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SPECIFIC ANTISERA TO ANDROSTANEDIOLS: INFLUENCE OF THE TYPE OF BRIDGE AND POSITION OF COUPLING

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SUMMARY

In order to obtain antibodies of high specificity for two androstanediols (5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol) different forms of linkage to the carrier protein were used. 5α-Androstane-3α,17β-diol was linked at position 1α and subsequently at 15α via a carboxymethyl group; 5α-androstane-3β,17β-diol was linked at position 1α via a carboxymethyl group and subsequently at position 7 via a carboxymethyloxime substituent. Bovine serum albumin (BSA) was used as the carrier protein throughout. Linkage at position 1α resulted in antisera of very poor specificity in each case. The use of the carboxymethyl bridge at position 15α elicited, in the case of 5α-androstane-3α,17β-diol, antisera of high specificity.

INTRODUCTION

Previous work has shown that for preparation of highly specific antibodies to steroids, the hapten should be coupled to the antigenic carrier without involvement of its natural functional groups. Thus the B ring (C_6, C_7) or the C ring (C_{11}) have usually been used for linkage; both of these are distant from the functional groups on the A or D rings. In general, antibodies obtained in this way are more specific than those elicited by immunogens prepared by forming the hapten–protein link through a functional group[1–10]. However, we have previously shown that antibodies to dihydroepiandrosterone (DHA) obtained using DHA-15α-carboxymethyl-BSA as immunogen were of higher specificity than those raised to DHA-7-carboxy-methyloxime-BSA[4, 5]. We decided to follow the same approach for preparing highly specific antibodies to other androgens.

5α-androstane-3α,17β-diol (A-3α-diol) and 5α-androstane-3β,17β-diol (A-3β-diol) were coupled to the same carrier, bovine serum albumine (BSA), through a carboxymethyl group in position 1α. In addition, A-3α-diol was conjugated through the same bridge in position 15α and A-3β-diol through a carboxymethyloxime group in position 7. In this work we present the parameters of binding and specificity studies of different antibodies obtained.

MATERIALS AND METHODS

Chemicals and apparatus. [1,2^{3}H]-5α-Androstane-3α,17β-diol (40 Ci/mmol) was purchased from New England Nuclear Corp. and [1,2^{3}H]-5α-androstane-3β,17β-diol (43 Ci/mmol) from Amersham (England). In addition, samples of both tritiated steroids (175 Ci/mmol) were kindly given by Dr. Robel and C. Corpechot (INSERM Unit 33, France). PPO, POPOP and Triton X-100 were obtained from Hopkin and Williams (Essex, England) and di-sodium phosphate, mono-sodium phosphate, sodium chloride and sodium azide from Merck (analytical grade). Dextran T 70 was purchased from Pharmacia Fine Chemicals. Charcoal and toluene were obtained from Prolabo, 12 rue Pelée, Paris, France. The reference steroids were purchased from Steraloids or Roussel Uclaf, Romainville, France. The radioactivity was determined with 30% efficiency in a liquid scintillation counter Intertechnique SL32.

Immunogens. The following immunogens were used in this study: 5α-androstane-3α,17β-diol-1α-carboxymethyl-BSA, (A-3α-diol-1-BSA); 5α-androstane-3α, 17β-diol-15α-carboxymethyl-BSA, (A-3α-diol-15-BSA); 5α-androstane-3β,17β-diol-1α-carboxymethyl-BSA, (A-3β-diol-1-BSA); 5α-androstane-3β,17β-diol-7-carboxymethyloxime-BSA, (A-3β-diol-7-BSA).

They were kindly provided by R. Condom (Laboratory of Structural Organic Chemistry, University of Nice, France). The synthesis and determination of the configuration of these immunogens have been reported by Condom and Emiliozzi [11, 12] and the reader is referred to these papers for further details.

Immunization. Antisera were raised in female Bouscat rabbits, weighing 2.5-3 Kg at the time of immunization. Three to five animals were injected with each immunogen. Immunization was carried out using a modification of the procedure described by Vaitukaitis *et al.*[13]. For the primary injection, 1 mg antigen solubilized in 0.5 ml of 0.9% saline was emulsified with an equal vol. of Freund's complete adjuvant

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Table 1. Serum sensitivity (amounts of cold steroid expressed in pg resulting in 50% inhibition of the labeled steroid binding)

A-3α-diol-15-BSA			A-3α-diol-1-BSA				
R* (1–2)	R (3–4)	R (5–6)	R (89–90)	R (91–92)	R (93–94)	R (95–96)	R (97–98)
400	80	95	66	40	42	52	40

* R: rabbit.

The affinity constants of the antisera for the A-3α-diol were between 6.9×10^9 and 3.5×10^{10} M^{-1}

Table 2. Specificity of anti-5α-androstane-3α,17β-diol antisera

Rabbits / Steroids	Cross reaction (%)*							
	Androstane-3α-diol-15-BSA			Androstane-3α-diol-1-BSA				
	1–2	3–4	5–6	89–90	91–92	93–94	95–96	97–98
5α-androstane-3α,17β-diol	100	100	100	100	100	100	100	100
5α-androstane-3β,17β-diol	<0.36	0.06	0.06	33	21	40	25	38
Androsterone	12	10	8	0.70	0.40	0.80	0.60	0.60
Testosterone	2.50	0.05	0.13	100	53	52	85	100
Dihydrotestosterone (DHT)	2.20	0.14	0.40	180	64	100	200	130
Epiandrosterone	0.60	0.20	0.09	0.38	0.40	0.40	0.09	0.06
5-androsten-3β,17β-diol	<0.50	0.17	0.06	2.30	1.20	4.20	3.80	2.70
5β-androstane-3β,17β-diol	<0.50	<0.01	<0.01	0.19	0.58	0.28	0.30	0.50
5β-androstane-3α,17β-diol	1.60	0.13	0.50	1.80	0.90	1.60	2.50	5
Dehydroepiandrosterone	<0.50	0.06	0.06	0.20	0.14	0.13	0.10	0.05
4-androsten-3,17-dione	1.50	0.03	0.09	1.70	3	1.15	1.60	2

* Defined [14] as $100\ X/Y$, where X is the mass of unlabeled steroid and Y the mass of the heterologous compound required to produce 50% inhibition of the binding of tritiated steroid by antibody.

(FCA), and injected intradermally at 20–30 separate points in the animal's dorsal region. One ampoule of *B. pertussis* (Vaccin DT Perthydral, Institut Pasteur) was injected subcutaneously on the same day.

Starting the second week after the primary injection the rabbits were bled every week. The antibody titer was calculated as that dilution capable of binding approximately 50% of 6000–7000 d.p.m. of tritiated hapten. Booster injections were carried out when there was a drop in the titer (1/500–1/1000) after the peak of the primary response; this occurred on average 6–8 weeks after the first injection. Each animal received 0.3 mg of antigen administered on 3 consecutive days according to following schedule: 1st day: 0.1 mg in 0.15 M NaCl and FCA intramuscularly, 2nd day: 0.1 mg in 0.15 M NaCl intravenously, 3rd day: 0.1 mg in 0.15 M NaCl intravenously.

Maximum titers were obtained 8–21 days after the booster injections. All blood samples were stored at 4°C, after addition of sodium azide (final concentration 0.1%).

Characterization of antisera. Changes in the titer, specificity and affinity constant of the antibodies obtained, were studied using the radioimmunoassay technique described below.

In propylene test tubes we added (final vol. 0.3 ml): 0.1 ml standard or buffer* solution, 0.1 ml tritiated hapten (≃6000 d.p.m.), 0.1 ml diluted antiserum. The tubes were gently stirred and allowed to equilibrate for 30 min at room temperature and then 10 min at 0°C (ice-cold bath). One ml of dextran-charcoal solution† was added to all tubes in the assay and 10 min later the tubes were centrifuged at 2200 *g* for 10 min. The supernatant was decanted into a scintillation vial, a scintillation mixture‡ added and the vials were counted. Non specific binding was less than 4%.

Affinity constants were calculated using the Scatchard plot.

RESULTS

1. *Antisera to 5α-androstane-3α,17β-diol.*

Titer and sensitivity. Sera from rabbits immunized with A-3α-diol-1-BSA diluted 1/35000 to 1/60000 (initial dilution) bound 40–50% of the tritiated hapten in the conditions of the radioimmunoassay described above. Antisera to A-3α-diol-15-BSA had titers 1/5000 to 1/22000. Inhibition of the binding of labeled steroid by 5α-androstane-3α,17β-diol is shown in Table 1.

Specificity in relation to the position of coupling. As is shown in Table 2, antibodies obtained using the

* Phosphate buffer: 0.1 M, pH 7.4, 0.15 M NaCl, 1 g/l gelatin, 1 g/l sodium azide.

† Norit A 2.5 g, Dextran T 70 0.25 g in 1000 ml buffer.

‡ PPO 4 g, POPOP 0.1 g, toluene 1 liter, Triton X-100 0.5 liter.

Table 3. Serum sensitivity (amounts of cold steroid expressed in pg resulting in 50% inhibition of the labelled steroid binding)

A-3β-diol-7-BSA			A-3β-diol-1-BSA		
R (39–40)	R (41–42)	R (43–44)	R (3 310)	R (3 312)	R (3 315)
25	40	90	70	45	25

* R: rabbit.
The affinity constants of the antibodies for A-3β-diol were between 5.8×10^9 and 2.4×10^{10} M^{-1}.

immunogen coupled in position 1α were much less specific than those obtained when the immunogen was coupled at position 15α. All antibodies to the 1-conjugate cross-reacted to a great extent with A-3β-diol, so could not be used for the radioimmunoassay of A-3α-diol without a preliminary chromatography step. In some cases (testosterone, dihydrotestosterone) they recognized the heterologous steroid at least as well, or even better. However, the anti-A-3α-diol-15-BSA antisera possessed a very high specificity. For the same inhibitors the cross reactions were less than 3%, except for androsterone with which the antibodies cross-reacted at 8–12%.

Antisera to 5α-androstane-3β,17β-diol

Titer and sensitivity. Titers of antisera to A-3β-diol-7-BSA were between 1/12000 and 1/70000 and those of anti A-3β-diol-1-BSA sera were in the range of 1/1000 to 1/7500 (initial dilution). Amounts of cold steroid inhibiting the binding of the tritiated hapten are given in Table 3.

Specificity in relation to position of coupling. Comparison of the specificity of the two types of anti-A-3β-diol sera shows that the cross-reactions are heterogeneous (Table 4). The difference in specificity related to the position of coupling is much less clear than in the case of anti-A-3α-diol antibodies. Despite this, it is possible to say that the anti-A-3β-diol-7-BSA antisera are more specific than those obtained using A-3β-diol-1-BSA. They could eventually be used for assay of the two androstanediols without preliminary separation.

DISCUSSION

In rabbits, the two kinds of immunogens derived from each type of androgen induced antibodies of comparable affinities. However, the specificity of antisera was found to differ markedly as a function of the coupling position.

Fixation of a carboxymethyl group to A-3α-diol and A-3β-diol in position 1α resulted in both cases in immunogens which induced the formation of antibodies exhibiting very poor specificity. As shown in Table 2 and Table 4, some cross reactions (testosterone, DHT) of those antibodies which were obtained using the 1α-carboxymethyl substituted immunogens, were very important. To account for this discrepancy it could be assumed that coupling in position 1α may prevent the antibody from coming within close vicinity of the A ring.

In the case of A-3α-diol one could postulate the existence of an intramolecular association between the carboxymethyl bridge in 1α and the -OH group in 3α, which would modify the spatial arrangement of the latter. Consequently, the antibodies directed to the altered configuration would no longer be able to fit the natural configuration.

When the coupling is through a carbon atom of the B ring, there is an increase in the specificity of

Table 4. Specificity of anti-5α-androstane-3β,17β-diol antisera

Rabbits / Steroids	Cross reaction (%)*					
	Androstane-3β-diol-7-BSA			Androstane-3β-diol-1-BSA		
	39–40	41–42	43–44	3 310	3 312	3 315
5α-androstane-3β,17β-diol	100	100	100	100	100	100
5α-androstane-3α,17β-diol	1.30	1	2.70	10.90	4.20	7.40
Dihydrotestosterone (DHT)	3.90	4.50	10.50	50	5.80	58
Testosterone	1.70	0.50	6.50	22	1.80	19.20
5-androsten-3β,17β-diol	12	28.50	21.50	17	9.50	23.80
Epiandrosterone	4.20	1	2.90	1.90	4.70	1.40
Dehydroepiandrosterone	1.20	0.60	2.30	0.60	0.55	0.75
5β-androstane-3α,17β-diol	0.90	<0.04	<0.10	4.40	0.50	0.36
Androsterone	0.30	<0.04	0.90	0.75	0.85	0.09
4-androsten-3,17-dione	0.10	<0.04	0.09	0.75	0.20	0.20
5β-androstane-3β,17β-diol	55	0.40	5	11	12.50	3.20

* See definition under Table 2.

antibodies. For A-3β-diol, we have studied the effect of the fixation of the carboxymethyloxime group at position 7. It was verified that antibodies elicited with the A-3β-diol-7-BSA immunogen showed a better specificity than did the anti A-3β-diol-1-BSA antibodies.

Comparison of the two types of anti A-3α-diol antisera shows that antibodies obtained using A-3α-diol substituted with the carboxymethyl group in position 1α were of poor specificity, whereas those directed to the immunogen substituted in position 15α were of high specificity. The relatively high cross reaction (8–12%) of anti A-3α-diol-15-BSA antibodies with androsterone could be explained by the proximity of the bridge in position 15α to the -OH group in position 17, which may result in steric hindrance. Another possible reason for this cross reactivity would be that the immunogen was contaminated with small amounts of androsterone-15α-carboxymethyl, this compound being the penultimate stage in the synthesis of the immunogen A-3α-diol-15 carboxymethyl. If this were so, separation of the anti-androsterone antibodies by affinity chromatography should increase the specificity of the anti-A-3α-diol antisera. Experiments are under way in the laboratory to ascertain if this is the case.

In conclusion, it can be said that coupling of A-3α-diol through a carboxymethyl bridge at position 15α preserves the specificity of the antibodies for the native steroid configuration. However, this has still to be demonstrated for the A-3β-diol. The preparation of specific antisera to androstanediols using as immunogens steroids coupled to protein through a carboxyethylmercapto bridge at position 15β was recently reported by Kohen and Lindner[15] and Rao *et al.*[16]. The results of these investigators together with ours provide convincing evidence that coupling of steroids to protein at position 15 through bridges of different chemical nature and showing either α or β orientation do result in the production of specific antibodies. It thus seems to us that position 15 is likely to be the position of choice to elicit the production of high specificity antibodies with this type of androgens.

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