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ANTIBODIES AGAINST SYNTHETIC PEPTIDES RECOGNIZE THE HUMAN AND RAT ANDROGEN RECEPTOR

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Summary—Antibodies against two synthetic peptides (aa 299–311 and aa 544–559) selected in different immunogenic domains of the human AR, were induced in rabbits. Antiserum reactivity against the native receptor was investigated by gel permeation chromatography and sucrose density gradient centrifugation using [^{3}H]mibolerone-labeled rat prostate cytosol and [^{3}H]5α-dihydrotestosterone-labeled T-47D cytosol as a source of AR. The absence of cross-reactivity of the antisera with estrogen, progesterone and glucocorticoid receptor was confirmed by density gradient centrifugation of rat uterus cytosol labeled with [^{3}H]E2 or [^{3}H]ORG 2058 and rat liver cytosol labeled with [^{3}H]dexamethasone.

After partial proteolytic breakdown of rat prostate AR by endogenous proteases the steroid-labeled receptor was recognized only by the second peptide (aa 544–559) antibody. This proteolytic breakdown could be prevented to a large degree by addition of a high concentration of soybean trypsin inhibitor.

The specific AR antibodies provide new tools for the functional analysis of AR, since they interact selectively with specific domains of the receptor.

INTRODUCTION

Like other steroid hormones, androgens exert their activities through a specific intracellular receptor. Purification and characterization of the androgen receptor (AR) by classical chemical methods have proven difficult both because of its low tissue concentration and because of its lability. Recently the cDNA sequences of human [1–4] and rat [3, 5] AR were reported. This opens the possibility to produce antibodies against synthetic peptides covering different domains of the AR. Such antibodies may be used for purification of the receptor as well as for morphological and functional studies. In this paper we describe the production and characterization of antibodies, directed against two different immunogenic domains of the AR. The reactivity of these antibodies was studied against human and rat androgen receptor and also against proteolytic fragments of the latter.

MATERIALS AND METHODS

Materials

[^{3}H]Mibolerone (57.0 Ci/mmol), mibolerone, [^{3}H]5α-dihydrotestosterone (58.4 Ci/mmol), [^{3}H]-dexamethasone (44.7 Ci/mmol) and [^{3}H]estradiol (107.8 Ci/mmol) were obtained from New England Nuclear. [^{3}H]ORG 2058 (57.0 Ci/mmol) and ORG 2058 were purchased from Amersham. 5α-Dihydrotestosterone (DHT), dexamethasone and estradiol (E2) was purchased from Sigma. All the materials for cell culture were obtained from Gibco. All other materials were of analytical grade.

Synthesis of peptides and preparation of antisera

The peptides AR299 (hAR299–311) and AR544 (hAR544–559) were synthesized using Fmoc-polyamide chemistry [6]. Coupling was achieved using pentafluorophenylesters of the amino acids and hydroxybenzotriazole as catalyst. The purity of the peptides exceeded 90% as estimated from analytical reverse-phase HPLC. Their amino acid composition was confirmed by quantitative amino acid analysis.

A two step glutaraldehyde method [7] was used to couple each peptide (2 mg) to bovine thyroglobulin (12.5 mg). The peptide AR544 was also coupled to thyroglobulin using 3-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBS) as coupling agent [8].

Two New Zealand White rabbits were immunized with each peptide. Preimmune serum was

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collected before immunization. The peptide-thyroglobulin conjugate (1 mg) was mixed with complete Freund's adjuvant and injected intradermally at multiple sites on the back of the rabbit. After three weeks a booster injection of the same mixture was administered. From the third injection on incomplete Freund's adjuvant was used. Blood was collected from an ear artery 5 days after each booster injection.

Preparation of cytosols

Four ventral prostates obtained from adult male Wistar rats, castrated 18 h earlier, were homogenized in 5 ml TEDG buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1.5 mM EDTA, 1.25 mM dithiotreitol, 10% glycerol, 2 mg/ml soybean trypsin inhibitor, supplemented or not with 20 mM sodium molybdate). The homogenate was centrifuged at 8000 *g* for 10 min and the supernatant was recovered. After addition of [^{3}H]mibolerone (15 nM), the supernatant was centrifuged again for 45 min at 100,000 *g*. The resulting cytosol was incubated further for 1 h and treated with dextran-coated charcoal.

Cytosol of ovariectomized rat uterus labeled with [^{3}H]E2 or [^{3}H]ORG 2058 and cytosol of rat liver labeled with [^{3}H]dexamethasone were prepared in the same way.

Cell culture

T-47D cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.16 IU/ml insulin. For each preparation ten 15-cm Petri dishes were seeded with 3×10^7 cells. After 3 days the medium was replaced by RPMI 1640 medium without fetal calf serum. Incubation was continued overnight. Thereafter, the dishes were rinsed twice with ice-cold phosphate buffered saline. The cells were harvested using a rubber policeman and collected by centrifugation at 800 *g*. The cytosol was prepared in the same manner as prostate cytosol, but [^{3}H]DHT (15 nM) was used instead of [^{3}H]mibolerone to avoid interference by high amounts of progesterone receptor present in these cells [9].

Density gradient centrifugation

150 μl aliquots of [^{3}H]mibolerone-labeled rat prostate cytosol or [^{3}H]DHT-labeled T-47D cytosol were incubated for 1 h with 25, 10 or 1 μl of the antisera, preimmune sera or TS buffer (25 mM Tris, pH 7.4, 300 mM NaCl). When [^{3}H]DHT was used as a label (T-47D cells), the rabbit sera were preincubated with 10 μM DHT to reduce the binding of labeled DHT to sex-hormone binding globulin [10]. The density gradient centrifugation was performed as described previously [11] using [^{14}C]-ovalbumin (3.7 S) as an internal marker.

Superose-12 gel permeation chromatography

750 μl aliquots of [^{3}H]mibolerone-labeled rat prostate cytosol or [^{3}H]DHT-labeled T-47D cytosol were incubated 1 h with 25, 15, 10, 5 or 2 μl of the antisera, preimmune sera or TS buffer. After incubation for 1 h the mixtures were applied on a Superose-12 column (Pharmacia) equilibrated with TS buffer, supplemented or not with 20 mM sodium molybdate. The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected and counted for radioactivity.

ELISA for detection of peptide antibodies

Nunc Maxisorb microtiter plates were coated overnight with synthetic peptides (0.1 μg/ml) in PBS (0.1 M Na_2HPO_4, pH 7.4, 150 mM NaCl). The plates were rinsed with PBS containing

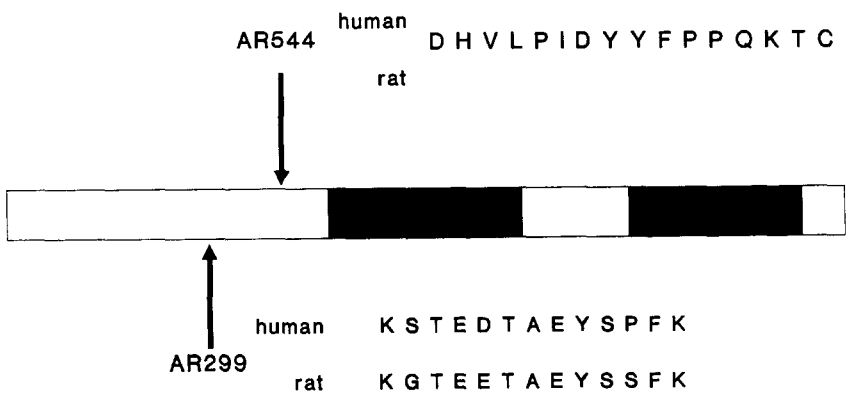

Fig. 1. Sequences of peptides AR299 and AR544 and their location in the human AR molecule. The sequence of AR299 is also compared with the corresponding sequence of rat AR.

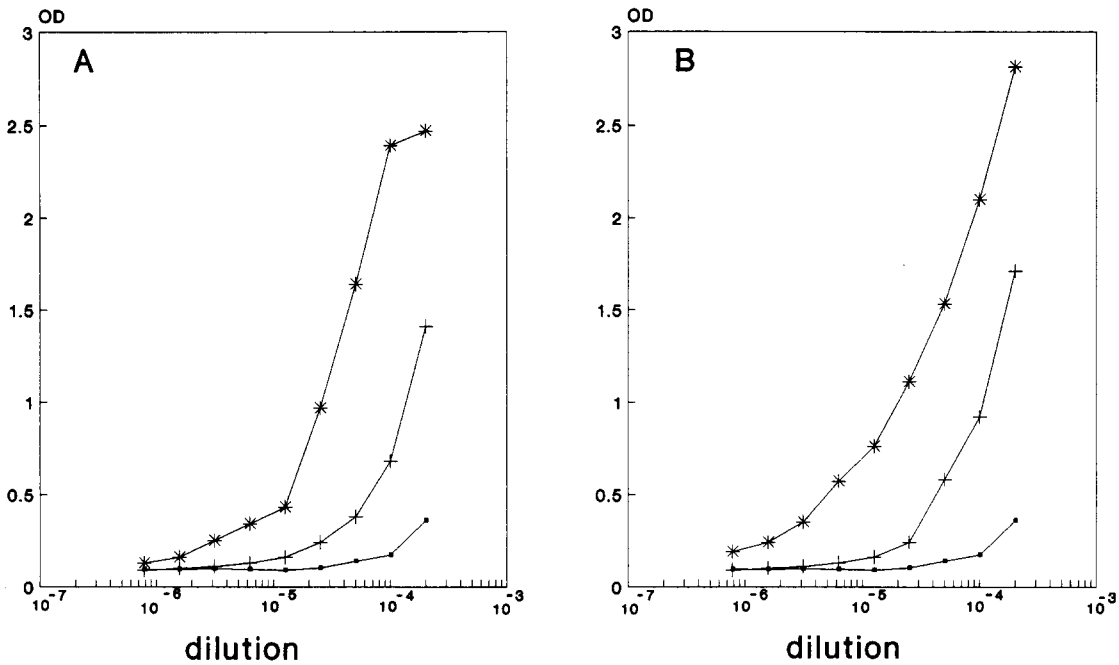

Fig. 2. ELISA assay of preimmune serum and antisera. Serial dilutions of serum were tested by ELISA described in the Methods section: preimmune serum (□), serum from the first (+) and from the third bleeding (*). In Fig. 2A and B the data obtained respectively with the peptide AR299 and AR544-2 are shown.

0.05% Tween 20 and incubated for 3 h with serial dilutions of the antisera or preimmune sera. Retained antibodies were detected using donkey-anti-rabbit immunoglobulins labeled with peroxidase (Nordic, 1/4000, 1 h). Peroxidase activity was measured in 0.1 M citric acid (pH 5.6) using ortho-phenylenediamine as substrate.

RESULTS

Selection of the antigenic peptides

To obtain antibodies specifically directed against the AR, the cDNA deduced amino acid sequence of the human AR [2] was compared to that of all other steroid receptors [12–14]. The largest differences were observed in the N-terminal domain and the hinge region between the DNA- and steroid-binding domains. The amino acid sequence of the N-terminal domain of the human AR [3] was then subjected to hydrophilicity analysis [15]. A large hydrophilic region was found between amino acid 287 and amino acid 342. Within this region peptide AR299 (Fig. 1) was chosen for its N- and C-terminal lysine, which allow efficient coupling to a carrier protein; it differs in 3 amino acids from the corresponding region of the rat AR. The second peptide (AR544) is largely identical to that used by Tan *et al.* [5] for immunization against AR. Its sequence is conserved in rat and human AR but has no homology with other known steroid receptors.

Induction of peptide antibodies

Induction of antibodies was observed with thyroglobulin conjugates of both AR299 and AR544. ELISA screening revealed measurable titers of antibodies already after the second injection and these titers increased during immunization (Fig. 2). For convenience antisera raised against AR299 will be indicated further as AS299. Antisera raised against the AR544-conjugate prepared respectively with glutaraldehyde and MBS are indicated as AS544-1 and AS544-2.

Study of antiserum reactivity against steroid receptors by sucrose density gradient centrifugation

The reactivity of the antisera against AR was studied by sucrose density gradient centrifugation of [^{3}H]mibolerone-labeled rat prostate cytosol as a source of rat AR and [^{3}H]DHT-labeled T-47D cytosol as a source of human AR. Density gradient centrifugation of rat (Fig. 3A) as well as human (Fig. 3B) AR preparations in medium of high ionic strength revealed a 4.6 S peak of radioactivity which disappeared when cytosol was incubated with an excess of unlabeled DHT or mibolerone

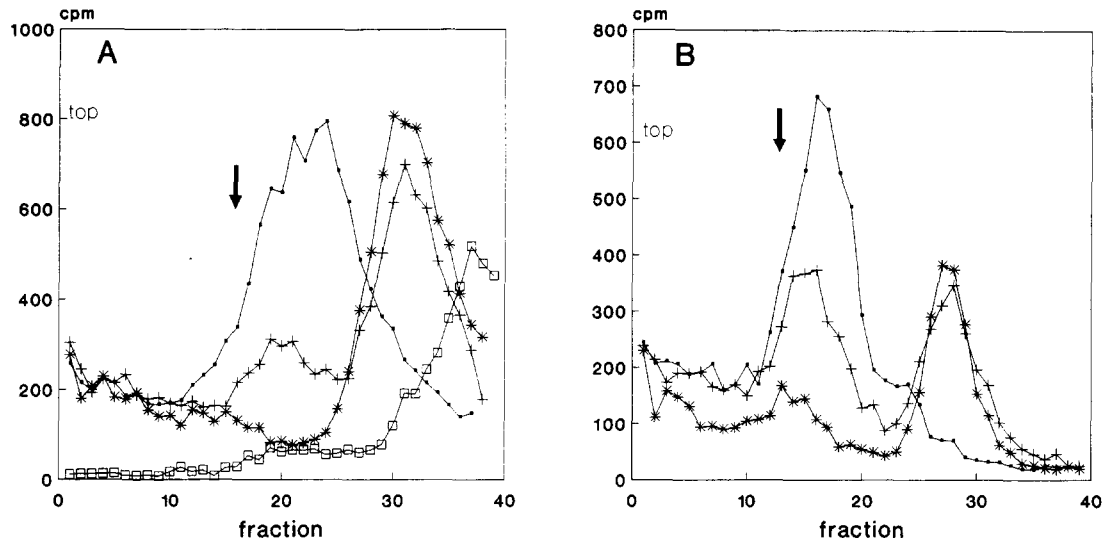

Fig. 3. Sucrose density gradient analysis of human and rat AR, with and without AR antibodies. Rat prostate cytosol, labeled with [^{3}H]mibolerone (rat AR, Fig. 3A) or T47-D cytosol, labeled with [^{3}H]DHT (human AR, Fig. 3B) were prepared as described in the Methods section. 200 μl aliquots were preincubated with preimmune serum (●), AS299 (+), 544-2 (*) or AS299 combined with As544-2 (□) are applied on a 5–20% sucrose gradient, containing 0.3 M NaCl. The position of the internal standard [^{14}C]ovalbumin (3.7 S) is indicated (arrow). The elution profile in the absence of serum was identical to that obtained with preimmune serum.

(data not shown). In the presence of immune sera this peak shifted to the 7.2 S region. Such a shift was not observed with preimmune sera. The displacement was nearly complete with AS544-1 and AS544-2. With AS299, however, a residual peak of radioactivity was consistently observed at 4.2 S, even in the presence of an excess of antiserum. When both antisera (AS299 and AS544) were combined, the increase in sedimentation was larger than that observed with each of them individually (Fig. 3A). This finding indicates that the antisera can bind simultaneously without steric hindrance. The absence of cross-reactivity of the antisera with estrogen, progesterone and glucocorticoid receptor was demonstrated by density gradient centrifugation of uterus labeled with [^{3}H]E2 or [^{3}H]ORG 2058 and liver cytosol labeled with [^{3}H]dexamethasone. In none of these experiments the antisera displaced the peaks of receptor-bound radioactivity (not shown).

Study of antiserum reactivity against AR by gel filtration

Comparable results were obtained with Superose-12 gel permeation chromatography. Indeed, in the absence of molybdate the major AR peak eluted at a position corresponding to a mol. wt of approx. 120 kDa. When immune antiserum was added, a marked shift was observed into the 250 kDa mol. wt region. This shift was observed with all antisera tested and with the human (T-47D-cytosol; not shown) as well as with the rat receptor (rat prostate cytosol; Fig. 4B). A 50% displacement into the higher mol. wt region required, respectively, 1 μl of AS544 and 5 μl of AS299. When the experiment was performed in the presence of molybdate, the AR was already eluted at a higher mol. wt position (approx. 300 kDa) in the absence of the antisera, most probably by stabilization of a complex of the AR with the 90 kDa heat shock protein (hsp 90) [21]. Preincubation with AS299 or AS544 resulted in a further increase in the size of the AR to a mol. wt or approx. 450 kDa (Fig. 4A).

Interaction of the antisera with proteolytic fragments of the AR

Routinely, cytosol from the rat ventral prostate was prepared in buffer containing a high concentration (2 mg/ml) of soybean trypsin inhibitor. Indeed, this addition largely prevents the proteolytic breakdown of the AR, usually observed in this organ. When the protease inhibitor was added only after the ultracentrifugation step, the AR was cleaved largely into a much smaller product (mol. wt approx. 50 kDa), as indicated by its increased elution volume from the Superose-12 column. This receptor fragment results most probably from proteolytic cleavage between both antigenic

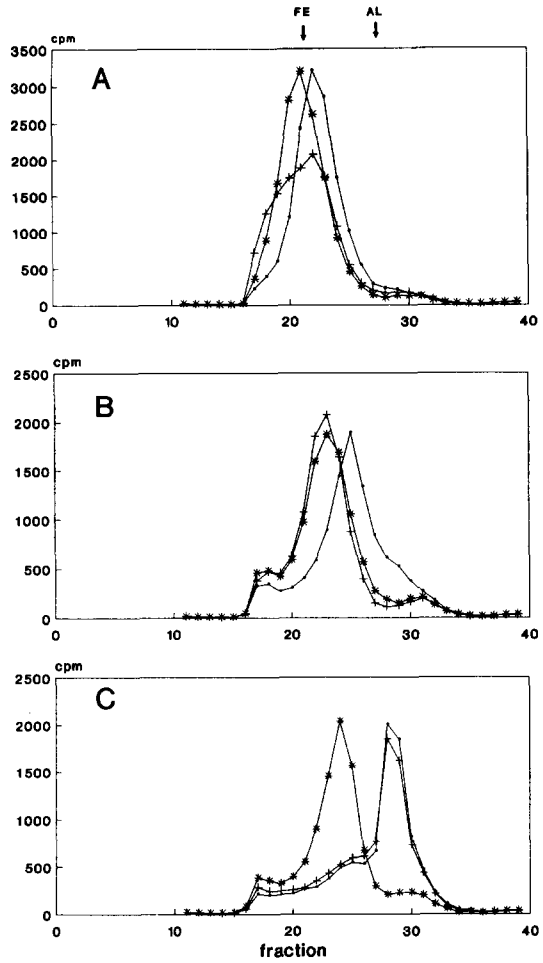

Fig. 4. Superose-12 gel permeation chromatography of rat AR, performed with and without AR antibodies. Rat ventral prostate (750 μl), labeled with [^{3}H]mibolerone was incubated with 25 μl of preimmune serum (●), AS299 (+) or AS544-1 (*) and 0.5 ml was applied on a Superose-12 column. Gel filtration was performed on Pharmacia FPLC equipment at a flowrate of 0.5 ml/min and fractions of 0.5 ml were collected. An aliquot of 0.1 ml was counted for radioactivity. In Fig. 4A and B the whole procedure was performed, respectively, in the presence or absence of 20 mM sodium molybdate. Figure 4C shows a similar experiment, performed in the absence of molybdate, but with addition of soybean trypsin inhibitor only after the ultracentrifugation step. The positions of albumin (66 kDa; AL) and ferritin (450 kDa; FE) are indicated.

sites used for immunization. Indeed, addition of AS299 no longer produced a shift of the mol. wt of the AR, whereas addition of AS544 still resulted in formation of a receptor antibody complex, as indicated by a change in elution position of the AR to the 200 kDa mol. wt region (Fig. 4C).

The effect of other inhibitors of proteolysis was also examined. After addition of aprotin (1 mg/ml), bacitracin (100 μg/ml) and leupeptin (100 μg/ml) the AR consisted mainly of its low MW form, which reacted only with AS544. When phenylmethylsulfonylfluoride (1 mM) was added, the AR showed a similar behavior, but a markedly lower yield of steroid binding was observed.

DISCUSSION

Although the use of antibodies may offer considerable benefits for the purification and characterization of the AR, such techniques have not been applied widely, because the AR has not yet been purified in significant amounts. Liao and Witte [18] and Young *et al.* [19] described AR antibodies occurring spontaneously in the serum of older men. Young *et al.* [18] succeeded in isolating permanent cell lines producing these antibodies, but their usefulness was limited. Another approach, which may overcome the lack of purified receptor is the immunization with synthetic peptides, whose amino acid sequence corresponds to specific immunogenic regions of the AR or to fusion proteins obtained by recombinant DNA techniques. Indeed, such approaches have become possible by the recent elucidation of the amino acid sequence of the human [1–4] and rat AR [3, 5], as derived from their cDNA sequence.

In the present communication two different polypeptides, both located in the amino terminal half of the AR were used for immunization. The antisera, induced by either peptide, recognized the AR, but did not interact with rat glucocorticoid, estradiol or progesterone receptors. Surprisingly, all of our antibodies recognized the human as well as the rat AR. This is remarkable for AS299, since the corresponding region in the rat AR differs by 3 out of 13 amino acids. One may hypothesize that the central "Thr-Ala-Glu-Tyr-Ser" sequence has acted as immunogenic determinant. Indeed, the oligopeptide was linked to thyroglobulin by its terminal lysines and peptide-conjugates usually do not elicit a specific immune response against amino acids close to the conjugation place [16].

With respect to the titer of the obtained antisera markedly different results were obtained by the ELISA technique, which estimates antiserum reactivity against the peptides used for immunization and the results obtained with native receptor. This may indicate that only a small fraction of the peptide antibodies actually recognize the receptor or alternatively that the interaction with the AR is of much lower affinity. We favor the latter hypothesis, since

the degree of antibody binding was affected little or not by the concentration of AR.

As expected, the antisera raised against both peptides react with different antigenic sites on the AR and this interaction occurs without mutal steric hindrance as shown by the larger increase in size after addition of both antisera. This observation may open the way for the development of a sandwich immunoassay. The difference in specificity is also clearly shown by the results obtained when the AR was not protected against proteolytic breakdown. Indeed, under these conditions the major receptor cleavage product in rat ventral prostate cytosol was recognized only by AS544, but not by AS299. Consequently, this smaller receptor form has lost an important part of its aminoterminal half, but still includes the DNA-binding domain. It should be stressed that only soybean trypsin inhibitor at very high concentration prevented this degradation and that most classically used inhibitors of proteolysis were ineffective in agreement with previous reports [22–24].

Another interesting aspect is the observation that the antisera also produce a further increase in size, when added to receptor prepared in the presence of molybdate. Under these conditions a high mol. wt form of steroid receptors is stabilized, most probably consisting of a receptor–hsp90 complex. Consequently, the interaction of the antisera with the receptor is not impaired by the interaction with hsp90. This observation is in agreement with the hypothesis that the aminoterminal half of the receptor is not involved in the interaction with hsp90, as already demonstrated for the glucocorticoid receptor [17].

Our results may be compared with those obtained recently by other authors by means of a similar approach [5, 20]. With regard to displacement of receptor-bound steroid our results are essentially similar, but we confirmed the antibody receptor interaction both by sucrose density centrifugation and gel permeation chromatography and extended these observations to molybdate stabilized receptor and androgen receptor after partial proteolytic breakdown. Our antisera were also tested by Western blotting. No significant protein bands were detected with AS544, but a clearcut 110 kDa protein band was observed, both in rat ventral prostate cytosol and in cytosol from T-47D cells from human origin (results not shown). Although the position of this band is similar to that reported by van Laar *et al.* [20], it seems unlikely that the 110 kDa band detected by our antisera corresponds to the androgen receptor. Indeed a similar signal was observed in cytosol prepared from a number of rat tissues, without any correlation with the actual concentration of androgen receptor as estimated by steroid binding. Furthermore, upon fractionation of [^{3}H]mibolerone-labeled cytosol from rat ventral prostate by mono Q anion exchange chromatography in the presence of molybdate, the immunoblot signal was eluted before the [^{3}H]steroid-labeled androgen receptor. In view of the lack of signal (AS544) and the doubtful specificity (AS299) of our antisera no immunocytochemical studies were undertaken, although van Laar *et al.* [20] and Tan *et al.* [5] demonstrated that their antisera could be used for that purpose.

We may conclude that antisera against synthetic peptides offer important perspectives for the functional analysis of ARs, since they interact selectively with specific domains of the receptor protein. For that reason further efforts in that direction may be worthwhile, to obtain antibodies with higher affinity and to extend the range of epitopes recognized by antibodies.

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