

Flow Cytometric Analyses of Antibody Binding to Chinese Hamster Ovary Cells Expressing Human Thyrotropin Receptor*

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ABSTRACT

To develop a method that can be used to directly detect binding of antibodies to TSH receptor (TSHr), we employed Chinese hamster ovary (CHO) cells permanently transfected with a human TSHr complementary DNA (CHOR). These cells showed increased cAMP production when treated with either human TSH or thyroid-stimulating antibodies and decreased TSH-mediated cAMP production when treated with stimulation-blocking antibodies. We employed flow cytometry and rabbit antibodies against the extracellular domain of the TSHr (ETSHr) to test whether these cells can be used to directly detect and quantitate the binding of anti-TSHr antibodies. Rabbit anti-ETSHr bound specifically to CHOR cells, and the binding could be blocked with purified ETSHr. To test the feasibility of using these cells for epitope mapping, we tested the binding of rabbit antibodies raised against several synthetic TSHr peptides. Rabbit anti-peptide 92

(amino acids 12–30) and 91 (amino acids 32–46) showed little or no binding to the CHOR cells. In contrast, antibodies raised against peptides 93 (amino acids 316–330), 95 (aa 325–345), 3A (aa 357–372), 367 (aa 367–386), and 1B (aa 362–376) showed significant binding to the CHOR cells. The specificity of binding of anti-peptide antibodies was demonstrated by a complete inhibition of binding by corresponding peptides. When TSH-binding inhibitory Ig-positive sera from 15 patients with hyperthyroidism were tested, 8 of them showed specific binding to the CHOR cells compared to their relative binding to normal CHO cells; sera from all normal individuals tested did not exhibit specific binding to CHOR cells. These studies showed the usefulness of CHOR cells and flow cytometry in epitope mapping using sera with known specificities and the potential usefulness of the technique to detect anti-TSHr antibodies in patient sera. (*J Clin Endocrinol Metab* 82: 1885–1893, 1997)

AUTOANTIBODIES to the TSH receptor (TSHr) have been implicated in the pathogenesis of several autoimmune thyroid disorders. Antibodies, due to different affinities or binding specificities, mediate diverse functional effects on the thyroid, leading to different clinical syndromes (1, 2). Previous studies by us and others have identified several epitopes on the TSHr with which stimulatory and blocking TSHr antibodies interact (2–15). Some of these studies suggested that TSH and stimulatory antibodies interact with conformational epitopes; other studies have indicated that some of the stimulatory and blocking antibodies might react with linear epitopes because they either interact or can be induced by immunization with some peptides (2, 4, 8, 13–15).

A RRA is routinely used to detect TSH binding inhibitory Igs (TBII) in which the ability of serum antibodies to displace TSH is measured (16). Bioassays that are generally used to detect thyroid-stimulating antibodies (stimulating TSHrAbs or TSAs) and thyroid stimulation-blocking antibodies (blocking TSHrAbs) rely on the ability of sera to stimulate or

block TSH-mediated cAMP production by FRTL5 cells, respectively (1, 2, 4, 17, 18). Results obtained using these assays are highly reliable and of diagnostic value.

More recently, to understand the structure/function relationship of TSHr, a number of laboratories have carried out transfection studies with various mutants or LH/CG receptor chimeras of human TSHr (2, 9–12, 19, 20). These cell lines were tested for their ability to bind [¹²⁵I]TSH or produce cAMP in response to TSH or autoantibodies. This has allowed generation of significant new information concerning the structure of TSHr and epitopes of TSHr autoantibodies. However, in none of these studies was direct binding of autoantibodies measured due to the unavailability of an appropriate assay (17, 18). The availability of an assay to detect antibody binding to native TSHr could expand our ability to interpret results from functional and [¹²⁵I]TSH binding inhibition assays. Therefore, in this study we employed Chinese hamster ovary (CHO) cells permanently transfected with a human TSHr (CHOR) and flow cytometry to detect the binding of antibodies to TSHr in sera from patients and rabbits, immunized with either extracellular domain of the TSHr (ETSHr) or peptides derived from ETSHr.

Materials and Methods

Cell culture

Stable expression of full-length human TSHr in CHO cells was previously described (9, 12). CHOR cells and untransfected CHO (CHON)

Received November 1, 1996. Revision received February 20, 1997. Accepted March 10, 1997.

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* This work was supported in part by the James W. McLaughlin Fellowship Fund (to S.A.P.) and NIH Grants DK-47417 and DK-44972 (to B.S.P.).

cells were routinely grown in 75-cm² culture flasks in Ham's F-12 medium supplemented with antibiotics and 10% FCS and maintained with 5% CO₂ in an incubator at 37 C. The transfected cells (CHOR) were maintained by adding 400 µg/mL geneticin to the medium.

cAMP assay

The effect of TSH on CHOR cells was evaluated by measuring the ability of TSH to elevate cAMP levels in the cells using a modification of previously described methods (9, 12). CHOR cells were seeded in 96-well plates and grown to confluency. The cells were washed twice with Hanks' Balanced Salt Solution without NaCl, which contained 10 mmol/L HEPES, 0.4% BSA, and 220 mmol/L sucrose (hypotonic HBSS; pH 7.4). The hypotonic HBSS containing 0.5 mmol/L isobutylmethylxanthine (IBMX) and human TSH (10⁻⁹-10⁻¹¹ mol/L; NIDDK hTSH-1-7; AFP-8644P; National Hormone and Pituitary Program, NIDDK, Baltimore, MD) was added to triplicate wells and incubated for 3 h at 37 C. Supernatants were collected from individual wells and assayed in duplicate for cAMP using a commercially available kit (DuPont, Boston, MA).

To test for stimulatory effects, human sera (1:8) were added individually to triplicate wells in hypotonic HBSS containing 0.5 mmol/L IBMX and incubated at 37 C in 5% CO₂ for 3 h. To test for inhibitory effects, rabbit antipeptide 3A and 95 IgG were added individually to triplicate wells in hypotonic HBSS. After incubation at 37 C for 30 min, HBSS containing 0.5 mmol/L IBMX along with human TSH were added to the wells and incubated for 3 h at 37 C. Supernatants were collected and assayed for cAMP.

Flow cytometry

Cells were dislodged from the culture flasks using No-zyme (JRH Biosciences, Lenexa, KS). The cells were washed twice with PBS containing 2% FBS and 0.02% azide (staining buffer). Flow cytometric analysis was used to detect binding of rabbit anti-ETSHr (T1 and T2) and antipeptide antibodies 92, 91, 93, 95, 3A, 367, and 1B. CHOR or CHON cells (1 × 10⁶) were incubated with 100 µL rabbit sera (diluted 1:100 or 1:200 in staining buffer) for 30 min at 4 C. Cells were washed and incubated with 100 µL fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG (diluted 1:100 in staining buffer) for 30 min at 4 C. Subsequent to washing, the cells were fixed in 0.5% paraformaldehyde before analysis on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To test for specificity of binding, 100 µL diluted antisera were preincubated with either 100 pmol ETSHr or 5 nmol of the respective peptide for 30 min at room temperature and then used to stain CHOR cells for fluorescence-activated cell sorter (FACS) analysis, as described above.

To detect binding of human sera, 1 × 10⁶ CHOR or CHON cells were incubated with 100 µL human sera (diluted 1:20 in staining buffer) for 30 min at 4 C. The cells were washed three times with staining buffer and then incubated with 100 µL (1:100) biotinylated antihuman IgGγ for 30 min at 4 C, washed, and further incubated with 100 µL (1:100) FITC-avidin for 30 min at 4 C. After washing, the cells were fixed in 0.5% paraformaldehyde. To test for specificity of binding, 100 µL sera diluted 1:20 were preincubated with 2 × 10⁶ Sf9 cells

infected with the recombinant glycosylated ectodomain of ETSHr (ETSHr-gp) virus and then used for staining the CHOR cells.

Specific binding to CHOR cells detected by flow cytometry was calculated using CELLQuest (Becton Dickinson, San Jose, CA) to create histograms. For each serum sample the CHON histogram was subtracted from the CHOR histogram to create subtracted histogram (CHOR-CHON). Histogram statistics were used to express CHOR-CHON as a percentage of the CHOR histogram to calculate the percentage of positive cells (see Table 4). A value of 15% or less was considered to be negative for binding to CHOR cells.

Production of recombinant ETSHr and ETSHr-gp proteins

Recombinant ETSHr was produced using the baculovirus system as described previously (21). Briefly, the crude extracts obtained from recombinant virus-infected Sf9 cells were sequentially treated with lysis buffer, nuclease buffer, and high salt buffer. The protein was dissolved in Tris buffer (0.05 mol/L Tris, pH 7.5, and 0.1% SDS) and was subjected to SDS-PAGE under reducing conditions. Pieces of gel corresponding to a 50-kDa molecular mass were excised, and protein was eluted from gels using bicarbonate buffer (50 mmol/L ammonium bicarbonate and 0.1% SDS) and lyophilized. The lyophilized protein was extracted with cold 80% acetone. The supernatant containing SDS was discarded, and the pellet containing the ETSHr protein was resuspended in 0.05 mol/L Tris buffer. The ETSHr-gp was produced as described previously (22) and purified as described above for ETSHr protein.

Synthesis of peptides and preparation of rabbit antibodies to recombinant ETSHr and synthetic peptides

The synthetic peptides used in the study, derived from two regions of the ETSHr [amino acids (aa) 22-46 and 316-386], were peptides 92 (aa 12-30), 91 (aa 32-56), 93 (aa 316-330), 95 (aa 325-345), 3A (aa 357-372), 367 (aa 367-386), and 1B (aa 362-376). The methods for peptide preparation and generation of rabbit antibodies to ETSHr and synthetic

TABLE 2. cAMP response of CHOR cells to normal and Graves' sera

Dilution	Normal human sera cAMP (pmol/mL)		Graves' sera cAMP (pmol/mL)	
	1	2	21	22
1:2	11 ± 0.4	15 ± 2	111 ± 23	123 ± 12
1:4	8 ± 2	10 ± 1	65 ± 11	88 ± 20
1:8	7 ± 2	13 ± 3	60 ± 12	62 ± 8
1:16	10 ± 1	9 ± 3	46 ± 5	49 ± 8

cAMP production of CHOR cells in response to two normal human sera (1 and 2) and two Graves' patient sera (21 and 22) were tested using four different dilutions. Values represent mean of triplicate determinations with SD.

TABLE 3. Inhibition of TSH-mediated cAMP production in CHOR cells by rabbit anti-3A and -95 IgG

IgG conc. (µg/mL)	cAMP (pmol/mL)		
	Normal rabbit IgG	Anti-3A IgG	Anti-95 IgG
30	171	36	35
10	192	36	49
3	186	38	87
1	185	50	102
0.3	193	99	113
0.1	182	130	174

Blocking of TSH-mediated cAMP production in CHOR cells by normal rabbit IgG, rabbit anti-3A, and rabbit anti-95 IgG was tested using six concentrations of IgG in the presence of 10⁻⁹ mol/L TSH. Supernatants were pooled from triplicate wells and used to assay for cAMP. The experiment was repeated with very similar results.

TABLE 1. cAMP response in CHOR cells to TSH

Human TSH (M)	cAMP (pmol/mL)
None	13.3 ± 0.07
10 ⁻¹¹	46 ± 5
5 × 10 ⁻¹¹	59 ± 10
10 ⁻¹⁰	143 ± 0
5 × 10 ⁻¹⁰	274 ± 4
10 ⁻⁹	362 ± 21
5 × 10 ⁻⁹	503 ± 15

cAMP production in CHOR cells was tested in the presence of different concentrations of human TSH. cAMP was assayed in duplicate by a commercially available kit (DuPont, Boston, MA). Values represent the mean of triplicate determinations with SD. The experiment was repeated with very similar results.

peptides have been described previously (7, 8). Polyclonal rabbit antibodies raised against peptides 91, 92, 93, 3A, 367, and 1B are designated antibodies 91, 92, 93, 3A, 367, and 1B, respectively.

Patients

Sera were collected from patients with Graves' disease who were TBII positive and from healthy normal volunteers with no family history of thyroid autoimmunity. All sera were obtained according to institutional guidelines.

Results

Characterization of CHO cells transfected with a human TSHr complementary DNA

Table 1 shows the response of CHOR cells to stimulation by human TSH in a dose-dependent manner. These data

show that the TSHr is functional, and 10^{-10} mol/L TSH could stimulate cAMP production in these cells. Table 2 shows that Graves' sera, but not normal human sera, could stimulate significant amounts of cAMP production. These data showed that the cell line can be used to directly measure bioactivity in the sera without having to purify IgG. Table 3 shows the inhibition of TSH-mediated (10^{-9} mol/L) cAMP production by normal rabbit, rabbit anti-3A, and anti-95 IgGs. cAMP production in the presence of 10^{-9} mol/L TSH was 190 pmol/mL. Rabbit anti-peptide 3A and anti-peptide 95 inhibited TSH-mediated cAMP production in CHOR cells in a dose-dependent fashion when used at concentrations ranging from 0.1–30 μ g/mL. Together, the data from Tables 1–3 confirm that CHOR cells can be used to detect functional

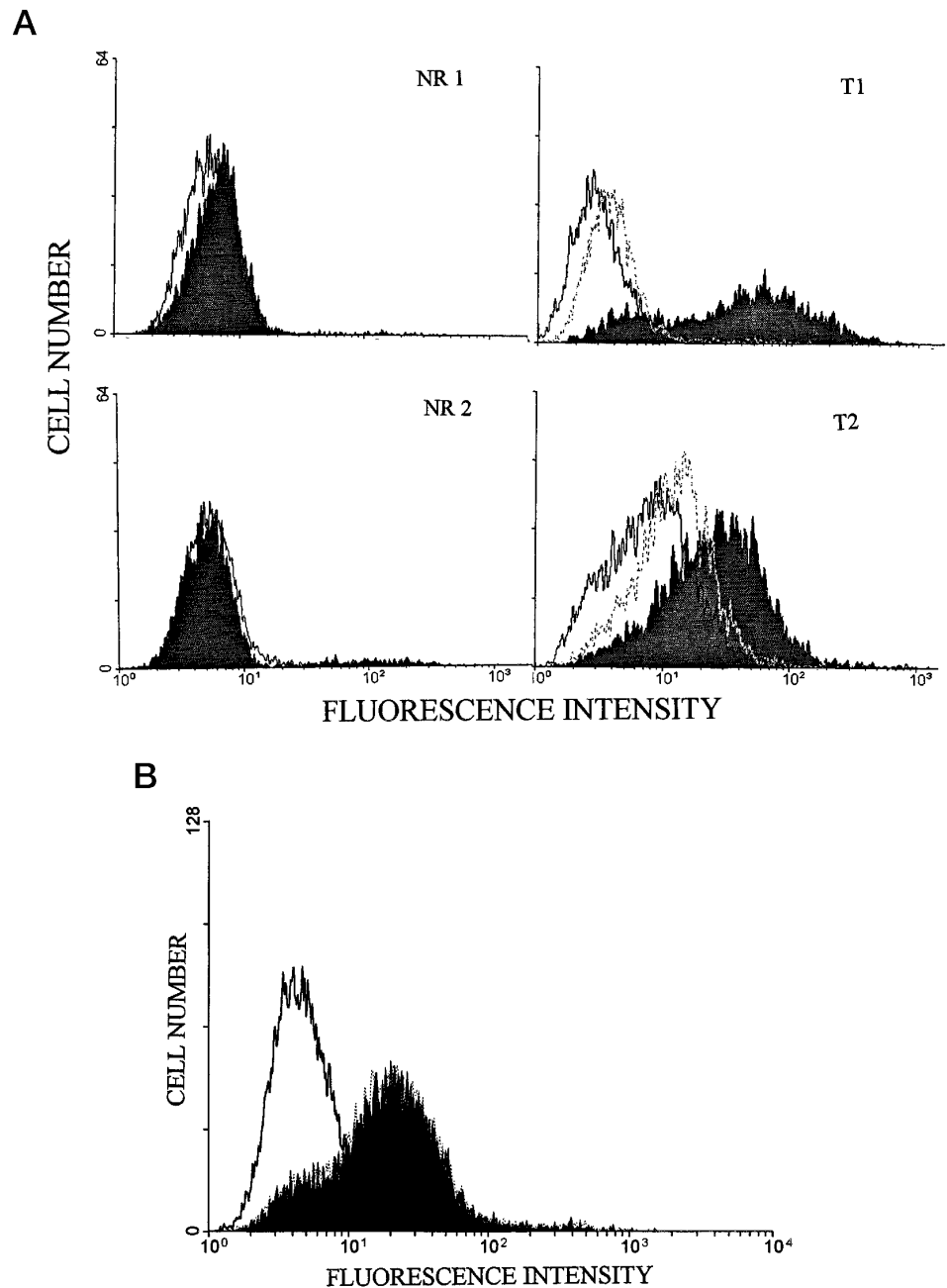
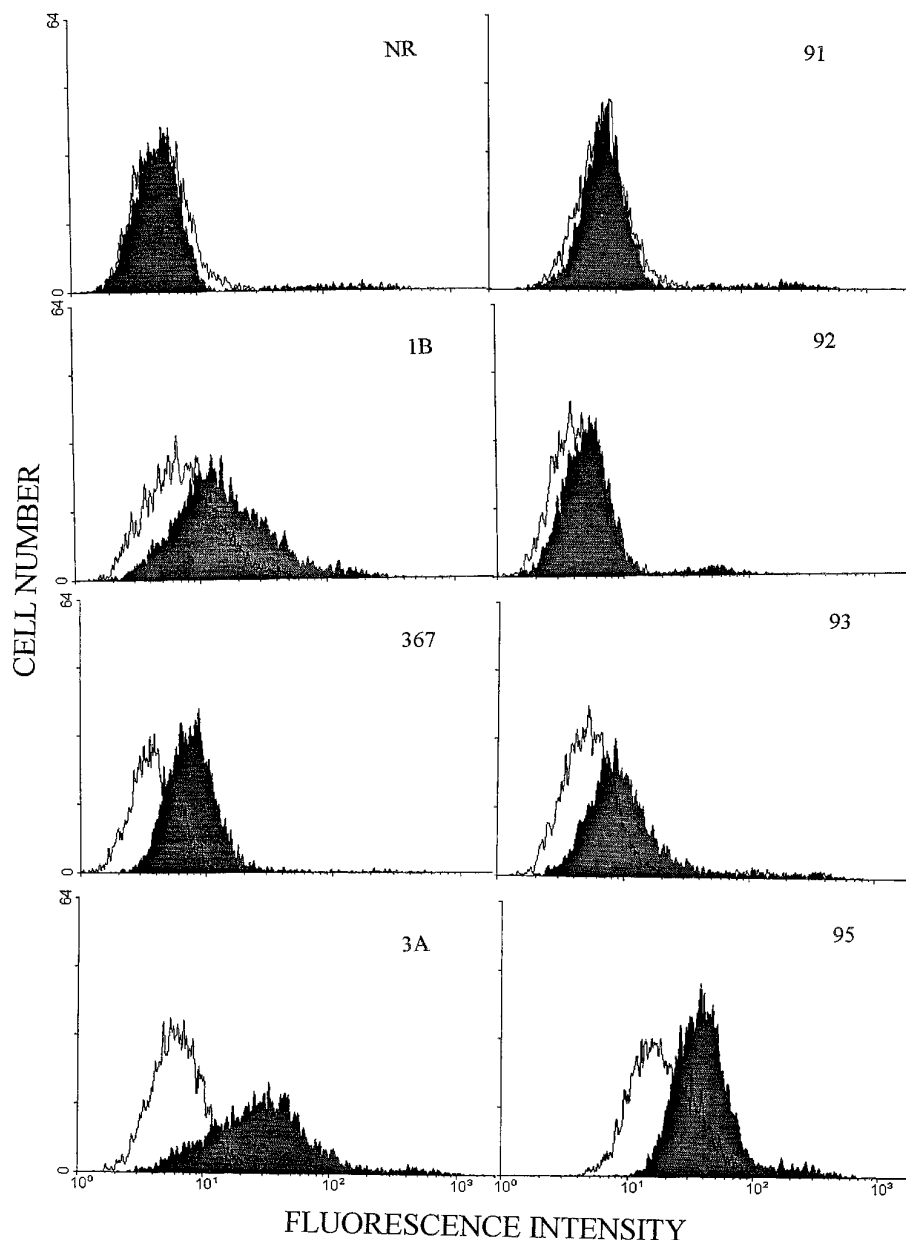


FIG. 1. Binding of normal rabbit sera and rabbit anti-ETSHr to CHOR or CHON cells. a, Fluorescence was measured after staining CHOR or CHON cells with normal rabbit sera (NR1 and NR2) and rabbit anti-ETSHr sera (T1 and T2). The binding was detected using a FITC-labeled goat antirabbit IgG. The binding of T1 and T2 was reversed by preincubating the sera with ETSHr protein (\cdots). ■, Binding to CHOR cells; —, binding to CHON cells. The experiment was repeated twice with very similar results. b, Fluorescence was measured after staining CHOR with T1 serum (■), T1 serum preincubated with Sf9 cells producing ETSHr-gp protein (\cdots), and T1 serum preincubated with uninfected Sf9 cells (\cdots).

FIG. 2. Binding of rabbit anti-peptide sera to CHOR or CHON cells. Fluorescence intensity was measured by flow cytometry after staining CHOR or CHON cells with normal rabbit sera or rabbit anti-peptide sera at a 1:100 dilution. NR, normal rabbit serum; 1B, aa 362–386; 367, aa 357–372; 3A, aa 357–372; 92, aa 12–30; 91, aa 32–46; 93, aa 316–330; 95, aa 325–345. ■, Binding to CHOR cells; —, binding to CHON cells. The experiment was repeated twice with very similar results.



effects of TSH, as well as blocking and stimulatory antibodies.

Binding of rabbit anti-ETSHr to CHOR or CHON cells by flow cytometry

Six sera from normal rabbits failed to react with CHOR cells when tested by flow cytometry (Fig. 1). Sera from two rabbits immunized with ETSHr, T1 and T2, showed significant binding to CHOR cells relative to CHON cells (Fig. 1a). IgG extracted from 3A serum showed binding to CHOR comparable to that seen when the whole serum was used (not shown). The specificity of this binding was shown when purified ETSHr protein prevented the binding of anti-ETSHr (T1 and T2) sera (Fig. 1a). This was further confirmed by failure of T1 serum to bind when preincubated with Sf9 cells

producing ETSHr-gp, but not when incubated with uninfected Sf9 cells (Fig. 1b).

Binding of rabbit anti-peptide serum to CHOR or CHON cells by flow cytometry

To test the feasibility of using these cells for epitope mapping, the binding of rabbit anti-peptide antibodies was tested. Antipeptides 93, 95, 1B, 367, and 3A, but not 91 or 92, showed significant binding to the CHOR cells compared with their binding to CHON cells (Fig. 2). The specificity of binding of antibodies to peptides 93, 95, 1B, 367, and 3A to CHOR cells was demonstrated by complete reversal of binding by the corresponding peptides; this is illustrated for peptide 3A (Fig. 3).

FIG. 3. Reversal of binding of rabbit anti-3A serum to CHOR cells. Cells were stained with 100 μ L anti-3A serum and analyzed by flow cytometry. ■, Binding to CHOR cells; —, binding to CHON cells. For reversal of binding, the anti-3A serum preincubated with the 3A peptide was used for staining CHOR cells (\cdots). The experiment was repeated twice with very similar results.

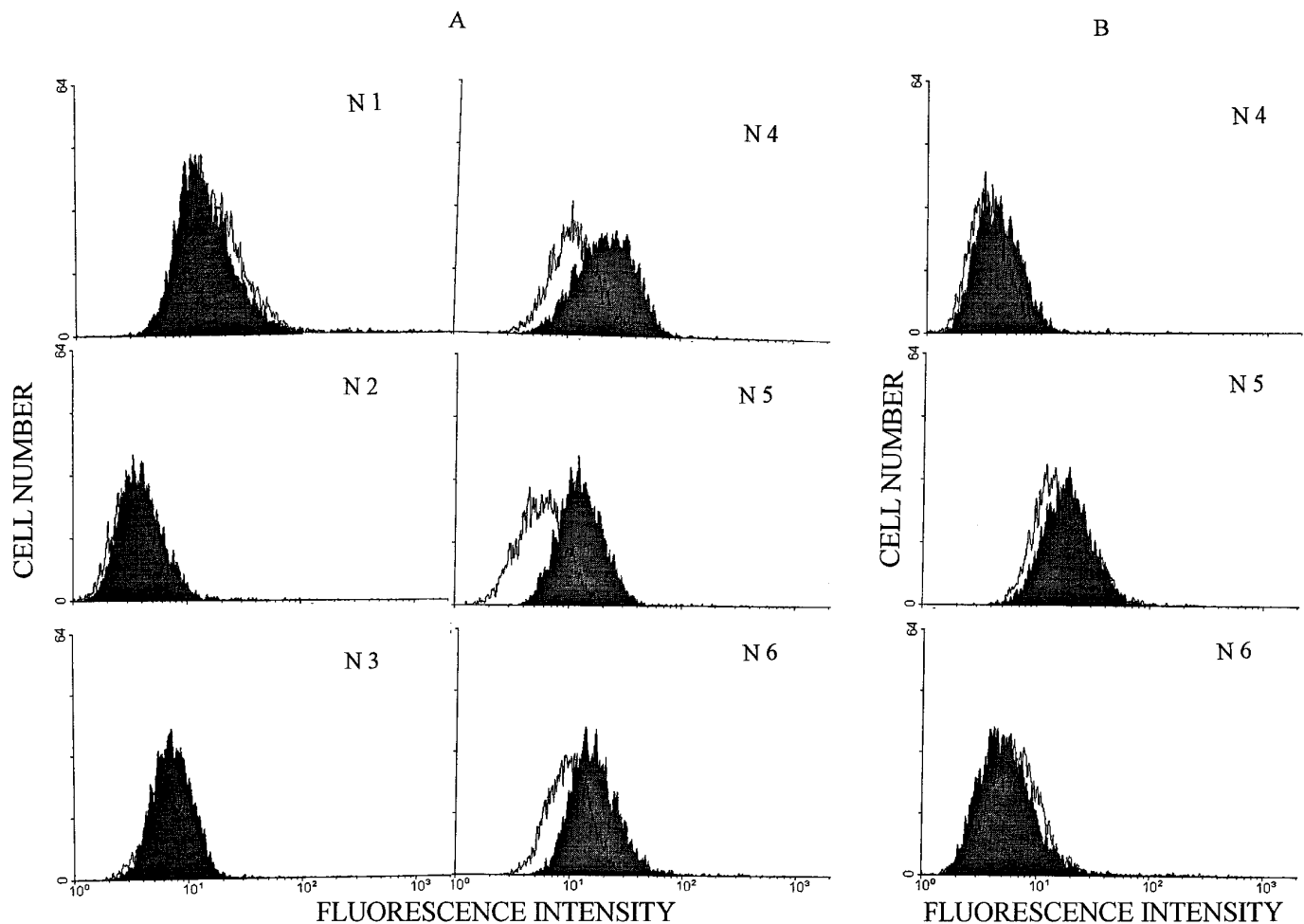
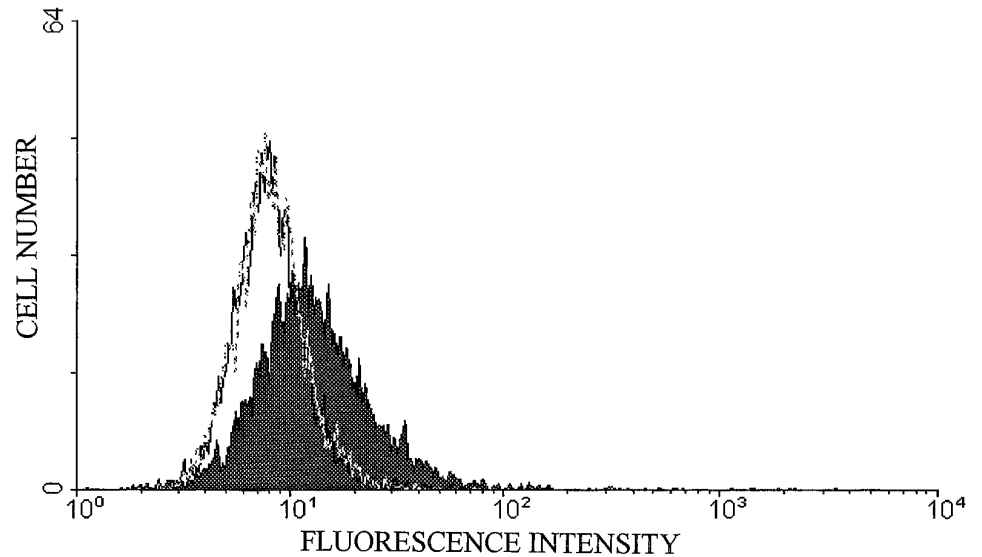


FIG. 4. A, Binding of normal human sera to CHOR or CHON cells. Cells were stained with 100 μ L normal sera (N1, N2, N3, N4, N5, and N6) and analyzed by flow cytometry. B, Binding of normal human sera to CHOR or CHON cells after preincubation of the sera with CHON cells; 100 μ L normal sera diluted 1:20 (N4, N5, and N6) were individually preincubated with 2×10^6 CHON cells and then tested for binding to CHOR or CHON cells as described above. ■, Binding to CHOR cells; —, binding to CHON cells. The experiment was repeated with very similar results.

Binding of human sera to CHOR or CHON cells by flow cytometry

Next, we used human sera to test their binding to CHOR cells by flow cytometry. The binding of human sera was either weak or undetectable when a FITC-labeled second antibody was used (data not shown). However, use of a biotinylated antihuman IgG, followed by the addition of FITC-avidin, allowed detection of antibody binding. Initially, sera from six normal individuals were tested for binding to CHOR cells. As shown in Fig. 4A, three of six normal sera (N4, N5, and N6) showed some binding to CHOR cells relative to their binding to CHON cells. To test for specificity, we preincubated the human sera with CHON cells, tested for its binding to CHOR cells, and found that they no longer bound to CHOR cells (Fig. 4B). We concluded from these experiments that some sera exhibit nonspecific binding, and it was important to use appropriate controls.

Next, we tested TBII-positive sera from 5 Graves' patients (G1, G2, G3, G4, and G5; Table 4) for binding to CHOR cells before and after preincubation with CHON cells (Fig. 5). Four of 5 patient sera (G1, G2, G3, and G4) retained their binding to CHOR cells (Fig. 5B). In all future experiments, we preincubated the sera with an excess of CHON cells before testing their binding to CHOR cells. When the binding of an additional 10 hyperthyroid (G6–G15) sera with known TBII activity was similarly tested, 4 of them reacted with CHOR cells (Table 4). The binding of patient sera to CHOR cells did not correlate with the degree of TBII or TSAb activity in the sera (Table 4). There was no increase in the sensitivity when the experiments were carried out using IgG from patients G1, G3, G6, G7, and G8 (not shown).

Previously we had shown that ETSHr-gp reverses the TBII activity of patient sera, whereas ETSHr does not (22). There-

fore, we tested the specificity of binding of human sera G1, G2, and G10 in the presence of ETSHr-gp and found that the binding was considerably reduced (Fig. 6).

Discussion

In the present study we used CHO cells permanently transfected with a human TSHr complementary DNA and flow cytometry to show specific binding of antibodies to TSHr. A number of studies have used CHO cells transfected with human TSHr to either detect TSH binding or carry out bioassays (9, 12, 23–28). Harfst *et al.* have shown that TSHr can be detected on CHO cells using a polyclonal rabbit antiserum (26). Furthermore, these studies showed that the binding was higher in CHO cells expressing a higher number of receptor molecules per cell. In another study, Forteza *et al.* used patient sera containing potent TBII activity to visualize TSHr expression on two different cell types employing an indirect immunofluorescence staining technique (29). In the present study, similar to the earlier studies, we used CHOR cells that had been previously developed (9, 12) and showed that this cell line is very sensitive to the effects of human TSH and autoantibodies. Using flow cytometry, we showed significant staining with the rabbit polyclonal antibodies to TSHr. These results are similar to those reported by Harfst *et al.* However, we extended these observations and further showed that the binding was specific and could be reversed by preincubating the antibodies with the ETSHr protein.

Results obtained previously with peptide antibodies have met with skepticism because of the concerns about the ability of antibodies raised against linear epitopes to react with the native molecule. The results of the FACS analysis using anti-peptide sera showed that they can specifically bind to native TSHr in CHOR cells, and the cells could be used to detect binding of peptide-specific antibodies. Thus, rabbit antibodies to peptides 93, 95, 3A, 367, and 1B from the C-terminal region of TSHr, which is associated with TSH binding and the binding of blocking TSHrAb (2–6, 9–12), showed specific binding to CHOR cells. In contrast, antibodies to peptides 91 and 92 from the N-terminal region of TSHr, which is associated with stimulating TSHrAb activity and TSH binding (2–6, 9, 12), did not bind to the CHOR cells. In our earlier studies, all anti-peptide sera that were used in this study showed reactivity to ETSHr or the corresponding peptide in an enzyme-linked immunosorbent assay (8). Anti-peptides 92 and 91 are negative for TBII activity when tested using porcine thyroid membranes. However, they show modest TSH-blocking activities of 18.2% and 35.6%, respectively, when tested for their ability to block TSH-mediated uptake of ^{125}I by rat thyroid cells (13). The inability of anti-peptides 91 and 92 to bind to CHOR cells and yet show a modest TSH-blocking effect on rat thyroid cells could be either due to species differences in the receptor structure or the lower sensitivity of FACS relative to the bioassay. Anti-peptides 95 [TSHr antibody-blocking activity (TSBAb) of 17.6%], 3A (TSBAb of 76.9%), 1B (TSBAb not known), and 367 (TSBAb of 53.9%) showed specific binding to CHOR cells by FACS analysis.

Unlike the normal sera, sera from Graves' patients showed strong binding even after preincubation with CHON cells.

TABLE 4. Percent TBII and binding obtained by FACS analysis of patient serum

Sample	% TBII	TSAb (pmol cAMP)	% Positive cells by FACS	Binding by FACS on CHOR cells
Exp 1				
G1	62	31	54	+
G2	80	7	52	+
G3	54	>200	49	+
G4	56	31	48	+
G5	79	48	15	–
Exp 2				
G6	54	12	9	–
G7	46	62	0	–
G8	49	32	13	–
G9	71	76	10	–
G10	54	175	59	+
G11	61	29	55	+
G12	87	>200	35	+
G13	54	21	31	+
G14	78	>200	14	–
G15	53	52	13	–
Pooled normal sera	<10	<15	–	–

TBII activity of the patient samples (G1–G15) was determined using a commercial RRA. Thyroid-stimulating antibody activity was tested in a bioassay using CHOR cells as described in *Materials and Methods*. The percentage of positive cells was determined using CELLQuest as described in *Materials and Methods*.

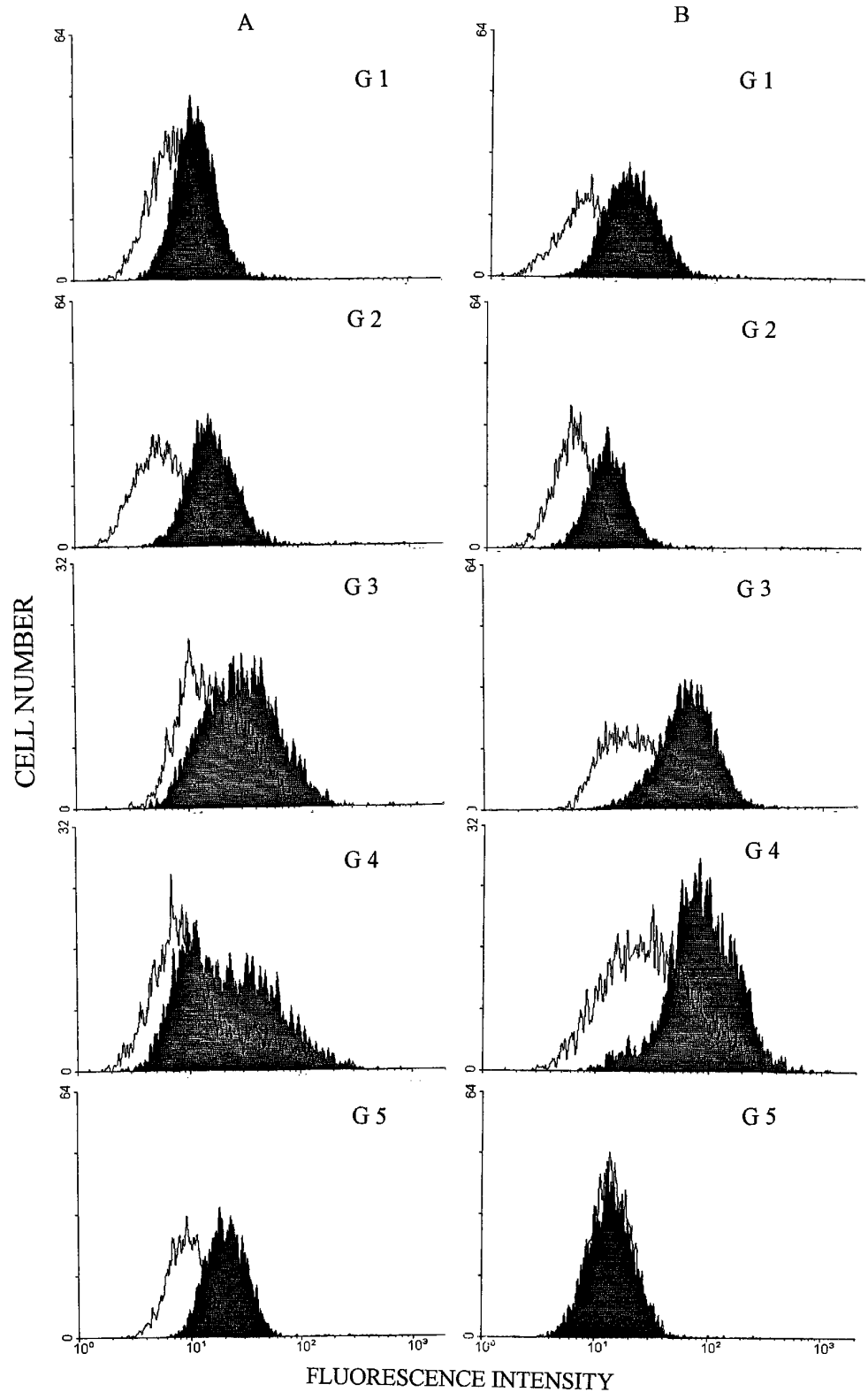


FIG. 5. A, Binding of Graves' patient sera to CHOR or CHON. Cells were stained with 100 μ L Graves' patient sera diluted 1:20 (G1, G2, G3, G4, and G5) and then used for flow cytometric analysis. B, Binding of Graves' patient sera to CHOR or CHON cells after preincubation of the sera with CHON cells; 100 μ L of sera diluted 1:20 (G1, G2, G3, G4, and G5) were individually preincubated with 2×10^6 CHON cells and then tested for binding to CHOR or CHON cells. ■, Binding to CHOR cells; —, binding to CHON cells. The experiment was repeated with very similar results.

The specificity of binding was further demonstrated by loss of reactivity of sera when they were preincubated with cell lysates containing ETSHR-gp, which had been previously shown to reverse the TBII, blocking, as well as stimulatory antibody activities in the sera of patients with thyroid dis-

eases (22, 30). Together, these studies showed that we can detect some patient antibodies that specifically react with the human TSHr using flow cytometry.

Our results were, nevertheless, somewhat disappointing, because sera from only 50% of the patients showed specific

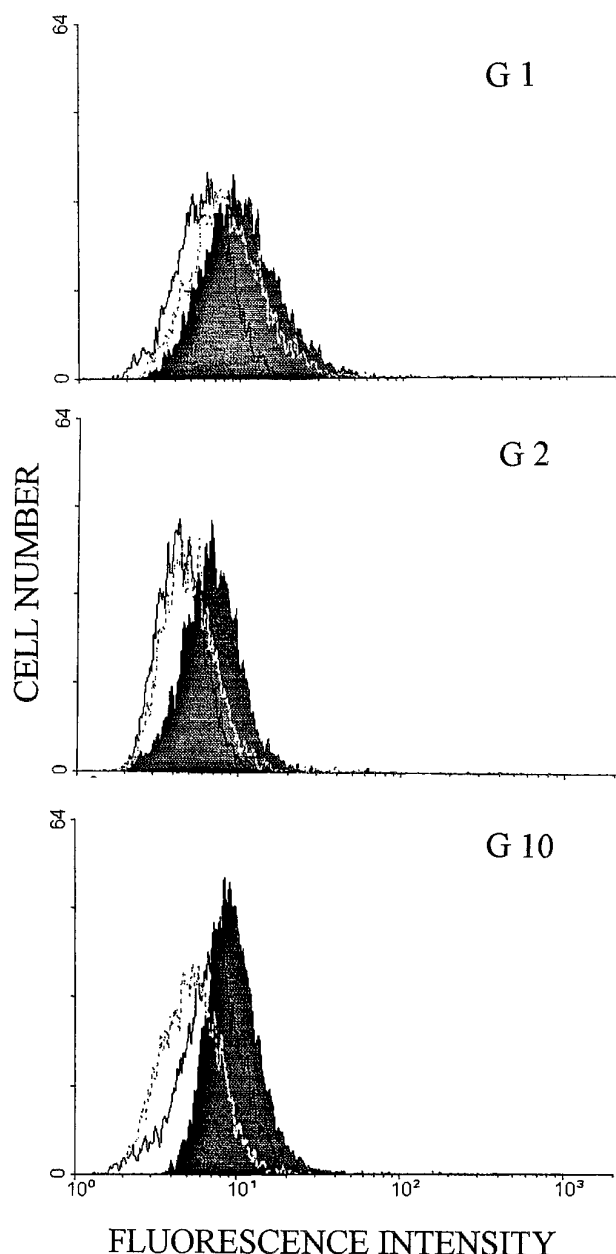


FIG. 6. Reversal of binding of human sera to CHOR cells. Sera (100 μ L; G1, G2, and G10) diluted 1:20 were preincubated with 2×10^6 lysed cells expressing the ETSHr-gp protein. The sera were then incubated with CHOR cells and stained for analysis by flow cytometry (···). ■, Binding to CHOR cells; —, binding to CHON cells.

binding to CHOR cells. Unlike previous studies from other laboratories, we had increased the sensitivity of detection by incorporating a biotinylated second antibody and FITC-labeled avidin. Despite this enhanced sensitivity, we were unable to detect antibodies in a substantial number of cases. This could be due to limiting concentrations of TSHr-specific antibodies in the sera of patients with autoimmune thyroid diseases (28), a limited number of receptors expressed on permanently transfected CHO cells, or antibody heterogeneity. The lack of correlation between the FACS data and the TBII assay, indicates that our ability to detect binding by

FACS was not dependent on the level of TBII activity in the sera, but perhaps on the qualitative nature of the antibody. This possibility is further supported by a number of earlier studies, including a recent report, which showed that there is considerable heterogeneity in the antibody population among patients (9, 11, 12, 22, 30).

Together, the data suggest that the flow cytometric assay described here will be useful in detecting antibodies in experimental sera. It will be of particular interest to test other antipeptide antibodies with their presumed specificity for linear epitopes to determine whether such epitopes are accessible on the native TSHr. The clinical significance and relation of binding to antibody activity remain to be determined, but the opportunity to measure TSHr antibody binding with relative ease may afford new insights into TSHr structure and, thus, disease expression.

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