Purification and Characterization of Donkey Chorionic Gonadotrophin

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(Received 6 August 1979)

SUMMARY

Serum of the pregnant donkey, like that of the mare, contains a gonadotrophin of chorionic origin. The chorionic gonadotrophin of the donkey (dCG) has been isolated in purified form from the serum of pregnant donkeys using methodology previously employed for the purification of pregnant mare chorionic gonadotrophin (eCG). Unlike eCG, dCG is predominantly an LH in biological tests. In the in-vitro rat Leydig cell assay, dCG was as active as eCG, but in the in-vitro rat seminiferous tubule assay for FSH and in the augmentation assay, dCG was considerably less potent than eCG (1–10%). Specific rat testis radioreceptor assays for LH and FSH also showed dCG to be at least nine times more potent in LH than in FSH activity. Chemically, dCG was found to be similar to eCG in fractionation behaviour and glycoprotein nature. However, dCG had significantly less carbohydrate (31%) than had eCG (45%) and several differences were noted in a comparison of amino-acid compositions. A single amino-terminal residue, phenylalanine, was detected in dCG. Immunologically, dCG cross-reacted in homologous radioreceptor assays for eCG, equine LH and equine FSH, but its inhibition curves were all non-parallel with those of the respective equine gonadotrophin standards.

INTRODUCTION

Pregnant mares produce a gonadotrophin of chorionic origin (Allen, Hamilton & Moor, 1973) which is present in high concentration in the serum during early pregnancy (Cole & Hart, 1930). This material has been referred to as pregnant mare serum gonadotrophin (PMSG). Since it is of trophoblastic cell origin and thus homologous to human chorionic gonadotrophin (hCG), a more appropriate designation for this hormone is equine chorionic gonadotrophin (eCG). Unlike the well-characterized human chorionic gonadotrophin, which exhibits almost exclusively luteinizing hormone (LH)-like biological activity (Brody, 1969; Louvet, Harman, Nisula, Ross, Birken & Canfield, 1976), eCG is approximately equipotent in follicle-stimulating hormone (FSH) and LH bioassays (Papkoff, 1974, 1978; Stewart, Allen & Moor, 1976). Other equids, including donkey (Bell, Loraine, Jennings & Weaver, 1967) and zebra (King, 1965) also secrete appreciable quantities of gonadotrophin during early pregnancy. Although the physicochemical properties of these other equine hormones have not been studied, the use of rat testicular radioreceptor assays has demonstrated a major difference in the biological activities of the chorionic gonadotrophins.

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secreted by horses and donkeys, the former expressing a much higher ratio of FSH-like to LH-like activity than the latter (Stewart, Allen & Moor, 1977).

This paper describes the isolation and purification of donkey chorionic gonadotrophin (dCG) from pregnant donkey serum and compares the chemical and biological properties of this material with those of the equivalent horse chorionic gonadotrophin (eCG) purified from pregnant mare serum.

MATERIALS AND METHODS

Purification procedure

A lyophilized sample of pooled serum (2812 ml), collected in Cambridge from five donkeys between 60 and 71 days of gestation, was sent to the Hormone Research laboratory, San Francisco, California, U.S.A., and reconstituted to the original volume with water at 4 °C. The gonadotrophin was then extracted and purified in a similar manner to that previously described for eCG (Gospodarowicz & Papkoff, 1967; Schams & Papkoff, 1972). The pH of the serum was initially adjusted to 3.0 by the dropwise addition of a fresh solution of 0.2 M-metaphosphoric acid and the resulting precipitate was discarded after centrifugation. The pH of the supernatant fluid was then readjusted to 7.5 with 1 M-NaOH before slowly adding 1 vol. cold (−20 °C) absolute ethanol. After 30 min at 4 °C the precipitate was again removed by centrifugation and the pH of the supernatant fluid adjusted to 4.5 with 1 M-HCl. A further 3 vols of chilled (−20 °C) ethanol were added and a precipitate was formed which was harvested 12 h later when it was dialysed and lyophilized (Fraction 1). This fraction contained the gonadotrophin activity.

This crude gonadotrophin extract was subjected to cation exchange chromatography on a column of sulphoethyl Sephadex C-50 equilibrated with 0.01 M-sodium acetate at pH 4.0. Approximately 1 g protein per 100 ml gel was loaded onto the column. After elution of unadsorbed material at pH 4.0, adsorbed material was eluted with 0.05 M-ammonium acetate at pH 6-0 and subsequently the active fraction was eluted with 1 M-NH₄HCO₃ (Fraction 2). This fraction was refractionated with HPO₃ and ethanol as described above except that the 50% ethanol precipitation step was carried out at pH 4.5 rather than at pH 7.5. The fraction that precipitated at 85% ethanol (Fraction 3) was then chromatographed on a column of Sephadex G-100 (2.5 × 90 cm) equilibrated with 0.05 M-NH₄HCO₃ for final purification.

Hormone assays

The activity of the various fractions obtained throughout the purification procedure was monitored by an homologous double-antibody radioimmunoassay (RIA) for eCG (Farmer & Papkoff, 1979) using highly purified eCG as standard (15 000 i.u./mg; Schams & Papkoff, 1972). In addition, the LH and FSH biological activities of the more purified fractions were measured using a modification of the in-vitro rat Leydig cell assay for LH (Ramachandran & Sairam, 1975; Licht, Bona Gallo, Aggarwal, Farmer, Castelino & Papkoff, 1979) with highly purified ovine LH (G3-222B; 2.7 × NIH-LH-S1; Papkoff, Gospodarowicz, Candiotti & Li, 1965) as standard and a cyclic AMP rat seminiferous tubule assay for FSH (Rao & Ramachandran, 1975) with highly purified ovine FSH (G4-150C; 25 × NIH-FSH-S1; Papkoff, Gospodarowicz & Li, 1967) as standard. The hCG augmentation assay was performed as described by Steelman & Pohley (1953). The FSH and LH receptor-binding activities of purified dCG were measured in rat testicular radioreceptor assays as described by Stewart et al. (1976) using highly purified preparations of human FSH (LER 1575C; 144 × NIH-FSH-S1; Reichert, 1972) and human LH (DEAE-I 27/1/69; 2.83 × NIH-LH-S1; Stockell-Hartree, 1966) as standards.
**Chemical characterization**

Chemical characterization of purified dCG included determination of amino-acid composition in an automatic amino-acid analyser by the method of Spackman, Stein & Moore, 1958; amino-terminal residue analysis by the dansyl technique (Gray, 1967; Woods & Wang, 1967), and carbohydrate determination by micro-colorimetric techniques as described earlier (Licht, Farmer & Papkoff, 1976).

**RESULTS**

**Hormone purification**

The method used in the present study for purifying dCG was very similar to that employed for eCG except that the initial 50% ethanol precipitation step was carried out at pH 7-5 rather than 4-5. This modification prevented partial precipitation of the hormone at this step.

![Gel filtration of donkey chorionic gonadotrophin (dCG) on a column of Sephadex G-100 in 0.05 M-NH₄HCO₃. The column size was 2.5 x 90 cm and the tube volume was 3.6 ml. The dCG eluted with a ratio of elution volume to void volume (Vₑ/V₀) of 1.27 (peak A). The yield was 1.15 mg. The material pooled from tubes 17 to 21 (peak B, 1.5 mg) had dCG activity (0.6 x), and the third fraction (peak C, 5.1 mg) was virtually inactive as assessed by equine chorionic gonadotrophin radioimmunoassay.](image)

The material eluting from Sephadex G-100 with a ratio of elution volume to void volume (Vₑ/V₀) of 1.27 (Fig. 1, peak A) had the highest specific activity in the eCG RIA and was used for studies to characterize dCG. The final yield of dCG was 1.15 mg which represented 0.4 mg/l starting serum. There was also significant activity associated with the area designated B (Fig. 1). This material had a potency of 0.6 relative to the dCG eluted in peak A, in both the eCG RIA and the rat Leydig cell assay. This activity could have resulted from the presence of the predominant form of dCG (peak A) in fraction B or of a somewhat different form of dCG. The fraction designated peak C had only 4% activity compared to dCG, peak A.

**Chemical characterization**

Tables 1 and 2 show the carbohydrate and amino acid composition data obtained for dCG compared with eCG. Table 1 shows that dCG had appreciably less total carbohydrate (31%) and sialic acid (6-8%) than eCG (45 and 10-2% respectively). Amino-terminal analysis indicated the presence of only one amino-terminal residue, phenylalanine, rather than the
Table 1. Comparison of the carbohydrate composition of equine chorionic gonadotrophin (eCG) and donkey chorionic gonadotrophin (dCG)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>eCG*†</th>
<th>dCG*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose</td>
<td>14·3</td>
<td>13·2</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>20·6</td>
<td>11·2</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>10·2</td>
<td>6·8</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>45·1</td>
<td>31·2</td>
</tr>
</tbody>
</table>

* g/100 g glycoprotein, uncorrected for moisture or ash, single determinations were made, each employing 100 µg.
† Taken from Schams & Papkoff (1972).

Table 2. Comparison of the amino-acid composition* of equine chorionic gonadotrophin (eCG) and donkey chorionic gonadotrophin (dCG)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>eCG†</th>
<th>dCG</th>
<th>Amino acid</th>
<th>eCG†</th>
<th>dCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>5·4</td>
<td>6·1</td>
<td>Alanine</td>
<td>7·5</td>
<td>6·5</td>
</tr>
<tr>
<td>Histidine</td>
<td>2·5</td>
<td>1·9</td>
<td>½ Cystine</td>
<td>7·5</td>
<td>5·3</td>
</tr>
<tr>
<td>Arginine</td>
<td>6·1</td>
<td>4·5</td>
<td>Valine</td>
<td>4·6</td>
<td>5·0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5·5</td>
<td>5·2</td>
<td>Methionine</td>
<td>1·7</td>
<td>1·6</td>
</tr>
<tr>
<td>Threonine</td>
<td>8·6</td>
<td>9·2</td>
<td>Isoleucine</td>
<td>4·8</td>
<td>3·2</td>
</tr>
<tr>
<td>Serine</td>
<td>8·0</td>
<td>9·0</td>
<td>Leucine</td>
<td>6·3</td>
<td>5·7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8·4</td>
<td>7·3</td>
<td>Tyrosine</td>
<td>2·4</td>
<td>6·6</td>
</tr>
<tr>
<td>Proline</td>
<td>12·5</td>
<td>11·6</td>
<td>Phenylalanine</td>
<td>4·0</td>
<td>4·0</td>
</tr>
<tr>
<td>Glycine</td>
<td>5·1</td>
<td>7·4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Amino-acid analysis: hydrolysis for 18 h, not corrected for hydrolytic destruction, calculated as residues per 100 residues analysed, numbers are the average of two determinations employing 100 µg each, ½ cystine and methionine values were obtained with performic acid oxidized preparations.
† Taken from Schams & Papkoff (1972).

two (phenylalanine and serine) found in eCG. Amino-acid analysis of dCG revealed a great similarity with eCG in composition but significantly more tyrosine and somewhat fewer arginine residues were observed in dCG (Table 2).

**Immunological, biological and receptor assays**

Although dCG in immunological assays showed significant cross-reaction in the eCG RIA, its dose–response curve was non-parallel to that of purified eCG (Fig. 2). Donkey chorionic gonadotrophin was also quite active in both equine LH and FSH radioimmunoassays (Farmer & Papkoff, 1979), and the inhibition curves were again non-parallel to the respective standards in both assays (data not shown). Therefore, potency estimates relative to the horse gonadotrophin standards could not be calculated. However, each of the principal fractions obtained during purification was assayed in a single eCG RIA as shown in Fig. 2. An increase in activity was observed as purification proceeded and the final product (dCG) was considerably purified compared with unfractionated pregnant donkey sera.

In-vitro bioassays specific for LH and FSH were used to determine the biological activity of the purified dCG (Table 3). In the LH assay, in which testosterone production in rat Leydig cells was measured, dCG was essentially equipotent to eCG. However, dCG had very low FSH activity in the assay measuring cyclic AMP production in an immature rat tubule cell preparation, having 4% of the activity of eCG. The FSH activity of a dCG preparation purified from another pool of sera was also very low in the in-vivo hCG augmentation assay: no detectable response being observed with 200 µg (<0·3 × NIH-FSH-S1). The low yield of the dCG preparation described in this paper precluded an extensive hCG augmentation assay of this material.
In the radioreceptor assays (Table 3) dCG had significant LH activity, although it was much lower than that of eCG, and very low FSH activity. Thus, in both bioassays and receptor assays, dCG had a very low FSH : LH activity ratio.

Table 3. Comparison of the LH and FSH biological and binding activities of equine chorionic gonadotrophin (eCG) and donkey chorionic gonadotrophin (dCG) (Values are means ± S.E.M. of several assays)

<table>
<thead>
<tr>
<th></th>
<th>eCG*</th>
<th>dCG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In-vitro bioassays</strong> (%) ovine standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>0.85 ± 0.32</td>
<td>0.03†</td>
</tr>
<tr>
<td>LH</td>
<td>2.9 ± 0.4</td>
<td>1.3 (1.1-1.5)‡</td>
</tr>
<tr>
<td>FSH : LH ratio</td>
<td>0.29</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Radioreceptor assays</strong> (%) human standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>61.2 ± 3.6</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>LH</td>
<td>60.2 ± 2.6</td>
<td>17.5 ± 1.3</td>
</tr>
<tr>
<td>FSH : LH ratio</td>
<td>1.02</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Highly purified eCG (15 000 i.u./mg). Data for bioassays taken from Licht et al. (1979).
† Data obtained from a single point.
‡ Data obtained from a single assay, numbers in parentheses are 95% confidence limits.

**DISCUSSION**

Our study describes, for the first time, the isolation and partial characterization of a highly purified preparation of donkey chorionic gonadotrophin. The yield was 0.4 mg/litre which is considerably lower than that obtained when eCG is similarly purified, when 3–5 mg/litre are obtained (Gospodarowicz & Papkoff, 1967). However, the levels of chorionic gonadotrophin in pregnant donkey sera are also much lower than the levels in pregnant mare sera (Allen, 1975).

The dCG was judged to be purified on the basis of its symmetrical elution profile from gel filtration and the presence of a single amino-terminal residue. It was found to be similar to...
eCG in terms of purification behaviour and glycoprotein nature, and is of a similar molecular size, as shown by the fact that both chorionic gonadotrophins elute from Sephadex G-100 with a $V_e/V_o$ ratio of 1.27. There were, however, significant differences in chemical composition between the two hormones. Both content of total carbohydrate (31%) and sialic acid (6-8%) in dCG were appreciably lower than the levels in eCG (45 and 10-2% respectively) and were more similar to the levels of these constituents found in hCG (30 and 10% respectively; Bahl, 1973). This in turn suggests that the in-vivo half-life of dCG may be shorter than that of eCG (26 h in the rat; Parlow & Ward, 1961; 6 days in the horse, Catchpole, Cole & Pearson, 1935) and perhaps closer to that of hCG (4-9 h in the rat; Parlow & Ward, 1961). If this is the case, it may explain the lower concentrations of gonadotrophin in serum of donkeys at 60 days of gestation than those in serum of mares at the same stage of pregnancy (10-20 v. 50-150 i.u./ml; Allen, 1975).

Despite the differences in carbohydrate content noted above, no significant differences were seen in the amino-acid compositions of dCG and eCG except for the higher tyrosine content in the former. Comparison of the amino-acid composition of dCG with that of equine LH and FSH (Licht et al. 1979) did not indicate that dCG was more closely related to either pituitary gonadotrophin. Sufficient material was not available to determine whether dCG is composed of subunits. This is likely, however, as all gonadotrophins studied to date have a subunit nature. The finding of a single amino terminus in dCG does not preclude the presence of two subunits. The $\alpha$ and $\beta$ subunits of dCG could both have amino terminal phenylalanine residues, as is the case with bovine thyroid-stimulating hormone (Pierce, Liao & Carlsten, 1973).

The immunological data obtained also indicated differences between dCG and eCG. Although dCG showed significant cross-reaction in the eCG RIA (Farmer & Papkoff, 1979), the slope of the inhibition curve was much lower, indicating differences in antisera recognition sites between the two molecules.

The bioassay and radioreceptor assay results obtained with highly purified dCG confirmed the observation of Stewart et al. (1977) that, unlike eCG, which has considerable FSH-like activity, the donkey hormone is primarily LH-like in biological activity. It was very active in both the LH radioreceptor assay and rat Leydig cell testosterone assays but relatively inactive in both in-vitro and in-vivo FSH assays. However, since this FSH-like activity was marked and considerably greater than that expressed by highly purified hCG (Louvet et al. 1976), dCG is more similar to eCG than to hCG in this regard.

Recent studies in our laboratory (Papkoff, 1978; Farmer & Papkoff, 1979; Licht et al. 1979) have shown that eCG is predominantly an LH-like molecule in terms of its chemistry and immunochemistry, and that equine pituitary LH shares with eCG the feature of significant intrinsic FSH activity. Our data with dCG has demonstrated that a gonadotrophin isolated from another species of equid shares this characteristic of dual gonadotrophic activity, although dCG has a much higher LH : FSH activity ratio than has eCG.

We thank Drs Ted Hayashida and Selna Kaplan and Mrs Eleanor Rowley for assistance with the radioimmunoassays, Mr Kenway Hoey for expert technical assistance, and Dr G. D. Niswender for testosterone antibody. We thank the NIAMDD for standard preparations of ovine LH and FSH. The encouragement and interest of Professor Choh Hao Li in these studies is appreciated. This work was supported in part by a grant from the National Institutes of Health (HD-05722) and the Thoroughbred Breeders' Association of Great Britain. One of us (B. B. A.) was a Fellow of the Population Council.

REFERENCES

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