Generation of an Auto-anti-idiotypic Antibody That Binds to Glucocorticoid Receptor*

Eftihia Cayanis, Raghavan Rajagopalan, W. Louis Cleveland, Isidore Edelman, and Bernard F. Erlanger

From the Department of Microbiology and of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10027

A monoclonal antibody (8G11-C6) that is directed to a region near the ligand-binding site of the glucocorticoid receptor was obtained by an auto-anti-idiotypic route, using a derivative of triamcinolone coupled to thyroglobulin to immunize a mouse. The resulting hybridomas were screened for anti-idiotypic antibody (anti-antisteroid) with Fab fragments of affinity-purified polyclonal rabbit anti-triamcinolone antibody. The anti-idiotypes were then screened for binding to rat cytosol glucocorticoid receptor by a depletion procedure, yielding a clone, 8G11-C6, whose specificity for receptor was verified by sucrose density and Western blot analyses. Depletion was not significantly reduced by prelabeling the cytosol with [3H]triamcinolone acetonide. The anti-idotype (8G11-C6) bound to Fab fragments of antisteroid and to partially purified receptor in a concentration-dependent manner. Both binding reactions were inhibited only by rabbit serum albumin conjugates of steroids known to bind to the glucocorticoid receptors. Triamcinolone derivatives of lysine and of oligopeptides containing up to six amino acids inhibited the binding of the anti-idotype to the Fab fragments but not to the receptor, implying that the target epitope of the antisteroid antibody may be closer to its glucocorticoid-binding site than the cross-reacting epitope of the receptor. Our findings demonstrate further the versatility of the auto-anti-idiotypic route for the preparation of anti-receptor antibodies.

Highly purified, but nonhomogeneous, preparations of glucocorticoid receptor have been used to raise specific polyclonal and monoclonal antibodies (1-9). In the preparations of the antigen, the receptor has been isolated with bound ligand because of the unstable nature of the glucocorticoid receptor in the absence of ligand. The resulting antibodies (with one exception (7)) were directed at epitopes other than those located at the combining site of the glucocorticoid receptor.

The intention of the present study was to raise antibodies to epitopes at or near the combining site of the receptor. Such antibodies could serve a variety of purposes, including use as reagents in purifying the receptor, as probes for mapping functional domains, and as reagents to assess intracellular distribution of receptor, with and without bound ligand.

This paper describes the application of a new method of preparing anti-glucocorticoid receptor antibodies, which does not require prior purification of the receptor for immunization purposes. An anti-idiotypic route, previously used to obtain monoclonal antibodies to other receptors (10-18), was employed, based on the one step auto-anti-idiotypic methodology described by Cleveland et al. (11) for obtaining monoclonal antibodies to the combining site of the acetylcholine receptor.

A triamcinolone derivative (TKH) linked to thyroglobulin served as the immunogen in the present study.

MATERIALS AND METHODS

New Zealand White rabbits were obtained from Pocono Farms (Canadensis, PA), BALB/c mice from West Seneca Labs (West Seneca, NY) and Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA). Acetylbutyric acid, N-hydroxysuccinimide, and dicyclohexylcarbodiimide were from the Aldrich. Norit A and Celite 545 were from Fisher Scientific (Springfield, NJ), Silica Gel 60 from Merck AG (Darmstadt, Germany), AH-Sepharose 4B, Sephadex G-100, and Protein A were from Pharmacia Fine Chemicals (Uppsala, Sweden). Typing sera (i.e. rabbit and goat antisera to mouse, “Gl(1-6)”, IgG, IgM, and light chain) were all obtained from Bionetics, Inc. (Kensington, MD). Goat anti-mouse IgG3 and the peroxidase-conjugated goat anti-mouse IgG + IgM antibodies were obtained from Tago, Inc. (Burlingame, CA). Iscoves' modified Dulbeco's medium, the nutrient mixture F-12 (Ham's), and penicillin-streptomycin were from Grand Island Biological Co. (Grand Island, NY). Fetal calf serum was purchased from Sterile Systems (Logan, UT).

The steroid derivative, 16α,17α-(4-carboxy-1-methylbutylidene)-bis(oxy)1-9-fluoro-11β,21-dihydroxyprogren-3,20-dione, was a gift from The Upjohn Co. The labeled steroids [1,2,4-3H]triamcinolone acetone ([3H]TA) and [6,7-3H]dexaethamethasone were purchased from Amersham-Buchler (Braunschweig, Germany).

Synthesis of Ketohexanoic N-Hydroxysuccinimide Ester (KHS)

4-Acetylbutyric acid (0.024 mol) in approximately 60 ml of dioxane was added to a solution of 0.036 mol of N-hydroxysuccinimide in dioxane. More dioxane was added slowly until all of the N-hydroxysuccinimide was in solution. Finally, 0.025 mol of dicyclohexylcarbodiimide in dioxane was added and the solution, a total volume of 100 ml, was stirred overnight at room temperature. The insoluble dicyclohexylurea was removed by filtration and the filtrate concentrated by evaporation in a rotary evaporator. The product was extracted with 200 ml of methylene chloride and washed with water 5 times to remove dioxane and unreacted N-hydroxysuccinimide. Solid magnesium sulfate and Norit A were then added to the methylene chloride solution to dry and decolorize it. The solids were removed by filtration through Celite 545, and the filtrate was concentrated by rotary evaporation. The product was examined for the presence of the ester by TLC using analytical silica gel plates with 2% methanol in methanol.

1 The abbreviations used are: TKH, triamcinolone δ-ketohexanoyl moiety; ELISA, enzyme-linked immunosorbent assay; TA, triamcinolone acetone; [3H]TA, [1,2,4-3H]triamcinolone acetone; RSA, rabbit serum albumin; KHS, δ-ketohexanoic N-hydroxysuccinimide ester; TKHS, triamcinolone δ-ketohexanoic N-hydroxysuccinimide ester; BSA, bovine serum albumin; PBS, 0.01 M phosphate buffer, 0.14 M NaCl (pH 7.4); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.
ylene chloride as the developing solvent. The ester was identified by the Fe-hydroxamate test (19).

**Purification of KHS**

Approximately 9 g of the concentrate containing the KHS was chromatographed on a column containing 160 g of Silica Gel 60 in chloroform. After washing with 100 ml of chloroform, the KHS was eluted with 3% methanol in chloroform. The first 250 ml were discarded, after which 20-ml fractions were collected, until all the color was eluted from the column. The purity of KHS in the various fractions was assessed by TLC as described above. The pure fractions were combined and evaporated to dryness.

**Coupling of KHS to Triamcinolone**

Triamcinolone (0.8 g) and 0.9 g of KHS were suspended in 16 ml of dioxane with stirring, followed by careful addition of 0.3 ml of perchloric acid. The mixture was stirred overnight at room temperature in order to obtain a clear solution. The reaction was terminated by the addition of Na2CO3 and the product extracted with 100 ml of methylene chloride. After washing with 200 ml of H2O, the upper water phase was discarded and the methylene chloride phase was dried over solid MgSO4. After filtration and evaporation to dryness, the product was chromatographed by TLC using 10% methanol in chloroform as the developing solvent. The product was detected as an ester (19) and distinguished from the starting material by its strong UV absorbance.

**Purification of Triamcinolone-O-Ketohexanoyl Hydroxysuccinimide Ester (TKHS)**

The product, in CHCl3, was passed through a Silica Gel 60 column (100 g) that had been equilibrated with chloroform. Development was with 7.5% methanol in chloroform, using a slow flow rate. Ten-ml fractions were collected and analyzed by TLC chromatography on Silica Gel 60 using 10% methanol in chloroform as the solvent. The fractions containing the TKHS (i.e. UV absorbance and positive Fe-hydroxamate test) were combined, concentrated by rotary evaporation, and purified further by high pressure liquid chromatography, using a Zorbax silica column of 21.2 mm x 25 cm dimensions. Methanol (5%) in chloroform at a flow rate of 16 ml/min produced 2 major peaks which were concentrated by rotary evaporation. Pure steroid ester (TKHS) was identified in the second peak by TLC chromatography as described above.

**Coupling of TKHS to BSA, RSA, or Thyroglobulin**

The steroid ester, at a concentration of 1.4 x 10-4 mol in 2 ml of tetrahydrofuran, was added dropwise to 2.8 x 10-4 mol of protein that was dissolved in 5 ml of 0.2 N Na2CO3/NaHCO3 buffer (pH 8.0). Additional tetrahydrofuran was added to clarify the solution. After standing overnight at 4°C, the solution was dialyzed against several changes of distilled water. A white precipitate formed, most of which was dissolved by dropwise addition of 0.2 ml of Na2CO3. Additional distilled H2O was added to bring the volume to 35 ml, and the suspension was centrifuged at 4°C to remove denatured protein. The amount of steroid covalently bound was calculated from the extinction coefficient of the steroid ester, which was previously determined to be 1.2 x 104 at 280 nm. The yield of bound steroid was 18-23 mol of steroid/mol of protein, and the other steroid conjugates, namely 17β-estradiol-RSA, testosterone-RSA, cortisone-RSA, and deoxycorticosterone-RSA were previously synthesized (20,21). The overall reaction scheme is shown in Fig. 1.

**Purification of the Polyclonal Antisteroid Antibody**

Immunization Protocol—New Zealand White rabbits were immunized by multiple intradermal injections with a total of 1 ml of TKHS-BSA (2 mg/ml) in saline emulsified with an equal volume of complete Freund's adjuvant. Booster injections were given 3 weeks later and then at monthly intervals. Animals were bled bimonthly from the ear vein and the sera stored at 4°C until required.

Affinity Chromatography of the Antisteroid Preparation on AH-Sepharose 4B Triamcinolone Column—AH-Sepharose 4B (1 g) was suspended in 100 ml of 0.5 N NaCl. After 15 min, the slurry was filtered on a Buchner funnel and washed with 250 ml of 0.5 N NaCl and then with 250 ml of water. The gel was transferred to a centrifuge tube and washed five times with 10 ml of 0.2 N NaHCO3, pH 8.15. After the last wash, the supernatant was discarded, an equal volume of 0.2 N NaHCO3 (pH 8.15) was added, followed by the addition of 58.6 mg of TKHS in 3-5 ml of tetrahydrofuran. The suspension was mixed overnight at 4°C, and the gel was washed sequentially with methanol/H2O solution (1:1) and distilled H2O, and then equilibrated with PBS. The gel was poured into a 10-ml Luer-lock plastic syringe to provide a 4-ml bed volume.

**Purification of the Polyclonal Antisteroid Antibody**—Thirty to 40 mg of serum was passed slowly (~3.5 ml/h) through the AH-Sepharose 4B triamcinolone column. The column was washed with PBS to remove unbound protein, and the antibody was eluted with 0.2 M glycine, pH 2.2 or 2.8. The eluate was dialyzed against 2 liters of 0.01 M PBS buffer (pH 7.4). The buffer was changed at least 4 times before the product was concentrated by vacuum dialysis at 4°C.

**Preparation of Fab Fragments**—Fab fragments were prepared essentially by the method of Porter (26). Affinity-purified antisteroid antibody (20 mg/0.5 ml) was dialyzed for 2 h at 4°C against 0.1 M potassium phosphate buffer, pH 7.0, containing 0.08 M NaCl for 2 h at 4°C. To the dialysate was added 0.1 ml dithiothreitol (15 μl), EDTA (2 mg), and 0.2 mg of mercaptoethanol (5 μl). The solution was incubated overnight at 37°C and then dialyzed against 4 liters of distilled H2O for 3 h and against 1 liter of PBS for 2 h. The dialysate was applied to a Sephadex G-100 column (1.5 x 90 cm) and eluted at 5 ml/h with 0.01 M PBS (pH 7.4); 1-ml fractions were collected. Fractions 53-79 were combined, and the protein content was estimated by absorbance at 280 nm. Undigested IgG and Fc fragments were removed by passage through a protein A-Sepharose column (5-
ml bed volume); unfractionated Fab was eluted with 0.01 M PBS, pH 7.4. 

The eluate was concentrated by vacuum dialysis.

Preparation of Monoclonal Anti-idiotype Antibody

Two female Balb/c mice were immunized intraperitoneally with 0.1 ml of a 1 mg/ml solution of triamcinolone-thyroglobulin conjugate in complete Freund’s adjuvant. The mice were boosted twice at 3-week intervals, with the same antigen, by intraperitoneal injection. Four days later, one of the mice was splenectomized and 2 x 10^6 spleen cells were fused with 2 x 10^7 cells of a nonsecreting myeloma line (P3X63-Ag 8.653) (27) according to the procedure of Köhler and Milstein (28) as modified by Sharon et al. (29). Supernatants from the hybridomas were obtained by a replica transfer technique and screened with the monoclonal anti-idiotype antibodies for anti-idiotype activity by ELISA. Monoclonal antibody-producing hybridomas were obtained by cloning the cells of interest on soft agar or by micromanipulation. The class and subclass of the heavy chain of the monoclonal antibodies were determined by Ouchterlony and by ELISA using anti-mouse Ig class and subclass antibodies. The clones of interest were expanded by growing them in 75-cm² T-flasks. The antibodies were partially purified by precipitation with 50% saturated (NH₄)₂SO₄.

The relevant hybridomas were injected intraperitoneally into EAFK mice previously treated with pristane. Ascitic fluid was harvested and the anti-idiotype purified on AH-Sepharose 4B glutaraldehyde gel (30) coupled to affinity-purified antisteroid.

Purification of the Anti-idiotype by Affinity Chromatography

Ten mg of affinity-purified antiserum in a total volume of 5 ml of 0.2 M Na₂CO₃/NaHCO₃ buffer (pH 8.8) was added to approximately 3 x 10⁷ AH-Sepharose 4B glutaraldehyde gel (30), mixed for 4 h at room temperature and then overnight at 4°C. The supernatant was decanted and the gel washed with 10 ml of 0.2 M Na₂CO₃/NaHCO₃ buffer (pH 8.8). Three ml of 0.2 M glycine (pH 8.5) was added to the gel and mixed for 4 h at room temperature. The gel was washed with 200 ml of 0.2 M Na₂CO₃/NaHCO₃ buffer (pH 8.8) poured into a 10-ml Luer-lock plastic syringe and equilibrated with 10 mM PBS (pH 7.3-7.4).

Five ml of ascites fluid was passed slowly through the AH-Sepharose 4B glutaraldehyde antiidiotype column. After washing with PBS to remove all unbound protein, the anti-idiotype was eluted with 0.2 M glycine buffer (pH 2.2). The eluate was concentrated by vacuum dialysis at 4°C and dialyzed twice against 2 liters of 0.01 M PBS (pH 7.4). The AH-Sepharose 4B glutaraldehyde antiidiotype column bound 3-5 mg of protein.

ELISA

The presence of anti-idiotype antibodies in the hybridoma culture medium was assayed by a double antibody sandwich ELISA. Polystyrene microplates (Corning 25855) were prepared by coating each well with 200 μl of 50 mg/ml affinity-purified rabbit antibody anti Fab fragments in 0.1 M NaHCO₃ (pH 9.3) and incubating overnight at 4°C. After washing twice with 0.01 M phosphate buffer, 0.14 M NaCl (pH 7.4) containing 0.05% Tween (PBS-Tween), the culture medium from the hybridomas was added and incubated at 37°C for 2 h. The wells were washed three times with PBS-Tween. Then 200 μl of a 1:3000 dilution in PBS-Tween of goat anti-mouse IgM + IgG coupled to horseradish peroxidase was added. After incubating for 1 h at 37°C, the wells were washed three times with PBS-Tween, and 200 μl of substrate (7 mg of o-phenylene diamine dichloride in 10 ml of 0.1 M citrate-phosphate buffer, (pH 4.8) containing 5 μl of 30% H₂O₂) was added. Depending on the intensity of the color, the reaction was stopped after 5-10 min by the addition of 50 μl of 8 N H₂SO₄ and the absorbance determined at 492 nm.

Cytosol Preparation of the Glucocorticoid Receptor

Male Sprague-Dawley rats (150-200 g, body weight) were anesthetized with ether 4-6 days postadrenalectomy and the livers perfused in situ through the portal vein with 50 ml of cold isotonic saline. The livers were removed, homogenized with a Teflon-glass homogenizer in 1.1 volumes of 10 mM HEPES, containing 1 mM Na₂EDTA, 1 mM dithiothreitol, and 10% glycerol (pH 7.6). The crude homogenates were centrifuged for 1 h at 250,000 x g at 4°C. The upper fatty layer was discarded. The supernatant was either frozen immediately in liquid nitrogen and used for assays or labeled by incubating with 75 μM [³H]TA for 2 h at 4°C. Unbound steroid was removed with 5% dextran-coated charcoal. The [³H]TA-labeled glucocorticoid receptor was partially purified by the method of Gametchu and Harrison (9), except that phosphocellulose was omitted from the first purification step.

Receptor Depletion with Monoclonal Anti-idiotype Antibody Bound to Rabbit or Goat Anti-mouse IgM Sepharose 4B

Preparation of the CNBr-Sepharose 4B Immunoadsorbent—Affinity chromatography-purified rabbit or goat anti-mouse IgM was coupled to cyano gen bromide-activated Sepharose 4B as described by Westphal et al. (6) and in the Affinity Chromatography Handbook of Pharmacia as follows CNBr-activated Sepharose (500 mg) was swelled in 1 ml HCl and washed on a sintered funnel with 200 ml of 1 M HCl. The gel was washed twice with coupling buffer (0.55 M NaHCO₃ containing 0.5 M NaCl, pH 8.6) and suspended immediately in affinity-purified anti-mouse IgM solution (4 mg/3 ml). The suspension was mixed gently by rotating either for 2 h at room temperature or overnight at 4°C. The mixture was then centrifuged in a clinical centrifuge and the gel sediment allowed to react with 1 M ethanamine (pH 8.2) either for 1 h at room temperature or overnight at 4°C. It was then washed with 15 ml of 0.1 M acetate buffer containing 1 mM NaCl (pH 4.0) followed by 0.1 M borate buffer (pH 8.0), and then with PBS several times, and suspended in 3 volumes of PBS.

Binding of Glucocorticoid Receptor to Mouse Anti-idiotype Antibody—Hybridoma culture medium (1-3.5 ml) containing antibody was mixed with 125 μl of anti-mouse IgM-Sepharose 4B overnight at 4°C. The Sepharose 4B beads coated with mouse anti-idiotype antibodies were centrifuged and washed 3 times with 2 ml of PBS. Receptor supernatant (300 μl containing 0.06-0.18 pmol of receptor) was added and mixed for 2 h at 4°C. The gel was centrifuged, and 200 μl of the supernatant was assayed for unbound glucocorticoid receptor by incubation at 4°C for 2 h with 25 μl of 25 nM [³H]TA (in methanol). Unbound [³H]TA was removed by adding 100 μl of dextran-coated charcoal (5 g of activated charcoal and 50 mg of dextran in 100 ml of 20 mM HEPES, 50 mM NaCl, 1 mM EDTA (pH 7.6)) and allowing it to stand at 4°C for 5 min. Cold PBS (1 ml) was added and after 5 min, the charcoal-dextran was sedimented by centrifugation. The supernant was assayed in a liquid scintillation counter.

Sucrose Density Gradient Analysis of Binding of Anti-idiotype Antibodies to Glucocorticoid Receptor

Heptica extracts containing glucocorticoid receptor were radiolabeled by incubating 0.5 ml of rat liver cytosol with 10 nM [³H]TA for 2 h at 4°C. Dextran-coated charcoal (0.25 ml) was added and the suspension allowed to stand for 10 min at 4°C. After centrifugation, 50 μl of radiolabeled cytosol (0.16 pmol of [³H]TA/50 μl) was incubated with either 190 pl of mouse anti-idiotypic antibodies, 5B5-B6 or 8Gll-C6 (∼9.4 mg/ml) at 4°C overnight. The reaction was stopped, and the samples were subjected to sucrose gradient centrifugation as described under “Cytosol Preparation of the Glucocorticoid Receptor,” with the exception that the gradient was made 10 mM Tris-HCl buffer, 1 mM EDTA, and 0.4 M KCl, pH 7.4. The gradient was overlaid on a 2-20% sucrose cushion and centrifuged at 4°C overnight. Two to 3 drop fractions were obtained by puncturing the bottom of the tube.

Immunoblot of the Glucocorticoid Receptor

One ml of rat liver cytosol was prepared and partially purified by ammonium sulfate precipitation as described under “Cytosol Preparation of the Glucocorticoid Receptor,” the only difference being that 50 mM sodium molybdate was included in the homogenization mixture. The ammonium sulfate precipitate was resuspended in 0.6 ml of 20 mM HEPES buffer containing 50 mM NaCl, 1 mM EDTA (pH 7.6). 0.3 ml of sample buffer was added to the latter, and the sample was denatured by heating by 20 min at 60°C. It was layered onto an SDS-polyacrylamide gel that was prepared by the Laemmli method (31) using 5.0% stacking gel and 7.5% running gel. High and low molecular weight proteins (obtained from Pharmacia calibration kits) were included as standards. Electrophoresis was carried out overnight at 50 V/alpha.

The proteins were electrotransferred onto nitrocellulose paper using electrophoretic transfer apparatus (Transblot, Bio-Rad) and 20 mM Tris base, 150 mM glycine, and 20% methanol at 0.5 A for 3 h.
After blotting, the portion of the nitrocellulose containing the protein standards was stained with Amido Black (0.1% in 45% methanol, 10% acetic acid) (32) and destained with 90% methanol, 2% acetic acid (33). The remainder of the nitrocellulose was blocked with PBS containing 1% BSA by incubating overnight at 4°C. Purified monoclonal antibodies (0.66 mg/ml) were diluted 500-1000 times in washing the nitrocellulose 3 times for 20 min at 4°C of different monoclonal antibodies overnight at 4°C. Purified monoclonal antibodies (0.66 mg/ml) were diluted 500-1000 times in PBS containing 1% BSA, 0.1% Triton X-100, and 0.02% SDS. The nitrocellulose strips were washed once in PBS containing 1% BSA, 0.1% Triton X-100, and 0.02% SDS it was incubated for 3h with rabbit anti-mouse alkaline phosphatase (Sigma) diluted 1:1000 in PBS containing 1% BSA, 0.1% Triton X-100, and 0.02% SDS. The nitrocellulose strips were washed once in PBS containing 1% BSA, 0.1% Triton X-100, and 0.02% SDS for 30 min. Finally, they were washed 2 times for 1 min with 0.14 M Veronal-acetate buffer, pH 9.6. The nitrocellulose was then developed with 0.14 M Veronal-acetate buffer, pH 9.6 (63 ml) containing 2 M MgCl₂ (250 µl), 3-indoxyl phosphate, 10 mg/ml (700 µl), and Nitro Blue tetrazolium, 10 mg/10 ml (7 ml). The reaction was stopped by washing the nitrocellulose with water.

RESULTS

Characterization of TKH-BSA Conjugate—The apparent affinity of TKH-BSA for the hepatic glucocorticoid receptor was assessed by a competitive binding assay. Fifty per cent inhibition of binding of 10 nM [³H]TA occurred at a concentration of TA in the conjugate of 2.5 x 10⁻⁴ M (Fig. 2). This is 100-fold greater (calculated as triamcinolone) than the concentration of TA required for 50% inhibition of the binding of [³H]TA. Since the Kᵰ for binding of TA to the hepatic glucocorticoid receptor was found to be 2.2 x 10⁻⁵ M, the apparent Kᵰ for the TA conjugate is 3.4 x 10⁻⁷ M.

The TKH-BSA conjugate is immunogenic and induces polyclonal antibodies specific for triamcinolone. Significant precipitating titer were detected in rabbit serum shortly after the second booster injection. Precipitation was detected only with TKH-BSA and not with RSA. No precipitation of conjugate was seen with preimmune serum. The antisteroid antibodies were purified by affinity chromatography on a TKH-BSA-Sepharose column. Forty ml of serum yielded 15–20 mg of purified antisteroid antibody.

Characterization of Polyclonal Antisteroid Antibodies by Radioimmunoassay—Both the affinity-purified antisteroid an-

Fig. 4. Competitive inhibition of binding of [³H]TA to the glucocorticoid receptor (9) and to antisteroid (unprocessed rabbit serum) 540). See the legend to Table I for a description of the experimental procedure. The steroids used were TA ( ), dexamethasone ( ), corticosterone ( ), hydrocortisone ( ), progesterone ( ), and dihydrotestosterone ( ).

bodies as well as their Fab fragments bound [³H]TA; the Kᵰ for binding of [³H]TA to Fab fragments of the antisteroid, as determined from Scatchard plots ranged from 9.3 x 10⁻⁹ to 4.3 x 10⁻⁹ M (Fig. 3). The curved plot clearly indicates the heterogeneous nature of the antisteroid population. In contrast, a linear Scatchard plot was obtained with [³H]dexamethasone, with a Kᵰ of 1.5 x 10⁻⁷ M (not shown). These results imply that [³H]dexamethasone bound to only one of the subclasses of the antisteroids.

A comparative study was made of the steroid-binding properties of the polyclonal antibodies and those of the glucocorticoid receptor. With [³H]TA as the ligand, the rank order of inhibition of binding by various steroids was similar for the glucocorticoid receptor and the antibody. Triamcinolone was the most potent inhibitor and dexamethasone > corticosterone > hydrocortisone > progesterone > dihydrotestosterone. In fact, dihydrotestosterone had no effect on the
Depletion of glucocorticoid receptor from rat liver cytosol by auto-anti-idiotype antibodies

Either 2 × or 4 × dilutions of rat liver cytosol were mixed with candidate anti-idiotype bound to CNBr-Sepharose-IgM beads or CNBr-Sepharose-IgM beads previously treated with basal medium containing 10% FCS. Rat liver cytosol was labeled with [3H]TA to a final concentration of 2.8 × 10^(-8) M for 2 h at 4 °C. Free steroid was removed with charcoal-dextran. These assays are described in detail under "Materials and Methods." The means of duplicate assays for [3H]TA in solution after removal of the Sepharose-bound constituents are listed as counts/min.

**Table II**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Glucocorticoid receptor</th>
<th>Rabbit serum 540</th>
<th>Rabbit serum 541</th>
<th>Fab fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Triamcinolone acetone</td>
<td>5 × 10^(-9)</td>
<td>3.2 × 10^(-9)</td>
<td>1.5 × 10^(-8)</td>
<td>1.5 × 10^(-8)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>7.9 × 10^(-9)</td>
<td>1.2 × 10^(-7)</td>
<td>6.2 × 10^(-7)</td>
<td>6.3 × 10^(-7)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>3.1 × 10^(-6)</td>
<td>1.0 × 10^(-4)</td>
<td>&gt;10^(-4)</td>
<td>1.0 × 10^(-4)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.2 × 10^(-7)</td>
<td>1.0 × 10^(-5)</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.6 × 10^(-6)</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
<td>&gt;10^(-4)</td>
</tr>
</tbody>
</table>

**Additions**

- + BM + 10% FCS^a^: 4723/1682
- + 5B5: 4851/1884
- + 8G11: 3989/1013

^a^10% FCS was added to the basal medium as the hybridomas 5B5 and 8G11 were grown in basal medium containing 10% FCS.

Binding of [3H]TA to either the glucocorticoid receptor or to the rabbit antiserum (Fig. 4 and Table I). The apparent K_d of TA for the antiserum in unprocessed rabbit serum was 3.2 × 10^(-7) M, similar to that of the glucocorticoid receptor (5 × 10^(-9) M) whereas those of the other steroids were about 1 order of magnitude higher. Although the apparent equilibrium dissociation constants of the various steroids for the affinity-purified antibodies were similar to those of their respective Fab fragments, they were considerably higher than those of the unprocessed serum, indicating that some of the high affinity antibodies were lost during the affinity purification procedure.

To maximize the probability of detecting appropriate anti-idiotype antibodies in the hybridoma system, Fab fragments of the affinity-purified antiserum antibodies were used in screening for anti-idiotype antibody production in mice, by ELISA.

Isolation of Anti-idiotype Antibodies to Fab Fragments and Characterization of the Subsets That Reacted with the Glucocorticoid Receptor—Auto-anti-idiotype antibodies to the anti-steroid antibodies were raised by immunizing a mouse with a TKH-thyroglobulin conjugate. After the mouse spleen cells were fused with the nonsecreting myeloma cell line P3X63-Ag8.653, 17 cell lines produced antibodies that bound to the Fab fragments of anti-TKH. Only 5 of these, however, remained positive after the cell lines were expanded.

Antibodies cross-reacting with the glucocorticoid receptor were selected from among the anti-idiotypes by their ability to deplete glucocorticoid receptor from rat liver cytosol. The anti-idiotype antibodies were first immobilized on beads of Sepharose coupled to goat anti-mouse Ig. After incubation of the coated beads with rat liver cytosol, unbound receptor was assayed by measuring binding of [3H]TA.

Of the anti-idiotype antibodies tested, only that of clone 8G11, an IgM, depleted the liver cytosol of the glucocorticoid receptor. As can be seen from Table II, rat liver cytosol (diluted 2 times) treated with 8G11 antibody immobilized on Sepharose anti-mouse IgM beads was depleted of 15% of its high affinity [3H]TA-binding capacity. The extent of receptor depletion by 8G11 was increased from 15 to 40% by diluting the cytosol fraction 4-fold, i.e., counts bound remained about the same. In contrast, neither the untreated nor the beads coated with antibody from hybridomas 5B5 had any significant effect on the [3H]TA-binding capacity of the liver cytosol.

Clone 8G11 was subcloned, and the clones 8G11-C5 and 8G11-C6 were derived from this line. Their respective antibodies were partially purified by precipitation in 50% saturated (NH_4)_2SO_4. From Table III, it can be seen that CNBr-Sepharose anti-mouse beads treated with basal medium containing 1% FCS or with PBS, or with partially purified antibody 5B5 or 5B5-B6, depleted liver cytosol [3H]TA binding capacity minimally (<10%). Partially purified antibody from 8G11, 8G11-C5, and 8G11-C6 depleted rat liver cytosol of 61, 59, and 35% of its [3H]TA binding capacity, respectively. As more [3H]TA receptor was removed from rat liver cytosol by 8G11-C6 than by 8G11-C5, it was used in all the remaining characterization studies.

Preincubation of rat liver cytosol with either 2.5, 13, or 50 nM [3H]TA (2 h at 4 °C) had no significant effect on depletion of receptor by the anti-idiotype bound to CNBr-Sepharose beads (compare Tables III and IV). (As in the earlier experiments, the 5B5-B6 preparations were inactive.) These results indicate that occupancy of the receptor-binding site by TA does not hinder attachment of the anti-idiotype. The epitope recognized by the 8G11-C6 anti-idiotype, therefore, might be near but is not in the ligand-binding site of the receptor. This was further confirmed by the finding that preincubation of
The effect of relabeling of rat liver cytosol with various concentrations of [3H]TA on depletion of glucocorticoid receptor by auto-anti-idiotypic antibodies

The methods are described in the legend of Table II. The results are means of duplicate assays for [3H]TA (cpm) in solution after removal of the Sepharose-bound constituents. The parental hybridomas are 5B5 and 8G11. The subclones are designated by -B6, -C6, -C5.

<table>
<thead>
<tr>
<th>Additions</th>
<th>cpm</th>
<th>% depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PBS</td>
<td>2328</td>
<td>0</td>
</tr>
<tr>
<td>+ BM + 1% FCS</td>
<td>2210</td>
<td>5</td>
</tr>
<tr>
<td>+ 5B5</td>
<td>2115</td>
<td>9</td>
</tr>
<tr>
<td>+ 5B5-B6</td>
<td>2225</td>
<td>4</td>
</tr>
<tr>
<td>+ 8G11</td>
<td>304</td>
<td>61</td>
</tr>
<tr>
<td>+ 8G11-C6</td>
<td>961</td>
<td>59</td>
</tr>
<tr>
<td>+ 8G11-C5</td>
<td>1514</td>
<td>35</td>
</tr>
</tbody>
</table>

* 1% FCS was added to the basal medium as the hybridomas were grown in basal medium + 1% FCS.

TABLE V

Effect of anti-idiotypic on the binding of [3H]TA to the hepatic glucocorticoid receptor

50 μl of rat liver cytosol were preincubated for 2.5 h at 4°C with either PBS or partially purified by precipitation with 50% saturated (NH₄)₂SO₄ anti-idiotypic of 5B5-B6 or 8G11-C6. [3H]TA was then added to a final concentration of 3.7 × 10⁻⁸ M in a final volume of 200 μl, and the solution was incubated for further 2 h at 4°C. Free steroid was removed with charcoal-dextran as described in the legend of Fig. 1.

<table>
<thead>
<tr>
<th>Additions</th>
<th>2.5 nm [3H]TA</th>
<th>18.0 nm [3H]TA</th>
<th>50.0 nm [3H]TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>cpm % depleted</td>
<td>cpm % depleted</td>
<td>cpm % depleted</td>
</tr>
<tr>
<td>+ PBS</td>
<td>1124</td>
<td>1222</td>
<td>0</td>
</tr>
<tr>
<td>+ 5B5</td>
<td>1354</td>
<td>0</td>
<td>1354</td>
</tr>
<tr>
<td>+ 5B5-B6</td>
<td>503</td>
<td>55</td>
<td>961</td>
</tr>
<tr>
<td>+ 8G11</td>
<td>449</td>
<td>65</td>
<td>31</td>
</tr>
<tr>
<td>+ 8G11-C6</td>
<td>934</td>
<td>31</td>
<td>646</td>
</tr>
</tbody>
</table>

Receptor Antibodies

5099

The anti-idiotypic with the glucocorticoid receptor had no effect on the subsequent binding of [3H]TA to the glucocorticoid receptor (Table V).

Additional evidence of binding of the 8G11-C6 anti-idiotypic to the glucocorticoid receptor was obtained by sucrose density gradient analysis. Incubation of [3H]TA-labeled receptor with the anti-idiotypic caused a shift in and broadening of the sedimentation profile of the glucocorticoid receptor, indicating the presence of aggregates (Fig. 5). To confirm the inference of specific binding of the auto-anti-idiotypic, 8G11-C6, to the native receptor, the density gradient centrifugation experiment was repeated with an additional control, namely the auto-anti-idiotypic, 5B5-B6 which does not bind receptor. The presence of 5B5-B6 shifted the buoyant density of the receptor toward the top of the tube making the downward shift of the receptor in the presence of the auto-idiotypic, 8G11-C6 even more significant and confirming the specificity of the binding of the 8G11-C6 anti-idiotypic to the glucocorticoid receptor.

The anti-idiotypic of 8G11-C6 is an IgM with a molecular mass of 10^6 daltons. Under the centrifugation conditions used in the present study, the free antibody was found at the bottom of the tube, without any associated [3H]TA-labeled receptor. This implies that the receptor-antibody complex dissociated during centrifugation.

The Western blot of the glucocorticoid receptor with the anti-idiotypic (8G11-C6) showed a major reactive band having a molecular weight of about 87,000-90,000 (Fig. 6). The other major low molecular weight band seen was also found in the control samples that were treated with antibody-free medium or with unrelated IgM monoclonal antibodies.

Characterization of 8G11-C6 Anti-idiotypic—Fig. 7 depicts the concentration dependence of the binding of the anti-idiotypic (8G11-C6) to the Fab fragments of the rabbit anti-steroid antibodies. Preincubation with the ligand, triamcinolone acetonide, or with rabbit serum albumin did not inhibit this binding reaction. The steroid conjugate, TKH-RSA however, inhibited strongly (Fig. 8). (The carrier alone, i.e. RSA, did not inhibit.) Deoxycorticosterone-RSA also inhibited the binding of the anti-idiotypic to the Fab fragments (Fig. 9). In contrast cortisone-RSA, testosterone-RSA, and estradiol-17β-RSA inhibited poorly (Fig. 9). The pattern of inhibition of binding of the anti-idiotypic to antisteroid Fab is in accord with the relative affinities of the various steroids for the binding site of the antisteroid antibodies (cf. Figs. 4 and 9). The nonderivatized steroids, TA, dihydrotestosterone, and estradiol-17β had no effect on the binding reaction. In fact, they appeared to produce a slight activation of binding.

The possibility of homology in regions of the steroid-binding sites of the antisteroid antibodies and of the glucocorticoid receptor was examined by competition experiments. The results indicate that the binding of the anti-idiotypic, (8G11-C6), to antisteroid was completely inhibited by rat liver cytosol (Fig. 10). As it could be argued that substances in cytosol other than receptor might have caused this inhibition, the glucocorticoid receptor was partially purified (=3000-fold) from rat liver cytosol. As shown in Fig. 10, partially purified glucocorticoid receptor preparations almost completely inhibited the binding of the anti-idiotypic to Fab fragments of the antisteroid, even though protein content was 600-fold less than in the crude cytosol.

The anti-idiotypic antibody, 8G11-C6, bound to partially purified glucocorticoid receptor preparations (from rat liver cytosol) in a concentration-dependent manner as determined by ELISA (Fig. 11). The binding of the anti-idiotypic to receptor was inhibited by TKH-RSA but not by TA or rabbit serum albumin (Fig. 12). Binding was also inhibited by deoxycorticosterone-RSA (not shown). Dihydrotestosterone-RSA inhibited only slightly, estradiol-17β-RSA had no effect.

The patterns of inhibition of binding of the anti-idiotypic to either the antisteroid or to the glucocorticoid receptor by steroid-RSA conjugates were similar and dependent upon the steroid moiety of the steroid-RSA conjugate (Table VI). How-
Sucrose density gradient analysis of \[^3H\]TA-labeled glucocorticoid receptor. Rat liver cytosol was labeled with \(10^{-8}\) M \[^3H\]TA for 2 h at 4 °C and then incubated with either (a) PBS (0) or the auto-anti-idiotypic (8G11-C6) (Δ), or (b) PBS (0) or auto-anti-idiotypic 5B5-B6 (0) or 8G11-C6 (Δ). The experimental details are described in the text under “Materials and Methods.”
Receptor Antibodies

Fig. 8. The effects of TA, RSA and TKH-RSA conjugate on the binding of anti-steroid Fab fragments to the auto-anti-idiotypic (8Gll-C6). The experimental details are given in the text under "Materials and Methods." The inhibitors were preincubated with the Fab fragments for 10-20 min at room temperature and then added 1.045 pg of partially purified auto-anti-idiotype for 2 h at 37 °C. The results on the ordinate are given in units of absorbance at 492 nm as a function of the concentration of the steroid (a) and of RSA (b). ○, TA; •, TKH-RSA; △, RSA.

Fig. 9. Inhibition of the binding of the auto-anti-idiotype (8Gll-C6) to rabbit antisteroid Fab fragments by various steroid-RSA conjugates. The experimental details are given in the legend of Fig. 8 and in the text under "Materials and Methods." Idiotype-anti-idiotype interactions occurred close to the combining site of the idiotype. The results implied that this anti-idiotype might have ligand-like binding properties. Support for this inference was provided by the finding that it cross-reacted with the glucocorticoid receptor in crude rat liver preparations.

Fig. 10. Inhibition of the binding of the auto-anti-idiotype (8Gll-C6) to rabbit antisteroid Fab fragments by rat liver cytosol. Inhibition of the reaction was assessed with rat liver cytosol (△) (crude preparation of glucocorticoid receptor) or cytosol partially purified for the glucocorticoid receptor (■). Microtiter wells were coated with 10 ng of purified antisteroid Fab fragments to which were added 1.045 pg of the anti-idiotype (partially purified by precipitation with 50% saturated (NH4)2SO4) and of rat liver cytosol or the partially purified preparation of glucocorticoid receptor, serially diluted with PBS, as indicated on the abscissa. The mixture was incubated for 2 h at 37 °C, and the reaction was assessed by ELISA.

Fig. 11. Binding of the auto-anti-idiotype (8Gll-C6) to partially purified glucocorticoid receptor. Microtiter wells were coated by adding 200-μl aliquots each containing 1.08 pg (A) or 0.54 pg (•) in 0.1 M NaHCO3 (pH 9.3) of the partially purified receptor preparation to each well and incubating overnight at 4 °C. The wells were then washed 2 × with Tween-20 PBS, and serial dilutions of the anti-idiotype (purified by (NH4)2SO4 precipitation) were added to the wells, as indicated on the abscissa. The experimental details are given in the text under "Materials and Methods." The A492 values are the readouts of the ELISA assays.

Partially purified glucocorticoid receptor preparations inhibited binding of the anti-idiotype (8Gll-C6) to the anti-steroid antibody (idiotype). Moreover, binding of the anti-idiotype to partially purified glucocorticoid receptor was inhibited by triamcinolone-RSA conjugates. These results are consistent with affinity of the anti-idiotype for a site in the region of the steroid-binding RSA conjugates of the receptor. Also consistent with this conclusion was the pattern of inhibition by various steroid-RSA conjugates of the binding of the anti-idiotype (8Gll-C6) to purified glucocorticoid receptor, i.e. conjugates of steroids with the highest affinity for the receptor inhibited best. On the other hand, unlike the idiotype-anit-
idiotypic antibody. Thus, although the anti-idiotype interaction was binding site associated (as seen from the experiments with steroid conjugates: Fig. 12 and Table VI), the cross-reaction epicope on the receptor that is recognized by the anti-idiotypic antibody might be at a greater distance from the binding site than is the epitope it recognizes on the idiotypic antibody. Alternative possibilities are that the epitope is on a "reverse aspect" of the combining site of the receptor (38) or that the local conformation of the two combining sites (i.e., the anti-idiotype and the receptor) differ.

The anti-idiotype, 8G11-C6, was raised as a secondary response to idiotypic antibodies that were induced by TKH complexed to thyroglobulin. The binding of the anti-idiotype to the antisteroid was inhibited by the TKH-RSA, TKH peptides, and TKH with varying degrees of effectiveness, but not at all by TA. As TA is a low molecular weight compound it may not occupy all or even most of the total combining surface of the antisteroid. This is consistent with the possibility that the idiotope is near but not co-extensive with the combining site. Similarly Sepharose-immobilized anti-idiotype (8G11-C6) depleted [3H]TA-labeled as well as unlabeled receptor from rat liver cytosol. Further evidence of binding of the anti-idiotype to [3H]TA-labeled glucocorticoid receptor was obtained from the sucrose density gradient studies (Fig. 5, a and b). In addition, the Western blots in the present study showed that the anti-idiotype, 8G11-C6, interacted predominantly with a single rat liver cytosol of M, 87,000-90,000 (Fig. 6). This is consistent with the findings that purified transformed glucocorticoid receptor has a M, 78,000-90,000 as determined by SDS-polyacrylamide electrophoresis (1, 39-41).

Taken together the above data support the conclusion that the anti-idiotype of clone 8G11-C6 binds to the glucocorticoid receptor in a binding site-related manner. Therefore, this antibody may serve as an immunochemical reagent to purify the glucocorticoid receptor by affinity chromatography provided that there is no cross-reaction with other glucocorticoid-specific receptors. The auto-anti-idiotypic antibodies have other potential uses as well, including: 1) the possibility of assaying tumor cells for steroid receptors and their relative distributions between nucleus and cytoplasm, 2) an antibody system for the recognition of tumor cell surface antigens, 3) a system for the purification of tumor cell surface antigens, and 4) a system for the purification of antibody systems. These results support the conclusion that the anti-idiotype of clone 8G11-C6 binds to the glucocorticoid receptor in a binding site-related manner.

**TABLE VI**

<table>
<thead>
<tr>
<th>A comparison of the inhibition of binding of the anti-idiotype of 8G11-C6 to either the antisteroid or to the glucocorticoid receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I</strong>&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>A. Steroid-RSA conjugate</td>
</tr>
<tr>
<td>TKH-RSA</td>
</tr>
<tr>
<td>Deoxycorticosterone-RSA</td>
</tr>
<tr>
<td>Cortisone-RSA</td>
</tr>
<tr>
<td>Testosterone-RSA</td>
</tr>
<tr>
<td>Estradiol-17β-RSA</td>
</tr>
<tr>
<td>B. TKH-peptide conjugate</td>
</tr>
<tr>
<td>TKH Lysine</td>
</tr>
<tr>
<td>TKH Gly-Lys-Oh</td>
</tr>
<tr>
<td>TKH Lys-Lys-Gly-Oh</td>
</tr>
<tr>
<td>TKH Lys-Lys-Lys-Oh</td>
</tr>
<tr>
<td>TKH Ala-Ala-Ala-Ala-Oh (5L)</td>
</tr>
<tr>
<td>TKH Ala-Ala-Ala-Ala-Ala-Oh (6L)</td>
</tr>
<tr>
<td>TKH Ala-Ala-Ala-Ala-Ala-Oh (6L)</td>
</tr>
<tr>
<td>C. TKHS</td>
</tr>
</tbody>
</table>

**FIG. 12.** The effects of TA (●), RSA (▲), and TKH-RSA conjugate (○) on the binding of the auto-anti-idiotype (8G11-C6) to preparations containing partially purified glucocorticoid receptor. The experimental details are given in the text under "Materials and Methods." The partially purified glucocorticoid receptor preparations (0.45 μg of protein in 0.1 M NaHCO<sub>3</sub>, pH 9.3) were used to coat microtiter wells, as described in the legend of Fig. 11. The anti-idiotype was added in aliquots of 0.83 μg (partially purified by precipitation with 50% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) in 200 μl, 20 min after preincubation with serial dilutions of the various inhibitors, as indicated on the abscissa. The results on the ordinate are given in units of absorbance at 492 nm as a function of the concentration of the steroid (a) and of RSA (b).
and 2) the use of these antibodies to define various functional domains of purified receptors.

The auto-anti-idiotypic strategy has now been successfully used to prepare antibodies specific for receptors of acetylcholine (11), adenosine (42, 43), and glucocorticoids (present studies). Thus, it is a strategy that should be considered seriously for the production of anti-receptor antibodies, particularly in cases in which receptor is difficult to purify or is impractical. An example of the latter would be one in which the ligand, e.g. adenosine, binds to many kinds of macromolecules in addition to receptor.

REFERENCES