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SPECIFICITIES OF ANTIBODIES TO OESTROGENS

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SUMMARY

Until recently attempts to endow antigenicity to 17β-oestradiol have invariably been made by conjugating protein (usually bovine serum albumin) via a hemisuccinate bridge to functional groups. Coupling in this manner produced antisera which unfortunately gave serious cross-reactions with other naturally occurring oestrogens. Antisera for 17β-oestradiol prepared by injecting rabbits with 17β-oestradiol-6-(O-carboxymethyl) oxime-bovine serum albumin produced antisera with very little cross-reaction (2 per cent or less) with oestrone, oestriol or 17α-oestradiol, and had extremely low cross-reactions with non-oestrogenic compounds. The specificities of antibodies to oestrogens in general are discussed in the light of both this and information from other workers.

INTRODUCTION

REASONABLY specific antisera now exist for testosterone and progesterone [1] but as yet no highly specific antisera for 17β-oestradiol (E_2-17β) have been reported. Previous attempts to endow antigenicity to this oestrogen have invariably been made by coupling protein to one of its functional groups [1–4], and this produced antisera with serious cross-reactions of the order of 35–100 per cent to oestrone and 17α-oestradiol. These cross-reactions with other important physiological hormones meant that radioimmunoassay could only be performed on plasma processed by extraction and chromatographic separation. In order to produce a simpler radioimmunoassay it was necessary to obtain more specific antisera. Speculation that more specific antisera may be produced if coupling to protein was made at a point distal to structurally unique regions, thus enabling antigenic recognition of both functional groups of 17β-oestradiol, led us [5] to prepare and characterise a hapten which involved coupling bovine serum albumin via the C_6 position.

The present paper gives details of the specificity of the antisera produced by this antigen, and discusses the specificities to oestrogens in general.

EXPERIMENTAL

Methods

Injection of antigen. Five rabbits were injected with the antigen (17β-oestradiol-6-(O-carboxymethyl) oxime-bovine serum albumin). The antigen was dissolved in 0·9 per cent sodium chloride (4 mg/ml). This solution (5 ml) was homogenised with freshly prepared Fruend's complete adjuvant (5 ml) by means of a Silverson mixer. 10 ml of this emulsion was suspended in 5 ml of 1 per cent Tween 80 (w/v).

Each animal received 3 ml (= 4 mg of antigen): 1 ml of the preparation (1.33 mg) was injected intra-muscularly into the thigh of each leg and the remaining 1 ml distributed among 8 subcutaneous sites in the animal's back. Booster injections were administered as above using half dose (2 mg per animal). The first boost was given one month after the primary injection. Boosts were given monthly.

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Collection of antisera. 25–30 ml of blood were drawn from the ear vein of the animals, collected in a tube containing one drop of heparin and centrifuged to give 12–15 ml plasma per animal. The first collections (Bleed 1) were made 10 days after the second boost. Bleed 2 was made 10 days after the third boost. Antisera produced by the various rabbits and bleeds were kept separate and are identified by the following nomenclature: L (Liverpool), R (rabbit), $6KE_2$ (antibodies produced by 17β-oestradiol-6-(O-carboxymethyl) oxime-bovine serum albumin) and B (bleed number). Plasma was BSA adsorbed and Rivanol treated [6]. The final dilution of sera was 1/5 after this treatment.

Polystyrene tubes. Small disposable 0·6 ml volume polystyrene test tubes (LP/2), Luckham Ltd., Surrey, U.K., were used for solid radioimmunoassay. They were cleaned in a Decon solution, (2% v/v), rinsed out with distilled water and dried in an oven at 50°C before use. Since no isotope effect between [6,7-^{3}H]-17β-oestradiol ([6,7-^{3}H]E_2-17β) and [2,4,6,7-^{3}H]E_2-17β could be observed a testing titre of 5000 c.p.m. (mass = 13·8 pg) of the latter was used.

Solid radioimmunoassay. A modified Abraham[6] technique of solid radioimmunoassay was used for all this work. Prepared assay buffer solution of steroids etc. were made up such that 0·5 ml volume contained the required mass of non-radioactive compound (if any) to be tested together with 5000 c.p.m. of [2,4,6,7-^{3}H]E_2-17β. All assays were performed in duplicate.

Cross-reaction test. The specificities of antisera were tested by determining the percentage cross-reaction of various steroids by the solid radioimmunoassay. Various amounts of the steroids to be tested were dissolved in 0·5 ml assay buffer containing 5000 c.p.m. [2,4,6,7-^{3}H]E_2-17β and the binding vs. mass curve of the compound compared with the curve given by E_2-17β.

Calculation of the cross-reaction was made as indicated by Abraham [6]:
i.e.: Mass of E_2-17β to displace 50% of bound [2,4,6,7-^{3}H]E_2-17β = x.

Mass of cross-reacting steroid required to displace 50 per cent of bound [2,4,6,7-^{3}H]E_2-17β = y.

Percentage cross-reaction = $\frac{x}{y} \times 100$

RESULTS

Table 1 presents the results of our cross-reaction study using second bleeds, setting 17β-oestradiol (E_2-17β) at 100 per cent. The compounds are arranged in order of descending cross-reactivity. The results show that apart from 6-keto-E_2-17β, the highest cross reaction is only 2 per cent. (17α-oestradiol, (E_2-17α)). Oestrone (E_1) and oestriol (E_3) have only about 1 per cent cross-reactions with this bleed.

DISCUSSION

The LR($6KE_2$) antisera are similar to anti-17β-oestradiol antisera produced by previous workers[1,3,4,6] in that these antisera have virtually no cross-reaction with non-oestrogenic steroids, despite the fact that these workers conjugated the steroids to one of the functional groups of E_2-17β. The specificity of the Liverpool antisera towards oestrogens containing functional groups at C_{17} other than β-hydroxyl (E_1 and E_2-17α) is entirely different from that previously reported, being superior in its low cross-reactivity (1–2 per cent) towards these compounds compared with the 35–100 per cent cross-reaction of the other antisera to E_2-17β. The fact that all five rabbits injected produced reasonably high titres of highly specific

Table 1. Percentage cross-reactions of LR(6KE$_2$) *antisera (bleed 2)

Steroid or Compound	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
(A) Antigen	400	316	400	800
(B) 6-O-(Carboxymethyl) oxime 17β-Oestradiol	100	50	150	125
(C) 6-keto-17β-oestradiol	136	77	100	110
(D) 17β-oestradiol	100	100	100	100
(E) 17α-oestradiol	2·1	2·4	1·7	2·0
(F) Oestriol	0·7	2·0	1·2	1·0
(G) Oestrone	1·1	0·7	1·2	0·9
(H) Equilin	0·4	0·5	0·5	0·6
(I) Equilenin	0·4	0·4	1·0	0·2
(J) Ethynyl-17β-oestradiol	0·08	0·02	0·05	0·02
(K) Testosterone	0·05	0·05	<0·01	<0·01
(L) Dehydroepiandrosterone	—	—	<0·01	<0·01
(M) Cortisol	0·01	0·01	<0·01	<0·01
(N) Progesterone	0·01	0·01	<0·01	<0·01
(O) Bovine Serum Albumin	<0·01	<0·01	<0·01	<0·01
(P) Cholesterol	<0·01	<0·01	<0·01	<0·01
(Q) Phenol	<0·01	<0·01	<0·01	<0·01

*Reciprocal dilutions of antisera used. Rabbit 1 = 15,000, Rabbits 2, 3, 4 = 10,000.

antisera suggests that free access to the C_3 phenolic and C_{17} functional groups is important for specificity in the sense that there is no serious cross-reaction with other C_{17} oestrogenic compounds.

The LR(6KE$_2$) antisera cross react with 6-keto-E$_2$-17β (77–140 per cent). Thus both in the case of these anti-E$_2$-17β antisera and those produced by previous workers, the major cross-reactivities occur with these oestrogenic compounds which have substituents at the point of covalent linkage to the protein. At first it is tempting to suggest that there will invariably be a cross-reaction with those compounds which have substituents at the point of attachment and could indicate that conjugation may be most profitably made at those positions of a steroid or compound which do not normally occur in nature.

The proposal that cross-reactions occur at the point of coupling seems to hold for the functional groups of E$_2$-17β. However, the large cross-reactions with E$_2$-17α, E$_1$ and E$_3$ with either a 3 phenolic or 17β-hydroxyl conjugation do not occur with E$_1$ conjugated with protein via the 17-keto groups Midgley and Niswender [1] found only a 4 per cent cross-reaction with E$_2$-17β, and a 1·8 per cent cross-reaction for E$_2$-17α with antisera raised by an antigen possessing 17-ketoconjugation to E$_1$.

This interesting difference in specificity between the two C_{17}-conjugated steroids requires explanation. There is at least one major difference here: E$_1$ does not possess a 17α hydrogen, and when conjugated via its carboxymethyl-oxime, it still keeps the double bond at C_{17} intact. Hemisuccination of the 17-hydroxyl of E$_2$-17β leaves the 17α hydrogen intact.

Could it be that the absence of the 17α hydrogen leads to the ready antibody process recognition of the keto conjunction without confusion with 17α and 17β isomers, and that the orientation of the 17α hydrogen is difficult to distinguish because of changes in bond angle because of possible hydrogen bonding?

The cross-reaction with the 6-keto-$E_2\beta$BSA antigen is 400–800 per cent that of E_2-17β, suggesting, as expected, a higher affinity. The cross-reaction with 6-keto-E_2-17β is of about the same order as that of E_2-17β. The lower cross-reactions of the other compounds studied could be due to the lower binding affinities for the same population of antibodies. This concept thus need only involve a population of very limited heterogeneity of antibodies which possess an all-embracing recognition of the structural features of the complete hapten.

The cross-reactions against E_2-17β of the treated LR(6KE_2) antisera suggest antigenic recognition of probably seven structural features: (i) steroid plus carboxymethyl oxime bridge; (ii) the rigid planar steroid backbone; (iii) aromatic A ring; (iv) C_3 phenolic group; (v) the 17β-hydroxyl group; (vi) BSA plus carboxymethyl oxime bridge; (vii) BSA.

The fact that BSA adsorption removes antibodies to BSA may be due to the development of a separate antibody because there was some free BSA in the antigen preparation.

According to Pressman and Grossberg[7], antibodies produced by an animal are probably made up of a few distinct antibody populations. Sela[8] suggested that the basis of specificity could be the idea that antisera are in general heterogenous with respect to their binding affinity, reflecting configurational differences in the binding sites of the different antibody populations within a given preparation.

The question of degree of heterogeneity of antibody populations remains at present unresolved. Future work directed towards purification of antibody preparations should, however, help to throw some light on this problem.

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REFERENCES

1. A. R. Midgley, Jr. and G. D. Niswender: In *Steroid Assay by Protein Binding* (Edited by E. Diczfalusy), 2nd Karolinska Symposium on Research Methods in Reproductive Endocrinology, Geneva (1970) (*Acta Endocr. (Kbh.)* **67 Suppl. 147**) p. 320.
2. L. Goodfriend and A. Sehon: In *Immunological Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) p. 15.
3. B. F. Erlanger, S. H. Beiser, F. Borek, F. Edel, and S. Lieberman: In Methods in Immunology and Immunochemistry (Edited by C. A. Williams and M. C. Chase). Academic Press, New York, Vol. 1 (1967) p. 144.
4. M. Ferin, P. E. Zimmering, S. Lieberman and R. L. Vande Wiele: *Endocrinology* **83**, (1968) p. 565.
5. P. D. G. Dean, D. Exley and M. W. Johnson: *Steroids* **18** (1971) 593.
6. G. E. Abraham: *J. clin. Endocr.* **29**, (1969) 866.
7. D. Pressman and A. L. Grossberg: In *Immunological methods in steroid determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) p. 1.
8. M. Sela: *Naturwissschaften* **56** (1969) 206.

DISCUSSION

Kellie: With the covalent link in the 6 position you have a double bond to the nitrogen atom, so this is presumably in the same plane as the ring, but the oxime can exist in two forms, α and β. Do you have any idea what the orientation is at this particular point?

Exley: No.

Kellie: The reason I ask this question is that we prepared similar derivatives. We thought that all attempts to couple steroids to BSA through functional groups

were effectively masking a group and therefore masking a discriminant. With the 6-carboxymethyl compounds we prepared the specificity was disappointing in that the exposure of both the functional groups appeared to widen the specificity rather than narrow it. It has occured to me that all our 6-carbomethyl compounds have 6β-orientation and that when the steroid goes onto the BSA, it will, presumably, expose the α side of the molecule. This makes me wonder whether, if we were to prepare the 6α-carboxymethyl compounds and expose the 6β side, whether it would have a better effect on the specificity.

Exley: In fact I didn't know what your specificities were until I came to this meeting; I awaited your findings with interest.

Kellie: I regard the specificity as disappointing, but I think that there's still some room to manoeuver.

Exley: May I point out that your point of conjugation at C-6 is a single bond; it's not a double bond, is it?

Kellie: No the carbon–carbon bond is a single bond, whereas in your derivative the carbon–nitrogen link is a double bond.

Exley: Yes but I've pointed out the fact that there's still a hydrogen at C-6 which is available for masking in the way I have mentioned in regard to estrone.

Kellie: In your linkage the carbon atom at the 6 position has two valency bonds to the nitrogen and these must be rigid. It is not these particular valencies that I'm worried about but it's the third valency of the nitrogen which can be α or β.

Exley: Yes, I agree, but I am concerned about the fact that you have a hydrogen atom still free at the position of conjugation and, as I said this morning, this could lead to trouble, and has already lead to trouble at the 17β position. When you conjugate with estrogen with O carboxymethylation at C-17 the resultant double bond seems to be responsible for a high degree of specificity.

Kellie: No one would be more pleased than I if we can make some sense out of the available data. This antisera preparation to estradiol looks excellent.

Grant: One of the problems in this type of methodology must be specificity and knowing what estrogens are present on plasma. I think we guess this from what estrogens are present in urine and as you know, Dr. Marrian always claimed that the 16,17-ketols were urinary estrogens of considerable quantitive importance We missed them in urine because we destroyed them on acid hydrolysis. Does anybody know whether there is a 16β, 17-oxo estrogen in plasma, because I'm prepared to bet that if there is, and if it's there in any quantity, it'll interfere rather badly with your method.

Adlercreutz: I can tell you that there is and also that it's the free form. I presume you mean 16-keto estradiol?

Grant: Yes.

Adlercreutz: In the general discussion I can say some more about these things.