Gonadotrophin in the term placenta of the dolphin (*Tursiops truncatus*), the Californian sea lion (*Zalophus californianus*), the grey seal (*Halichoerus grypus*) and man

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Summary Chorionic gonadotrophin activity in extracts of the term placenta of a dolphin, a sea lion and a grey seal was measured by its effectiveness in increasing uterine weight in the mouse and by solid-phase RIA using hCG as immunogen and labelled antigen. Bioreactive (B) gonadotrophin was found in these placentae and, compared to the human term placenta, the concentration of CG in the dolphin was higher, in the sea lion similar and in the grey seal lower. The biological activity in each species was neutralized with a rabbit anti-hCG serum. All placental extracts contained material active in the hCG immunoassay (I). The ratio B/I was significantly higher for the CG in the placental extracts of the marine mammals compared with that of the human term placenta. Results of in-vivo bioassay, RIA, electrophoretic and gel-chromatographic studies indicate structural similarities between CG in the placentae of the marine mammals and human CG.

Introduction

Contributions to the reproductive endocrinology of marine mammals have been mostly of a morphological nature (Harrison, 1951, 1960, 1963; Harrison, Matthews & Roberts, 1952; Laws, 1956; Amoroso *et al.*, 1965; Harrison & Young, 1966; Sinha & Erickson, 1972; Bigg & Fisher, 1974; Boshier, 1977, 1981; Sinha, Erickson & Seal, 1977). However, in recent years some plasma steroids have been measured during pregnancy and *post partum* in a few species of seal (Daniel, 1974, 1975; Liggins, France, Knox & Zapol, 1979; Raeside & Ronald, 1981; Boyd, 1983). Until recently the concentration of protein and steroid hormones in placentae of marine mammals seems to be confined to a single estimation of progesterone (Harrison, 1960). Hobson & Boyd (1984) have shown that progesterone and a biologically active chorionic gonadotrophin (CG) is present in the placenta of the grey seal (*Halichoerus grypus*). These results point to the placenta as the source of a luteinizing hormone which Amoroso, Harrison, Matthews & Rowlands (1951) suggested was most probably responsible for the precocious enlargement of the gonads of fetal grey seals.

The present investigation is concerned with providing information about CG and its subunits in the term placenta of a grey seal, a bottle-nosed dolphin and a Californian sea lion. For comparison a human term placenta was included in the investigation.

Materials and Methods

A term placenta was obtained from a captive bottle-nosed dolphin (*Tursiops truncatus*), a captive Californian sea lion (*Zalophus californianus*), a grey seal (*Halichoerus grypus*) shot at the Farne Islands and a human placenta obtained after a normal delivery and gestation of 41 weeks. In the

dolphin, sea lion and seal gestation is approximately 12 months. Delayed implantation of about 3 months occurs in the sea lion and seal. The animal placentae and membranes were frozen at -20°C before being sent to the laboratory. The human placenta was not frozen and was dealt with soon after delivery.

Extraction of placental gonadotrophin

The frozen placentae were thawed, dissected free from umbilical cord and membranes and weighed. Half of each placenta was homogenized in ice-cold acetone and ice-cold diethyl ether (5:1, v/v, 6 ml/g). The human placenta was treated similarly except that all of it was used. The homogenized tissue was kept at 4°C overnight and collected by filtration on a Buchner funnel. The tissue residue was washed in acetone and ether during filtration after which drying was completed in a desiccator over Dryerite. This method of extraction has been shown to remove steroids present in the primate placenta (Hobson, 1972). The desiccated placental material was disintegrated in 0.9% (w/v) NaCl in a high-speed tissue grinder (Ultra turrax), left overnight at 4°C , centrifuged and the supernatant assayed.

In-vivo bioassay

The gonadotrophic activity of placental extracts and the hCG standard was measured by its effectiveness in increasing uterine weight of immature female mice. The method was essentially that of Levin & Tyndale (1937) in which mice were injected subcutaneously with one third of the total dose on 3 consecutive days and killed 72 h after the first injection. Uteri, dissected free from mesentery, fat, ovaries and oviducts, were cut at the uterocervical junction. Intrauterine fluid was expressed between filter paper before the uterus was weighed to the nearest 0.1 mg on a torsion balance. Five or 10 mice were used at each of 3 dose levels, a log dose interval of 0.301 of the unknown and the hCG standard preparation being assayed simultaneously. Estimations of relative potency of the placental extracts were expressed per g wet weight as International Unit equivalents of the Second International Standard for hCG (i.u. hCG, WHO code 61/16). The smallest amount of hCG which produced a significant ($P < 0.01$) response in the bioassay, compared with that in saline injected controls, was 0.0625 i.u. The response parameter was the logarithm of the mouse uterine weight.

Antiserum to hCG

The antiserum was a rabbit anti-hCG preparation raised against an hCG preparation (Gonadex, Leo, Sweden) having a biological activity of 5000 i.u./mg. This antiserum was used to see if it would neutralize the gonadotrophic activity of the 4 placental extracts. These and the hCG standard were assayed with the addition of antiserum or normal rabbit serum (NRS). The amount of antiserum used was 15 μl per total dose per mouse.

Gel chromatography

An amount of dolphin, sea lion, grey seal and human placental tissue extract equivalent to respectively 34.2 mg, 56.0 mg, 375.0 mg and 480.0 mg was dissolved in 0.9% (w/v) NaCl and dialysed before chromatography on a 26×930 mm Sephadex G-200 column, equilibrated with 0.1 M-Tris-HCl buffer of pH 7.5, 0.2 M-NaCl. The flow was against gravity with a rate of 9 ml/h and 3-ml fractions were collected and assayed by solid-phase radioimmunoassays for hCG, and the alpha and beta subunits of hCG. The degree of retardation on the column, expressed in K_{av} values (Laurent & Killander, 1964) where $K_{av} = (V_e - V_o/V_t - V_o)$ and V_e is the elution volume of the sub-

stance, V_0 is the void volume and V_t is the total bed volume, was compared with that of purified preparations of hCG and its subunits similarly treated.

Zone electrophoresis in agarose suspension

Zone electrophoresis in agarose of the placental extracts was performed in a column, 1.3 cm × 67 cm, with 0.17% agarose (Agarose C, Pharmacia Fine Chemicals, Uppsala, Sweden) suspension in 0.075 M-sodium veronal buffer (pH 8.6) according to the method of Hjertén (1963) with some modifications (Wide, 1981). Samples of 100–500 µl of the extracts, together with trace amounts of labelled hCG, labelled human serum albumin (HSA), were added to 0.3–0.5 ml serum from a child. One volume of the mixture was added to an agarose pellet obtained by centrifugation of 2 volumes agarose suspension. Application of the sample was performed by layering as described by Hjertén (1963). Electrophoresis was performed at a current of 50 mA for about 20 h. After electrophoresis the agarose suspension was pumped out through a funnel fitted with tubing to a sample collector and the agarose was then removed by centrifugation. The supernatant, without further treatment, was analysed for CG activity by RIA. The migration rate of labelled HSA was about 12 cm²·s⁻¹·V⁻¹. The migration rate of the activity of a hormone was expressed as a percentage of that of labelled HSA. The electrophoretic migration velocities in the agarose suspension are similar to those in a free solution and related to molecular charge in the particular buffer solution. A difference in migration rate is directly proportional to a difference in charge.

Radioimmunoassay

The immunoactivities of the saline extracts, before and after chromatography, were assayed by solid-phase radioimmunoassays (Wide, 1969) for hCG, and the alpha and beta subunits of hCG. An hCG preparation (13 000 i.u./mg by bioassay and 7500 i.u./mg by radioimmunoassay) was supplied by AB Leo (Sweden), and the hCG-alpha- and beta-subunits and their antisera (SA6 and SB6) by NIAMDD, National Institutes of Health, Bethesda, MD, U.S.A. The purified preparation of hCG and its subunits were labelled with ¹²⁵I by the chloramine T technique (Greenwood, Hunter & Glover, 1963).

The Second International Standard for hCG was used as a reference preparation in the hCG assays of the placental extracts before chromatography. The sensitivity of the hCG assays was about 0.1 mU per test tube. The hCG assays of the unfractionated extracts were made as 3 × 3 assays with 4 replicates at each dose level with a log dose interval of 0.3010 for the standard and the unknown. The immunoreactivity of the material after chromatography was expressed in µg/l using purified preparations as reference standards.

Statistical analysis

Results of all bioassay, immunoassays, and tests of validity (linearity, parallelism and index of precision expressed as λ) were calculated according to Gaddum (1953).

Results

CG activity in placental extracts

Extracts of a dolphin, a sea lion, a grey seal and a human placenta contained measurable amount of biologically (B) and immunologically (I) active gonadotrophin (Table 1). The concentration of biologically active CG in these placentae was higher in the dolphin, similar in the sea lion and lower in the seal compared with the human. The concentration of immunologically active

Table 1. Concentration of bio- and immuno-reactive CG in the term placenta of a dolphin, a sea lion, a grey seal and a woman

Material	Placenta dissected wet wt (g)	Assay results (i.u. CG/g placenta)		
		Bioassay (B)	Immunological assay (I)	B/I
Dolphin	1076	271 (251–293)*	55 (51.6–58.4)	4.93
Sea lion	745	43 (38–44)	8 (7.5–8.5)	5.30
Seal	1153	0.53 (0.46–0.61)	0.25 (0.23–0.27)	2.20
Human	360	27.7 (22.8–33.1)	32.1 (27.3–37.9)	0.86

*Estimates of potency and their 95% fiducial limits, in parentheses, expressed in i.u. equivalents of the Second International Standard for hCG (WHO code 61/6).

CG was higher in the dolphin, but lower in the sea lion and seal than it was in the human placental extracts. The ratio between the biological and immunological (B/I) CG activity in the extracts of the marine mammals placentae was significantly higher compared with that of the human term placenta (Table 1).

No significant ($P < 0.05$) departure from parallelism or linearity was observed between log dose and log response of the placental extracts and the hCG standard preparation in either the biological or immunological assays. An index of precision (λ) was calculated for each assay and the mean value for λ was 0.056 in the bioassays and the corresponding value for the immunoassays was 0.030.

Neutralization of placental CG activity

The effect of a rabbit anti-hCG serum upon the biological activity of the hCG standard preparation and upon the extracts from the placentae of the marine mammals and the human is shown in Table 2. The mean uterine weight of mice injected with these placental extracts or with the hCG standard plus hCG antiserum was, at all dose levels, significantly ($P < 0.0001$) smaller than those of mice injected with the hCG standard or placental extracts plus normal rabbit serum (NRS).

Table 2. Neutralization with an anti-hCG serum of the biological activity of term placental extracts and the hCG standard preparation

Material	Dose	Mean $\pm$ s.e.m. uterine wt (mg)*	
		With antiserum	Without antiserum
Dolphin placenta	0.045 mg	6.8 $\pm$ 0.09	12.0 $\pm$ 0.35
	0.090 mg	6.9 $\pm$ 0.07	22.2 $\pm$ 1.14
	0.18 mg	7.0 $\pm$ 0.14	45.2 $\pm$ 2.69
Sea lion placenta	0.60 mg	6.7 $\pm$ 0.11	10.2 $\pm$ 0.40
	1.20 mg	7.5 $\pm$ 0.18	20.0 $\pm$ 1.03
	2.40 mg	8.9 $\pm$ 0.36	40.5 $\pm$ 2.41
Seal placenta	42.5 mg	8.0 $\pm$ 0.45	11.0 $\pm$ 0.24
	85.0 mg	7.8 $\pm$ 0.53	20.6 $\pm$ 0.73
Human placenta	0.7 mg	6.8 $\pm$ 0.05	13.5 $\pm$ 0.98
	1.4 mg	7.4 $\pm$ 0.28	25.7 $\pm$ 2.53
hCG standard	0.0625 i.u.	7.2 $\pm$ 0.19	10.6 $\pm$ 0.30
	0.125 i.u.	7.5 $\pm$ 0.32	20.1 $\pm$ 0.73
	0.25 i.u.	8.6 $\pm$ 0.32	44.5 $\pm$ 2.09
Saline control	3.0 ml		7.1 $\pm$ 0.13
Mouse pituitary	8 glands	25.3 $\pm$ 1.46	26.6 $\pm$ 1.28

* $n = 5$ except for saline control for which $n = 10$.

Fig. 1. Elution patterns of activities after Sephadex G200 chromatography of extracts from the placenta of a dolphin, a sea lion, a seal and a human. K_{av} values for the CG activity are expressed in relation to purified human CG and its subunits.

Chromatographic studies

The elution patterns obtained after Sephadex G-200 chromatography of the placental extracts are shown in Fig. 1. The extracts from the 4 placentae had, with the exception of that from the seal, a significant activity after column chromatography in the RIAs for hCG and hCG alpha and beta subunits. The activities were expressed in relation to those of highly purified hCG and subunit preparations. The K_{av} values for the CG activity in the extracts from the 4 placentae were close, 0.40–0.43. The relative amount of beta subunit activity was high in the dolphin placental extract while the alpha subunit predominated in the human placental extract. The amounts of free subunits were very small in the sea lion and not detectable in the seal extract.

Zone electrophoresis

Elution patterns of immunoreactive CG activity after zone electrophoresis are shown in Fig. 2. The migration rate of each fraction was expressed as a percentage of that of labelled human serum albumin (HSA). The median migration rate of CG, calculated for the dolphin, sea lion, grey seal and human placental extracts, was 38, 39, 37 and 34 respectively.

Fig. 2. Elution patterns of immunological CG activity of extracts from the placenta of a dolphin, a sea lion, a seal and a human. Migration rate of each fraction is expressed as percentage of that labelled HSA.

Discussion

In the present study biologically and immunologically active CG was found in the placental extracts of the dolphin, sea lion, grey seal and the human. The concentration of bioreactive CG varied from 0.25 i.u./g for the seal to 271 i.u./g for the dolphin. The CG concentration in the dolphin term placenta is considerably higher than that of the human term placenta (Hobson & Wide, 1974). CG concentrations with high biological activity are found in the human placenta early in pregnancy (Diczfalusy, 1953; Hobson, 1971) or in abnormal trophoblastic tissue of gestational and non-gestational origin (Hobson, 1965). The concentration of immunological CG in the human term placenta was similar in amount to the biological CG activity. However, extracts of term placenta from the marine mammals contained a significantly lower concentration of immunologically active CG compared with the biological activity. The difference between these two activities may be due to a lower cross-reactivity of placental CG in the homologous RIA for hCG. A relationship between charge and metabolic clearance rate has been observed for pituitary FSH (Wide, 1984). Small differences in charge were observed on the CG molecules, and if a similar relationship to FSH exists the higher in-vivo biological activity of the placental extracts may be due to their longer half life.

Subunit activity was found in all the placental extracts except that of the seal. Of particular interest is the relatively large amount of free beta subunit activity in the dolphin compared with the large amount of alpha subunits in the human term placenta. Franchimont, Gaspard, Reuter & Heynen (1972) showed that when villi from human term placenta were cultured the amount of hCG and beta subunit in the medium was relatively low compared to the amount of alpha subunit present. They suggested that the synthesis of beta subunit might be the limiting factor in hCG production. Chatterjee & Munro (1977) synthesized hCG from polyribosomes prepared from 1st trimester and term placenta and concluded that the amount of hCG produced was limited by the availability of the beta subunit. It is known that the tissue of some trophoblastic tumours contains large amounts of biologically and immunologically reactive hCG and have the hCG beta subunit as the major component (Vaitukaitis & Ebersole, 1976; Vaitukaitis, 1978). It is remarkable that the dolphin placental CG is qualitatively and quantitatively similar to that of abnormal human trophoblastic tissue.

The results obtained in this investigation suggest that the gonadotrophin in the placental extracts is substantially the same as hCG and the following similarities were observed. In the biological and immunological assays there was no departure from parallelism and linearity between the hCG standard preparation and the CG activity in the placental extracts. This CG activity in the placenta of the dolphin, sea lion and seal was, like the hCG activity in the human placenta and hCG standard preparation, neutralized by an anti-hCG serum. The extracts of placenta, with the exception of the grey seal, were shown to have subunit activity when measured in the RIA for the alpha and beta subunits of hCG. K_{av} values for CG activity in the extracts from the placenta of the marine mammals and the human placenta after gel chromatography indicate an apparent similarity in molecular size between the CG and hCG. The median migration rate of CG activity in the placental extracts of the marine mammals and that of the human were not very different.

The relative amounts of CG subunits compared to CG varied between species indicating that differences may exist with respect to hormone synthesis. Until more is known about the physiological role of CG in pregnancy, an explanation for observed differences between species in placental concentration of gonadotrophin and its subunits can only be speculative.

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