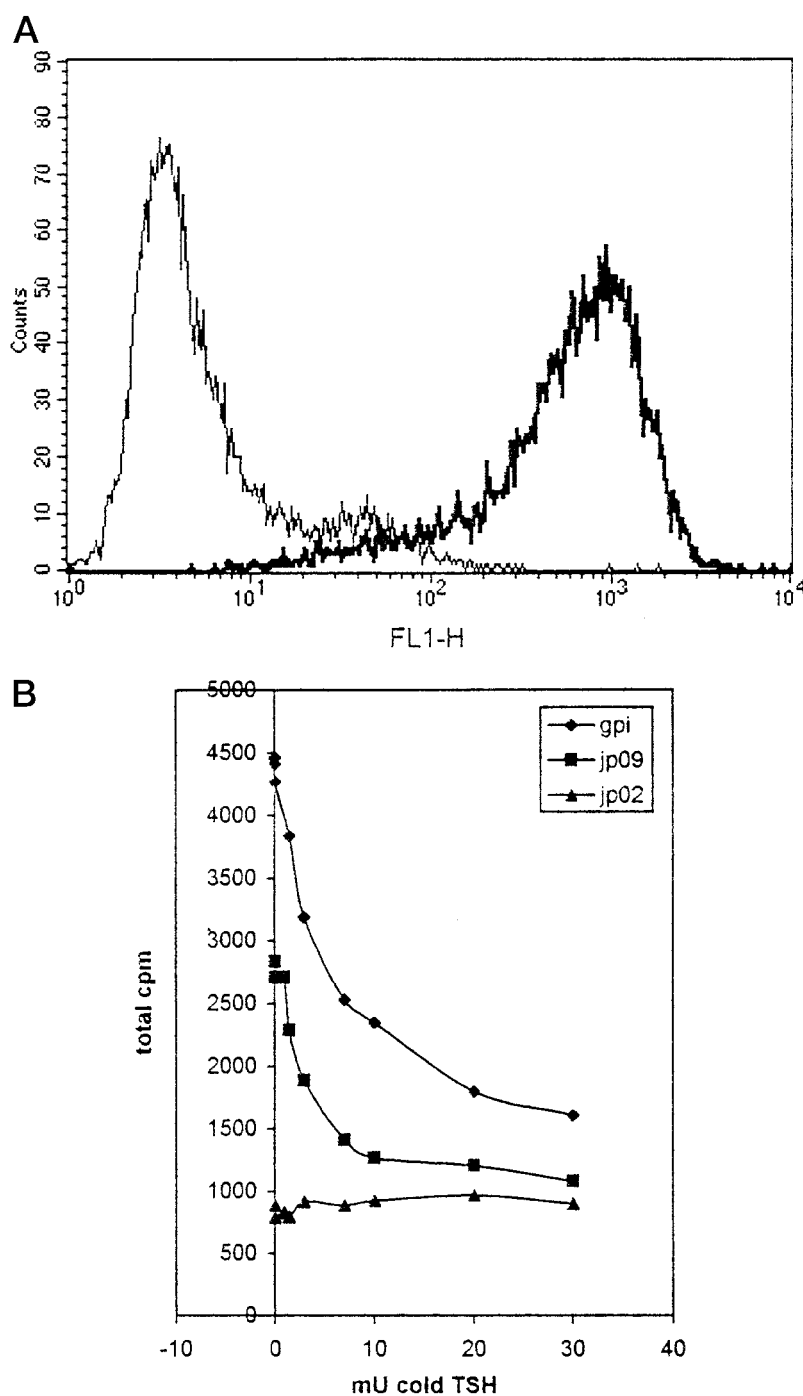


FIG. 1. Characterization of the GPI cell line. A, Typical profile of TSHR staining at the surface of GPI. The *faint trace* shows the fluorescence intensity using an isotype control monoclonal, and the *bold trace* shows the fluorescence intensity with TSHR monoclonal 2C11. The D value is 0.93. B, TSH binding to TSHR expressing CHO GPI and JP09 and the control JP02 cell lines. Binding was performed with 40,000 counts/well (JP09 and JP02) or 80,000 counts/well (GPI). The *x*-axis shows the concentration of added cold TSH in milliunits per ml, and the *y*-axis shows the total counts bound (mean of duplicates agreeing to within 10%).

cells expressing the neomycin resistance plasmid but no TSHR). Binding was performed in binding buffer comprised of NaCl-free Hanks' solution, 280 mM sucrose, and 0.2% BSA. Initially, saturation curves, using increasing volumes of [^{125} I]TSH (Brahms Diagnostica, Berlin, Germany) from 50–500 μ l/well and an equal volume of binding buffer, were carried out. Subsequently, the optimized volume of tracer was competed for by increasing concentrations of cold bovine TSH (Sigma, St. Louis, MO) for 2 h at room temperature. Cells were washed twice with the binding buffer and lysed with 0.5 ml 1 N NaOH/well, and bound radioactivity was determined in a γ -counter. All measurements were made in duplicate and performed at least three times to calculate average EC_{50} and binding capacity values from Scatchard analysis, reported as milliunits per ml TSH.

Flow cytometry

Seventy to 90% confluent cells (GPI and JP02) were detached from 75-cm ^{2} culture dishes using 5 ml 5 mM EDTA and 5 mM EGTA in PBS. The cells were washed three times in PBS containing 0.1% BSA and adjusted to 2×10^6 cells/ml in the same buffer. Aliquots (100 μ l) of cells were incubated with 2 μ l heat-inactivated test serum from the various patient and control groups for 1 h at room temperature. After three washes in PBS-BSA, they were incubated on ice in the dark for 30 min with antihuman IgA-FITC conjugate (1:32, from Sigma), antihuman IgE (1:50, from Sigma; 1:50, from Serotec) or antihuman IgG (1:50, from Sigma). They received an additional three washes in PBS-BSA and were resuspended in 1 ml of the same buffer, but containing propidium iodide

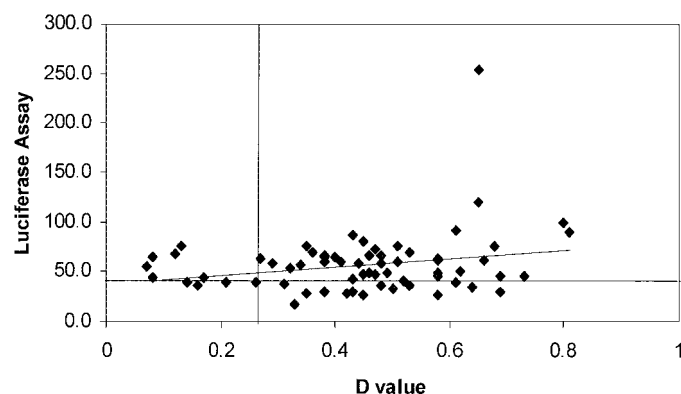


FIG. 2. TSHR autoantibodies in 65 patients with untreated GD. TSAB were assessed by luminescent bioassay, and results are reported as a percentage of a TSAB standard on the x-axis. Antibodies (IgG) binding directly to the TSHR were measured by flow cytometry on GPI cells, and results are reported as a D value on the y-axis. There is a poor correlation ($r = 0.22$) between the two methods. The dotted lines depict the normal cut-off (TSAB, $>40\%$; $D = >0.28$) for TSAB and direct TSHR binding, respectively.

for gating out dead cells. Cells were also analyzed in the same protocol, but omitting the first antibody (either TSHR monoclonal or patients' serum) to control for nonspecific binding of the mouse and human FITC conjugates. In addition, 12 of the samples positive for IgG binding to GPI were analyzed further by replacing the antihuman IgG-FITC with either an antihuman κ or an antihuman λ light chain-FITC.

Flow cytometric analysis was performed on a FACS Vantage from Becton Dickinson and Co. (Mountain View, CA), incorporating a Coherent Enterprise II laser emitting at 488 nm. Forward light scatter, 90° light scatter, and fluorescence emissions were collected for 1×10^4 cells, and the geometric mean fluorescence intensity values of GPI and JP02 were compared for all sera, including the normal samples. In addition, the Kolmogorov-Smirnov (K-S) two-sample test, which gives the greatest difference between the two histograms (GPI and JP02) and is quoted as the D value, was used (26), and cut-off values were defined based on the mean ± 2 SD of the normal sera.

Results

Characterization of the GPI cell line

Surface expression of TSHR ECD was confirmed by flow cytometry. Background staining with an isotype-matched control antibody showed a median fluorescence of 4.03 and peak channel fluorescence of 3 compared with 667 and 889 when using the 2C11 monoclonal ($D = 0.93$). Similar results were obtained with the other three TSHR monoclonal antibodies tested, which together recognize epitopes at the extreme N- and C-termini of the TSHR ECD and the native conformation of the human TSHR. A typical experiment is shown in Fig. 1A.

Saturation curves for [^{125} I]TSH binding demonstrated that JP09 reached saturation with 150 μ l (40,000 cpm) tracer, but GPI required 300 μ l (80,000 cpm) to achieve this, the first indication of the greater surface receptor expression in the latter cells (data not shown). TSH binding curves are shown in Fig. 1B. The affinity of the ECD TSHR expressed in the GPI cells is similar to that of the full-length TSHR in JP09, with EC_{50} values of 4.3 (range, 2–6) and 3.4 (range, 3–5) mU/ml TSH, respectively. Scatchard analysis of the GPI and JP09 cell lines produced B_{max} values of 76 and 19 mU/ml TSH respectively, indicating approximately 4 times as many receptors expressed at the surface of GPI compared with JP09.

TABLE 2. Lambda light chain predominance in direct binding IgGs to the TSHR

GD sample	'D' value kappa	'D' value lambda
GD 2	0.51	0.81
GD 3	0.26	0.65
GD 4	0.44	0.60
GD 5	0.55	0.39
GD 6	0.55	0.53
GD 7	0.57	0.83
GD 8	0.55	0.46
GD 9	0.46	0.77
GD 10	0.42	0.55
GD 11	0.53	0.74
GD 12	0.69	0.67
GD 13	0.49	0.74

Based on the mean ± 2 SD of the group as a whole, D values for kappa <0.3 and for lambda <0.37 were considered negative. On this basis, patient 3 displays complete lambda restriction because the kappa value is negative.

Flow cytometry

Each patient or control serum was assayed against both the JP02 and GPI cell lines, because of variable reactivity of human sera with the control receptor negative cell line, *e.g.* when detecting IgG binding the geometric mean ranged from 14–92. The distributions of fluorescence and background fluorescence were similar for the receptor-negative and -positive populations.

Based on the mean ± 2 SD of the normal control serum, a D value of more than 0.28 was considered positive, and 55 of 65 untreated GD sera displayed specific positive binding to the GPI cell population. As shown in Fig. 2, there was a poor correlation ($r = 0.22$) between autoantibodies binding directly to the TSHR and TSAB measured in a luminescent bioassay.

Twelve of the sera positive on GPI were tested for κ or λ light chain restriction in their TSHR binding antibodies. From the mean ± 2 SD of the 12 sera and the difference in D value between κ and λ , 1 sample displayed complete λ restriction, and 7 of the other 11 displayed more binding associated with λ than κ light chains, as summarized in Table 2.

Positive specific IgG binding to the ECD TSHR of the GPI cells was also observed in 3 of 25 normal and 4 of 8 of the TSAB/TBII-negative TED sera. Representative histograms are shown in Fig. 3.

IgA binding to the control receptor-negative cells had a range of geometric mean from 48–112. A D value greater than 0.35 was taken as the cut-off, and positive IgA receptor autoantibodies were demonstrated in 1 of 12 normal sera, 1 of 7 GD with TED, and 3 of 8 of the TSAB/TBII-negative TED sera. Representative results are shown in Fig. 4.

Circulating IgE levels are considerably lower than those for IgG and IgA, making the production of specific antisera more difficult. We applied two different IgE-FITC conjugates, both polyclonal antibodies raised in goat that produced cut-offs for the D values of 0.41 (Sigma) and 0.25 (Serotec), respectively. Positive IgE binding was demonstrated, using both IgE-FITC conjugates, in 1 of 12 normal sera (28-yr-old male), 2 of 6 GD without TED, 1 of 8 GD with TED, and 0 of 8 atypical TED sera. Representative results are shown in Fig. 5.

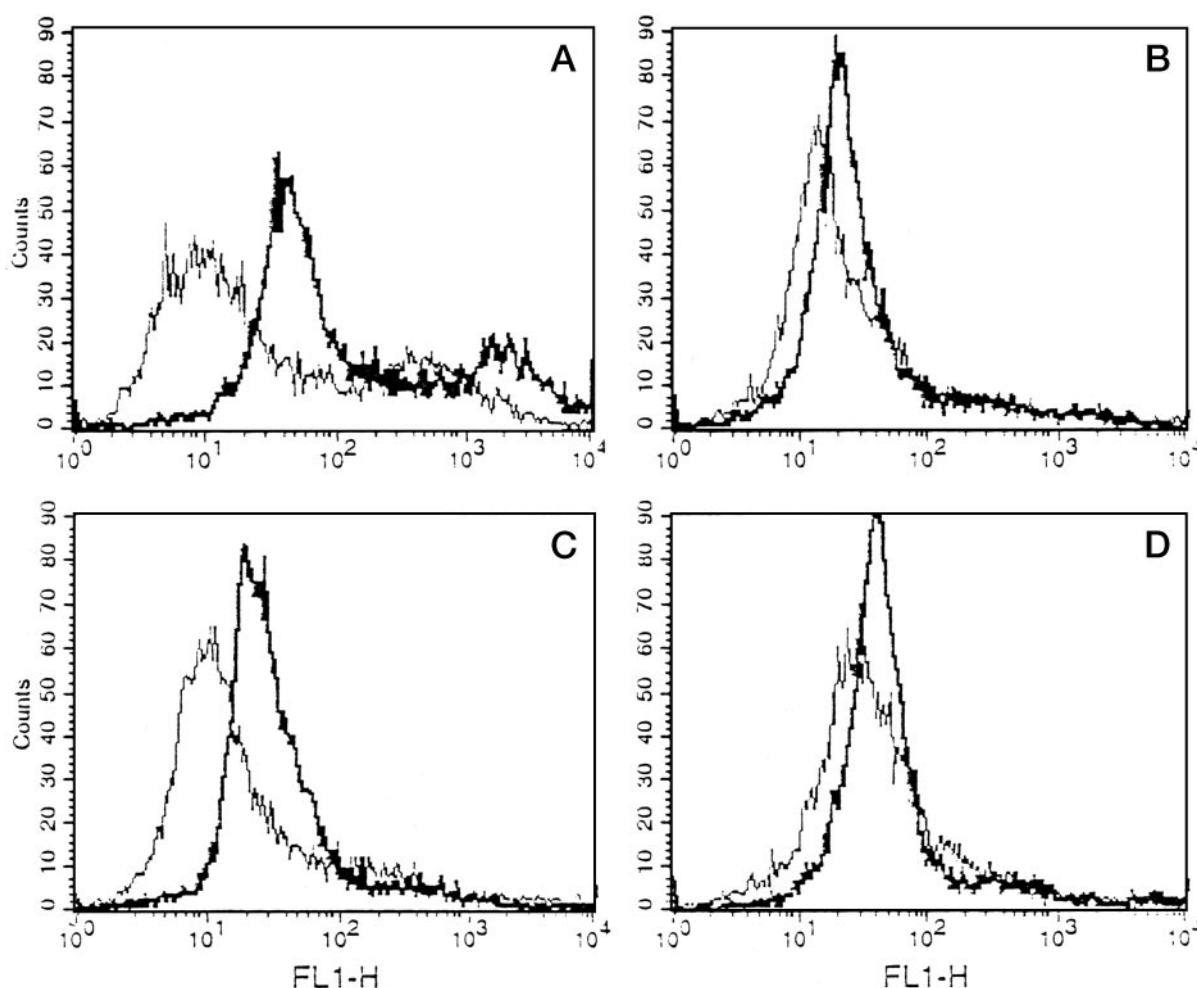


FIG. 3. IgG autoantibodies binding directly to the TSHR. Flow cytometry with fluorescence intensity shown on the x-axis and cell counts on the y-axis. The **bold trace** was obtained from GPI cells, and the *faint trace* was obtained from JP02 receptor-negative control cells. A, Atypical TED P4 ($D = 0.5$); B, atypical TED P1 ($D = 0.2$); C, normal 5 ($D = 0.44$); D, normal 7 ($D = 0.19$). A D value more than 0.28 is considered positive.

Flow results for IgG, IgA, and IgE binding, showing the presence of receptor antibodies of more than one isotype in some individuals, are summarized in Table 1.

Discussion

We have produced a CHO cell line stably expressing the ECD of the TSHR linked to the cell surface via a GPI anchor. The characteristics of the GPI line are similar to those in two earlier reports (7, 8) and indicate that the ECD has an affinity for TSH comparable to that of the full-length TSHR and is sufficient to detect binding of TSHR autoantibodies.

We have demonstrated IgG antibodies binding directly to the receptor in the majority of untreated patients with GD, in agreement with some (8, 10), but not all (9), previous studies that have applied flow cytometric analysis. We found a poor correlation between these direct binding antibodies and TSAB, as reported by others (10). Our data lend further support to the concept that TSAB comprise a small proportion of TSHR autoantibodies. The results also indicate that TSHR autoantibodies in general display a preference for the IgG λ isotype and that it is not a feature confined to TSAB (1).

The presence of TSHR autoantibodies in GD is not sur-

prising, but we have also obtained positive binding of IgG and/or IgA antibodies to GPI cells in 50% of patients with atypical TED and a small proportion of normal euthyroid controls, all of them women. Binding of IgE antibodies to the GPI cells, apart from 3 of 14 GD sera, was detected only in one 28-yr-old normal male subject and in none of the TSAB/TBII-negative TED patients.

The etiopathogenesis of TED remains a puzzle to endocrinologists, although most of the signs and symptoms can be attributed to an increase in orbital volume as the consequence of glycosaminoglycans production, edema, and fat hypertrophy. A variety of immune cells and cytokines are present in TED orbits, and TSHR-specific T cell lines have been reported (27). Several additional lines of evidence favor the TSHR as a common antigen in GD and TED, as described above. The present study has demonstrated immunoreactivity to the TSHR in a subset of patients with TED previously assumed to be free of receptor autoimmunity. Their euthyroid state is explained by the neutral nature of their TSHR autoantibodies, which lack TSAB or TBII activity. Given the size of an Ig, it is perhaps surprising that an antibody that is

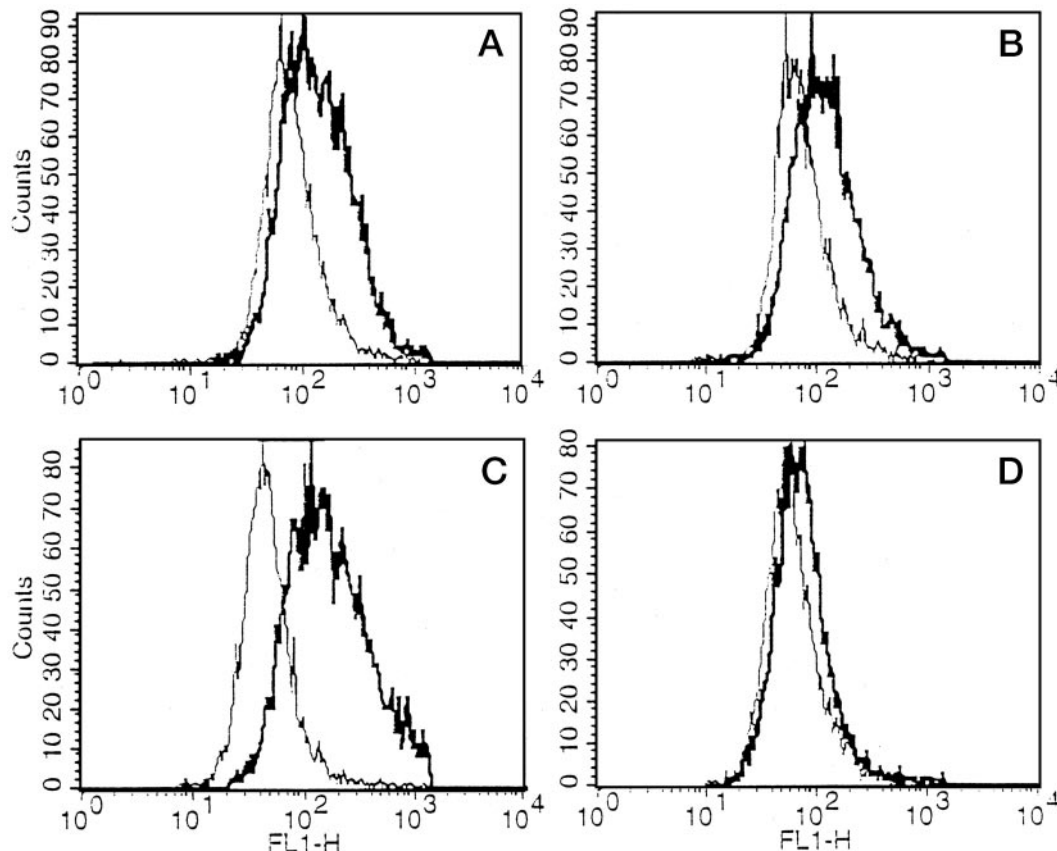


FIG. 4. IgA autoantibodies binding directly to the TSHR. Flow cytometry with fluorescence intensity shown on the x-axis and cell counts on the y-axis. The **bold trace** was obtained from GPI cells, and the **faint trace** was obtained from JP02 receptor-negative control cells. A, GD with TED GD1 ($D = 0.38$); B, atypical TED P8 ($D = 0.35$); C, normal 6 ($D = 0.72$); D, normal 7 ($D = 0.15$). A D value more than 0.35 is considered positive.

able to bind the receptor does not inhibit TSH binding, although this has been reported previously (4), as have TSAB that lack TBII activity (11). Measurement of direct binding TSHR antibodies could be applied to confirm the diagnosis if TED is suspected in an individual negative for TSAB and TBII.

There are a few reports indicating TSHR autoreactivity in normal healthy subjects, including the demonstration of receptor-specific T cell lines (28) and peripheral blood mononuclear cells (29). More recently Atger and colleagues (3) applied an ELISA, using a solubilized TSHR preparation to coat the plates, and found neutral antibodies in a surprisingly high proportion of euthyroid controls. In our series of normal subjects, TSHR antibodies were confined to three women, aged 46–52 yr, one of whom displayed both IgG and IgA reactivity.

The idea of autoantibodies in healthy subjects is not novel, and the other major thyroid autoantigens, Tg and TPO, are associated with incidences of approximately 15% and 10%, respectively; they are highest among middle-aged women (2). It seems that the TSHR is not so different, although studies using a larger panel of euthyroid male and female subjects spanning all age ranges are required to determine the prevalence of TSHR autoreactivity in the absence of disease.

GD is very common, and our data imply that immunoreactivity to the target antigen may also be common. Is its immunogenicity due to molecular mimicry of the TSHR by structures on the surface of commensal bacteria, or does receptor processing release/unmask an immunodominant epitope? Immunodominant epitopes have been implicated in a variety of autoimmune diseases, and indeed, there is a suggestion that the development of thyroiditis requires removal of immunodominant regions in a receptor-induced animal model (30).

We were able to detect IgE immunoreactivity to the receptor by flow cytometry in patients with GD and TED. Interest in this arm of the immune response has been kindled by an animal model of TED induced by passive transfer of TSHR-primed T cells, which one of us has reported (16). In this model, mast cell infiltration was one of the earliest indicators of orbital pathology. Development of TSHR autoantibodies of the IgE subclass by isotype switching would provide a neat explanation for the severe TED found in some, but not all, GD patients. Recently, elevated IgE levels have been reported in GD patients, although whether the antibodies were directed to the TSHR was not investigated (20), although IgE antibodies to another major thyroid autoantigen, TPO, have been reported (31). Further studies, using a larger series of

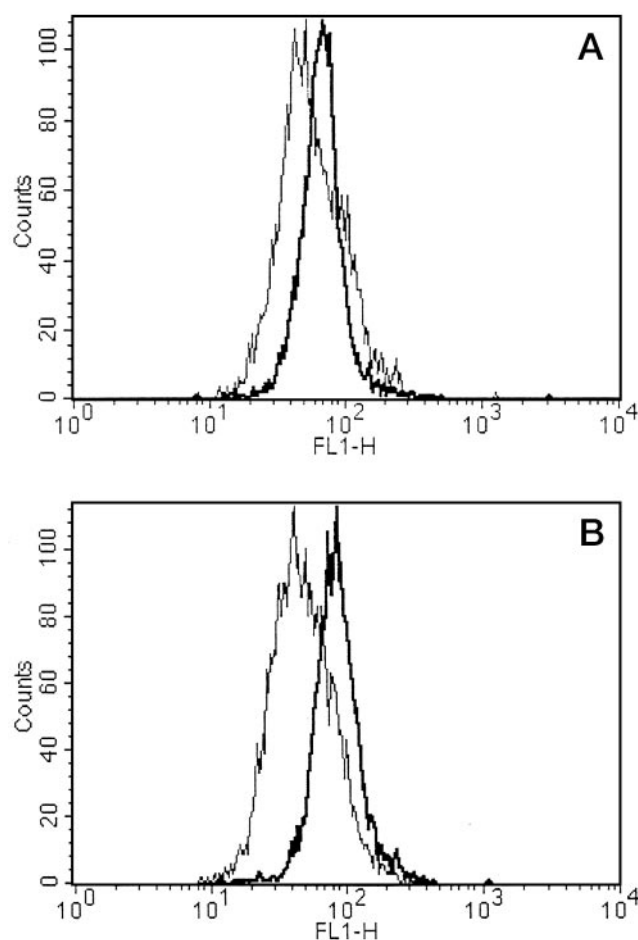


FIG. 5. IgE autoantibodies binding directly to the TSHR. Flow cytometry with fluorescence intensity shown on the *x*-axis and cell counts on the *y*-axis. The *bold trace* was obtained from GPI cells, and the *faint trace* was obtained from JP02 receptor-negative control cells. A, normal 15 ($D = 0.29$); B, GD with TED GD1 ($D = 0.56$). A D value more than 0.41 is considered positive.

patients, particularly GD patients before the onset of TED, are warranted to resolve the issue of IgE antibodies to the receptor.

In conclusion, we report neutral IgG antibodies, predominantly of the λ class, in the majority of patients with untreated GD and in a small proportion of middle-aged euthyroid female controls. We have clearly demonstrated IgG and/or IgA antibodies recognizing receptor conformation in a high proportion of patients with TED who are negative for TSHR autoantibodies with biological activity measured as TSAB or TBII.

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References

1. Rees Smith B, McLachlan S, Furmaniak J 1998 Autoantibodies to the thyrotropin receptor. *Endocr Rev* 9:106–120
2. Weetman AP, McGregor AM 1994 Autoimmune thyroid disease; further developments in our understanding. *Endocr Rev* 15:788–830
3. Atger M, Misrahi M, Young J, Jolivet A, Orgiazzi J, Schaison G, Milgrom E 1999 Autoantibodies interacting with purified native thyrotropin receptor. *Eur J Biochem* 265:1022–1031
4. Morgenthaler NG, Hodak K, Seissler J, Steinbrenner H, Pampel I, Gupta M, McGregor AM, Scherbaum WA, Banga JP 1999 Direct binding of thyrotropin receptor autoantibody to in vitro translated thyrotropin receptor: a comparison to radioreceptor assay and thyroid stimulating bioassay. *Thyroid* 9:466–475
5. Prentice L, Sanders JF, Perez M, Kato R, Sawicka J, Oda Y, Jaskolski D, Furmaniak J, Smith BR 1997 Thyrotropin (TSH) receptor autoantibodies do not appear to bind to the TSH receptor produced in an *in vitro* transcription/translation system. *J Clin Endocrinol Metab* 82:1288–1292
6. Bobnikova Y, Graves PN, Vlase H, Davies TF 1997 Characterization of soluble di-sulphide bond stabilized, procaryotically expressed human thyrotropin receptor ectodomain. *Endocrinology* 138:588–593
7. DaCosta CR, Johnstone AP 1998 Production of the thyrotropin receptor extracellular domain as a glycosylphosphatidylinositol-anchored membrane protein and its interaction with thyrotropin and autoantibodies. *J Biol Chem* 273:11874–11880
8. Costagliola S, Khoo D, Vassart G 1998 Production of bioactive amino-terminal domain of the thyrotropin receptor via insertion in the plasma membrane by a glycosylphosphatidylinositol anchor. *FEBS Lett* 436:427–433
9. Jaume JC, Kakinuma A, Chazenbalk GD, Rapoport B, McLachlan SM 1997 Thyrotropin receptor autoantibodies in serum are present at much lower levels than thyroid peroxidase autoantibodies: analysis by flow cytometry. *J Clin Endocrinol Metab* 82:500–507
10. Patibandla SA, Dallas JS, Seetharamaiah GS, Tahara K, Kohn LD, Prabhakar BS 1997 Flow cytometric analysis of antibody binding to chinese hamster ovary cells expressing human thyrotropin receptor. *J Clin Endocrinol Metab* 82:1885–1893
11. Tonacchera M, Costagliola S, Cetani F, Ducobu J, Stordeur P, Vassart G, Ludgate M 1996 Patient with monoclonal gammopathy, thyrotoxicosis, pretibial myxedema and thyroid-associated ophthalmopathy: demonstration of direct binding of autoantibodies to the thyrotropin receptor. *Eur J Endocrinol* 134:97–103
12. Perros F, Kendall-Taylor P 1995 Thyroid-associated ophthalmopathy: pathogenesis and clinical management. *Bailliere Clin Endocrinol Metab* 9:115–135
13. Crisp M, Lane C, Halliwell M, Wynford-Thomas D, Ludgate M 1997 Thyrotropin receptor transcripts in human adipose tissue. *J Clin Endocrinol Metab* 82:2003–2005
14. Bahn R, Dutton C, Natt N, Joba W, Spitzweg C, Heufelder A 1998 Thyrotropin receptor expression in Graves' orbital adipose/connective tissues; potential autoantigen in Graves' ophthalmopathy. *J Clin Endocrinol Metab* 83:998–1002
15. Crisp MS, Starkey KJ, Lane C, Ham J, Ludgate M 2000 Adipogenesis in thyroid eye disease. *Invest Ophthalmol Vis Sci* 41:3249–3255
16. Many MC, Costagliola S, Detrait M, Denef JF, Vassart G, Ludgate M 1999 Development of an animal model of autoimmune thyroid eye disease. *J Immunol* 162:4966–4974
17. Costagliola S, Many MC, Denef JF, Pohlenz J, Refetoff S, Vassart G 2000 Genetic immunization of outbred mice with thyrotropin receptor cDNA provides a model of Graves' disease. *J Clin Invest* 105:803–811
18. Arnold K, Metcalfe A, Weetman AP 1995 Immunoglobulin A class fibroblast antibodies in patients with Graves' disease and pretibial myxoedema. *J Clin Endocrinol Metab* 80:3430–3436
19. Khalil HA, Dekeizer RJW, Bodelier VMW, Kijlstra A 1989 Secretory IgA and lysozyme in tears of patients with Graves' ophthalmopathy. *Doc Ophthalmol* 72:329–334
20. Sato A, Takemura Y, Yamada T, Ohtsuka H, Sakai H, Miyahara Y, Aizawa T, Terao A, Onuma S, Junen K, Kanamori A, Nakamura Y, Tejima E, Ito Y, Kamijo K 1999 Possible role of immunoglobulin E in patients with hyperthyroid Graves' disease. *J Clin Endocrinol Metab* 84:3602–3605
21. Evans C, Morgenthaler N, Lee S, Llewellyn DH, Clifton-Bligh R, John R, Lazarus JH, Chatterjee VK, Ludgate M 1999 Development of a luminescent bioassay for thyroid stimulating antibodies. *J Clin Endocrinol Metab* 84:374–377
22. Watson PF, Ajjan RA, Phipps J, Metcalfe R, Weetman AP 1998 A new chemiluminescent assay for the rapid detection of thyroid stimulating antibodies in Graves' disease. *Clin Endocrinol (Oxf)* 49:577–581
23. Costagliola S, Swillens S, Niccoli P, Dumont J, Vassart G, Ludgate M 1992 Binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. *J Clin Endocrinol Metab* 75:1540–1544

24. **Nicholson L, Vlase H, Graves P, Nilsson M, Molne J, Huang GC, Morgenthaler NG, Davies TF, McGregor AM, Banga JP** 1996 Monoclonal antibodies to the human TSH receptor: epitope mapping and binding to the native receptor on the baso-lateral plasma membrane of thyroid follicular cells. *J Mol Endocrinol* 16:159–170
25. **Costagliola S, Rodien P, Many MC, Ludgate M, Vassart G** 1998 Genetic Immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. *J Immunol* 160:1458–1465
26. **Young IT** 1977 Proof without prejudice: use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. *J Histochem Cytochem* 25:935–941
27. **Pappa A, Calder V, Ajjan R, Fells P, Ludgate M, Weetman AP, Lightman S** 1997 Analysis of extraocular muscle-infiltrating T cells in thyroid-associated ophthalmopathy. *Clin Exp Immunol* 109:362–369
28. **Molteni M, Zulian C, Scrofani S, Della Bella S, Bonara P, Kohn LD, Scorza R** 1998 High frequency of T cell lines responsive to immunodominant epitopes of thyrotropin receptor in healthy subjects. *Thyroid* 8:241–247
29. **Tandon N, Freeman M, Weetman AP** 1992 T cell responses to synthetic TSH receptor peptides in Graves' disease. *Clin Exp Immunol* 89:468–473
30. **Wang SH, Carayanniotis G, Zhang Y, Gupta M, McGregor AM, Banga JP** 1998 Induction of thyroiditis in mice with thyrotropin receptor lacking serologically dominant regions. *Clin Exp Immunol* 113:119–125
31. **Guo J, Rapoport B, McLachlan SM** 1997 Thyroperoxidase autoantibodies of IgE class in thyroid autoimmunity. *Clin Exp Immunol* 82:157–162

FIRST WORLD CONGRESS: HORMONAL AND GENETIC BASIS OF SEXUAL DIFFERENTIATION DISORDERS

May 17–18, 2002
Tempe, Arizona

Participants: Maria I. New (Chairman), New York, NY; Jean Wilson (Chairman), Dallas, TX; Richard Behringer, Houston, TX; Camerino Giovanna, Pavia, Italy; Cheryl Chase, San Francisco, CA; Patricia Donahoe, Boston, MA; Maguelone Forest, Lyon, France; James Griffin, Dallas, TX; Melvin Grumbach, San Francisco, CA; Nathalie Josso, Montrouge, France; Ursula Kuhnle-Krahl, Munich, Germany; Robin Lovell-Badge, London, UK; Berenice Mendonca, Sao Paulo, Brazil; Heino Meyer-Bahlburg, New York, NY; Claude Migeon, Baltimore, MD; Dix Poppas, New York, NY; Martin Ritzen, Stockholm, Sweden; Jacques Simard, Quebec, Canada; Garry Warne, Victoria, Australia; Amy Wisniewsky, Baltimore, MD.

The Conference lectures will include: Genetics of Sex, Anti-Müllerian Hormone Mutations, Animal Models of Intersex, Normal Sexual Differentiation, Ambiguous Genitalia in Humans, Hormones and Sexual Behavior, Rationale for Gender Assignment, Legal Aspects of Gender Assignment, Ethical Issues in Gender Assignment, Role of Advocacy Groups, Prenatal Therapy in Congenital Adrenal Hyperplasia, Surgical Reconstruction in Patients with CAH and Ambiguous Genitalia, Medical Management at Puberty, Questions Frequently Asked by Patients and Their Families, Support Groups for CAH and Androgen Insensitivity Syndrome, Outcome Studies in CAH, Choosing Gender in the Indian Cultural Context, Partial Androgen Insensitivity Syndrome and Partial Gonadal Dysgenesis, Complete AIS and Congenital Micropenis, Males with 17 β -Hydroxysteroid Dehydrogenase Deficiency, 5 α -Reductase Deficiency, True Hermaphrodites.

For Congress registration and scientific information, please contact: Maria New, M.D., Professor and Chairman, Department of Pediatrics New York Presbyterian Hospital, Weill Cornell Medical College, 525 E. 68th Street, New York, NY 10021. E-mail: minew@med.cornell.edu; Phone: (212) 746-3450.

This conference was previously scheduled to occur in Gubbio, Italy, on September 12, 2001, as "Update on Androgen Disorders," but was postponed due to the September 11 disaster.