Characterization of a monoclonal antibody that probes the functional domains of the glucocorticoid receptor

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Monoclonal antibodies to the rat hepatic glucocorticoid receptor (GR) were produced by using 4000-fold-purified unactivated rat hepatic GR as the immunogen in an immunization *in vitro*. Hybridomas were screened for anti-GR antibody production by using an enzyme-linked immunosorbent assay. The antibody, ³A6, described here, is an IgM (λ). The interaction of ³A6 with the purified GR was explored by sedimentation analysis, where a shift of the 9 S GR to a form with a higher *s*_{20,w} value was demonstrated. Binding specificity and sensitivity were demonstrated by protein immunoblotting. ³A6 cross-reacted with all rat tissue glucocorticoid receptors (GRs) examined, except those of the brain. Species cross-reactivity was observed with other mammalian GRs (from human CEM-C7 cells and from pig and mouse liver). Immunocytochemical localization of the GR was assessed by indirect immunofluorescence in intact fixed cells, which demonstrated intense cytoplasmic staining in the absence of pretreatment with glucocorticoids and nuclear localization when cells were pretreated with glucocorticoids. This monoclonal antibody significantly inhibited steroid binding to unoccupied receptor and DNA binding of activated steroid–receptor complexes. Furthermore, preincubation of the purified activated GR complex with ³A6 prevented phosphorylation of the GR *in vitro*. Thus ³A6 differs from previous monoclonal antibodies to the GR in its capacity to cross-react with the human GR and by its specificity for an epitope on or near a functional domain of the GR.

INTRODUCTION

Glucocorticoid hormones influence a wide spectrum of tissues, eliciting unique responses specific to the target tissues or cell types involved (Munck *et al.*, 1984; Ringold, 1985). Glucocorticoid effects are mediated by binding to an intracellular receptor protein with high specificity and affinity. Cytoplasmic hormone-bound receptor complexes undergo a two-step process which ultimately leads to the transcription of specific genes. The first step, activation, involves a conformation change to a less negatively charged DNA-binding form. This activated complex undergoes a second step, translocation, which involves the movement of the activated complex from the cytoplasm to the nucleus, where it binds to acceptor sites within the chromatin (Ringold, 1985; Schmidt & Litwack, 1982).

Monoclonal antibodies have proven to be valuable tools for the study of biological processes (Milstein, 1980). Previous reports of monoclonal antibodies to the glucocorticoid receptor (GR) have demonstrated immunoactivity to the 'DNA modulation' domain of the receptor (Westphal *et al.*, 1982; Carlstedt-Duke *et al.*, 1982); however, no monoclonal antibodies which inhibit steroid binding or DNA binding have been developed.

In order to further define structural and functional

aspects of the GR, we have generated a monoclonal antibody immunoactive to the 'functional' domain of the GR using an immunization *in vitro*. We describe here the characterization of this monoclonal antibody, which will aid in further understanding the structure and function of this receptor, the apparent cellular location of the unoccupied receptor and provide insights into the mechanism of action of glucocorticoid hormones.

EXPERIMENTAL PROCEDURES

Chemical reagents and buffers

[6,7-³H]Triamcinolone acetonide ([³H]TA), (44 Ci/mmol), [1,2-³H]dihydrotestosterone (123 Ci/mmol), [6,7-³H]β-oestradiol (54 Ci/mmol), [1,2-³H]progesterone (84 Ci/mmol), [1,2-³H]aldosterone (81 Ci/mmol), [1,2-³H]cortisol (91.5 Ci/mmol) and [γ-³²P]ATP [tetra(triethyl)ammonium salt; aqueous solution; 2900 Ci/mmol] were obtained from New England Nuclear, Boston, MA, U.S.A. Non-radioactive steroids were from Sigma, St. Louis, MO, U.S.A. Agarose (Bio-Gel A1.5m), hydroxyapatite (HAP; DNA grade), all reagents for the Bradford protein assay and SDS/polyacrylamide-gel electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. Sephacryl G-200 and

Trivial names and abbreviations used: [³H]TA, [6,7-³H]triamcinolone acetonide (9α-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione); [γ-³²P]ATP, adenosine 5'-[γ-³²P]triphosphate; PBS, phosphate-buffered saline (g/l; anhydrous CaCl₂, 0.1; KCl, 0.2; KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; NaCl, 8.0; Na₂HPO₄·7H₂O, 2.16), pH 7.4; BSA, bovine serum albumin; DTT, dithiothreitol; pristane, 2,6,10,14-tetramethylpentadecane; ABTS, 2,2-azinodi-(3-ethylbenzthiazolinesulphonic acid); HAP, hydroxyapatite; GR, glucocorticoid receptor; e.l.i.s.a., enzyme-linked immunosorbent assay; (S)DMEM, (supplemented) Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; NGS, normal goat serum; HAT selection medium, hypoxanthine (100 μM)/thymidine (16 μM)/aminopterin (0.4 M)/glycine (3 μM) (to volume with DMEM).

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electrophoresis low- M_r protein standards were from Pharmacia, Piscataway, NJ, U.S.A.; DNA-cellulose was from P-L Biochemicals, Milwaukee, WI, U.S.A.; DEAE-cellulose (Whatman DE52) was obtained from Thomas Scientific, Philadelphia, PA, U.S.A., and nitrocellulose membranes (0.45 μ m pore size) from Schleicher and Schuell, Keene, NH, U.S.A. Peroxidase-conjugated goat anti-mouse IgM, peroxidase-conjugated goat anti-mouse IgM+IgG+IgA and rhodamine-conjugated goat anti-mouse IgM were purchased from Kirkegaard and Perry, Gaithersburg, MD, U.S.A. Vectastain ABC kit was from Vector Laboratories, Burlingame, CA, U.S.A. MOPC 104.E purified mouse IgM (λ) was purchased from Litton Bionetics, Charleston, SC, U.S.A., and used as non-specific antibody (NsIgM). Saponin was obtained from Kodak Laboratories, Rochester, NY, U.S.A. All tissue-culture medium and supplements were from Hazleton Dutchland, Denver, PA, U.S.A. All chemicals were reagent grade and were obtained from Fisher Scientific Co., King of Prussia, PA, U.S.A.

Buffers used were: (A) 50 mM-potassium phosphate/10 mM- Na_2MoO_4 /10 mM-thioglycerol, pH 7.0; (B) 10 mM-potassium phosphate/10 mM- Na_2MoO_4 /10 mM-thioglycerol/10% (v/v) glycerol, pH 7.0; (C) 10 mM-Mes/0.1 mM-DTT/10% glycerol, pH 6.8 at 0 °C.

Preparation of cytosols

Tissue cytosols (brain, testes, thymus, spleen, liver, kidney cortex, heart and lung) were prepared from adrenalectomized male rats (150–200 g) as described previously (Webb *et al.*, 1985). Distal- and proximal-colon cytosols were prepared as described previously (Bastl *et al.*, 1984). Uterine tissue was obtained from young female Sprague-Dawley rats and prepared as described above.

CEM-C7 cells (Norman & Thompson, 1977) were grown in DMEM supplemented with 10% (v/v) FCS (SDMEM). DMEM contained glucose (4.5 g/l) supplemented with 26 mM- NaHCO_3 , 18 mM-Hepes, 10 mM-MEM non-essential amino acids, 1 mM-sodium pyruvate, 50 μ M- β -mercaptoethanol, 30 μ M-hypoxanthine, 3 μ M-thymidine and 2 mM-glutamine. Steroid-stripped FCS was substituted for FCS 24 h before experimentation. CEM-C7 cell cytosol was prepared by harvesting cells from ten T-75 flasks by centrifugation, washing with PBS and homogenizing in Buffer A with 5 mM-DTT.

Receptor purification

The rat hepatic GR was purified by using a large-scale modification of the three-step procedure which our laboratory has developed for the purification of the unactivated GR (Grandics *et al.*, 1984). Large-scale modification of this procedure was necessary in order to obtain sufficient quantities of the highly purified GR. All chromatographic procedures were conducted at 4 °C, and after each purification step a sample of the pooled peak was assayed for specific binding of [^3H]TA by the HAP technique (Erdos *et al.*, 1970; Norman & Thompson, 1977), DNA-cellulose binding (Kalimi *et al.*, 1975; Alberts & Herrick, 1971) and protein concentration (Bradford, 1976). A 200 ml portion of rat hepatic cytosol was incubated with 75 ml of a deoxycorticosterone-derivatized agarose for 2 h at 0–4 °C with gentle stirring. The resin was washed with Buffer A, then filtered on a

Teflon filter. This was repeated ten times to remove non-specifically absorbed proteins. Receptors were eluted from the resin by the addition of an equivalent volume of 2 μ M-(200 nM-[^3H]- + 1.8 μ M non-radioactive) TA in Buffer B overnight at 0–4 °C. The eluted receptors (affinity-gel eluate) were obtained by vacuum filtration. The affinity-gel eluate (~100 ml) was then loaded on to a Bio-Gel A1.5 m column (2-litre bed volume; 5 cm $\times$ 100 cm) pre-equilibrated with Buffer A. Fractions (9 ml) were collected and portions were measured for radioactivity. The receptor was eluted as a single peak of radioactivity. Peak fractions were pooled, pumped on to a DEAE-cellulose column (100 ml bed volume; 2.5 cm $\times$ 50 cm) and the unactivated GR complexes were eluted with a linear salt gradient (50–500 mM-potassium phosphate, containing 10 mM-sodium molybdate and 10 mM-thioglycerol, pH 7.0 at 4 °C). The unactivated receptor complexes were eluted as a single peak at 250 mM-potassium phosphate. Peak fractions were pooled and frozen in liquid N_2 or used immediately. For immunization, the DEAE-cellulose peak fractions were concentrated by vacuum dialysis.

Immunization *in vitro*

Immunization in culture was performed as previously described by using thymocyte-conditioned medium (Reading, 1982; Pardue *et al.*, 1983).

Hybridization was done as described previously (Reading, 1982; Kohler & Milstein, 1976).

E.l.i.s.a.

To detect anti-GR antibody, an e.l.i.s.a. modified from a previously published procedure was performed (Voller *et al.*, 1980). A portion (100 ml) of purified GR, (100–300 ng) was added to each well in an e.l.i.s.a. plate and incubated overnight at 4 °C. Antigen was reapplied twice to increase the concentration of the GR to 300–900 ng/well. Plates were washed five times with PBS containing 0.02% Tween 20 (PBS/Tween). Non-specific binding sites were blocked with a solution of 1% BSA in PBS overnight at 4 °C. Plates were washed twice, and 100 μ l of conditioned supernatant from hybridoma cells were added to each well and to a control well containing BSA as a non-specific protein antigen. Medium for the SP2/0 cells was used as a negative antibody control. Plates were incubated for 3–4 h at room temperature and then washed five times with PBS/Tween. Peroxidase-labelled goat anti-mouse IgG/IgM/IgA was diluted 1:500 in PBS containing 10% NGS and 1% BSA; 100 μ l portions were added to each well and incubated for 3–4 h at room temperature. Plates were washed five times in PBS/Tween and colour development was obtained by allowing wells to react with a peroxidase substrate solution, ABTS/ H_2O_2 . Absorbance was measured by using a Biotek spectrophotometer at 405 nm.

To ascertain the cross-reactivity of the monoclonal antibody with other rat tissue GR, portions (100 μ l) of equivalent concentrations of GR (0.02–1.0 pmol/mg) of the appropriately diluted tissue cytosol were applied as antigen and the e.l.i.s.a. was performed as described above.

Determination of the affinity constant

The affinity constant was also estimated by an e.l.i.s.a. (Carson *et al.*, 1983; Makikainen & Lehtonen, 1984). To determine the affinity constant, background absorbance

from the control wells was subtracted from the absorbance of the $^3\text{A6}$ wells to generate specific absorbance. The reciprocals of the specific absorbance and of $^3\text{A6}$ concentrations were analysed by linear regression and plotted (double-reciprocal plot; Lineweaver & Burk, 1934).

Cloning and expansion of hybridomas

A monoclonal-specific-antibody-secreting line was established by cloning under conditions of limiting dilution using non-immune spleen cells as a feeder layer (Kennett, 1980). Larger amounts of monoclonal antibodies were generated as ascites in Balb/c mice pretreated with pristane 7 days before injection of hybridoma cells (2×10^7 cells/ml in $200 \mu\text{l}$ of PBS). Ascites fluid was purified as described previously (Oxenham *et al.*, 1984).

Classification of antibody

Class and subclass of the monoclonal antibody purified from ascites were verified using the Ouchterlony technique as described previously (Oxenham *et al.*, 1984). Also, a mouse immunoglobulin subtype identification kit was utilized in an e.l.i.s.a. according to the directions supplied by the manufacturer (Boehringer-Mannheim Biochemicals).

Specific binding of tritiated steroids

Specific glucocorticoid binding in the appropriate tissue or cell cytosols was determined by incubating with 50 nM - ^3H]TA for 2 h at 0°C in the presence or absence of a 1000-fold excess of non-radioactive competing steroid (Schmidt *et al.*, 1984). Aldosterone receptors were measured in the distal colon cytosol as previously described (Schulman *et al.*, 1985). Briefly, the GRs in the distal-colon cytosol were saturated first by incubating for 20 min at 0°C with RU26988, a synthetic glucocorticoid that does not bind to the aldosterone receptor (Teutsch *et al.*, 1981). Distal-colon cytosol was incubated with 50 nM - ^3H]aldosterone at 0°C for 1 h, in the presence or absence of non-radioactive competing steroid (Schulman *et al.*, 1985). Other hormone-receptor complexes (those of oestrogen, progesterone and androgen) and serum transcortin were labelled as described previously (Schmidt *et al.*, 1984).

Tissue or cell cytosols were incubated with $^3\text{A6}$, NsIgM or Buffer A for 2 h at 0°C . At this time the samples were labelled with the appropriate tritiated ligand in the presence or absence of an excess of non-radioactive competing steroid. Specific steroid binding was determined using the HAP technique described by Erdos *et al.* (1970).

DNA-cellulose binding assay

Binding of GR complexes to DNA-cellulose was determined as described previously (Alberts & Herrick, 1971; Kalimi *et al.*, 1985). This assay was used to distinguish unactivated from activated GR complexes.

Rat liver cytosol prepared in Buffer A without Na_2MoO_4 was labelled with ^3H]TA for 2 h at 0°C . Portions ($250 \mu\text{l}$) of the GR complexes were incubated with $250 \mu\text{l}$ of either $^3\text{A6}$, NsIgM or buffer overnight at 0°C . Samples were then warmed at 25°C for 30 min to thermally activate the GR complexes and the specific ligand binding and DNA-cellulose binding were assessed.

In a parallel experiment, GR complexes were thermally activated before incubation with $^3\text{A6}$, NsIgM or buffer.

Sucrose-gradient ultracentrifugation

Partially purified GR (approx. 600-fold through the affinity-gel and Bio-Gel steps) was incubated for 3 h at 0°C with either $^3\text{A6}$ or NsIgM. A $200 \mu\text{l}$ portion of each sample was layered on a 5 ml 5–20%-(w/v)-sucrose density gradient in Buffer B containing BSA (2 mg/ml). After centrifugation at 4°C for 16 h at $100000 g$ in a SW 50 rotor, the samples were assayed by puncturing the bottom of the nitrocellulose tubes. Three-drop fractions were collected and dissolved in 5 ml of Liquiscint scintillation fluid. BSA (4.6 S) and catalase (11.2 S) were run as standards, and the positions of these protein standards were determined by measuring absorbance at 280 nm. Sedimentation coefficients were determined as described by Martin & Ames (1961).

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Electrophoresis was performed at 4°C using 1.5 mm-thick linear 10%-polyacrylamide slab gels. Receptor samples were concentrated by precipitation with trichloroacetic acid as described previously (Grandics *et al.*, 1984; Oraffell, 1975). The M_r standards were phosphorylase *b* (97000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α -lactalbumin (14400). A highly sensitive double stain of Coomassie Blue and silver was employed to detect proteins on the gel (Morrisey, 1981; Irie *et al.*, 1982). After electrophoresis, proteins were transferred from the polyacrylamide gel to nitrocellulose paper and immunoblotted as previously described (Towbin *et al.*, 1979; Burnette, 1981).

Inhibition of phosphorylation

GR was phosphorylated *in vitro* as described previously (Miller-Diener *et al.*, 1985). Purified GR was eluted from a Sephadex G-75 column with Buffer C to remove Na_2MoO_4 . Peak radioactivity fractions were concentrated 5–10-fold by centrifugation on Amicon Centricon 30 filters. The GR complexes were thermally activated (25°C for 30 min), loaded on to a DEAE-cellulose column and eluted with a 5–500 mM-KCl gradient. A portion ($100 \mu\text{l}$) of the activated peak (0.3 pmol) was incubated with $100 \mu\text{l}$ of either $^3\text{A6}$, NsIgM or buffer C without NaMoO_4 , for 2 h at 0°C . Samples were then incubated with 50 pmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $100 \mu\text{M}$ - CaCl_2 at 25°C for 30 min. The reaction was stopped by trichloroacetic acid precipitation.

Samples were electrophoresed on 10%-SDS/polyacrylamide slab gels and stained as described previously (Morrisey, 1981; Irie *et al.*, 1982). They were then washed with distilled water for 15 min, processed with Autofluor for 2 h at room temperature and autoradiographed for 12 h at -70°C . No intensifying screens were used.

Immunofluorescence

Human CEM-C7 cells (2×10^6) were placed into T-25 flasks containing 4 ml of Joklik's medium and incubated in the presence or absence of $1 \times 10^{-6} \text{ M}$ non-radioactive TA for 10 min at 37°C as previously described (Govindan, 1980; Papamichail *et al.*, 1980). Portions

Fig. 1. Electrophoretogram of purified GR

Lane 1, standard M_r proteins: phosphorylase *b* (M_r 97000); bovine serum albumin (M_r 67000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 30000); soybean trypsin inhibitor (M_r 20100); α -lactalbumin (M_r 14000). Lane 2, GR purified 4000-fold and stained with Coomassie Blue and silver.

(1.5×10^5 cells/ml) were centrifuged for 6 min at 1400 *g* on to glass slides using a Shandon cytocentrifuge. The intact cells were fixed and permeabilized using a glutaraldehyde/borohydride/saponin procedure described previously (Willingham, 1983). The fixed permeabilized cells were processed for immunofluorescence.

Miscellaneous

Proteins were measured by the Bradford (1976) method. Radioactivity was measured in an Inter-technique model SL 30 spectrometer with an average counting efficiency of 30% for tritium. Conductivity was determined on a Markson model 10 conductivity meter.

RESULTS

Receptor purification

A large-scale version of the purification of the unactivated GR (Grandics *et al.*, 1984) enabled us to produce larger quantities (120–300 μ g) of the highly purified receptor with each preparation. The receptor was purified approx. 4000-fold as calculated by specific radioactivity. By using a highly sensitive double stain

Fig. 2 Sedimentation profile of GR in the presence or absence of specific antibody

Unactivated GR (partially purified 600-fold) was incubated with 3 A6 for 3 h at 0°C and analysed by sucrose-density-gradient ultracentrifugation as described in the Experimental procedures section. + Ab, GR- 3 A6 complex (●); - Ab, GR-NsIgM complex (○). The standard proteins were BSA (4.6 S) and catalase (11.2 S).

Table 1. Cross-reactivity of 3 A6 demonstrated by e.l.i.s.a.

Results are means for three experiments.

Antigen source*	GR (pmol/mg)†	A_{405} ‡
Rat tissues		
Liver	0.16	0.643
Kidney	0.19	0.594
Heart	0.02	0.458
Brain	0.09	0.067
Lung	0.12	0.431
Distal colon	0.59	0.462
Proximal colon	0.98	0.715
Spleen	0.06	0.428
Thymus	0.13	0.376
Other species		
Pig liver	0.10	0.364
Mouse liver	0.15	0.471
Human (CEM-C7 cells)	0.90	0.471

* Cytosols from all tissues were diluted to give normalized glucocorticoid receptor concentrations.

† pmol of GR [based on specific bindings of (3 H)TA d.p.m./100 μ l]/total cytosolic protein (mg)] as determined by the Bio-Rad protein assay.

‡ The e.l.i.s.a. values represent changes in receptor concentration and not simply variations in the e.l.i.s.a. assay, since measurements were from the linear portion of the absorbance-versus-receptor-concentration curve.

of Coomassie Blue and silver, a predominant band of M_r 94000 was detected (Fig. 1).

Characterization of the hybridoma

After propagation of hybridomas in HAT selection medium 1, the hybrids were screened for the production of anti-GR antibody using an e.l.i.s.a., as described in the

Fig. 3. Immunoblot analysis of purified GR with monoclonal 3A6

Purified GR (10 $\mu\text{g/ml}$) was electrophoresed on SDS/10%-(w/v)-polyacrylamide gel, transferred to nitrocellulose paper and incubated with 3A6 (50 $\mu\text{g/ml}$) (lane 1) or NsIgM (50 $\mu\text{g/ml}$) (lane 2). The blots were incubated with a horseradish peroxidase-labelled goat anti-mouse IgM (1:500) and allowed to react with a peroxidase substrate until colour developed.

Experimental procedures section. Of the total number of wells, 240 (33%) contained hybridomas. By using 4000-fold-purified GR as the antigen, 42 hybridomas (17%) gave positive results after the first screening. After two additional screenings, five clones remained strong positives. One of these clones, 3A6 , was recloned under conditions of limited dilution and rescreened. Characterization of 3A6 using class-, subclass- and chain-specific anti-mouse immunoglobulins revealed that 3A6 was an IgM (λ). The affinity (K_d) of 3A6 for the GR was determined to be 1.1×10^{-8} M (results not shown).

Sedimentation of the antibody-receptor complex

The interaction of 3A6 with the GR was explored by using sucrose-density-gradient analysis, since this is a classical receptor identification technique (Toft & Sherman, 1975). As demonstrated in Fig. 2, after centrifugation at 100000 g for 16 h the sedimentation constant of the 3A6 -GR complexes was 19 S, whereas in the absence of 3A6 antibody the receptor sedimented as a 9 S protein. A 10-fold increase (relative to control) in radioactivity was observed for the 3A6 -GR complexes at the 19 S position.

Tissue and species cross-reactivity

The presence of GRs has been demonstrated in nearly all tissues examined to date (Ballard *et al.*, 1973; Antakly *et al.*, 1985). The data in Table 1 shows that 3A6 recognized GR from rat liver, kidney cortex, thymus,

spleen, heart, distal-colon and proximal-colon cytosols. However, 3A6 did not cross-react with GR from the rat brain.

In addition, antibody affinity for other mammalian GRs was elicited. Table 1 illustrates that 3A6 cross-reacts with GR from mouse liver, human CEM-C7 cell and pig liver cytosols.

Immunoblotting of the GR with 3A6

Rat liver GR was purified (Grandics *et al.*, 1984), electrophoresed on SDS/polyacrylamide gels and immunoblotted as described in the Experimental procedures section. The immunoblot shown in Fig. 3 revealed that, in the presence of 3A6 , a single band of M_r 94000 identical with the steroid-binding subunit of the GR was identified, whereas a duplicate lane incubated with NsIgM antibody demonstrated no immunoreactivity.

Immunofluorescence microscopy

Immunofluorescence microscopy using 3A6 with CEM-C7 cell preparations as described in the Experimental procedures section is shown in Fig. 4. When whole cells were incubated in the absence of TA, the immunofluorescence staining obtained with 3A6 was exclusively cytoplasmic; however, in the presence of 10^{-6} M-TA for 10 min at 37 $^{\circ}\text{C}$, the immunofluorescence is located exclusively in the nucleus and is no longer detected in the cytoplasm. The intense fluorescence demonstrated with the specific antibody indeed represents specific binding to the GR.

Effects of 3A6 on steroid binding

We decided to determine whether 3A6 was binding to an epitope on or near the steroid-binding site of the GR. As shown in Table 2, the specific binding of [^3H]TA was significantly inhibited by preincubation of unbound rat hepatic or CEM-C7 cell cytosols with 3A6 . When a non-specific antibody was used in a parallel incubation, no inhibition of binding was detected. The dose-response curve for the inhibition of [^3H]TA binding to rat hepatic receptors is depicted in Fig. 5.

In order to verify the specificity of 3A6 for the GR, the interaction with other steroid receptors and serum transcortin was examined. An e.l.i.s.a. could not be utilized, since the presence of GR in these crude tissue cytosols would have elicited a false positive reaction, and purification of the other steroid receptors would be a vast undertaking. To test its effect on the ligand binding of other steroid receptors and serum transcortin, a steroid inhibition assay was utilized. As demonstrated in Table 3, preincubation of 3A6 with other rat steroid receptors (aldosterone, androgen, oestrogen, and progesterone) or serum transcortin had no inhibitory effect on the subsequent binding of the appropriate ligand. These data indicated that 3A6 did not block ligand binding with the other receptors tested or with serum transcortin.

Effects of 3A6 on phosphorylation

Since the purified activated [^3H]TA-GR preparation has been reported to contain kinase activity (Miller-Diener *et al.*, 1985), it was crucial to determine whether 3A6 could affect this activity. As shown in the autoradiograph in Fig. 6, preincubation of this purified activated GR preparation with 3A6 resulted in complete inhibition of phosphorylation of the receptor, whereas

Fig. 4. Fluorescent micrographs of CEM-C7 cells

Cultured CEM-C7 cells were incubated in the absence (*a, b* and *c*) or presence (*d* and *e*) of hormone (1×10^{-6} M-TA) for 10 min at 37 °C. Cells were fixed and permeabilized using a glutaraldehyde/borohydride/saponin procedure described in the Experimental procedures section. Cells were incubated with 3 A6 (*b, c* and *d*) or NsIgM (*a* and *e*) and labelled indirectly with rhodamine-labelled goat anti-mouse IgM.

Table 2. Monoclonal antibody inhibition of specific steroid binding in rat liver or CEM-C7 cytosols

Rat liver or CEM-C7 cell cytosols were incubated with buffer A, NsIgM or 3 A6 (approx. 200 molecules of monoclonal antibody per molecule of GR) at 0 °C for 30 min. The preincubated cytosol was incubated for 2 h with 50 nM-[3 H]TA in the presence or absence of a 500-fold excess of non-radioactive TA. Specific binding was determined by using the HAP technique. All values represent the mean of triplicate determinations. The s.d. was less than 5% unless otherwise noted. Abbreviations: RLC, rat liver cytosol; MAb, monoclonal antibody.

Expt.	Specific binding (d.p.m./100 μ l)	Specific steroid binding (%)*	Inhibition of steroid binding (%)†
RLC			
+ Buffer A	96 000	100	0
+ Control NsIgM	100 500	100	0
+ 3 A6 MAb	44 285	44	56
CEM-C7			
+ Buffer A	2700	100	0
+ Control NsIgM	2480	91	9
+ 3 A6 MAb	278	9	91

* Specific steroid binding = [(Specific binding RLC + experimental sample)/(specific binding RLC + NsIgM)] $\times$ 100.

† Inhibition of steroid binding (%) = 100 - specific steroid binding (%).

Fig. 5. Inhibitory effect of ³A6 on the binding of [³H]TA by rat liver GR

Rat liver cytosol was incubated with an appropriate concentration of ³A6 (▲) or NsIgM (○) for 2 h at 0 °C. Portions were labelled with 50 nM-[³H]TA in the presence and absence of 1000-fold excess of non-radioactive TA for 2 h at 0 °C. Specific binding was determined by using the HAP technique.

NsIgM or buffer had no effect. The resultant lack of ³²P incorporation could not be attributed to proteolysis of the GR by the ³A6 preparation since the GR appeared as the intact *M_r*-94000 steroid-binding entity on SDS/polyacrylamide-gel electrophoresis when incubated with ³A6, NsIgM or buffer alone (Fig. 7). In addition, scanning laser densitometry of the gel demonstrated equal intensity of GR, which indicated that the GR remained intact when preincubated as described above. Furthermore, proteolytic activity of the purified monoclonal-antibody preparation was not observed when [¹⁴C]casein was used as substrate (results not shown).

Effects of ³A6 on DNA-cellulose binding

Table 4 summarizes the results of two experimental conditions demonstrating the effect of ³A6 on the binding of GR complexes to DNA-cellulose. Preincubation of unactivated GR complexes with ³A6 before thermal activation (25 °C, 30 min) inhibited the subsequent DNA-cellulose binding of these complexes by approx. 38% relative to a NsIgM control. Likewise, when previously activated GR complexes were incubated with ³A6, 47% inhibition was obtained. Because equivalent inhibition of DNA binding was obtained when the antibody was added before, or after, heat activation, ³A6 must inhibit DNA binding without affecting the process of activation.

Fig. 6. Effects of ³A6 on phosphorylation of the purified activated GR

The activated GR peak was incubated with either ³A6, NsIgM or buffer C without NaMoO₄ for 2 h at 0 °C. Samples were incubated with 50 pmol of [³²P]ATP and 100 mM-CaCl₂ at 25 °C for 30 min. The reaction was stopped by trichloroacetic acid precipitation. Samples applied to each lane of an SDS/10%-polyacrylamide gel were: lane 1, GR + CaCl₂ + NsIgM; lane 2, GR + CaCl₂ + buffer C; lane 3, GR + CaCl₂ + ³A6. Autoradiograms were developed for 12 h at -70 °C.

Fig. 7. Electrophoretogram of purified activated GR

The activated GR peak was preincubated with either buffer C without NaMoO₄ (lane 1), NsIgM (lane 2) or ³A6 (lane 3) for 2 h at 0 °C. Samples were boiled for 5 min at 100 °C in Laemmli sample buffer, electrophoresed on a 1.5 mm-thick SDS/10%-polyacrylamide slab gel and double stained with Coomassie Blue and silver.

Table 3. Effects of monoclonal antibody on ligand binding

Tissue cytosols were incubated with $^3\text{A6}$, NsIgM or buffer A for 2 h at 0 °C. After the preincubation, portions were labelled with the appropriate tritiated ligand in the presence or absence of an excess of non-radioactive competing steroid. Specific binding was determined using the hydroxyapatite technique. All values are means.

Expt.		Specific binding ($\pm$ S.D.) (d.p.m./100 μ l)	Specific steroid binding (%)*	Inhibition of steroid binding (%)†
A.	^3H]TA binding to rat hepatic cytosol			
	Buffer A	—	—	—
	NsIgM	82 139	100	4
	$^3\text{A6}$	32 919	40	60
B.	[6,7- ^3H]Oestradiol binding to uterine receptors			
	Buffer A	19 415 ($\pm$ 268)	—	—
	NsIgM	22 814 ($\pm$ 663)	100	0
	$^3\text{A6}$	23 217 ($\pm$ 173)	101	0
C.	[1,2- ^3H]Progesterone binding to uterine receptors			
	Buffer A	67 113 ($\pm$ 996)	—	—
	NsIgM	81 327 ($\pm$ 3336)	100	0
	$^3\text{A6}$	68 049 ($\pm$ 4117)	84	16
D.	[1,2- ^3H]Dihydrotestosterone binding to testicular receptors			
	Buffer A	26 868 ($\pm$ 872)	—	—
	NsIgM	27 144 ($\pm$ 1058)	100	0
	$^3\text{A6}$	41 814 ($\pm$ 116)	154	0
E.	[1,2- ^3H]Cortisol binding to serum transcortin			
	NsIgM	21 003 ($\pm$ 454)	100	0
	$^3\text{A6}$	25 564 ($\pm$ 886)	121	0
F.	[1,2- ^3H]Aldosterone binding to distal-colon receptors			
	Buffer A	19 591 ($\pm$ 331)	—	—
	NsIgM	21 832 ($\pm$ 835)	100	0
	$^3\text{A6}$	24 $\pm$ 202 ($\pm$ 585)	110	0

* Specific steroid binding (%) = [(Specific binding of sample)/(specific binding of control NsIgM)] $\times$ 100.

† Inhibition of steroid binding (%) = 100-specific binding (%).

Table 4. Effects of monoclonal antibody on DNA-cellulose binding

Data are means for three experiments. The S.D. was less than 5% unless otherwise noted. Abbreviations: RLC, rat liver cytosol; MAb, monoclonal antibody

Expt.	Specific [^3H]TA binding (d.p.m./100 μ l)	DNA-cellulose binding (d.p.m./100 μ l)	Binding to DNA-cellulose (%)*	Inhibition of DNA-cellulose binding (%)†
A. UA-RLC‡				
+ Control NsIgM	75 410	44 687	59	0
+ $^3\text{A6}$ anti-GR MAb	76 951	29 134	37	38
B. ACT-RLC§				
+ Control NsIgM	79 281	23 871	30	0
+ $^3\text{A6}$ anti-GR MAb	83 724	13 895	16	47

* Binding to DNA-cellulose (%) = {[DNA-cellulose bound [^3H]TA (d.p.m.100 μ l)]/[specific [^3H]TA binding (d.p.m./100 μ l)]} $\times$ 100.

† Inhibition of DNA-cellulose binding (%) = 100 [(RLC+experimental sample bound to DNA-cellulose)/(RLC+NsIgM bound to DNA-cellulose)].

‡ Unactivated (UA) GR complexes were preincubated with $^3\text{A6}$ or control antibody (NsIgM) before thermal activation (25 °C, 30 min).

§ Activated (ACT) GR complexes.

DISCUSSION

The unactivated rat hepatic GR was purified 4000-fold as previously described (Grandics *et al.*, 1984). This purification method entailed a three-step procedure involving affinity chromatography, agarose-gel filtration, and DEAE-cellulose chromatography. The purified GR (45 μ g) was used as the immunogen for an immunization *in vitro*. An immunization *in vitro* was chosen because this methodology allows the production of monoclonal antibodies against highly conserved proteins that by standards *in vivo* may be weakly antigenic. Furthermore, nanogram amounts of soluble protein antigens are sufficient quantities to produce specific antibody detectable within 2–4 weeks after fusion (Reading, 1982; Pardue *et al.*, 1983).

As shown in Fig. 2, 3 A6 was able to interact directly with the unactivated GR to produce a shift on sucrose density gradients of the 9 S GR to a 19 S GR- 3 A6 complex. This observed alteration of the sedimentation property of the GR has been a critical method of demonstrating specific anti-GR antibody (Westphal *et al.*, 1982; Okret *et al.*, 1984; Toft & Sherman, 1975). Furthermore, the ability of 3 A6 to stabilize the steroid-receptor complex, observed as less steroid dissociation in the presence of 3 A6, is consistent with our suggestion that 3 A6 may recognize an epitope in close proximity to the ligand-binding site.

In addition, an immunoblotting technique was used to demonstrate the specificity of 3 A6 for a protein of M_r 94000 previously characterized as the steroid-binding moiety of the GR. Upon presaturation of the cytosol with non-radioactive TA before incubation with the affinity resin, no detectable band of M_r 94000 was observed (Grandics *et al.*, 1984; Webb *et al.*, 1985).

Tissue cross-reactivity studies demonstrated that 3 A6 recognized the GR from all rat tissues tested with the exception of that from brain. This result differs from those obtained in other anti-GR monoclonal- or polyclonal-antibody cross-reactivity experiments (Westphal *et al.*, 1982; Okret *et al.*, 1984), where cross-reactivity with the brain was observed in addition to all other tissues tested. To evaluate this absence of cross-reactivity with the brain GR, the brain regions were analysed for immunoactivity with the antibody. Our preliminary data showed that 3 A6 reacts strongly with only the cerebellum and the anterior pituitary and is weakly positive or totally negative to the other regions tested (results not shown). These results suggest two possibilities: (1) that the antigenic determinant recognized by this antibody is present in specific brain regions but may be effectively diluted below the level of detection in whole-brain cytosol; or (2) the antigenic determinant is absent from, or is different in, other brain regions. DeKloet *et al.* (1975) have previously suggested that the hippocampal receptor is different from the traditional GR and have termed it a 'corticosterone receptor'.

Species cross-reactivity studies demonstrated that pig liver, human CEM-C7 cell and mouse liver receptors were recognized by 3 A6. Cross-reactivity with mammalian species has also been observed previously with polyclonal (Dougherty *et al.*, 1984; Renoir *et al.*, 1984) and monoclonal antibodies (Westphal *et al.*, 1982; Gametchu & Harrison, 1984; Okret *et al.*, 1984) raised against the GR. However, 3 A6 is the only reported anti-GR monoclonal antibody, to date, which demon-

strates immunoreactivity with the human GR, although it has been reported that monoclonal antibodies generated against the rabbit progesterone receptor (Logeat *et al.*, 1981) and bovine oestrogen receptor (Moncharmont *et al.*, 1982) interact with other mammalian receptors tested, including human receptor.

When rat aldosterone, oestrogen, progesterone and androgen receptors and rat serum transcortin were preincubated with 3 A6, no inhibition of steroid binding was observed. However, the binding of dihydrotestosterone to androgen receptors was stimulated. Since the genes for progesterone, oestrogen and glucocorticoid receptor have shown sequence homology, it is not unlikely that the androgen-receptor gene, when sequenced, will show regions similar to the glucocorticoid-receptor gene. Thus the possibility exists that 3 A6 may cross-react with the androgen receptor and stimulate ligand binding. The lack of cross-reactivity with other steroid-hormone receptors and serum transcortin has been reported with other anti-GR monoclonal (Okret *et al.*, 1984) and polyclonal (Eisen, 1980) antibodies. These data are consistent with the suggestion that the glucocorticoid receptors in different tissues and species contain analogous antigenic determinants, although they differ immunologically from other steroid-hormone receptors and serum transcortin (Okret *et al.*, 1981).

In the present study, immunofluorescence localization of the GR was examined. As demonstrated in Fig. 4, in the absence of hormone, the immunofluorescence was cytoplasmic, whereas in the presence of hormone, exclusively nuclear immunofluorescence was observed. Comparable immunofluorescence results were obtained by other investigators using polyclonal anti-GR antibodies (Govindan, 1980; Papamichail *et al.*, 1980). Also, analogous localization of the GR was observed with both polyclonal and monoclonal anti-GR antibodies using an immunoperoxidase technique (Antakly & Eisen, 1984). These immunocytochemical data corroborate the various biochemical studies which have demonstrated that the unoccupied receptor is a soluble cytoplasmic protein which, upon association with an active glucocorticoid, ultimately becomes bound to nuclear acceptor sites on the chromatin (Ringold, 1985). In contrast with the glucocorticoid receptor, recent evidence on the unoccupied oestrogen and progesterone receptors is suggestive of a primarily nuclear, rather than a cytoplasmic, location (King & Greene, 1984; Parrot-Appianet *et al.*, 1985; Welshons *et al.*, 1984).

In contrast with other monoclonal anti-GR antibodies (Westphal *et al.*, 1982; Okret *et al.*, 1984), this study demonstrated that 3 A6 significantly inhibited steroid binding and DNA-cellulose-binding of the GR. Although Eisen *et al.* (1985) have demonstrated that anti-GR antibody (BUGR-1) recognized a region associated with the DNA-binding domain of the receptor, this antibody did not significantly block the binding of activated GR to DNA-cellulose. Additional experiments are needed to examine further the structural and functional domains of the GR by immunoblotting receptor proteolytic digests with 3 A6. However, the biochemical data presented here suggest strongly that the epitope recognized by 3 A6 lies on, or close to, the 'functional' domain of the GR. Other investigators have reported monoclonal anti-GR antibodies directed against antigenic determinants located on the non-steroid binding and non-DNA-binding 'modulation' region of

the GR (Carlstedt-Duke *et al.*, 1982; Westphal *et al.*, 1982). In numerous studies with glucocorticoid-resistant and -sensitive cell lines, the absence of this 'modulation' domain was detected immunologically in certain glucocorticoid-resistant variant cells (Okret *et al.*, 1983; Westphal *et al.*, 1984). However, the development of this monoclonal antibody (³A6) which probes the 'functional domain' will be conducive to an immunological analysis of the structure and function of the GR not previously possible.

Our laboratory recently reported that the steroid-binding component of the activated rat hepatic glucocorticoid receptor undergoes Ca²⁺-stimulated phosphorylation *in vitro* (Miller-Diener *et al.*, 1985). This kinase activity is specifically inhibited by preincubation of the activated receptor with ³A6; however, no effect on the kinase activity is observed with NsIgM. Although the biochemical mechanism underlying this effect is presently unknown, it is tempting to speculate that the antibody induces a conformational change in the receptor which inhibits kinase activity, or in fact, binds at the receptor's ATP-binding site. This result, in addition to the inhibition of DNA-binding data also demonstrated that ³A6 was able to recognize the activated form of the GR in spite of the conformational change in the receptor which occurred upon activation, showing that the antigenic determinant remained immunoreactive.

Although this monoclonal antibody was generated using the unactivated GR, these data demonstrate that ³A6 interacts with both the unactivated and activated forms of the GR. Essentially, a ³A6 differs from other anti-GR monoclonal antibodies by its ability to interact with the human GR, its lack of cross-reactivity with rat brain GR and its inhibitory effects *in vitro* on the functional aspects of the GR. Thus this monoclonal antibody should prove to be a unique probe and an invaluable tool for future analysis of GR structure and function on both the cellular and molecular levels.

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REFERENCES

- Alberts, B. & Herrick, G. (1971) *Methods Enzymol.* **21**, 198-217
- Antakly, T. & Eisen, H. J. (1984) *Endocrinology* (Baltimore) **115**, 1984-1989
- Antakly, T., Sasaki, A., Liotta, A., Palkovits, M. & Krieger, D. (1985) *Science* **229**, 227-229
- Ballard, P. L., Baxter, J. D., Higgins, S. J., Rousseau, G. G. & Tomkins, G. M. (1973) *Endocrinology* (Baltimore) **94**, 998-1002
- Bastl, C. P., Barnett, C. A., Schmidt, T. J. & Litwack, G. (1984) *J. Biol. Chem.* **259**, 1186-1195
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195-203
- Carlstedt-Duke, J., Okret, S., Wrangé, O. & Gustafsson, J. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4260-4264
- Carson, S. D., Carson, S. M. & Konysberg, W. H. (1983) *J. Biol. Chem.* **258**, 9510-9513
- DeKloet, R., Wallach, G. & McEwen, B. S. (1975) *Endocrinology* (Baltimore) **96**, 598-609
- Dougherty, J. J., Puri, R. K. & Toft, D. O. (1984) *J. Biol. Chem.* **259**, 8004-8009
- Eisen, H. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3893-3897
- Eisen, L. P., Reichman, M. E., Thompson, E. B., Gametchu, B., Harrison, R. W. & Eisen, H. J. (1985) *J. Biol. Chem.* **260**, 11805-11810
- Erdos, T., Best-Belpomme, M. & Bessada, R. (1970) *Anal. Biochem.* **37**, 244-252
- Gametchu, B. & Harrison, R. W. (1984) *Endocrinology* (Baltimore) **114**, 274-279
- Govindan, M. V. (1980) *Exp. Cell Res.* **127**, 293-297
- Grandics, P., Miller-Diener, A. S., Schmidt, T. J., Mittman, D. & Litwack, G. (1984) *J. Biol. Chem.* **259**, 3173-3180
- Irie, S., Sezaki, M. & Kato, Y. (1982) *Anal. Biochem.* **126**, 350-354
- Kalimi, M., Colman, P. & Feigelson, P. (1975) *J. Biol. Chem.* **250**, 1080-1086
- Kennett, R. H. (1980) in *Monoclonal Antibodies* (Kennett, R. H. & McKern, T. J., eds.), pp. 365-375, Plenum Press, New York
- King, W. J. & Greene, G. L. (1984) *Nature* (London) **307**, 745-747
- Kohler, G. & Milstein, C. (1976) *Eur. J. Immunol.* **6**, 511-519
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 668-676
- Logeat, F., VuHai, M. T. & Milgrom, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1426-1430
- Makikainen, M. T. & Lehtonen, O. P. (1984) *J. Immun. Methods* **72**, 341-347
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372-1379
- Miller-Diener, A. S., Schmidt, T. J. & Litwack, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4003-4007
- Milstein, C. (1980) *Sci. Am.* **243**, 66-74
- Monchamont, B., Su, J.-L. & Parikh, I. (1982) *Biochemistry* **21**, 6916-6921
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310
- Munck, A., Guyre, P. M. & Holbrook, N. J. (1984) *Endocr. Rev.* **5**, 25-44
- Norman, M. R. & Thompson, E. B. (1977) *Cancer Res.* **37**, 3785-3791
- Okret, S., Carlstedt-Duke, J., Wrangé, O., Carlstrom, K. & Gustafsson, J.-A. (1981) *Biochem. Biophys. Acta* **677**, 205-219
- Okret, S., Stevens, Y.-W., Carlstedt-Duke, J., Wrangé, O., Gustafsson, J.-A. & Stevens, J. (1983) *Cancer Res.* **43**, 3127-3131
- Okret, S., Wilkstrom, A. C., Wrangé, O., Anderson, B. & Gustafsson, J.-A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1609-1613
- Oraffell, P. H. (1975) *J. Biol. Chem.* **250**, 4007-4021
- Oxenham, E. A., Matsumoto, K., Gasser, D. L. & Litwack, G. (1984) *Cancer Res.* **44**, 772-776
- Papamichail, M., Tsokos, G., Tsawdaroglou, N. & Sekeris, C. E. (1980) *Exp. Cell Res.* **125**, 490-493
- Pardue, R. L., Brady, R. L., Perry, G. W. & Dedman, J. R. (1983) *J. Cell. Biol.* **96**, 1149-1154
- Parrot-Appianet, M., Logeat, F., Groyer-Picard, M. T. & Milgrom, E. (1985) *Endocrinology* (Baltimore) **116**, 1473-1484
- Reading, C. L. (1982) *J. Immunol.* **53**, 261-291
- Renoir, J. M., Mester, J., Buchow, T., Catelli, M. G., Touhimaa, P., Binart, N., Joab, I., Radanyi, C. & Baulieu, E. E. (1984) *Biochem. J.* **217**, 685-692
- Ringold, G. M. (1985) *Annu. Rev. Pharmacol. Toxicol.* **25**, 259-266
- Schmidt, T. J. & Litwack, G. (1982) *Physiol. Rev.* **62**, 1131-1192

- Schmidt, T. J., Miller-Diener, A. & Litwack, G. (1984) *J. Biol. Chem.* **259**, 9536–9543
- Schulman, G., Miller-Diener, A., Litwack, G. & Bastl, C. (1985) *J. Biol. Chem.* **260**, 12102–12108
- Teutsch, G., Costerusse, G., Peraedt, R., Benzoni, J., Fortin, M. & Philibert, D. (1981) *Steroids* **38**, 651–665
- Toft, D. & Sherman, M. N. (1975) *Methods Enzymol.* **36**, 156–165
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Voller, A., Bidwell, D. & Bartlett, A. (1980) in *Manual of Clinical Immunology* (Rose, N. R. & Friedman, H., eds.), pp. 359–371, American Society for Microbiology, Washington, DC
- Webb, M. L., Miller-Diener, A. S. & Litwack, G. (1985) *Biochemistry* **24**, 1946–1952
- Welshons, W. V., Lieberman, M. E. & Gorski, J. (1984) *Nature (London)* **307**, 747–749
- Westphal, H. M., Moldenhauer, G. & Beato, M. (1982) *EMBO J.* **1**, 1467–1471
- Westphal, H. M., Mugele, K., Beato, M. & Gehring, U. (1984) *EMBO J.* **3**, 1493–1498
- Willingham, M. C. (1983) *J. Histochem. Cytochem.* **6**, 791–798

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