Production and Properties of Antisera to Dexamethasone-Protein Conjugates

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Antibodies to dexamethasone 21-hemisuccinate conjugated to bovine serum albumin were produced in rabbits. Antisera diluted 1:3000 bound 50% of 90 pg of [1,2-³H]dexamethasone. The cross-reaction of the antisera with other synthetic and natural corticosteroids was measured.

In recent years the technique of radioimmunoassay has been developed as a very sensitive method for the determination of small amounts of steroids in biological fluids. The antisera produced to date have been mainly to protein conjugates of naturally occurring steroids. The highly potent, synthetic, anti-inflammatory steroids are used widely in both human and veterinary medicine. Determination of these steroids by g.l.c. is difficult because of the labile C-17 side chain. Radioimmunoassay appears to offer a suitable technique for the determination of these compounds, and this paper describes the production and properties of antisera to protein conjugates of dexamethasone (9 α -fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione.)

Materials and methods

Animals. Male lop-ear rabbits, weighing about 4 kg, were obtained from Goodchild and Bros., Crawley, Sussex, U.K. They were fed *ad libitum* on rabbit pellets from C. Hitchcock Ltd., Bures, Suffolk, U.K., and they were allowed free access to water.

The rabbits were bled by incision of a marginal ear vein and the blood was collected into glass bottles containing heparin (250 units/ml of blood). The blood was centrifuged within 1 h of being collected and the plasma stored at -20°C.

Chemicals. Bovine serum albumin (crystallized) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, Mich., U.S.A., and Tween 80 (polyoxyethylene sorbitan mono-oleate) from George T. Gurr Ltd., London S.W.6, U.K. Dexamethasone was a gift from Merck Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A., and [1,2-³H]dexamethasone (22 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All reagents were of A.R. grade.

The following trivial names of steroids are used: aldosterone (11 β ,21-dihydroxy-3,20-dioxopregn-4-

en-18-al), beclomethasone dipropionate (9 α -chloro-11 β -hydroxy-16 β -methyl-17,21-dipropionyloxy-pregna-1,4-diene-3,20-dione), betamethasone (9 α -fluoro-11 β ,17,21-trihydroxy-16 β -methylpregna-1,4-diene-3,20-dione), chlormadinone acetate (17-acetoxy-6-chloropregna-4,6-diene-3,20-dione), corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione), cortisol (11 β ,17,21-trihydroxypregn-4-ene-3,20-dione), cortisone (17,21-dihydroxypregn-4-ene-3,11,20-trione), equilenin [3-hydroxyoestra-1,3,5(10),6,8-pentaen-17-one], fludrocortisone (9 α -fluoro-11 β ,17,21-trihydroxypregn-4-ene-3,20-dione), flumethasone (6 α ,9 α -difluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione), fluocinolone (6 α ,9 α -difluoro-11 β ,21-dihydroxy-16 α ,17 α -isopropylidenedioxy-pregna-1,4-diene-3,20-dione), flucortolone (6 α -fluoro-11 β ,21-dihydroxy-16 α -methylpregna-1,4-diene-3,20-dione), fluorometholone (9 α -fluoro-11 β ,17-dihydroxy-6 α -methylpregna-1,4-diene-3,20-dione), fluperolone acetate (21-acetoxy-9 α -fluoro-11 β ,17-dihydroxy-21-methylpregna-1,4-diene-3,20-dione), fluprednisolone (6 α -fluoro-11 β ,17,21-trihydroxypregna-1,4-diene-3,20-dione), flurandrenolone (6 α -fluoro-11 β ,21-dihydroxy-16 α ,17 α -isopropylidenedioxy-pregn-4-ene-3,20-dione), paramethasone acetate (21-acetoxy-6 α -fluoro-11 β ,17-dihydroxy-16 α -methylpregna-1,4-diene-3,20-dione), prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione), progesterone (pregn-4-ene-3,20-dione), testosterone (17 β -hydroxyandrost-4-en-3-one) and triamcinolone (9 α -fluoro-11 β ,16 α ,17,21-tetrahydroxypregna-1,4-diene-3,20-dione).

Preparation of dexamethasone 21-hemisuccinate. Dexamethasone 21-hemisuccinate was prepared by a method similar to that used by Erlanger *et al.* (1959) for deoxycorticosterone 21-hemisuccinate (21-hydroxypregn-4-ene-3,20-dione 21-hemisuccinate). The compound was recrystallized from aqueous methanol to give white needles, m.p. 137–139°C. The yield was 33% of theoretical. The purity and identity of the compound were confirmed by t.l.c., i.r. spectroscopy and n.m.r. spectroscopy.

Preparation of dexamethasone-protein conjugate. Dexamethasone 21-hemisuccinate was conjugated to bovine serum albumin by the mixed-anhydride method of Erlanger *et al.* (1959). The conjugate was purified by dialysis against water and then freeze-dried. U.v. spectroscopy indicated a ratio of 6mol of dexamethasone to 1mol of bovine serum albumin, assuming a molecular weight of 70000 for the protein. Acidic hydrolysis of the conjugate and t.l.c. of the hydrolysate confirmed the presence of covalently bound dexamethasone.

Injection of rabbits. The dexamethasone-protein conjugate (4mg) was dissolved in 0.9% NaCl (1 ml) and homogenized with Freund's complete adjuvant (1 ml) and aq. 1% (v/v) Tween 80 (1 ml). Each of five rabbits received the equivalent of 4mg of conjugate. A third of the homogenized conjugate was injected intramuscularly into each hind-leg and the final third was injected subcutaneously into five sites along the back. A sixth rabbit was used as a control. Booster doses of 2mg of conjugate, homogenized as described above, were administered similarly to each rabbit at monthly intervals after preliminary immunization. Blood was collected 10 days after each booster dose had been given.

Detection and titre of antibodies. The ability of the sera from immunized rabbits to bind dexamethasone was compared with that from a non-immunized rabbit. Serial dilutions of plasma in 0.1 M-phosphate

buffer, pH6.6 (Tulchinsky & Abraham, 1971), were prepared from 1:100 to 1:25600.

The titre of the antibodies was checked by two different, complementary, methods. The first method was based on that of Tulchinsky & Abraham (1971) in which the unbound steroid was adsorbed on charcoal, the bound radioactive steroid being measured in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. The efficiency of counting was 31%. The second method was based on that of Arnold & James (1971), in which the bound steroid was precipitated with $(\text{NH}_4)_2\text{SO}_4$, the unbound radioactive steroid being measured by liquid-scintillation counting.

Determination of specificity. The specificity of the antisera to dexamethasone was tested by cross-reaction of other steroids. Various amounts (20–1000g) of the steroid to be tested were dissolved in ethanol and treated with a 1:800 dilution of plasma from rabbit C, bleed 2, referred to below as plasma C₂. The unbound steroid was adsorbed on charcoal and the bound radioactive dexamethasone was measured as described above.

Results and discussion

The rabbits lost weight and developed large abscesses at the sites of injections. Although these

Fig. 1. Percentage of 14000 d.p.m. (90 pg) of $[1,2-^3\text{H}]$ dexamethasone bound by (a) various dilutions of rabbit antisera and (b) 1:800 dilution of antiserum (plasma C₂) in the presence of non-radioactive dexamethasone

For experimental details see the text. The antisera used in (a) were: $\square$, plasma B₂; $\blacksquare$, plasma C₂; $\triangle$, plasma D₁; $\blacktriangle$, plasma E₃; $\bullet$, plasma F₁.

Table 1. *Cross-reaction of antisera raised against dexamethasone-protein conjugates with other steroids*

For experimental details see the text. The percentage cross-reaction is expressed as $(x/y) \times 100$, where x is the mass of non-radioactive dexamethasone and y is the mass of cross-reacting steroid required to produce 50% inhibition of the binding of [1,2-³H]dexamethasone by the antisera. Antiserum (plasma C₂) was diluted 1:800. Negligible (<1%) cross-reaction was given by fluocortolone, fluocinolone acetonide, fluprednisolone, fluorometholone and triamcinolone, and zero cross-reaction by chlormadinone acetate, flurandrenolone acetonide and prednisone; the natural steroids aldosterone, corticosterone, cortisol, cortisone, equilenin, progesterone and testosterone also gave zero cross-reaction.

Steroid	Cross-reaction (%)
Dexamethasone	100
Paramethasone acetate	19
Flumethasone	11
Beclomethasone dipropionate	5
Betamethasone	4
Fludrocortisone	2
Fluprolone acetate	2

abscesses were initially sterile, they became infected when they ruptured. The abscesses healed and the rabbits put on weight after they had been treated and injections of conjugate had been stopped. Haning *et al.* (1972) reported that three of six sheep died while being immunized with aldosterone hemisuccinate conjugated to bovine serum albumin. There appear to have been very few reports of adverse reactions of animals to immunization with steroid-protein conjugates. Whether this is due to such reactions not occurring or to their not being reported is not known.

Three of the five rabbits produced a reasonable concentration of antibodies to the dexamethasone-protein conjugates. The two methods of detecting antibodies gave very similar results. The percentage of dexamethasone bound was dependent on plasma dilution. With the charcoal method, the plasma from the three rabbits bound 50% of the 14000 d.p.m. (90 pg) of the [1,2-³H]dexamethasone when diluted 1:1900 (plasma B₂), 1:3100 (plasma C₂) and 1:1600 (plasma F₁). The plasma from the control rabbit (plasma A₂) bound only 3% at a dilution of 1:3000. The percentage of [1,2-³H]dexamethasone bound fell to 25% at dilutions of 1:5100 (plasma B₂),

1:5700 (plasma C₂) and 1:4200 (plasma F₁), whereas the amount bound by the control remained the same on dilution to 1:6000 (Fig. 1a).

The concentrations of antibodies produced are comparable with those raised to conjugates of natural corticosteroids such as corticosterone (Underwood & Williams, 1972), deoxycorticosterone (Arnold & James, 1971; Rose & Newsome, 1972) and deoxycortisol (17,21-dihydroxypregn-4-ene-3,20-dione) (Abraham *et al.*, 1972), but are lower than those often raised to natural protein hormones (Kirkham & Hunter, 1971).

A plasma dilution of 1:800 was chosen for antisera from rabbit C (plasma C₂) for assessing the specificity. Addition of increased amounts of non-radioactive dexamethasone progressively lowered the percentage of radioactive dexamethasone bound.

The standard curve is linear over the range 25–200 pg of dexamethasone and should enable this amount of steroid to be measured (Fig. 1b).

The cross-reaction of the antisera with other synthetic and natural corticosteroids is given in Table 1.

Cross-reaction of the antisera with synthetic corticosteroids ranged from zero to 19% (as defined in Table 1). However, the most important fact is that none of the natural steroids examined exhibited any cross-reaction. This means that this antisera can be used to measure dexamethasone in biological fluids provided that any of the steroids with appreciable cross-reaction have not been administered as well.

Attempts to correlate immunological cross-reaction with structure of the steroids indicated that stereoisomerism was important. Conversion of a 16 α -methyl group into a 16 β -methyl group (betamethasone) diminished the cross-reactivity from 100 to 4%. Antiserum raised to 17 β -oestradiol behaves in a similar way when 17 α -oestradiol is tested (Midgley & Niswender, 1970). Our antiserum was very sensitive to changes in the D-ring of the steroid, and appreciable cross-reaction (>10%) occurred only when both 17 α -hydroxyl and 16 α -methyl groups were present (compare dexamethasone with betamethasone and triamcinolone). The antisera also 'recognized' changes in the B-ring of the steroid, because the addition of a 6 α -fluoro atom (flumethasone) diminished the cross-reaction from 100 to 11%.

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