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MONOCLONAL ANTIBODIES TO TESTOSTERONE: THE EFFECT OF IMMUNOGEN STRUCTURE ON SPECIFICITY

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Summary—A 15β-thioalkyl derivative of testosterone conjugated to bovine serum albumin (BSA) was synthesised and used to produce monoclonal antibodies. These antibodies were evaluated using 15β-(2-carboxyphenylthio)-testosterone [^{125}I]histamine as radioligand. Out of 1368 hybrids, 5 secreted anti-testosterone antibodies. These were compared with monoclonal antibodies derived from immunisation with testosterone-3-carboxymethyloxime-BSA. The first group of monoclonal antibodies all showed very low cross-reactivity with 5α-dihydrotestosterone (< 2.8%) indicating that this site of linkage is a good choice for discriminating between differences at the 4–5 position in the A-ring on the testosterone molecule. However they generally showed much higher cross-reactivity with progesterone and androstenedione than monoclonal antibodies raised to the 3-linked immunogen. Nevertheless within each fusion there were monoclonal antibodies with markedly different specificities. None of these antibodies could be considered suitable for use in a testosterone immunoassay, but it does suggest that an antibody with an improved specificity profile could be found using the monoclonal antibody approach.

INTRODUCTION

Despite recent advances in immunoassays, the accuracy of testosterone measurements is still compromised by the cross-reactivity of 5α-dihydrotestosterone (5α-DHT), particularly in samples from human females. Literature reports suggest that antisera raised to testosterone haptens conjugated to protein through the 15β-position exhibit low cross-reactivity with 5α-DHT. Rao and Moore [1] used a 15β-carboxyethylmercaptotestosterone–bovine serum albumin (BSA) conjugate to produce three antisera which gave less than 2% cross-reactivity, while Miyake *et al.* [2] raised antisera to a 15β-carboxymethyltestosterone–BSA conjugate which showed 2–4% cross-reactivity with 5α-DHT. However, the former workers observed cross-reactivity with progesterone and other C_{21}-steroids which again prevents accurate estimation of testosterone in human female plasma samples. We envisaged that this difficulty might be overcome by the production of monoclonal antibodies to testosterone conjugated through the 15 position, with the hope that among the various hybrids produced, one might secrete an antibody which did not recognise the C_{21}-steroids.

In the examples given above the antisera were evaluated using a tritiated tracer, but modern practice increasingly favours the use of radioiodinated tracers and we decided to gear our work towards these tracers. One of us has previously described a successful heterologous bridge combination to facilitate the use of radioiodinated tracers for progesterone immunoassay [3] and the same paper contained preliminary results with a 15β-linked heterologous bridge assay for testosterone. We have used the tracer described therein to evaluate our 15β-derived antibodies.

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EXPERIMENTAL

Materials

Testosterone-3(*O*-carboxymethyl)oxime-bovine serum albumin was a kind gift from Dr G. Read (Tenovus Institute, Cardiff, U.K.). [1,2,6,7-^{3}H]testosterone, testosterone-3(*O*-carboxymethyl)oximino-2-[^{125}I]iodohistamine and Na^{125}I were purchased from Amersham International. The steroids used in the antibody characterisation were purchased from Sigma (Poole, Dorset, U.K.).

Cell culture media were obtained from Flow Laboratories (Irvine, Ayrshire, U.K.) and Costar culture dishes (24 well No. 3424 and 96 well No. 3595) from Northumbria Biological Limited (Cramlington, Northumbria, U.K.). Polyethylene glycol (MW4000) for the fusion experiments was bought from Merck (Darmstadt) and pristane (2,6,10,14-tetramethylpentadecane) from Aldrich Chemical Company (Gillingham, U.K.). Polyethylene glycol (6000) for immunoassays was from BDH Chemicals Ltd (Poole, Dorset, U.K.). Donkey anti-mouse antibody-coated cellulose suspension (Sac-cel) was obtained from Wellcome Reagents Limited (Beckenham, Kent, U.K).

Preparation of derivatives

The overall chemical reactions followed the work by Rao and Moore [1] but differed in the sequence used (see Fig. 1). Oxidation of the Δ^5-3β-ol (**2**), either by Oppenauer oxidation for the aliphatic 15β-derivative or with pyridine-sulphur trioxide-

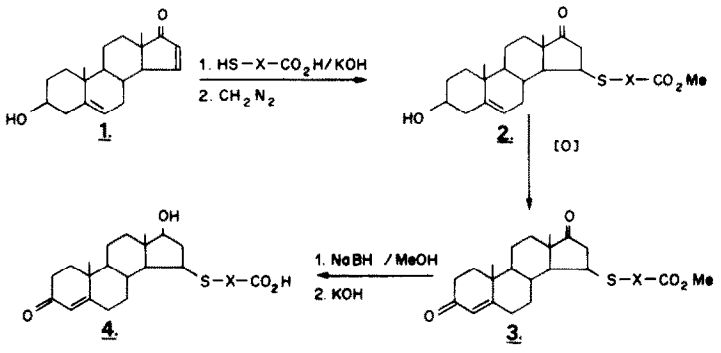

Fig. 1. Scheme for the synthesis of 15β-carboxyalkyl(or aryl)thiotestosterone. $X = Ch_2)_2$ for the immunogen or o-C_6H_4 for synthesis of the radioligand.

DMSO(4) followed by mild acid-catalysed Δ^5 to Δ^4 isomerisation for the aromatic 15β-compound, smoothly afforded the Δ^4-3,17-diones **(3)**. Selective reduction **(5)** of the 17-ketone followed by column chromatography readily gave good yields of pure Δ^4-17β-ol-3-ones which after mild alkaline hydrolysis afforded the required 15β-carboxyalkyl(or aryl)thiotestosterone **(4)**.

Δ^5-3β-ol-17-ones **(2)**

Methyl 3[(3β-hydroxy-17-oxo-androst-5-en-15β-yl)thio]propanoate. (*2*,X = CH_2CH_2), m.p. 119–120°C was prepared as described (1,m.p. 116–118°C).

Methyl 2[(3β-hydroxy-17-oxo-androst-5-en-15β-yl)thio]benzoate. (*2*,*X* = *o*-C_6H_4) 2-Mercaptobenzoic acid (1.3 g) was added to 3β-hydroxyandrost-5,15-dien-17-one **(6)** (1.37 g) as described for the above aliphatic analogue. The crude reaction product was treated with ethereal diazomethane and chromatographed on alumina (60 g) in dichloromethane–petroleum ether (1:1, v/v). Elution with dichloromethane gave a non-steroid material, mainly methyl 2-mercaptobenzoate by NMR, and dichloromethane–ethyl acetate (9:1, v/v) gave the ester (*2*,X = *o*-C_6H_4), which crystallised from ether–petroleum ether as needles (1.12 g, 56%), m.p. 185–7°C ν_{max} 3420, 1745, 1715 cm^{-1}: λ_{max} 261, 320 nm (ε 8700, 3200) ∂ 7.93 (d,1 × Ar-H), 7.1–7.6 (m,3 × Ar-H), 5.35 (m,H-6), 3.92 (s,OCH_3), 1.30 (s,H-18), 1.11 (s,H-19).

Anal. Found: C,71.2; H,7.4; S,7.2. $C_{27}H_{34}O_4S$ requires C, 71.3; H,7.5; S,7.05%.

Δ^4-3,17-diones **(3)**

Methyl 3[(3,17-dioxoandrost-4-en-15β-yl)thio]propanoate. (*3*,X = CH_2CH_2). A solution of cyclohexanone (3.8 ml) in toluene (575 ml) was heated under reflux in a Dean–Stark apparatus until no further water separated and compound **2**(X = CH_2CH_2, 0.83 g) and aluminium isopropoxide (0.29 g) were added. Reflux was continued for 3 h, when the solution was cooled and washed with dilute hydrochloric acid (70 ml). The organic phase was concentrated under reduced pressure and steam distilled to remove toluene and cyclohexanone. The residue was extracted with ethyl acetate and the extract was dried, evaporated and the residue crystallized from ether–petroleum ether to afford the dione **3**(X = CH_2CH_2) as needles (0.69 g, 83%), m.p. 140–2°C, ν_{max} 1740, 1725, 1675 cm^{-1}: λ_{max} 239 nm (ε 16,400): ∂ 5.76 (s,H-6), 3.73 (s,OCH_3), 1.26 (s,H-19), 1.16 (s,H-18).

Anal. Found: C,68.4; H,8.05; S,8.1. $C_{23}H_{32}O_4S$ requires C, 68.3; H, 8.0, S, 7.9%.

Methyl 2[(3,17-dioxoandrost-4-en-15β-yl)thio]benzoate (*3*,X = *o*-C_6H_4). A stirred solution of the alcohol **2**(X = *o*-C_6H_4; 1.1 g) in dry benzene (3.0 ml) and dry dimethyl sulphoxide (3.0 ml) was treated with triethylamine (2.3 ml) and a solution of pyridine-sulphur trioxide (1.17 g) in dry dimethyl sulphoxide (7.3 ml). The mixture was kept at approx 20°C during the latter addition by means of a water bath, then stirred at room temperature for 15 min and quenched by addition of ice-cold dilute hydrochloric acid (30 ml). The reaction mixture was extracted with ethyl acetate and the extract was washed with water and brine, dried and evaporated. The residue was dissolved in redistilled tetrahydrofuran (5 ml), diluted with a solution of concentrated hydrochloric acid (0.12 ml) in methanol (24.5 ml) and the solution kept at room temperature for 2 h, then diluted with ethyl acetate and washed with sodium bicarbonate solution, water and brine. The organic layer was dried and evaporated and the residue was chromatographed on silica gel (70 g) in dichloromethane. Elution with dichloromethane–ethyl acetate (3:1, v/v) gave the diketone **3**(X = *o*-C_6C_4) as a pale foam (0.78 g, 71%) which could not be crystallised, ν_{max} 1745, 1720, 1675 cm^{-1}: ∂ 7.95 (d,1xAr-H), 7.1–7.6 (m,3xAr-H), 5.76 (s, H-6), 3.93 (s,OCH_3), 1.33 (s,H-18), 1.28 (s,H-19).

Δ^4-17β-ol-3-ones **(4)**

3[(17β-Hydroxy-3-oxo-androst-4-en-15β-yl)thio]-propanoic acid (*4*,X = CH_2CH_2). Sodium borohydride (47 mg) was added to a stirred, ice-cold solution of

the dione **3** ($X = CH_2CH_2$, 0.78 g) in methanol (120 ml) and the solution was stirred for 2 h in an ice-bath. Acetic acid (0.1 ml) was added and the solvent was removed under reduced pressure. The resiude in ethyl acetate was washed with water and brine, dried and evaporated and chromatographed on alumina (24 g) in dichloromethane–petroleum ether (1:1, v/v). Elution with dichloromethane gave some unchanged dione and dichloromethane–ethyl acetate mixtures gave the 17β-ol methyl ester (0.51 g) as a gum which was saponified as described [1] to yield the acid **4** ($X = CH_2CH_2$) from acetone–petroleum ether as crystals (0.26 g, 34%) m.p. 199–201°C (lit. 202–4°C)

2[(17β-Hydroxy-3-oxo-androst-4-en-15β-yl)thio]benzoic acid (*4*X = *o*-C_6C_4)

The dione **3** ($X = o\text{-}C_6C_4$) was reduced with sodium borohydride exactly as above. Chromatography of the reaction product on alumina in dichloromethane, followed by dichloromethane–ethyl acetate mixtures was performed as rapidly as possible and afforded the 17β-ol methyl ester (41%) as fine needles, m.p. 157-9°C, from ethyl acetate–petroleum ether, δ 7.89(d,1xAr-H), 7.1–7.6 (m,3xAr-H), 5.74(s,H-6), 3.93(s,OCH_3), 1.26(s,H-19), 1.17(s,H-18).

Anal. Found: C,7.11; H,7.6; S,7.2. $C_{27}H_{34}O_4S$ requires C,71.2; H,7.5; s,7.05%.

When the chromatography was prolonged over several hours, the eluate contained two components, resolvable by careful TLC on silica gel. The more polar component was identical with the above material, while the less polar component was apparently the 15α-epimer, δ 7.98(d,1xAr-H), 7.0–7.6(m,3xAr-H) 5.72(s,H-6), 3.90(s,OCH_3), 1.22(s,H-19), 0.95(s,H-18).

The pure 15β-epimer (0.31 g) was dissolved in 1 M methanolic potassium hydroxide (20 ml) and tetrahydrofuran (10 ml), left overnight at room temperature and worked up as for compound **4**($X = CH_2CH_2$) to yield the acid **4** ($X = o\text{-}C_6C_4$) as an amorphous solid (0.12 g, 40%) which resisted recrystallisation. The nmr spectrum was similar to that of the pure 17β-ol methyl ester (see above) except for the lack of the methoxyl signal and gave no evidence of contaminating 15α-epimer.

Testosterone-15β-BSA conjugate

3[(17β-Hydroxy-3-oxo-androst-4-en-15β-yl)thio]-propanoic acid (**4** $X = CH_2CH_2$) (48 mg) was activated and coupled to bovine serum albumin (150 mg) by the general method previously described [7], to give a conjugate bearing 45 testosterone residues per protein molecule.

Testosterone-15β-(2[^{125}I]iodohistamine) conjugate **(5)**

The 15β-(2-carboxyphenylthio)testosterone–[^{125}I]-histamine conjugate **(5)** (Fig. 2) was prepared by activation of the testosterone derivative (**4**,X = *o*-C_6H_4,293 μg) using tri-*n*-butylamine and methyl chloroformate followed by addition of the iodinated histamine solution [8]. After extraction, the organic layer was chromatographed on a plastic-backed silica gel plate in benzene–ethanol (70:30, v/v). The less polar band was recovered by soaking in ethanol overnight at 4°C.

Immunisation

Balb/c mice received s.c. injections of 100 μg testosterone-15β-BSA or testosterone-3-carboxymethyloxime–BSA conjugate emulsified in Freund's complete adjuvant followed by multiple i.p. injections of 10 μg conjugate in Freund's incomplete adjuvant at monthly intervals, antiserum titre being assessed 14 days after the injection. Four days prior to the fusion, 10 μg conjugate in phosphate-buffered saline was given i.v. to the mouse with the highest antibody titre.

Sheep anti-mouse IgG antiserum was prepared by immunising a Kerry-Gritstone cross-bred ewe with 500 μg mouse IgG (Sigma I 5381) at monthly intervals and collecting serum 10–14 days after the 4th injection.

Hybridisation and antibody production

Spleen lymphocytes (2×10^8 cells) were fused with 1×10^7 P_3-NS1 1-Ag4 mouse myeloma cells [9] (donated by Dr C. Milstein) or X63-Ag8-653 mouse myeloma cells [10] (given by Dr J. Boyle), using polyethylene glycol 4000, by the protocol of Fazekas de St Groth and Scheidegger [11]. The resulting suspension was diluted in medium containing 20% foetal calf serum and HAT supplement [12] and distributed into 6 × 96-well plates on "feeder layers" of non-immunised spleen cells (1×10^5 well). After 6–10 days, hybrid colonies were visible and supernatants tested for antibody. Positive cultures were cloned by limiting dilution [13] on non-immune spleen cells (2×10^5 cells/well) and simultaneously passaged so that hybrids could be preserved in liquid nitrogen as quickly as possible. Primary clones were recloned at least once and hybrids chosen for further study were injected (1×10^7 cells) i.p. into pristane-primed Balb/c mice. Ascitic fluid containing antibody was isolated 10–14 days later.

Antibody detection assay

Culture medium from hybrids, mouse antisera or ascitic fluid (100 μl) were incubated with

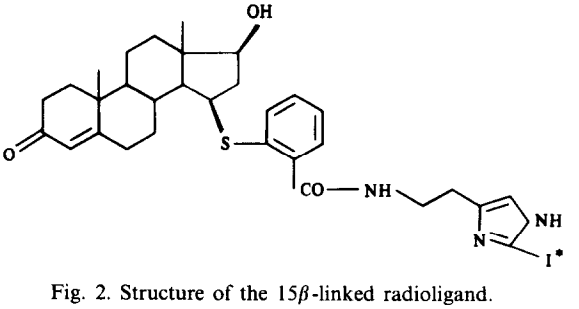

Fig. 2. Structure of the 15β-linked radioligand.

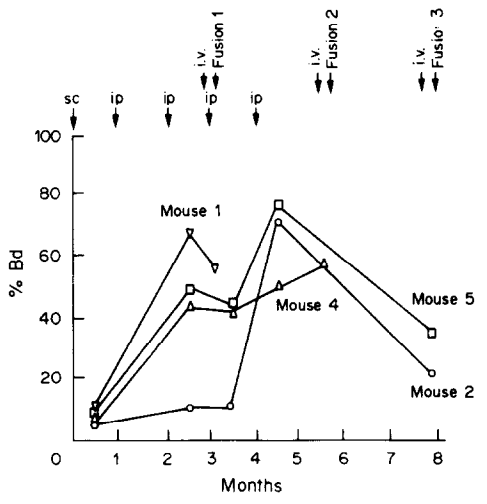

Fig. 3. Immunisation regime for testosterone 15β-BSA and antiserum production in mice prior to fusion. Ordinate represents binding of [^{3}H]testosterone to antiserum at a dilution of 1:100. s.c. injection administered 100 μg conjugate per mouse 1:1 in Freund's complete adjuvant. i.p. injection administered 10 μg conjugate per mouse 1:1 in Freund's incomplete adjuvant. i.v. injection administered 10 μg conjugate per mouse in phosphate buffered saline, 4 days prior to fusion.

[^{3}H]testosterone (150 fmol, 200 μl) or testosterone-3-CMO-histamine-[^{125}I] (10,000 cpm, 200 μl) for 2 h at room temperature, and separated by overnight incubation with sheep anti-mouse IgG antiserum (diluted 1:20) and addition of polyethylene glycol (5% w/v, 500 μl) immediately prior to centrifugation (2000 *g*, 20 min at 4°C). Background values were obtained by assaying culture medium, and a positive control of pooled mouse antiserum diluted in culture medium was included in each assay.

Determination of specificity and affinity constants

For assessment of the monoclonal antibodies derived from immunisation with testosterone 15β-thioalkyl derivatives, 15β(2-carboxyphenylthio)-testosterone-[^{125}I]histamine (10,000 cpm, 100 μl) was incubated with antibody (100 μl) and standards/samples (100 μl) for a 2 h at room temperature and a solid-phase double-antibody separation (Sac-cel) was employed. The antibodies derived from immunisation with the conjugates linked through the 3-position were characterised using the conditions described in the antibody detection assay. The cross-reactivity for each steroid was calculated using the criteria described by Abraham *et al.* [14]. The binding affinities were calculated by the method of Muller [15] from the competitive radioimmunoassay under equilibrium conditions.

RESULTS

To produce a range of hybridomas, groups of 5 mice were immunised with either the 15β- or 3-substituted testosterone immunogens. The serum antibody levels in the mice immunised with the testosterone 15β-BSA conjugate are shown in Fig. 3. Antibody levels achieved were less than those normally attained in generating polyclonal antibodies in other species, though the antibody levels at the time of fusion were suboptimal because of the necessity of a short period between intravenous boost and fusion. The details of the hybridomas produced from mice immunised with each agent are given in Table 1. The culture supernatants from a total of 1368 hybrids for the 15β-linked immunogen (fusions 1–4) were assessed for antibody binding. Five hybridomas were found to secrete antibody which bound 10–66% [^{3}H]testosterone, and three were successfully cloned. No antibody-secreting hybridomas were detected in fusions involving the 653 myeloma cell line. A mouse immunised with the 3-linked immunogen and with a titre of 1:110 at the time of fusion was utilised in fusion 5. This fusion yielded 454 hybridomas, of which 24 secreted antibody and of these, 14 were cloned at least twice.

All three monoclonal antibodies (1H8, 3C8, and 5E5) raised to the 15β-linked immunogen were characterised using 15β(2-carboxyphenylthio)testosterone-[^{125}I]histamine (**5**) as the radioligand (Fig. 2), and their properties are given in Table 2. The three antibodies had low binding affinity constants in the range $10^7\,M^{-1}$. This was similar to that of a polyclonal antiserum raised to the same immunogen ($5.7 \times 10^7\,M^{-1}$) and was consistent with the high concentrations of testosterone required to displace 50% labelled antigen. The specificity of the three antibodies for testosterone is characterised by a very low cross-reactivity with 5α-dihydrotestosterone (Table 3) but a high cross-reactivity with androstenedione and progesterone, except for 1H8 which exhibited very low progesterone cross-reactivity (0.07%).

In comparison, the studies utilising the 3-linked immunogen resulted in a series of hybrids with markedly different antibody profiles. Three hybrids (3H4, 3H8 and 5G12) were grown *in vivo* and their antibodies exhibited binding affinities for testosterone an order of magnitude higher than those obtained

Table 1. Fusion details

Fusion	Myeloma cell line	No. hybrids		No. antibody secreting hybrids
1	653*	168	(70%)	0
2	653	461	(89%)	0
3	NSI†	381	(66%)	4
4‡	NSI	358	(62%)	1
5§	NSI	454	(79%)	24

*The 653 myeloma cell line is X63-Ag8-653(10).
†NSI myeloma cell line is P_3NSI/1-Ag4(9).
‡For fusions 1–3 the immunogen was testosterone 15β-BSA and the antibody response in mice is given in Fig. 3. For fusion 4 a mouse with an antibody titre of 1:400 was used.
§Fusion utilising splenic lymphocytes from a mouse immunised with testosterone-3-carboxymethyl-oxime-BSA. After the fusion, cells were plated into 10 × 24-well plates (Fusion 1) or 6 × 96-well plates (Fusions 2–5).

Table 2. Properties of the monoclonal antibodies

	Monoclonal antibodies to T_{15} linked immunogen			Monoclonal antibodies to T_3 linked immunogen		
	1H8	3C8	5E5	3H4	3H8	5G12
Titre*	1:10,000	1:700	1:400	1:4,000	1:3,500	1:1,000
Displacement† (nmol l^{-1})	180	700	1150	20	16	24
K_a (10^8 M^{-1})	0.49	0.09	0.1	4.2	7.4	4.1
Antibody subclass	IgG_{2a}	IgG_1	IgG_1	nd	nd	nd

The properties were assessed for antibodies in ascitic fluid except for antibody subclass which was determined by Ochterlony double diffusion on culture supernatants. Monoclonal antibodies raised to the testosterone 15β-linked immunogen and the 3-linked immunogen were characterised using 15β(2-carboxyphenylthio) testosterone-[^{125}I]histamine and testosterone-3-CMO-[^{125}I]histamine respectively.

*Titre is expressed as the antibody dilution binding 50% labelled antigen.

†Displacement represents the concentration of testosterone required to displace 50% of labelled antigen.

with the 15β-linked immunogen (Table 2). The specificity of these three antibodies is given in Table 4. The 5α-dihydrotestosterone cross-reactivity is similar to that observed with polyclonal antisera raised to 3-linked immunogens [16]. The antibodies exhibited a wide range of cross reactivities with androstenedione and progesterone, this being lowest for the monoclonal antibody 5G12, which showed a stikingly different specificity profile from that of the polyclonal antiserum obtained from the immunised mouse at the time of fusion.

Table 5 shows data on the behaviour of the three antibodies derived from the 15β-linked immunogen with [^{3}H]testosterone and the ^{125}I-tracer (**5**) as radioligands. In each case the standard curves performed with the radioiodinated tracer were slightly more sensitive, which lends support to the notion discussed previously [3] on the desirable structural features of radioiodinated tracers for hapten assays. In particular the use of heterologous bridges for the immunogen and radioligand reduces the problems of bridge recognition which has previously been responsible for serious loss of sensitivity with iodinated tracers.

DISCUSSION

The factors which determine the formation of a highly specific antisteroid antibody are complex, but important requirements include the synthesis of a

Table 3. A comparison of the specificity of monoclonal and polyclonal antibodies resulting from immunisation with testosterone-15β-thioalkyl derivative

	Polyclonal antiserum*	Monoclonal antibodies		
		1H8	3C8	5E5
Cross-reactivity				
5α-Dihydrotestosterone	1.3	1.0	2.6	2.8
5β-Dihydrotestosterone	1.8	33	0.64	<0.1
Dehydroepiandrosterone	0.14	0.06	0.2	0.2
Cortisol	0.09	<0.01	0.64	0.76
5-Androstene-3β,17β-diol	<0.006	<0.07	<0.07	<0.07
Androstene-3,17-dione	47	90	70	100
Progesterone	6.4	<0.07	29	33

*Polyclonal antiserum was obtained from a mouse, hyperimmunised with testosterone 15β-thioalkyl derivative. The specificity was assessed using the [^{125}I]radioligand.

Table 4. Specificity of monoclonal and polyclonal antibodies derived from immunisation with testosterone-3-carboxymethyl-oxime-BSA

	Polyclonal antiserum	Monoclonal antibodies		
		3H4	3H8	5G12
Cross-reactivity				
5α-Dihydrotestosterone	27	22	16.7	96
5β-Dihydrotestosterone	100	100	95	10
Dehydroepiandrosterone	1.4	0.8	0.8	0.08
Androstene-3,17-dione	26	16	35	0.5
Progesterone	0.3	0.4	0.37	<0.03
Cortisol	0.03	<0.02	0.01	<0.02
5α-Androstane-3β,17β-diol	0.2	3.4	2.2	11
5-Androstene-3β,17β-diol	nd	0.9	0.4	7.3

Polyclonal antiserum was obtained from the mouse used as the spleen donor for the fusion. Antibody specificity was determined using the testosterone-3-CMO-[^{125}I]histamine radioligand.

Table 5. Comparison of antibody properties with different radioligands

Monoclonal antibody	[^{3}H]testosterone Titre	[^{3}H]testosterone Displacement (nmol l^{-1})	[^{125}I]testosterone* Titre	[^{125}I]testosterone* Displacement (nmol l^{-1})
1H8	1:100	300	1:10.000	180
3C8	1:50	1000	1:700	700
5E5	1:50	1700	1:400	1150

The monoclonal antibodies were derived from immunisation with testosterone-15β-BSA conjugate. Titre and displacement are defined in Table 2.
*15β-(2-carboxyphenylthio)testosterone-[^{125}I]histamine. (**5**).

chemically defined immunogen, the position and type of linkage of the carrier protein in the immunogen, and the ability to produce large numbers of different antibodies from which an antibody with the desired specificity might be selected. Published data describe a range of testosterone immunogens which have been linked to carrier proteins through various sites on the testosterone molecule, namely C_1[17], C_3[16], C_6[18], C_7[17], C_{11}[16], C_{15}[1, 2], C_{17}[19] and C_{19}[20]. None of these immunogens, with the exception of a polyclonal antiserum raised to a C_{19}-linked immunogen [20], has generated anti-testosterone antibodies with satisfactory specificity for accurate measurement of testosterone in samples from human females without chromatographic purification of the sample. Although antisera raised to testosterone conjugates linked through the 15β-position [1] have low cross-reactivity with 5α-dihydrotestosterone, they exhibit unacceptable cross-reactivity with progesterone and other C_{21} steroids.

In this paper we describe the synthesis of a 15β-thioalkyl derivative of testosterone and the application of monoclonal antibody techniques, to study the effect of immunogen structure on the specificity of single antibodies rather than the mixture found in a polyclonal antiserum. This derivative has not proved to be highly immunogenic in mice or in rabbits (unpublished data) and has generated relatively few antibody-secreting hybrids. Our results comparing the specificity of monoclonal antibodies raised against 15β- and 3-linked testosterone derivatives (shown in Tables 3 and 4) are broadly in line with previous experience of polyclonal antisera to steroids [1]. Thus structural changes proximal to the bridging position are poorly discriminated and distal changes are well recognised. All the monoclonal antibodies derived from immunisation with the 15β-linked testosterone conjugate have low cross-reactivity with 5α-dihydrotestosterone ($< 3\%$) but for most of the antibodies this is accompanied by a significant progesterone cross-reactivity. The recent report on monoclonal antibodies raised to a 17β-linked testosterone derivative by Fantl and Wang [19] describes similar problems.

Nevertheless, our observations and those of Kohen *et al.* [21] indicate that the specificities of individual monoclonal antibodies do exhibit striking departures from the general trend of other antibodies derived from the same fusion. This was most clearly seen in the very different specificity profile of our antibody 5G12 (Table 4). It is also indicated by the very low cross-reactivity for progesterone of our antibody 1H8 (Table 3) and with antibody F2 described by Kohen [21] which shows very low cross reactivity for 5α-dihydrotestosterone. It therefore remains entirely possible that future work will produce a monoclonal antibody with an overall improvement on the specificity of currently available polyclonal antisera. The ability to analyse 5–10 monoclonal antibodies from the immunisation of a single animal and the promise of an unlimited supply of the reagent are likely to promote continued efforts in this field.

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