EVIDENCE FOR THE PRESENCE OF TWO DISTINCT TYPES OF ANTIBODIES IN AN ANTISERUM RAISED AGAINST OESTRIOL-16α-GLUCURONIDE

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Received 5 April 1976

1. Introduction

The high concentrations of oestriol-16α-glucuronide ($E_3$16G) present in maternal plasma, during late pregnancy, are largely of foeto-placental origin [1] and therefore a direct radioimmunoassay for this steroid should be useful in assessing foetal well-being. Previous attempts [2–4] to raise specific antisera for $E_3$16G, using an antigen in which the steroid was linked to an immunogenic carrier via its native carboxyl group, gave extremely variable cross-reactions (from $< 1\%$ to 53%) for oestriol (E_3). However, even the most specific of these antisera appeared to give an overestimation of 24% when incorporated into an assay [4]. Using a similarly linked antigen we have obtained antisera which, by traditional criteria [5], are highly specific; the cross-reaction for unconjugated E_3 being only 0.03%. Nevertheless, it was found that, under assay conditions, the presence of unconjugated E_3 led to an overestimation of $E_3$16G. This apparently contradictory behaviour can be explained by the presence, in the antisera, of two distinct types of antibodies; binding to one of these types of antibody being dependent upon the presence of both the steroid and the glucuronic acid moieties, whereas for the other type of antibody the presence of the steroid alone is sufficient. The presence of these two types of antibodies, in varying ratios, would explain the findings of other workers [2–4].

In our antisera the more specific (for $E_3$16G) type of antibody was predominant and the effect of the non-specific antibodies could be suppressed by presaturation with E_3. This permitted the accurate measurement of $E_3$16G over a range of 0–200 pg in the presence of unconjugated E_3.

2. Materials and methods

2.1. *Reagents*

Steroid glucuronides, other than $E_3$16G were obtained from the MRC Reference Collection and unconjugated steroids were purchased from Steraloids Inc. Wilton, USA. Tritiated E_3-16G (sp. act. 30 Ci/mmole) and tritiated E_3 (sp. act. 70 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, UK. Scintillant (NE 260) was obtained from Nuclear Enterprises, Edinburgh and all samples were counted in 4 ml of this material. $E_3$16G was extracted from pregnancy urine and purified as the sodium salt [6]. General reagents were purchased from BDH and all solvents were redistilled prior to use. Freund's complete adjuvant was obtained from Difco Laboratories.

2.2. *Experimental*

The sodium salt of $E_3$16G was coupled to bovine serum albumin as previously described [7] and the resulting antigen injected into four adult, male New Zealand White rabbits using a previously described regimen [8]. Blood samples were removed ten days after the second booster injection and tested in the normal manner [9] using tritiated $E_3$16G as tracer. In addition one antiserum (R4B1) was also equilibrated with tritiated E_3 and the ability of cold E_3 and cold $E_3$16G to displace this tracer measured.

In order more nearly to simulate assay conditions, the displacing ability of $E_3$16G in the presence of E_3 (in a 1:2 ratio; w/w) was compared with that of $E_3$16G alone. This experiment was then repeated using antiserum that had been presaturated with E_3 (10 ng/ml of diluted antiserum).

3. Results

The molar steroid:protein ratio of the conjugate was determined spectrally to be 12:1. The characteristics of the antisera, obtained using this antigen, were tested using a tritiated $E_3$16G tracer and the results are presented in table 1. Fig.1 shows the detailed displacement curves for E_3 and $E_3$16G. The two responses are markedly non-parallel; E_3 showing an initial phase in which it displaced tritiated $E_3$16G and then a second phase in which it caused virtually no further displacement, whereas $E_3$16G showed continuing displacement down to low levels of binding.

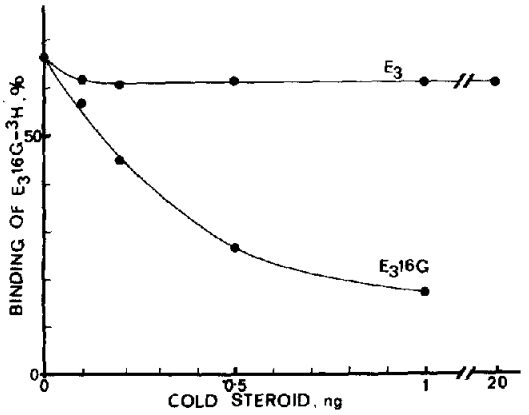

Fig.1. Displacement of tritiated $E_3$16G from antiserum R4B1 with unlabelled $E_3$16G and E_3.

When antiserum R4B1 was equilibrated at a dilution of 500:1 with a tritiated E_3 tracer, the displacing ability of cold E_3 relative to that of $E_3$16G was found to be 495%.

Table 2 shows the displacing ability of $E_3$16G in the presence of unconjugated E_3. The binding was read off from a standard curve, using $E_3$16G alone. It can be seen that, using untreated antiserum, the presence of E_3 consistently caused an overestimation of $E_3$16G. However, using antiserum presaturated with E_3, this problem was overcome.

The sensitivity of the standard curve, obtained using the pretreated antiserum (100 μl), was found

Table 2
Measurement of $E_3$16G in the presence of E_3, using antiserum both with and without pretreatment

$E_3$16G (pg)	E_3 (pg)	$E_3$16G Measured (pg)	
		Untreated antiserum	Treated antiserum
0	0	0	0
50	100	72	48
100	200	164	100
150	300	192	150
200	400	283	192

Table 1
Cross-reactions (as %) of oestriol-16α-glucuronide antisera and their working dilutions

Steroid	Antiserum			
	R1 B1	R2 B1	R3 B1	R4 B1
Oestrone		5.0		<0.01
Oestradiol		1.5		0.02
Oestriol	58	5.0	100	0.03
Oestriol-16α-glucuronide	100	100	100	100
Oestrone-3-glucuronide		<0.01		<0.01
Oestradiol-3-glucuronide		0.04		<0.01
Oestriol-3-glucuronide		<0.01		<0.01
Oestradiol-17β-glucuronide		2.7		0.05
Working Dilution	100:1	500:1	100:1	2000:1

Coding of antisera: R1-R4 refers to 4 individual rabbits and B1 refers to blood samples removed after the 3rd injection.

to be 10 pg (least quantity of $E_3$16G distinguishable from the zero point with $p < 0.001$).

4. Discussion

It is possible to prepare steroid-specific antisera using antigens in which the steroid is linked through an existing functional group, providing that the accessible part of the steroid is unique [10]. In this context, it was felt that conjugation of $E_3$16G via the carboxyl group to an immunogenic carrier should allow recognition of the entire steroid and the majority of the glucuronide moiety. This line of thought was supported by the results presented in table 1, although there was considerable interanimal variation which is consistent with the variable results of other workers [2–4].

Although, by traditional criteria [5], antiserum R4B1 was highly specific, a more detailed examination revealed that the displacement curve for E_3 was biphasic; a likely explanation of this being the presence of two distinct types of antibody; one type binding both E_3 and $E_3$16G, whereas the other binds $E_3$16G exclusively. Both sets of antibodies would be initially loaded with tritiated $E_3$16G; the first phase of the E_3 displacement curve reflecting the ability of E_3 to displace tritiated $E_3$16G from the less specific antibodies whereas in the second phase E_3 is unable to cause displacement from the specific antibodies.

The presence of two distinct types of antibody was clearly demonstrated by the following two experiments. First, the antiserum was loaded with tritiated $E_3$16G and the cross-reaction of E_3 assessed in the normal manner [5] to be 0.03%. The antiserum was then loaded with tritiated E_3 and, under these conditions, the cross-reaction for E_3 was 495%. When tritiated $E_3$16G is used as the tracer, E_3 gives a very low cross-reaction because much of this tracer is bound to the highly specific antibodies from which E_3 cannot cause displacement. However, when tritiated E_3 is used as tracer, it can only be bound by the less specific antibodies so that, under these conditions, the E_3 cross-reaction is very much higher. As this cross-reaction is much greater than 100%, these antibodies must show a preference for unconjugated E_3.

When antiserum R4B1 was used to measure $E_3$16G in the presence of E_3 there was a considerable overestimation of $E_3$16G which can be attributed to displacement of tritiated $E_3$16G from the less specific antibodies by E_3. This was remedied by presaturation of the antiserum with E_3 which prevented any initial loading of the less specific antibodies with tritiated $E_3$16G; under these conditions the tracer would only bind to the specific antibodies and displacement could then only be effected by cold $E_3$16G. It has been shown that the treated antiserum could completely discriminate against E_3 in a 2:1 ratio with $E_3$16G; this ratio being four times as great as that found in pregnancy plasma [11,12]. Late pregnancy plasma contains around 50 ng/ml of $E_3$16G [11] and so the sensitivity of the standard curve (10 pg) should be adequate.

In conclusion, we have prepared an antiserum which should be adequate for the clinical measurement of $E_3$16G in pregnancy plasma. We have also illustrated that the commonly used 50% displacement method for testing antiserum specificity [5] can be extremely misleading in cases of pronounced non-parallelism. The problem being that, although large amounts of steroid may be required for 50% displacement, a small amount of the same steroid may produce a disproportionately large displacement.

Acknowledgements

We would like to thank Dr G. Wilson for a gift of oestriol 16α-glucuronide. We are also grateful to G. D. Searle Ltd, the Wellcome Trust and the MRC for financial support

References

[1] Diczfalusy, E. and Mancuso, S. (1969) in: Foetus and Placenta (Klopper, A. and Diczfalusy, E., eds.), pp. 191–248, Blackwell, Oxford.
[2] Niswender, G. D., Nett, T. M., Meyer, D. L. and Hagerman, D. D. (1975) in: Steroid Immunoassay (Cameron, E. H. D., Hillier, S. G. and Griffiths, K., eds.), pp. 61–66, Alpha-Omega, Cardiff.
[3] Wright, K., Collins, D. C. and Preedy, J. K. R. (1974) J. steroid Biochem. 5, 303.
[4] Adlercreutz, H., Lehtinen, T. and Tikkanen, M. (1976) J. steroid Biochem. 7, 105–107.
[5] Abraham, G. E. (1969) J. clin. Endocr. Metab. 29, 866–870.

[6] Adlercreutz, H., Soltmann, B. and Tikkanen, M. J. (1974) J. steroid Biochem. 5, 163–166.
[7] Park, B. K., Rowe, P. H., Barrington, E. J. and Dean, P. D. G. (1975) IRCS Medical Science 3, 616.
[8] Park, B. K., Rowe, P. H. and Dean, P. D. G. (1976) FEBS Lett. 64, 300–302.
[9] Rowe, P. H., Lincoln, G. A., Racey, P. A., Lehane, J., Stephenson, M. J., Shenton, J. C. and Glover, T. D. (1974) J. Endocr. 61, 63–73.
[10] Cook, I. F., Rowe, P. H. and Dean, P. D. G. (1973) Steroids Lipids Res. 4, 302–309.
[11] Levitz, M., Jirku, H., Kadner, S. and Young, B. K. (1975) 6, 663–667.
[12] Goebelsmann, U., Katagiri, H., Stanczyk, F. Z., Cetrulo, C. L. and Freeman, R. K. (1975) J. steroid Biochem. 6, 703–708.