Rabbit anti-steroid antisera: a study of titers and specificities over a 22-week period

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Summary. Anti-equilin antisera titers were determined weekly in 4 rabbits over a 5-month immunisation period. Cross-reactions to equilin sulphate, oestrone and equilenin were also measured. There appeared to be no relationship between antiserum titer and specificity.

The use of radioimmunoassay (RIA) for the determination of steroids in biological material[3,4] has become widespread. Steroids require conjugation to immunogenic material in order to stimulate antisera production. Many procedures are employed differing in the type of immunogen, the immunisation procedure or the animal species used. Comparisons of techniques and results are useful in enabling improvement in RIA. Of particular importance are specificity and titer but few studies have documented their relationship. We report such data from a recent immunisation programme.

Experimental. Materials. Steroids were obtained from Ayerst Inc., Montreal, Canada, or Steraloids Inc., Wilton, USA. {2,4-^{3}H}-Equilin, sp. act. 41 Ci/mmole, was purchased from NEN, Boston, USA. Equilin-3-hemisuccinate-BSA was synthesized and characterized as described previously[5]. The molar steroid:BSA ratio was 26:1. Rabbits (male, New Zealand white) were obtained from Cheshire Rabbit Farms, UK.

Immunisation. Rabbits (R1-4) were injected with the equilin-conjugate suspended in saline plus Freund's complete adjuvant and Tween. 4 s.c. and 2 i.m. sites were injected[6], each rabbit receiving 3 mg of antigen. The procedure was repeated monthly. Blood samples (B1-21) were removed at weekly intervals from the marginal ear veins into heparinized tubes and the plasma stored at − 15 °C.

Radioimmunoassay. Antisera and steroids were diluted and dissolved in 0.1 M phosphate buffer (pH 7.0) containing sodium chloride (0.9% w/v) and BSA (0.1% w/v). The assay procedure, involving dextran-charcoal separation, has been previously described[5]. Antiserum titer was defined as the final serum dilution required to bind 50% of a given amount of labelled antigen[7]. Oestrone, equilin sulphate and equilenin were found to have the highest cross-reactions in bleed R4B15[5]. The cross reactions of these 3 steroids were then determined in other representative bleeds, at their appropriate titers, using the ratio of the mass of each steroid, giving 50% inhibition of zero binding, to the required mass of equilin[3].

Results and discussion. The observed development of titer in the 4 rabbits (table 1) showed the classical pattern of increasing titer following each injection, until the 4th injection when R1 and R2 failed to respond. This suggests that they had become tolerant to the antigen and one cause of the tolerance is probably the high doses of antigen used in the immunisation method. An oestrone-6-(*O*-carboxymethyl)oxime-BSA conjugate required 9 monthly booster injections before antisera of suitable titer were realized[6]. Tolerance might be avoided by the use of conjugates with steroid:carrier ratios of a different order of magnitude than those presently available.

Peak titers were observed to occur at various times between 7 and 31 days after an injection. It is clear that the maximum value occurs for a short period of time only, and that constant monitoring is necessary if the peak value is to be utilized. The observed titer development in all 4 rabbits showed no evidence of the episodic antibody release previously reported[8]. Though we were sampling at longer time

Table 1. The anti-equilin antibody titers of 21 bleeds of rabbits R1-4. The days of each bleed and each injection are also shown

	Bleed titers			
	R1	R2	R3	R4
Injection/Day 0				
B1/Day 7	–	–	–	–
B2/Day 14	4	–	–	–
B3/Day 22	–	–	–	8
B4/Day 27	18	6	2	10
Injection/Day 28				
B5/Day 35	180	60	80	660
B6/Day 42	240	80	120	560
B7/Day 50	380	100	120	480
B8/Day 55	380	160	100	800
Injection/Day 56				
B9/Day 63	1240	600	560	1380
B10/Day 70	1320	860	520	1240
B11/Day 80	1640	900	1120	1780
B12/Day 87	2800	580	1200	920
B13/Day 91	1360	480	740	1020
Injection/Day 92				
B14/Day 98	1620	900	2400	5640
B15/Day 105	1340	920	2460	5900
B16/Day 112	1320	1000	4900	8940
B17/Day 119	1200	700	3680	5900
B18/Day 126	580	500	3200	3020
B19/Day 133	460	360	900	1320
B20/Day 144	580	220	1680	1000
B21/Day 158	240	180	700	700

Table 2. The cross-reactions of equilin sulphate, oestrone and equilenin with anti-equilin antisera of various bleeds of rabbits R1-4. Booster antigen injections were given between bleeds B8 and B9, and between bleeds B13 and B14

Bleed	Cross-reaction (%)		
	Equilenin	Oestrone	Equilin sulphate
R1B9	0.40	1.10	2.60
R1B13	0.35	0.89	1.80
R1B14	0.52	2.04	2.70
R1B15	0.31	0.55	3.16
R1B16	0.37	0.72	2.60
R1B21	0.72	1.12	1.66
R2B9	0.95	0.72	2.19
R2B13	0.68	0.73	0.81
R2B14	0.95	1.29	2.04
R2B15	0.52	0.95	2.40
R2B16	0.40	1.80	2.40
R3B9	0.60	4.60	1.70
R3B13	0.60	1.00	1.40
R3B14	0.30	3.20	1.40
R3B15	0.20	3.80	1.10
R3B21	0.60	5.40	1.80
R4B9	0.03	0.60	3.55
R4B13	0.30	1.00	1.55
R4B14	0.03	0.70	4.15
R4B15	0.03	0.55	4.95
R4B16	0.04	0.85	3.80
R4B21	0.10	0.90	5.25

intervals than these authors, the random nature of the putative episodes should have ensured their detection. Similar studies using the same immunisation schedule also failed to observe episodic release[9].

Each of the antisera obtained showed comparatively high specificity for equilin. In addition, R3 and R4 produced antisera (R3 and R4 B14-18) of reasonable titer. An antiserum raised against equilin-17-(*O*-carboxymethyl)oxime-BSA[10] using the same immunisation procedure was less specific and showed, for example, a cross-reaction of 38% for equilenin[11]. The high specificity of all the present antisera confirms that position 3 is suitable for derivatisation for most oestrogens[6,12,13].

3 of the rabbits consistently produced antisera with similar interactions with equilin sulphate, oestrone and equilenin (table 2). The other rabbit (R3) produced antisera with a higher cross-reaction for equilin sulphate than oestrone. This clearly illustrates the possible variation in animal responses to identical antigens presented in the same manner. Furthermore it is difficult to decide whether the changes in specificity observed from bleed to bleed are the result of non-specific influences or are real changes induced either by changes in circulating antigen or antibody. Antisera cross-reactions have been seen to vary from week to week in an unpredictable fashion in a non-specific anti-testosterone antiserum[14], changes of $\pm 30\%$ being seen in the 5α-dihydrotestosterone cross-reaction. In the present study the variation is observed in antisera of high specificity. Therefore, it would appear that these cross-reactions are due to the production of antibody populations of differing specificity, and that they contain a smaller contribution due to errors in recognition of antigen by antibody than might have been supposed. Such a situation implies that high specificity antisera (as well as poorer antisera) could be further improved by suitable fractionation[15].

Although a previous report[7] showed an increase in specificity during immunisation, our data indicate no relationship between booster antigen injection and bleed specificity. There is also no apparent relationship between titer and specificity. Thus whilst constant monitoring (perhaps even on a daily basis) is required in order to obtain maximum titer using the immunisation procedure described, the situation as regards specificity is less clear.

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Studies on the relationships between biotin and the behaviour of B and T lymphocytes in the guinea-pig

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Summary. In biotin-deficient guinea-pigs the number of circulating neutrophils is increased; lymphocytes carrying B and T markers are decreased. Incubation with biotin increases significantly the number of lymphocytes carrying B and T markers, from biotin-deficient guinea-pigs; no increase was observed when the lymphocytes from normal guinea-pigs were incubated.

The importance of biotin in the defence mechanism of the organism is well known. The data in the literature indicate that in animals deficient in biotin, as with deficiencies of other vitamins[1-3], there is a reduction in the production of antibodies. The administration of biotin to the normal rat increases the immunological reactivity: an increase of hemolysin production in response to inoculation of sheep erythrocytes has been observed[4]; in the case of the splenectomized rat it exerts a protective action in the course of the infection from *Haemobartonella*[5]. Moreover, the administration of biotin to the normal rat produces an increase of the activity of the RES demonstrable either by means of the evaluation of the kinetics of the disappearance of colloidal carbon from the blood stream, or by the assessment of the granulomatous reaction to the inoculation of agar gel in the s.c. tissue[6]. The biotin increases the in vivo and in vitro

Table 1. Circulating leukocytes in normal and biotin-deficient guinea-pigs

Groups	Leukocytes (No./mm^3)	Leukocytes series (%) Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils
Normal guinea-pigs (10)	7632 ± 248	62.87 ± 1.41	2.98 ± 0.29	31.63 ± 1.72	1.95 ± 0.29	0.57 ± 0.15
Biotin-deficient guinea-pigs (10)	15384 ± 1561*	33.08 ± 1.21*	2.32 ± 0.20 NS	62.60 ± 1.30*	1.60 ± 0.20 NS	0.40 ± 0.12 NS

Numbers in parentheses indicate the number of animals. Values are expressed as mean ± SE. * $p < 0.001$, significant difference from normal (Student's t-test). NS, no significant difference from normal.