Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 147–150. Pergamon Press. Printed in Great Britain

THE DEVELOPMENT OF TITRE AND SPECIFICITY OF ANTIBODIES TO TESTOSTERONE

E. JEAN WICKINGS, CARLOS A. TAFURT,* HEDWIG HOOGEN and EBERHARD NIESCHLAG
Abteilung Experimentelle Endokrinologie, Universitätsfrauenklinik, Westring 11, D-4400 Münster, Germany

(*Received* 10 *June* 1976)

SUMMARY

The influence of sex and the presence of gonads on the development of titre and specificity of antibodies in testosterone-immunised rabbits was investigated. The one-time, multiple-site, intradermal immunisation technique was applied. All rabbits (normal males and females, and castrated males) responded to immunisation with titres ranging between 1/16,000 and 1/240,000. Female and castrated male rabbits tended to produce higher titres. Cross reactivity towards 5 α-dihydrotestosterone varied in all animals between 60 and 100% in an unpredictable fashion. The female rabbits showed higher cross reactivity towards androstenedione than the male animals, and this increased during immunisation. Serum testosterone levels in the normal male and female groups increased parallel to titre development.

INTRODUCTION

The use of steroid–protein conjugates as antigens for the production of antisera has led to an explosion in the field of steroid radioimmunoassay in the last ten years. However, workers have tended to rely on immunisation techniques which have previously proved successful in practice, without systematically investigating the factors influencing antibody production within the experimental animal. Each antiserum developed has individual properties which vary with the site of hapten-coupling, route and schedule of immunisation, and length of immunisation [1]. The most important properties of an antiserum to be used in a steroid radioimmunoassay system are its specificity, sensitivity, and hence affinity, and antibody concentration. Recent studies have attempted to elucidate some of the factors determining the antibody characteristics for various steroids [2–6].

In this study, we have investigated the effect of the animal's endogenous steroid production on the development of antisera against testosterone. It is known that in male rabbits immunised with testosterone, plasma levels of the steroid reach extremely high values [7, 8]. The amount of testosterone available in the free form is, however, very small, but it has been suggested that the presence of low levels of the endogenous steroid may provide a continuous stimulation of the immune system [9]. To this end we have followed the course of immunisation with testosterone in male, castrated male and female rabbits.

EXPERIMENTAL

Immunisation procedure. Three groups of 5 white New Zealand rabbits were used; 5 females, 5 normal males and 5 males which had been bilaterally castrated 2 days before immunisation. All rabbits received 100 μg immunogen intradermally at multiple sites, as previously described [10, 11]. No booster injections were given. Blood samples were collected either from the central ear vein or by heart puncture at weekly intervals commencing 3 weeks after immunisation. Serum was stored at −20°C until analysis.

Immunological analyses. Antiserum titre was determined by incubating serial dilutions of antiserum with 10,000 c.p.m. [1, 2-^{3}H]-testosterone (New England Nuclear Corporation, S.A. 50 Ci/mmol) at 4°C in a total vol. of 1 ml diluent [saline containing 1 mg/ml bovine γ-globulin (Behring, Marburg)] for 10 h. Bound and free testosterone were separated by incubating with 0.2 ml dextran-coated charcoal suspension (0.25 g Dextran T70 + 0.25 g Norit A Charcoal/100 ml diluent) for 12 min at 4°C, followed by centrifugation at 2500 rev./min for 12 min at 4°C. The bound fraction was decanted into a counting vial containing 3 ml dioxane, and 10 ml Instafluor scintillation fluid (Packard) were added. The vials were counted in a Packard scintillation counter (Model 2450). Antiserum titre was defined as that dilution binding 50% of the radioactive testosterone [12].

Testosterone concentration was determined in each sample using a specific radioimmunoassay [13]. Before extraction, the serum sample (1 ml, or 0.1 ml after the fourth week in the normal male group) was incubated overnight at 4°C with 1000 c.p.m. tritiated testosterone as an internal standard. The samples were extracted 3 times with 5 ml diethyl ether.

From data on antisera previously raised against this conjugate, we have seen that the only steroids cross reacting to any extent with testosterone were androstenedione and 5 α-dihydrotestosterone. Cross reactivity towards these two steroids was evaluated

* Present address: Hospital Militar Central, Bogotá, Colombia.

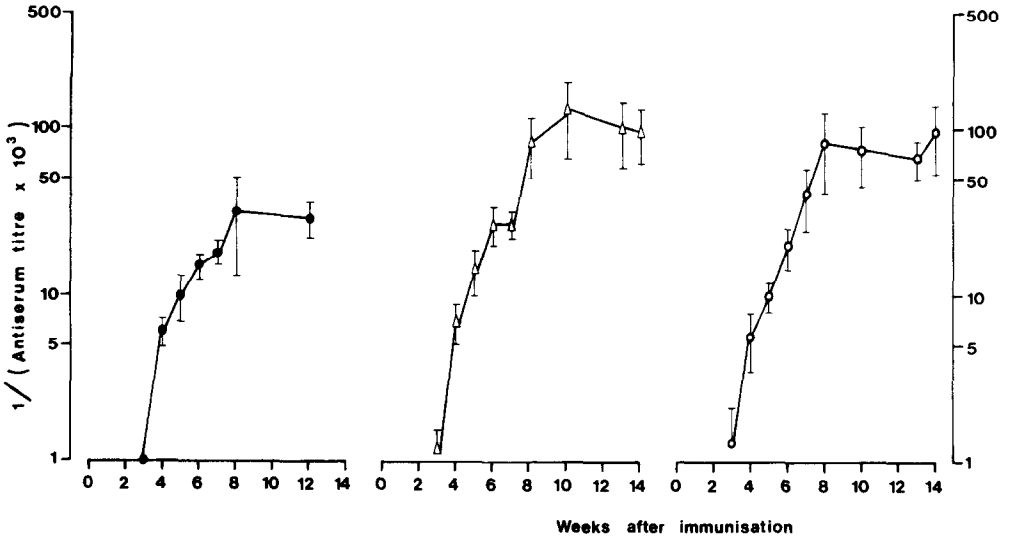

Fig. 1. Antiserum titre development in male (●), female (△) and castrated male (○) rabbits, following immunisation with testosterone (mean ± 1 S.E.M.).

in all samples by incubating the antiserum dilution giving 50% binding with 10,000 c.p.m. tritiated testosterone in the presence of increasing amounts of the cold steroid. The percent cross reaction was calculated from the ratio of the mass of immunogenic steroid required to displace 50% of the radiolabelled immunogenic steroid to the mass of the cross reacting steroid required to displace the same fraction of the labelled steroid [12].

RESULTS

The development of antiserum titres for the three groups are shown in Fig. 1. The maximum titre reached in the female group was 1/240,000, in the castrate group 1/200,000 and in the normal male group 1/105,000. All rabbits developed antiserum titres in response to the primary immunisation. After the seventh week of immunisation, antiserum titres in the normal male rabbits were lower than those in the female and castrate male groups ($P < 0.05$).

The cross reactivities of the antisera towards 5 α-dihydrotestosterone was high in all animals, and varied from week to week in an unpredictable fashion. However, the level of cross reaction in the normal male group decreased from 100% at week 6 to 65% at week 10. Cross reactivity against androstenedione developed steadily in the female group during the immunisation. The antisera from the male and castrate groups demonstrated only very low cross reactivity towards androstenedione over the 14 week observation period.

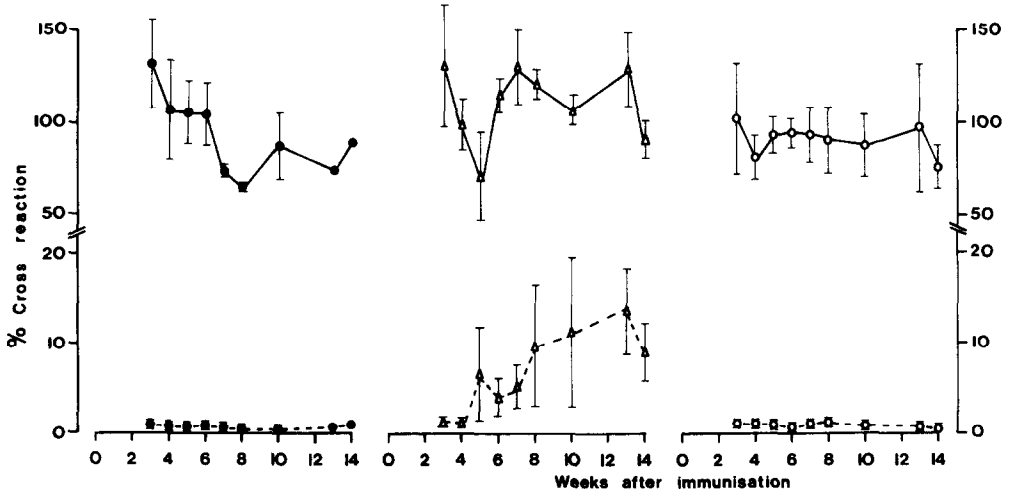

Fig. 2. Per cent cross reaction towards androstenedione (---) and dihydrotestosterone (——) in male (●), female (△) and castrated (○) rabbits, following immunisation with testosterone (mean ± 1 S.E.M.).

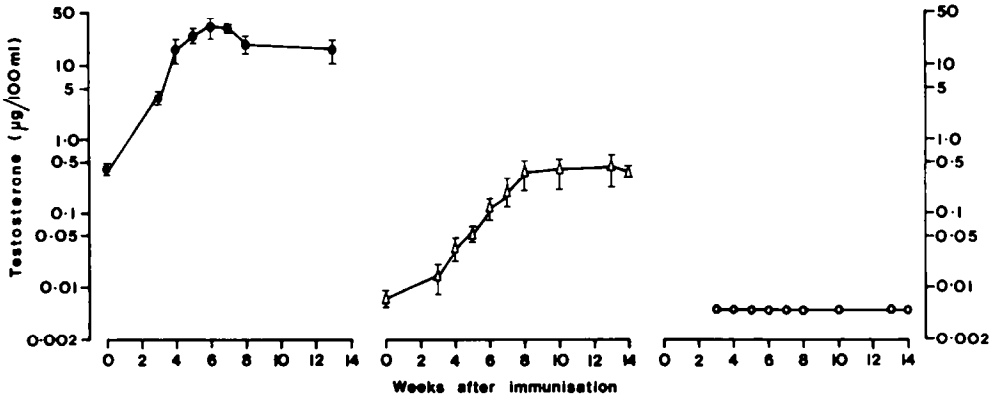

Fig. 3. Testosterone concentrations in the serum of male (●), female (△) and castrated male (○) rabbits, following immunisation with testosterone (mean ± 1 S.E.M.).

The testosterone levels of the immunised rabbits are shown in Fig. 3. Serum levels in the castrate rabbits remained almost undetectable throughout. In the female rabbits, testosterone levels increased steadily during immunisation, but never reached the same proportions as the concentrations found in the normal males; peak values in the female rabbits being 417 ± 191 ng/100 ml (mean ± 1 S.E.M.) at week 13, as compared to peak male values of 30,000 ± 3960 ng/100 ml at week 7. There was a significant correlation between the log(titre) and log(serum testosterone concentration) in the male rabbits ($r = 0.757$) and between titre and serum testosterone concentration in the female group ($r = 0.906$).

DISCUSSION

The one-time, multiple-site, intradermal immunisation technique [10, 11] has proved to be a very convenient method of producing antisera to both protein and steroid hormones [11, 14]. In this study all rabbits immunised showed titre development within 4 weeks of immunisation, and all animals produced antisera suitable for use in radioimmunoassay systems. A large individual animal variation was apparent. The production of IgM antibodies in response to intradermal immunisation is probably responsible for the rapid appearance of antibodies in the circulation. IgG antibodies are produced later in the immunisation process, as the immunity is transferred to the lymphocytes [15].

In the male and female groups, total serum testosterone concentrations increased parallel to the rise in antiserum titres. The higher titres produced in the female and castrated male rabbits indicate that the endogenous steroid concentration may have an influence on the antibody production. Other workers have made similar observations in oestrogen-immunised animals [16]. The male animals produced higher antibody titres than the females; it was proposed that the endogenous steroid level lowers the antibody titre. However, the authors did not observe any difference in the titres in intact and ovariectomised rabbits. The lower antiserum titres in the male rabbits may be an experimental artifact, caused by partial saturation of the binding sites by the very high circulating testosterone levels.

Several reports can be found in the literature, where specificity increases during immunisation [5, 6, 12], but this was not apparent from our results. The immunisation schedules used in these studies all included regular booster injections, and repeated challenges with the antigen affect the populations of antibodies being synthesized. A change from IgM immunoglobulins to IgG during the course of intradermal immunisation may account for the increase in cross reactivity towards androstenedione seen in the female rabbits. This increase could also be accounted for by the increasing levels of androstenedione measured in the serum of these animals. There is a slight indication of an increase in specificity in the male rabbits, as the level of cross reaction towards 5 α-dihydrotestosterone decreased from 100% to 65% at week 10, but by the end of the study, had increased to almost 100% again.

One practical conclusion which may be drawn from this study is that each experimental animal reacts differently to immunisation. Regular investigation is essential, to ensure that antisera of optimal antibody titre and suitable specificity are obtained.

Acknowledgements—This study was supported by the Deutsche Forschungsgemeinschaft. C.A.T. received a grant from the Deutsche Akademische Austauschdienst.

REFERENCES

1. Nieschlag E. and Wickings E. J.: *Z. klin. Chem. Klin. Biochem.* **13** (1975) 261–271.
2. Walker C. S., Clark S. J. and Wotiz H. H.: *Steroids* **21** (1973) 259–283.
3. Vetter W., Armbruster H., Tschudi B. and Vetter H.: *Steroids* **23** (1974) 741–756.

4. Schmied U., Vetter W., Nussberger J. and Siegenthaler W.: *Steroids* **26** (1975) 478–487.
5. Haning R., McCracken J., St. Cyr M., Underwood R., Williams G. and Abraham G.: *Steroids* **20** (1972) 73–88.
6. Schmied U., Siegenthaler W., Nussberger J., Beckerhoff R. and Vetter W.: *Horm. Res.* **7** (1976) 28–33.
7. Nieschlag E., Usadel K.-H., Schwedes U., Kley H. K., Schöffling K. and Krüskemper H. L.: *Endocrinology* **92** (1973) 1142–1147.
8. Wickings E. J., Becher A. and Nieschlag E.: *Endocrinology* **98** (1976) 1142–1146.
9. Frazer H. M.: in *Immunization with Hormones in Reproduction Research* (Edited by E. Nieschlag). North-Holland, Amsterdam (1975) p. 107.
10. Vaitukaitis J., Robbins J. B., Nieschlag E. and Ross G. T.: *J. clin. Endocr. Metab.* **33** (1971) 988–991.
11. Nieschlag E., Kley H. K. and Usadel K.-H.: in *Steroid Immunoassays* (Edited by E. D. H. Cameron, S. G. Hillier and K. Griffiths). Alpha Omega, Cardiff (1975) p. 87.
12. Abraham G. E.: *Acta endocr., Copenh.* **75** Suppl. 183 (1974).
13. Nieschlag E. and Loriaux D. L.: *Z. klin. Chem. klin. Biochem.* **10** (1972) 164–168.
14. Playfair J. H. L., Hurn B. A. L. and Schulster D.: *Brit. Med. Bull.* **30** (1974) 24–31.
15. Eshkol A.: In *Immunization with Hormones in Reproduction Research* (Edited by E. Nieschlag). North-Holland, Amsterdam (1975) p. 240.
16. Gross S. and Bullock D.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) p. 13.