On the raising of antibody to the synthetic anabolic steroid trienbolone, its partial characterization and preliminary application for radioimmunoassay

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Summary. The development of a radioimmunoassay method for the detection of trienbolone is described. It is dependent on antibody raised against a bovine serum albumin conjugate of trienbolone 17-hemisuccinate in sheep.

Trienbolone acetate (17β-acetoxyoestra-4,9,11-trien-3-one) with the formula shown in the inset of figure 2, has anabolic properties which have been exploited in improving the feed conversion rate and carcase characteristics of livestock intended for human consumption[3-8]. However, this work was undertaken as part of a programme for monitoring possible effects that anabolic steroids might have on the horse.

Methods. The preparation of trienbolone 17-hemisuccinate from trienbolone (540 mg) and succinic anhydride (800 mg) by a method modified from Abraham and Grover[9] yielded a yellow crystalline material (420 mg) from methanol. Melting point (155–7 °C), UV absorption characteristics (λ_{max} in ethanol, 351 nm, ε 21000), chromatographic behaviour on TLC plates with the system, chloroform/acetone (5:1 by volume) and mass spectrometry established the identity of the derivative.

The conjugation of trienbolone 17-hemisuccinate with bovine serum albumin was achieved by a method similar to the Jondorf[10] modification of the procedure of Abraham and Grover[9]. The reaction product was dialyzed and purified by column chromatography (Sephadex G-25) and lyophilization, to yield the light yellow conjugate (molar ratio of trienbolone to bovine serum albumin, 13:1). This material was administered s.c. to 2 Soay ewes under conditions described for the raising of antibody to 19-nortestosterone[10].

Titres of sequentially obtained plasma were determined under standard conditions of assay[10,11], using [6,7]-^{3}H-trienbolone (sp.act. 58 Ci/mmole Roussel Uclaf, Romainville, France) as the isotopically labelled steroid reference. The titres began to rise appreciably after the 4th monthly booster injections of antigen. The antibody with the highest titre obtained from the definitive bleed, designated II-152 was selected for a survey of its sensitivity to other anabolic steroids and to some endogenous steroids according to the general procedures and definitions previously given[10,12].

Results and discussion. As shown in figure 1, some of the cross-reactivity data obtained with II-152 plasma diluted 1:7100 before addition to the assay mixtures, indicate quite clearly that apart from trienbolone acetate, the other anabolic steroids tested do not cross-react with the antibody. The antibody is therefore rather specific and would not be likely to detect other anabolic steroids under the conditions of assay.

Figure 2 shows the depression of binding of ^{3}H-trienbolone to II-152 antibody by trienbolone and/or its metabolites, excreted in urines collected at various times after trienbolone acetate was administered i.m. in veterinary dosages (75 mg/435 kg, 90 mg/538 kg, i.e. 0.17 mg/kg) to each of 2 gelded thoroughbreds.

The analysis of chloroform/methanol (9:1 by volume) extracts of urine samples[13] equivalent to 1 ml urine shows that the timecourse for detecting urinary excretion products is very similar for both horses. The depression of binding tends to revert to preadministration levels by the 7th day. This leads to the conclusion that compared with various veterinary preparations of 19-nortestosterone[13], trienbolone acetate is excreted fairly rapidly by the horse.

However, these preliminary results do not in any way determine what might be retained in various tissues to be released more slowly over a prolonged period. The present method as here applied is merely concerned with analyses of solvent extracts equivalent to 1 ml urine, and does not predict what might happen if trienbolone acetate is administered by other routes, or as a course of treatment.

It remains to be seen how useful the II-152 antibody will be in determining residues of trienbolone and/or its metabolites in various tissues. The application of trienbolone for improved meat production presupposes that any exogenous steroid residues have to be reliably monitored.

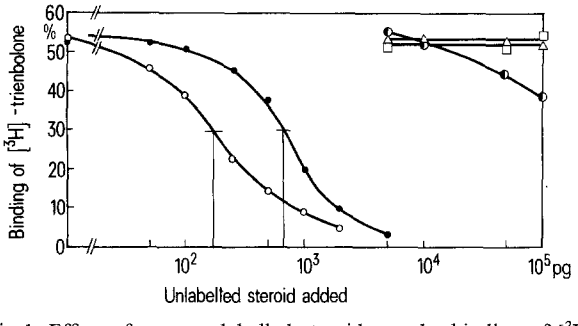

Fig. 1. Effect of some unlabelled steroids on the binding of [^{3}H]-trienbolone (90 pg) by antibody preparation II-152 (diluted 1:7100). Steroids added to the radioimmunoassay system in amounts 50–5000 pg: (○—○) trienbolone; (●—●) trienbolone acetate. In range 5000–100,000 pg: (□—□) methandienone; (△—△) 17α-methyltestosterone; (◐—◐) 19-nortestosterone. Not shown, but resembling curve obtained with methandienone: 1-dehydrotestosterone; testosterone. The cross-reactivities are calculated (Nieschlag and Wickings[12]) from the ratios of the amounts of trienbolone and other test steroids needed to displace the same fraction of [^{3}H]-trienbolone from binding to the antibody under standardized conditions[10].

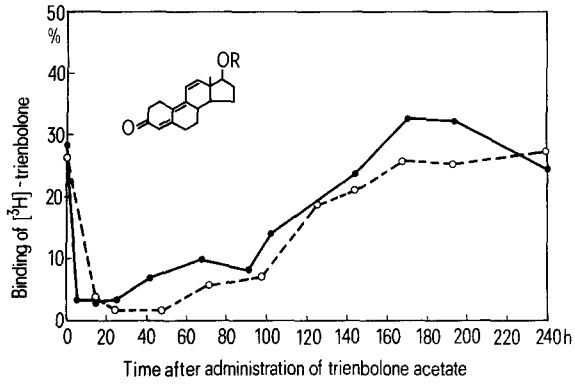

Fig. 2. Time course of urinary excretion of trienbolone and/or metabolites after administration of trienbolone acetate (0.17 mg/kg, i.m.) to 2 gelded thoroughbreds, as determined by radioimmunoassay with II-152 antibody used in conjunction with [^{3}H]-trienbolone. What is shown is the depression of binding (relative to preinjection control levels) of [^{3}H]-trienbolone with solvent-extracted (chloroform/methanol, 9:1 v/v) material from 1 ml urine samples. Inset, trienbolone (R = H), trienbolone acetate (R = $COCH_3$).

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Collagen breakdown by gingival collagenase and elastase

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Summary. The granule fraction of human polymorphonuclear leucocytes (PMNs), the concentrated product of gingival washing from 2 human volunteers and the culture fluid of samples of human gingiva were incubated with neutral salt soluble collagen from rat skin and the patterns of collagen degradation were studied by SDS polyacrylamide gel electrophoresis. Collagenase from human gingiva cleaved the collagen molecules in a fashion similar to that of the PMN granule fraction. Collagen was also attacked by elastase from human PMNs and, to a lesser extent, by elastase from the gingival washings.

Inflammation of the gingiva is accompanied by an increased migration of polymorphonuclear leucocytes (PMNs) toward the oral cavity through the gingival sulcus[3-5]. These cells discharge their endogenous enzymes in the gingival environment, either by phagocytosis or upon death[6]. Previous work from our laboratory has shown that inflamed gingivae contain more free lysosomal enzymes[7]. During experimental gingivitis, obtained by suppression of toothbrushing in human volunteers, free collagenase and elastase were found to increase in the washings obtained from marginal gingiva[8].

The aim of the present investigation was to clarify further the mode of action of gingival collagenase and elastase upon collagen molecules by using the technique recently described by Murphy et al.[9].

Materials and methods. The granule fraction of human PMNs was prepared by dextran sedimentation and differential centrifugation from 400 ml of heparinized human blood[10,11]. The granules were suspended in 2 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$. They were frozen and thawed 10 times, centrifuged at 30,000 × g for 40 min and the supernatant used as a crude enzyme solution.

Gingival washings were collected from 2 male subjects, 25 and 34 years old and with no visible clinical signs of inflammation, by circulating 5 ml of physiological saline for 15 min along the buccal and palatal marginal regions, using acrylic individual appliances connected to a peristaltic pump[12]. After centrifugation of the product of washing at 200 × g for 15 min, the supernatant was concentrated 40 times by dialysis under vacuum for several hours[10].

Samples of marginal gingiva were obtained from patients during periodontal therapy. After being cut into cubes of approximately 1 mm^3, the fragments were cultured in Eagle MEM medium[13] supplemented with 100 units/ml of penicillin G.potassium and 100 µg/ml of dihydrostreptomycin sulfate for up to 10 days in an atmosphere of 5% CO_2 and 95% O_2, with daily changes of the medium. Collagenase was assayed in the medium after ammonium sulfate fractionation[14].

Neutral salt-soluble collagen from rat skin[8-15] was incubated at 25 °C for 6–20 h with samples of the 3 preparations, i.e. granule fraction of PMNs, concentrated gingival washing and culture fluid of gingiva. The reaction mixture consisted of 50 µl of the collagen solution, 50 µl of the enzyme solution and 50 µl of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$. In some instances, the enzymes were inhibited by adding either

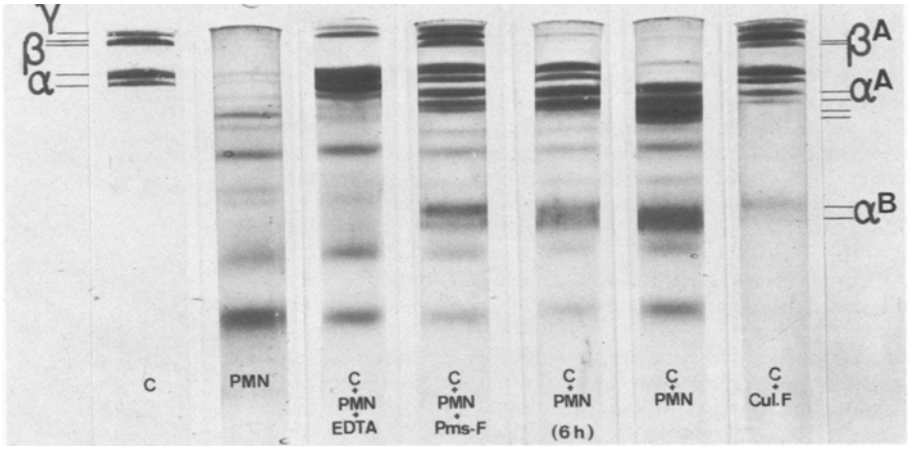

Fig. 1. SDS-polyacrylamide gel electrophoresis of collagen (C) digestion products. Aliquots of PMN granule fractions, alone (C+PMN) or in the presence of EDTA (C+PMN+EDTA) or Pms-F Pms-F (C+PMN+Pms-F), and culture fluid of human gingiva (C+Cul F) were incubated with rat skin collagen, as described in 'materials and methods'. The incubation time was 20 h, except for 1 of the assays (6 h). Native collagen (C) and granule fraction of PMN (PMN) in the absence of collagen are included as references for intact γ-, β- and α-chains of collagen and for bands of foreign proteins.