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Autoimmune anti-androgen–receptor antibodies in human serum

(prostate/steroid receptors/dimethylnortestosterone/immunoglobulins/sex difference)

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ABSTRACT **Circulating autoantibodies to human and rat androgen receptors are present at high titers in the blood sera of some patients with prostate diseases. The antibodies from some serum samples were associated with a purified IgG fraction and interacted with the 3.8S cytosolic androgen–receptor complexes of rat ventral prostate to form 9- to 12S units. Other serum samples, however, formed 14- to 19S units, suggesting that other immunoglobulins might be involved. In the presence of an anti-human immunoglobulin as a second antibody, the androgen–receptor–antibody complexes could be immunoprecipitated. The antibodies interacted with the nuclear and the cytosolic androgen–receptor complexes, either the DNA-binding or the nonbinding form, but not with receptors for estradiol, progestin, or dexamethasone from a variety of sources. Human testosterone/estradiol-binding globulin, rat epididymal androgen-binding protein, or rat prostate α-protein (a nonreceptor steroid-binding protein) also did not interact with the antibodies to form immunoprecipitates. About 37% of male and 3% of female serum samples screened had significant antibody titer. The chance of finding serum with a high titer is much better in males older than 66 years than in the younger males or females at all ages. The presence of the high-titer antibodies may make it possible to prepare monoclonal antibodies to androgen receptors without purification of the receptors for immunization.**

Steroid receptors are usually characterized by their ability to bind radioactive ligands. The isolation of polyclonal and monoclonal antibodies to estrogen (1, 2), glucocorticoid (3–5), and progestin (6–8) receptors in recent years, however, has provided alternative means to study these hormone receptors. An antibody against androgen receptors, however, has not been produced, mainly because of the difficulty in obtaining androgen receptors in sufficiently pure form and in amounts needed for efficient immunization. In this report, we show that anti-androgen receptor antibodies are present in high titers in blood sera of some male patients having clinical prostate problems.

MATERIALS AND METHODS

Materials. 5α-[1,2,3,4,5,6,16,17-^{3}H(N)]dihydrotestosterone ([^{3}H]DHT; 179 Ci/mmol; 1 Ci = 37 GBq), [6,7-^{3}H]dexamethasone (49 Ci/mmol), and 17β-[6,7-^{3}H]estradiol (53 Ci/mmol) were obtained from New England Nuclear. 7α,17α-[17α-*methyl*-^{3}H]Dimethyl-19-nortestosterone ([^{3}H]-DMNT; 76 Ci/mmol) was provided by Amersham. Carboxymethyl-Sephadex was a product of Pharmacia. Affinity-purified sheep or goat anti-human immunoglobulins (Ig) were purchased from Cappel Laboratories (Cochranville, PA). Rabbit antisera to purified human IgG were produced in female New Zealand White rabbits by the procedure of Vaitukaitis *et al.* (9). DNA-Sepharose adducts were prepared from calf thymus DNA and cyanogen bromide-activated Sepharose (10). Electrophoretically homogeneous human testosterone/estradiol-binding globulin (TEBG) was a gift of George Moll of this university.

Cytosolic Receptor Fractions. Male rats (300–400 g) of the Sprague–Dawley strain (SASCO-King Animal Laboratories, Madison, WI) were castrated 18 hr prior to sacrifice. For cellular preparation and isolation of steroid receptors, all manipulations were carried out at 0–4°C. For routine immunoprecipitation assays, the ventral prostates were minced and washed with 10 vol of buffer A [1.5 mM EDTA/10 mM Na_2MoO_4/2 mM dithiothreitol/10 mM NaF/10% (vol/vol) glycerol in 50 mM sodium phosphate, pH 7.2]. The washed prostates were homogenized with a Potter–Elvehjem homogenizer in 2 vol of buffer A supplemented with 0.1 mM bacitracin, 1 mM phenylmethylsulfonyl fluoride, and 1 trypsin inhibitor unit of aprotonin per ml as protease inhibitors (11). The homogenate was centrifuged at 2000 × *g* for 10 min. The supernatant was incubated with 10 nM [^{3}H]DMNT for 15 min and then centrifuged at 220,000 × *g* for 45 min. One-tenth volume of a dextran-coated charcoal suspension [1.25% (wt/vol) dextran T-70 and 12.5% (wt/vol) Norit-A charcoal in 20 mM Tris·HCl, pH 7.2] was added to the soluble (cytosol) fraction to remove free steroid. After the removal of the charcoal by centrifugation, an aliquot of the cytosol fraction was taken for assay of DMNT–receptor complexes (DMNT-R) by the hydroxylapatite–filter assay method (11).

Immunoprecipitation of Radioactive Steroid–Receptor Complexes. Human blood serum was obtained from clinical laboratories in The University of Chicago Hospitals. Fresh serum samples were also obtained at different periods of time from some of the individuals who had been positively identified as having anti-androgen-receptor antibodies. The amount of anti-human immunoglobulins needed for maximum immunoprecipitation in the assays was determined by titration of ^{125}I-labeled IgG.

For immunoprecipitation assays, all serum was centrifuged at 2000 × *g* for 10 min prior to use. Human serum (1–10 μl) was incubated with 50–100 μl of cytosol or other receptor preparations containing 10,000–60,000 dpm of the steroid–receptor complexes at 4°C for 4 hr. Anti-human immunoglobulins (200–400 μl) were then added as second antibodies and the mixture was incubated at 4°C for another 18 hr. For control tubes, the radioactive steroid receptor preparations were heated at 50°C for 20 min before use. The radioactivities obtained for the control tubes were deducted from experimental values. In the studies of antigenic specificities, antisera were present in excess so that all immunoprecipitable steroid–receptor complexes could be pelleted. For the comparison of antibody titers in serum samples, the [^{3}H]DMNT-R was present in excess. The mixture was then centrifuged at 2,000 × *g* for 10 min. The precipitate was washed twice with 1 ml of buffer A. The washed pellet was dissolved in 100 μl

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Abbreviations: DHT, 5α-dihydrotestosterone; DMNT, 7α,17α-dimethyl-19-nortestosterone or mibolerone; DMNT-R, DMNT–receptor complexes; TEBG, testosterone/estradiol-binding globulin.

of 0.1 M NaOH, and the solution was neutralized with 10 μl of 1 M HCl. The radioactivity was determined in a Packard scintillation counter with a counting efficiency of about 30% (12).

DNA-Binding and Nonbinding Androgen Receptor Fractions. For these preparations, the rat prostates were washed and homogenized in buffer B [1.5 mM EDTA/2 mM dithiothreitol/10 mM NaF/10% (vol/vol) glycerol in 50 mM sodium phosphate, pH 6.0]. The cytosol fraction was prepared as described above but without incubation with the radioactive androgen and charcoal treatment. For the removal of cationic and DNA-binding proteins the cytosol fraction was passed through a double-layer column (2.5 cm diameter) containing carboxymethyl-Sephadex (1 ml/ml of cytosol) layered on the top of DNA-Sepharose (0.25 ml/ml of cytosol). Practically all receptors passed through the column. The pH of this fraction was adjusted to 7.2 and the fraction was used as the "naked receptor." The receptors were labeled with 10 nM [^{3}H]DMNT for 30 min at 0°C. For the heat transformation (or activation) of these complexes to the DNA-binding forms, DNA-Sepharose (0.5 ml/ml of cytosol) equilibrated with buffer C (buffer B adjusted to pH 7.2) was added and the mixture was incubated for 45 min at 20°C. The heated mixture was packed into a column (13 × 2.5 cm) and washed with buffer C containing 50 mM KCl. The DNA-bound [^{3}H]DMNT-R (more than 80% of total) was then eluted with buffer C containing 0.4 M KCl (12–15).

The [^{3}H]DMNT-R was also precipitated from the cytosol fraction by ammonium sulfate (40% saturation). The precipitated complexes were resuspended in buffer C and desalted by Sephadex G-25 (14). The salt-precipitated complexes in this fraction (control) were separated into DNA-excluded and -bound fractions as described above but without heating at 20°C.

Cytosol and Nuclear Receptor Fractions of Minced Prostates Incubated with [^{3}H]DMNT. Ventral prostates were minced and incubated with 10 vol of Dulbecco's modified Eagles' medium and 20 nM [^{3}H]DMNT for 45 min at 37°C in a shaking water bath as described in our previous publications (13–15). The incubated prostates were homogenized in buffer D (0.32 M sucrose/3 mM $MgCl_2$/20 mM Tris·HCl, pH 7.5) and the cytosol fraction was prepared. The nucleus-bound receptor complexes were then extracted with buffer E (1.5 mM EDTA/0.4 M KCl/20 mM Tris·HCl, pH 7.5) as shown elsewhere (15). The procedures for gradient centrifugation and other techniques were described in our previous reports (13–15).

RESULTS

The radioactive androgen–receptor complexes in the cytosol fraction of rat ventral prostate sediment as 7- to 12S units or as heavier aggregates in low-salt media. The sedimentation properties can vary, depending on pH and temperature treatment of the receptor preparations as well as the presence of other cellular components, such as nucleic acids or nucleoproteins (12, 16), metal ions and thiols (11, 17), and proteases (18). In the media containing 0.4 M KCl, the receptor complexes are dissociated from aggregated materials and sediment rather uniformly at 3.8 ± 0.3 S (17). For this reason, we have carried out the gradient centrifugation and immunoprecipitation studies in the media containing 0.4 M KCl. The use of [^{3}H]DMNT, a synthetic androgen highly specific for androgen receptor (13), also minimize the difficulties that may be caused by the nonspecific interaction of blood or prostate proteins with natural androgens such as DHT (19).

As shown in Fig. 1, the radioactive androgen–receptor complexes had a sedimentation coefficient of about 4 S. In the presence of a serum sample from patient CJ, the complexes sedimented as 9- to 12S units. When the serum sample from

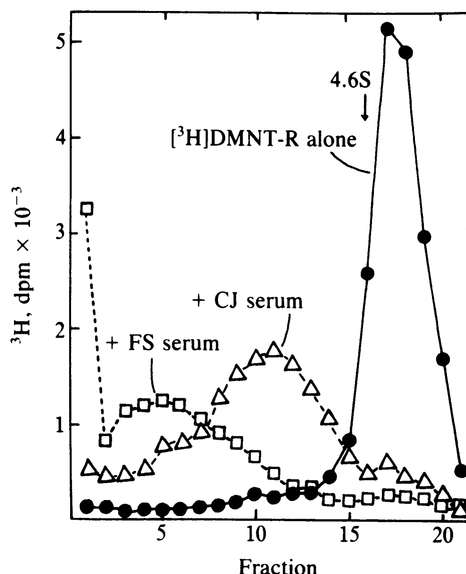

FIG. 1. Sedimentation profiles of androgen–receptor complexes in the presence of human sera containing autoantibodies. Salt-precipitated [^{3}H]DMNT-R (20,000 dpm) alone (●) or with 40 μl of serum from CJ (△) or FS (□) was incubated at 4°C for 4 hr. The incubated mixtures (100 μl) were layered on the top of sucrose gradients (5–20%) containing 1.5 mM EDTA, 2 mM dithiothreitol, 0.4 M KCl, and 20 mM Tris·HCl, pH 7.5. Centrifugation was performed at 34,000 rpm for 16 hr at 2°C in a Beckman SW 60 rotor. The gradient fractions (0.2 ml each) were collected and numbered from the bottom of each tube. Human IgG (7S), bovine serum albumin (4.6S), and [^{3}H]DMNT-R alone (3.8S) sedimented at the vicinity of fractions 13, 16, and 17, respectively.

patient FS was present, the major radioactive peak was at 14–19 S and some material sedimented to the bottom of the tube. When immunoprecipitation was carried out with the serum from CJ or FS according to the standard procedure, the radioactive receptor complexes precipitated only in the presence of anti-human immunoglobulins (Table 1). In the absence of the human serum the second antibodies did not precipitate the receptor complexes or the radioactive androgen.

Steroid binding, heating, and salt precipitation have been shown to promote the transformation (also called activation) of various steroid receptors from the forms that do not bind to DNA to the DNA-binding forms (19). The experiments shown in Table 2 indicated that the human antibodies recognized both DNA-binding and nonbinding forms of

Table 1. Anti-human immunoglobulin-dependent precipitation of the androgen–receptor complexes in the presence of human serum

Addition	Immunoprecipitable [^{3}H]DMNT-R, dpm
None	210
FS serum	53
Sheep anti-human Ig	90
FS serum + sheep anti-human Ig	19,283

Immunoprecipitation of the [^{3}H]DMNT-R (50 μl of prostate cytosol, containing about 20,000 dpm of hydroxylapatite-filter-assayable complexes) was carried out in the absence or presence of 10 μl of a human serum sample from patient FS, with or without 200 μl of sheep anti-human immunoglobulins as the second antibody. Similar results were obtained with affinity-purified goat or sheep anti-human IgG specific for heavy and light chains or affinity-purified rabbit anti-human IgG, but not with preimmune rabbit sera. In other experiments we also showed that the receptor complexes could be precipitated by goat anti-human IgM specific for μ chain.

Table 2. Immunoprecipitation of different forms of the androgen–receptor complexes

Receptor	Preparation	[^{3}H]DMNT-R, dpm Hydroxylapatite–filter-assayable	Immuno-precipitable
Naked receptor	Before heat activation	23,274	21,193
incubated with [^{3}H]DMNT	Heated and DNA-bound	20,274	15,383
Ammonium sulfate-	Unfractionated	19,940	16,130
precipitated [^{3}H]DMNT-R	DNA-bound	14,260	9,887
	DNA-excluded	10,420	11,833
Minced prostate	Cytosol	25,917	25,413
incubated with [^{3}H]DMNT	Nucleus-bound	19,440	29,765

Various radioactive receptor fractions were prepared and [^{3}H]DMNT-R was immunoprecipitated with 10 μl of a human serum sample from CJ and 200 μl of goat anti-human immunoglobulins.

[^{3}H]DMNT-R, although about 25–30% of the heat- or salt-activated DNA-binding form resisted immunoprecipitation. Both the cytosol and nucleus-bound receptor complexes extracted from minced prostates previously incubated with radioactive androgen were immunoprecipitated. The antibodies, however, recognized a significant portion of the nucleus-bound receptor complexes that were not measured by the hydroxylapatite-filter assay. In contrast, all cytosol [^{3}H]DMNT-R that was assayable by the hydroxylapatite-filter assay could be immunoprecipitated. The antibodies could immunoprecipitate DHT- or DMNT-R from different sources such as the rat ventral prostate, human prostate, and human breast tumor MCF-7 cells. With several antisera, no immunoreactivity was found with glucocorticoid receptors of rat liver, estrogen receptors of rat uterus, and MCF-7 cells, or progestin receptors of human breast tumor T-47D cells. The receptor selectivity was not due to cellular components that promoted or inhibited immunoprecipitation of specific steroid receptors, since mixing of cytosol fractions from different sources did not significantly alter the amount of immunoprecipitable radioactivity (Table 3). The antisera also did not immunoprecipitate radioactive steroids bound to other nonreceptor steroid-binding proteins, such as human TEBG (20), rat epididymal androgen-binding protein (21), or rat ventral prostate α-protein (22).

The anti-androgen receptor antibodies in the serum of CJ were apparently due to IgG, since essentially all of the antibody activity in the whole serum could be recovered in an IgG fraction (>95% γ-globulin on electrophoresis) purified from his serum by ammonium sulfate precipitation and DEAE-cellulose chromatography (23). The dissociation constant for the antibody–receptor complex appeared to be well below 0.1 nM so that, under the assay conditions with excess antibodies, there was complete precipitation of the receptor complexes. The presence of anti-androgen-receptor antibody is clearly more prevalent in males than in females. About 36.9% (124 of 336) of male and 2.6% (7 of 268) of female serum samples screened had significant antibody titer (immunoprecipitated 1 fmol or more of [^{3}H]DMNT-R per 10 μl of serum) (Table 4). Males under 45 years of age (16.0%) were less likely to have significant amounts of the antibody than those older than 45 (45%) and the chance of finding serum with an antibody titer of over 10 fmol/10 μl of serum is better in males older than 66 than in the younger males or females at all ages.

Table 3. Specificity of anti-receptor antibodies

Source of cytosol or TEBG	^{3}H-labeled steroid	Immunoprecipitable steroid, dpm
Prostate (0.5 ml)	DMNT	11,051
Prostate (0.5 ml)	17β-Estradiol	127
Prostate (0.5 ml)	Dexamethasone	166
Uterus (0.5 ml)	17β-Estradiol	183
Liver (0.5 ml)	Dexamethasone	554
Uterus/prostate (0.25 ml each)	17β-Estradiol	101
Uterus/prostate (0.25 ml each)	DMNT	6,610
Liver/prostate (0.25 ml each)	Dexamethasone	311
Liver/prostate (0.25 ml each)	DMNT	6,640
TEBG	DHT	56
TEBG	DMNT	126
TEBG	17β-Estradiol	259
TEBG/0.4 M KCl	DHT	67
TEBG/0.4 M KCl	DMNT	193
TEBG/0.4 M KCl	17β-Estradiol	147
No cytosol or TEBG	DHT	64
No cytosol or TEBG	DMNT	91

Cytosolic fractions from ventral prostate, liver, or uterus of rats deprived of steroid hormones (by castration, adrenalectomy, or ovariectomy) or purified TEBG (25 μg) were incubated with radioactive DMNT (10 nM), 17β-estradiol (10 nM), DHT (20 nM), or dexamethasone (30 nM), each at 40 Ci/mmol, for 60 min at 0°C. Immunoprecipitation of the radioactive steroid complexes was carried out in the presence of 10 μl of serum from CJ and a rabbit anti-human IgG serum. The amounts of hydroxylapatite–filter-assayable receptors in the cytosol preparations of prostate (for DMNT), uterus (for 17β-estradiol), and liver (for dexamethasone) were about 70 fmol (12,000 dpm)/0.5 ml of cytosol.

DISCUSSION

The autoimmune antibodies we have studied appeared to be highly specific for androgen receptors. Both nuclear and cytosolic androgen receptor complexes, either DNA-binding

Table 4. Anti-androgen–receptor antibody activities in human sera

[^{3}H]DMNT-R immunoprecipitated, fmol/10 μl serum	No. of serum samples: Males 2–45 yr	Males 46–65 yr	Males 66–90 yr	Females 2–45 yr	Females 46–65 yr	Females 66–90 yr
<1*	79	43	90	132	66	63
1–3	11	34	55	2	0	2
3–5	1	6	6	1	0	0
5–10	1	0	2	0	1	0
10–15	1	0	1	0	1	0
15–20	1	0	1	0	0	0
>20	0	1	3	0	0	0
Total	94	84	158	135	68	65

*Since the variation in the duplicate assays was about 0.5 fmol, this value was considered insignificant.

or nonbinding forms, reacted with the antibodies. Some of the cytosol receptor complexes, however, appeared to lose their ability to interact with the antibodies during heat or salt activation. This may be due to partial proteolysis of the receptor proteins. In contrast, the antibodies recognized a portion of the nucleus-bound androgen–receptor complexes that were not measurable by the hydroxylapatite-filter assay. By using both methods, it may be possible, therefore, to analyze different forms of the androgen–receptor complexes.

The anti-androgen–receptor antibodies in some sera have been identified as IgG but other immunoglobulins, such as IgM, may also be responsible for the antibody activity, since antibodies from different serum samples caused [^{3}H]DMNT-R to sediment differently during gradient centrifugation and were recognized by antiserum specific for the μ chain of human IgM.

Recent studies (24, 25) suggest that autoimmune antibodies may appear not only in pathologic conditions but also in the serum of healthy individuals, although normally at low titers. Our observation that many male serum samples have higher anti-androgen–receptor antibodies than most female samples do appears to reflect the difference in the levels of autoantigens, such as androgen receptors, in the two sexes. So far three serum samples with very high titers belonged to old males (73, 75, and 83 years old) with clinical symptoms of benign prostate hyperplasia or prostate cancers. Their antibody titers were about 150 $\pm$ 20 fmol of [^{3}H]DMNT-R precipitated per 10 μl of serum. This suggests that prostates were the sources of the antigens or receptors that caused the immune responses. Since many other patients with similar symptoms in the same age group had low titers ($<$3 fmol/10 μl of serum), further evaluation is required to establish the relationship between the antibody titers and clinical status or treatment history of patients. In addition, one cannot ignore the possibility that antigenic androgen receptors may be supplied by organs other than prostates. Although the tissue concentrations of androgen receptors in organs such as liver, kidney, skin, and muscles are low (17, 19), these organs, in total, contain more receptors than the prostates in an individual.

Virtually all female serum samples showed insignificant antibody titers. These samples included those from 57 patients with hirsutism. The individual with the highest antibody titer (14 fmol/10 μl of serum) among females was a heart transplant (female donor) patient (age 49). Among young males, the highest titer (16 fmol/10 μl of serum) belonged to a kidney transplant patient (age 36). Additional studies are necessary to determine whether organ transplants and/or medication that can cause alteration of immune systems are responsible for increased emergence of antibodies to certain autoantigens.

The gamma globulin fractions of many species have protein components (5S-CA) that can form complexes with 5S nuclear estrogen receptors of rat uterus but not with the 4S cytosolic form (26). 5S-CA is present in many animals immunized against an array of seemingly unrelated antigens but appears to be specific for estrogen receptor. 5S-CA-like components have also been found in humans, especially in females, and it was suggested that this component may result from an autoimmune response to target tissue cycling in the females (27). A spontaneous sheep antibody against chicken progesterone receptor has also been described. About one-third of the sheep tested had such an endogenous immunoglobulin activity (28). Both 5S-CA and the spontaneous sheep antibodies, however, were present in very small quantities in the sera. Judged on the basis of the titers and concentrations of IgG needed to show receptor–antibody interactions, the high-titer antisera shown in this paper had anti-receptor antibody activities about 100 times higher than those reported for 5S-CA or the sheep antibodies. In fact, the titers of many human serum samples we have employed were comparable to the titer of anti-estrogen receptor antibodies in the blood sera of animals (1) immunized with purified estrogen receptors. It may be feasible, therefore, to use lymphocytes from these individuals and hybridoma techniques to produce monoclonal antibodies against androgen receptors and to use them for the study of the structure, functions, and intracellular dynamics of androgen receptors.

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