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Secretion of Gonadotropic Factors by the Preimplantation Rabbit Blastocyst (40506)

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Haour and Saxena (1) described a factor in uterine fluids and blastoceolic fluid of the preimplantation rabbit blastocyst which inhibited the binding of ^{125}I-human chorionic gonadotropin (^{125}I-hCG) to receptor sites on the pregnant bovine corpus luteum, and suggested that this factor may be an luteinizing hormone (LY) or hCG-like molecule. This was supported by findings (2, 3) that concentrations of progesterone in serum were greater in pregnant that in pseudopregnant rabbits from day 5 through day 10 postcoitum. Fujimoto *et al.* (4) have also detected LH-like activity in rabbit blastoceolic fluid prior to implantation using an LH radioimmunoassay. In contrast, Holt *et al.* (5) found no differences in progesterone concentrations in serum when levels in pregnant and pseudopregnant rabbits were compared at any time prior to implantation, nor did they find LH or hCG-like activity in blastoceolic fluid using a variety of bioassays. In addition, Sundaram *et al.* (6) were unable to detect LH-like activity in blastoceolic fluid using a sensitive *in vitro* bioassay. An excellent review of the factors which regulate luteal function in rabbits is provided by Hilliard (7).

In light of these conflicting reports, we have attempted to establish whether the preimplantation rabbit blastocyst secretes a gonadotropic factor by: (a) Comparison of serum progesterone concentrations in pregnant and pseudopregnant rabbits; (b) examination of the molecular nature of the factor responsible for LH-like activity in radioreceptor assays by gel filtration chromatography; and (c) examination of the protein synthetic and secretory capability of the preimplantation blastocyst by culturing them *in vitro* in the presence of radioactive amino acids.

Materials and Methods. Serum concentrations of progesterone in pregnant and pseudopregnant rabbits. Thirty-two mature Dutch-belted or Dutch-belted New Zealand cross-bred rabbits were divided into two groups of 16 rabbits each. Rabbits in one group received a single iv injection of 2.5 mg LH (Reheis Chemical Co., Chicago, IL) to induce ovulation (designated day 0) and pseudopregnancy. Rabbits in the second group received the same dose of LH and were artificially inseminated within 30 min (8). Pregnancy was confirmed by laporotomy at mid-gestation or by birth of live young. Pseudopregnancy was indicated by elevated serum concentrations of progesterone from day 2 through day 13. Blood samples were taken from the marginal ear vein 4 hr after LH injection, every 12 hr until day 2, daily until day 9 and every other day until day 15. Progesterone in serum was quantified by radioimmunoassay (9).

Radioreceptor assay of blastoceolic fluid and uterine flushings. To collect large numbers of blastocysts, rabbits were superovulated with twice-daily injections of 0.5 mg follicle stimulating hormone (FSH) (Reheis Chemical Co., Chicago, IL) for 3 days, followed by artificial insemination. One-hundred twenty hours following LH administration, rabbits were anesthetized with sodium pentobarbital and blastocysts flushed from the uteri with sterile isotonic saline (10 ml/uterine horn). After transfer of the flushings to a conical centrifuge tube, excess saline was drawn off with a filter paper strip and the blastocysts were disrupted with a needle and centrifuged to obtain blastoceolic fluid. All procedures following collection of the blastocysts were carried out at 4°.

Rabbits from which only uterine fluids were collected were mated to intact or vasectomized bucks. Day of coitus was designated day 0 of pregnancy or pseudopregnancy. Each uterine horn was flushed with 10 ml sterile isotonic saline and flushings were placed on ice immediately. Any flush which contained visible amounts of blood was dis-

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carded. All uterine flushings were clarified by centrifugation at 100,000*g* for 90 min. Protein in the clear supernatants was concentrated to 1–4 mg/ml by ultrafiltration on Amicon PM-10 membranes (Amicon Corp., Lexington, MA) at 40 psi. These membranes have a molecular weight cut-off of approximately 10,000. Each preparation of uterine flushings contained the combined material from 3 to 4 rabbits.

Radioreceptor assays were carried out according to the method of Haour and Saxena (1) with several minor modifications. A crude membrane preparation was prepared from ovine corpora lutea homogenized in 5 vol 0.25 *M* sucrose containing 10 m*M* Tris, 1 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$ (pH 7.2). The homogenate was filtered through four layers of cheese cloth and centrifuged at 480*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 1 hr and the resulting pellet resuspended in 10 m*M* Tris, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% bovine serum albumin (BSA) (pH 7.2). The reaction was carried out at 23° with 20 μg membrane protein per tube. Highly purified hCG (CR-117, 10,000 IU/mg, kindly supplied by Dr. R. Canfield, Columbia University) was radioiodinated with ^{125}I to a specific activity of 60 μCi/μg using a lactoperoxidase method (10).

Blastoceolic fluid and concentrated uterine flushings from pregnant and pseudopregnant rabbits were fractionated on a 1.5 × 85 cm Sephadex G-200 column at 4° with 10 m*M* Tris, 10 m*M* NaCl (pH 7.4) as the eluant. Fractions (0.5 ml) were collected and assayed by radioreceptor assay for LH-like activity or for protein (11). Each fluid, except blastoceolic fluid, was fractionated twice; once for protein determination and once for LH-radioreceptor activity. Blastoceolic fluid was only fractionated once and LH-like activity determined due to the limited quantity of this fluid which was available.

Protein synthesis and secretion by the preimplantation blastocyst. Blastocysts collected 120 hr after insemination were incubated groups of 4, 8, and 16 for 24 hr with 0.5 mCi of a ^3H-amino acid mixture consisting of 15 uniformly labeled amino acids (New England Nuclear Corp., Boston, MA) in modified Ham's F-10 medium containing 1.5% BSA (12). All media contained 100 IU penicillin and 50 μg streptomycin per ml, and were sterilized by filtering through a 0.22 μm Millipore filter. Culture was carried out in an incubator (New Brunswick Scientific Model CO-20) maintained at 37° in an atmosphere of 95% air–5% CO_2 at 100% humidity. Sterile plastic culture trays (Linbro Disposo Trays, Flow Laboratories, Englewood, CA) with 1 ml wells were used. Final volume of incubation medium was 0.8 ml which was covered by 0.2 ml paraffin oil. Blastocyst diameters were measured before and after the culture period with an ocular micrometer so that growth rates could be determined. Six blastocysts were cultured without ^3H-amino acids, to be used for vital staining at the end of the culture period. Control incubations consisted of ^3H-amino acids only, incubated in culture medium for 24 hr.

At the end of the culture period blastoceolic fluid was expelled from each blastocyst into the culture medium. Broken blastocysts in each group were transferred to tubes containing 0.5 ml distilled water. The contents of each tube, containing 4, 8, or 16 blastocysts, were subjected to a series of 10 rapid freeze–thawings and proteins precipitated with 2 ml cold (4°) 10% TCA. The mixture was filtered over a Gelman type E fiberglass filter under slight vacuum and rinsed consecutively with 5 ml cold 5% TCA, 10 ml hot (80°) 5% TCA to remove tRNA bound ^3H-amino acids and 5 ml cold 70% ethanol to speed drying. After drying at 50°, each filter was counted in 10 ml of toluene-based scintillation fluid in a Nuclear Chicago ISOCAP/300 liquid scintillation counter with a 50% efficiency for tritium.

To determine if any large molecular weight proteins were secreted by the blastocysts, combined blastoceolic fluid and culture medium from each group was fractionated on a 0.9 × 17 cm Sephadex G-25 column at 4° with 10 m*M* Tris (pH 7.2) containing 1 m*M* $CaCl_2$ and 0.1% BSA as eluant. The BSA was included to minimize possible losses of small quantities of protein. In the second experiment a larger (1.5 × 30 cm) column with no BSA in the eluant was used. Aliquots (100 μl) of 0.5 ml fractions were counted in 4 ml Trition X-100 toluene-based scintillation fluid. After fractionation of the medium and fluid following culture of 16 blastocysts on

the 0.9 × 17 cm column, the combined void volume fractions (tubes 7–11) were fractionated further on a 1.5 × 85 cm Sephadex G-200 column at 4° with 0.02 *M* phosphate–citrate buffer (pH 7.4) as the elutant. Fractions (0.5 ml) were collected and counted as above.

Results. Serum concentrations of progesterone in pregnant and pseudopregnant rabbits. Concentrations of progesterone in serum from pregnant and pseudopregnant rabbits are depicted in Fig. 1. Values represent the mean ± SEM (shading) for 16 animals at each sampling time. Four hours following LH administration serum progesterone levels had reached approximately 60 ng/ml. Within 12 hr, concentrations had fallen to baseline and remained low until day 2, after which they gradually increased in both pregnant and pseudopregnant rabbits until day 11. The patterns diverge on day 11, with progesterone levels continuing to rise in pregnant rabbits but beginning to decline in pseudopregnant rabbits. There were no significant differences in concentrations of progesterone in serum between pregnant and pseudopregnant rabbits at any time prior to implantation, which occurs on day 7. These data are in agreement with those of Challis *et al.* (13) and Holt *et al.* (5) but in contrast to those of Fuchs and Beling (2).

Radioreceptor assay for blastoceolic fluid and uterine flushings. Blastoceolic fluid and uterine flushings from pregnant rabbits inhibited the binding of ^{125}I-hCG to ovine luteal membranes (Fig. 2). However, low but detectable quantities of a similar material

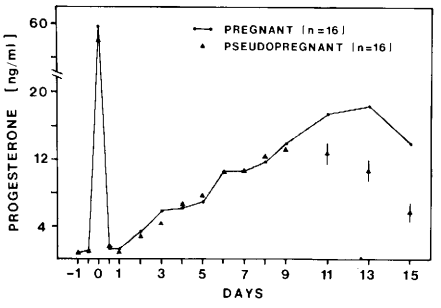

FIG. 1. Concentration of progesterone in serum from pregnant and pseudopregnant rabbits. Each point represents the mean for 16 determinations. The SEM is represented by shading for the data from pregnant and by a bar for data from pseudopregnant rabbits

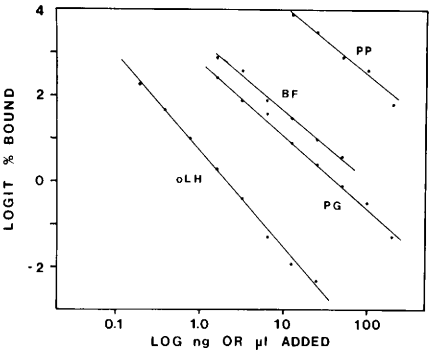

FIG. 2. Radioreceptor assay for LH-like activity in rabbit blastoceolic fluid and uterine flushings. Each point represents the mean of three determinations. Slopes of inhibition curves obtained with NIH-LH-S19, blastoceolic fluid (BF, 0.56 mg/ml) and uterine flushings from pregnant (PG, 4.9 mg/ml) and pseudopregnant (PP, 3.1 mg protein/ml) rabbits are −0.99, −0.69, −0.75 and −0.74, respectively.

were also observed in uterine flushings from pseudopregnant rabbits. Slopes of inhibition curves obtained with NIH-LH-S19, blastoceolic fluid and uterine flushings from pregnant and pseudopregnant rabbits were −0.99, −0.69, −0.75, and −0.74, respectively. Similar results were obtained with four preparations of blastoceolic fluid and uterine flushings from pregnant rabbits and 3 preparations of uterine flushings from pseudopregnant rabbits. Protein and radioreceptor activity elution profiles obtained after fractionation of uterine flushings and blastoceolic fluid on a 1.5 × 85 cm Sephadex G-200 column are depicted in Fig. 3. All detectable radioreceptor activity in uterine flushings (Panel A and B) eluted before bovine gamma globulin, indicating a molecular weight of greater than 150,000. The protein peak which elutes near cytochrome C probably represents uteroglobin or blastokinin (14) and has no radioreceptor activity. The radioreceptor elution profile obtained from 2 ml of blastoceolic fluid (257 blastocysts) which had been collected from eight superovulated rabbits is depicted in Panel C. The protein elution profile depicted in Panel C was obtained after fractionation of uterine flushings for the eight donor rabbits. These data indicate that the molecular weight of the factor in blastoceolic fluid responsible for radioreceptor activity is greater than 150,000. Thus, the elution profile

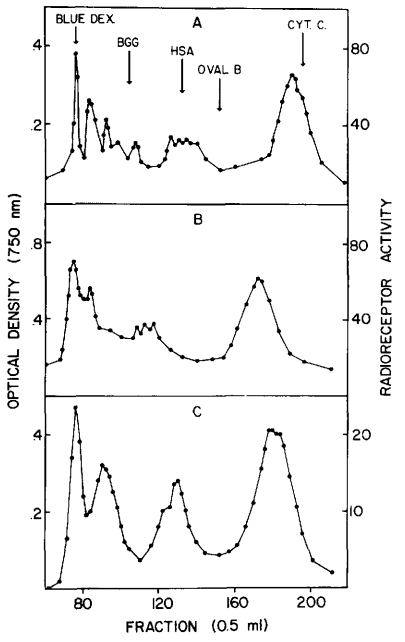

FIG. 3. Data following Sephadex G-200 column chromatography of rabbit blastoceolic fluid and uterine flushings. Solid line represents protein elution profile expressed at o.d. at 750 nm. Shading represents RRA activity expressed as percent inhibition of binding of ^{125}I-hCG to receptor. Panel A: Uterine flushings (4.2 mg) collected from pregnant rabbits on day 5 postcoitum. Elution volumes of blue dextran (mol wt ~ 2,000,000), bovine gamma globulin (mol wt 150,000), human serum albumin (mol wt 67,000), ovalbumin (mol wt 43,500) and cytochrome c (mol wt 12,400) are indicated. Panel B: Uterine flushings (9.5 mg) collected from pseudopregnant rabbits on day 5 postcoitum. Panel C: RRA profile from 2 ml blastoceolic fluid collected on day 5 of pregnancy. Protein profile is from 4.3 mg uterine luminal protein collected from eight rabbits during blastocyst collection.

for the material which inhibits in the radioreceptor assay is different from that for any of the known luteotropic hormones.

Protein synthesis and secretion by the preimplantation blastocysts. During the 24 hr culture period blastocysts increased in diameter from 1.16 ± 0.05 mm to 1.78 ± 0.06 mm (mean ± SEM). Blastocysts subjected to vital staining at the end of the culture period were judged to be alive by either the trypan blue (14) or neutral red (15) method. Essentially identical results were obtained in two additional experiments. The elution profile obtained when combined blastoceolic fluid and culture medium were fractionated on a 0.9 × 17 cm column of Sephadex G-25 is shown in Fig. 4A. For simplicity, only the profile obtained after fractionation of medium from control wells and medium and blastoceolic fluid after incubation of 16 blastocysts are shown. Approximately 2800 cpm of ^{3}H-amino acids became bound to the albumin (1.5%) in the culture medium, as indicated by the radioactivity recovered in the void volume after fractionation of the control medium. In the second experiment greater resolution was obtained by using a larger (1.5 × 30 cm) column with no BSA in the buffer (Fig. 4B).

The total amount of radioactivity recovered in the void volume (minus the amount recovered in the void volume of the control fractionation) following fractionation of medium from groups containing 4, 8, or 16 blastocysts is tabulated in Table I. There was a linear increase in radioactivity recovered in the void volume as the number of blastocysts in the incubation wells was increased. There was also a linear increase in the quantity of radioactivity recovered in the peak which eluted at 38 ml but the identity of this factor or factors is unknown. Also

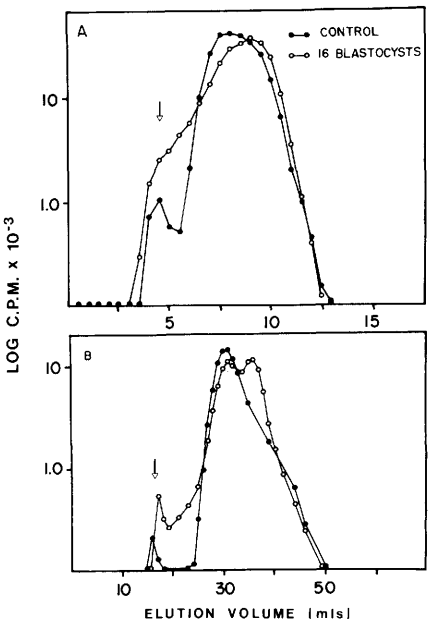

FIG. 4. Data following Sephadex G-25 column chromatography of supernatant (Panel A) and blastoceolic fluid (Panel B) from blastocysts incubated *in vitro* with ^{3}H-amino acids. A. 0.9 × 17 cm column; B. 1.5 × 30 cm column. Arrows indicate the void volume of each column.

TABLE I. RADIOACTIVITY INCORPORATED INTO TCA PRECIPITABLE BLASTOCYSTIC PROTEINS AND LARGE MOLECULAR WEIGHT PROTEINS IN BLASTOCEOLIC FLUID AND CULTURE MEDIUM DURING INCUBATION *in Vitro* WITH ^{3}H-AMINO ACIDS.[a]

Number of blastocysts in well	dpm in TCA precipitable blastocystic proteins ± SEM, $n = 3$	dpm in void volume of G-25 fractionation of blastoceolic fluid and culture medium ± SEM, $n = 2$
4	39,000 ± 9,340	25,000 ± 3,180
8	87,000 ± 19,000	55,300 ± 8,530
16	143,600 ± 4,320	82,500 ± 6,790

[a] The correlation coefficients between the number of blastocysts incubated and the dpm in TCA precipitable blastocystic proteins and dpm in the void volume after fractionation of blastoceolic fluid were 0.99 and 0.98, respectively.

shown in Table I is the amount of radioactivity incorporated into TCA precipitable blastocystic proteins. Again there was a linear increase in radioactivity incorporated, and the amount incorporated was highly correlated with the number of blastocysts.

The elution profile obtained when combined void volume fractions (7–11) following fractionation of the medium and fluid from 16 embryos on Sephadex G-25 columns were further fractionated on Sephadex G-200 is shown in Fig. 5. Calibration of this column is shown in Fig. 6. A small peak of radioactivity having an elution volume of 78 ml was observed, but 95% of the radioactivity was recovered near the column volume, indicative of a molecular weight of 5000 or less. A second peak which eluted at a volume of 78 ml is identical to the elution of albumin, and no radioreceptor activity was detectable in either of these peaks. These data suggest that the factor in blastoceolic fluid and uterine flushings having LH-like radioreceptor activity is not produced by the blastocyst or that the quantities produced are too low to detect under the experimental conditions employed.

Discussion. The data presented suggest that the preimplantation rabbit blastocyst does not secrete a gonadotropin similar to hCG or LH. If such a molecule is secreted, it does not appear to influence systemic progesterone levels during the preimplantation period, nor can its secretion by blastocysts be demonstrated *in vitro*. That the blastocysts cultured *in vitro* were growing and functioning normally seems likely since they remained viable, increased in volume by three- to fourfold and were metabolically active in terms of uptake and incorporation of ^{3}H-amino acids into proteins. Blastocysts cultured *in vitro* in this manner have been transferred to recipient does with resultant birth of live young (16, 17). In addition, the growth rate obtained for blastocysts in this study was identical to the *in vivo* growth rate observed between days 5 and 6 in pregnant rabbits by Daniel (18). However, the possibility still remains that the blastocyst may secrete factors while growing in its intrauterine environment that it would not secrete under *in vitro* conditions.

This study confirms the existence of a factor in blastoceolic fluid and uterine fluids of pregnant rabbits which inhibits the binding of ^{125}I-hCG to its receptor as reported by Haour and Saxena (1). However, the estimated molecular weight of ~150,000 is much greater than any known gonadotropin; and the factor is also found in uterine flushings

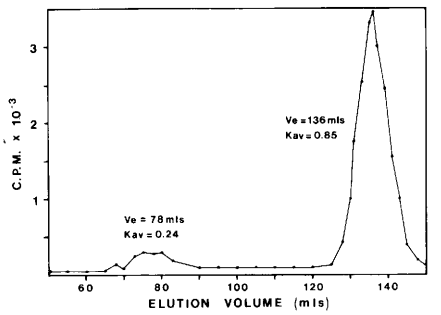

FIG. 5. Data following Sephadex G-200 column chromatography of combined void volume fractions (7–11) from Sephadex G-25 (0.9 × 17 cm column, 16 blastocyst group) chromatography of blastoceolic fluid and incubation medium.

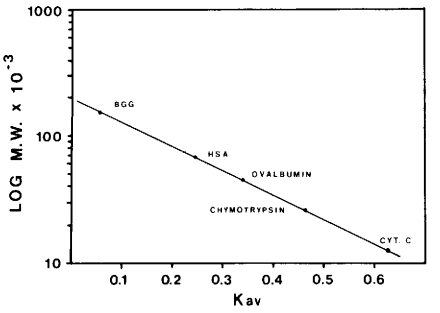

FIG. 6. Calibration of Sephadex G-200 column. Standard proteins are: BGG (mol wt 150,000), HSA (mol wt 67,000), ovalbumin (mol wt 43,500), chymotrypsinogen (mol wt 25,700) and cytochrome c (mol wt 12,400).

obtained from pseudopregnant rabbits. The material did not degrade ^{125}I-hCG when both were incubated together under conditions similar to those used in the radioreceptor assay. However, this fraction did appear to bind the ^{125}I-hCG under these conditions as indicated by gel filtration chromatography. These data, in conjunction with the findings of Holt *et al.* (5) who were unable to detect LH-like biological activity in rabbit blastoceolic fluid using a variety of bioassays, make it unlikely that the factor responsible for radioreceptor activity is a gonadotropin.

Considerable evidence has accumulated which indicates that any factor, up to the molecular weight of ferritin (mol wt 480,000) can, and will, be transported from the uterine lumen into blastoceolic fluid (19–23). We suggest that the factor responsible for radioreceptor activity is a uterine luminal protein. Whether it is synthesized by the endometrium or originates from the serum remains to be determined. Several unique uterine proteins are formed during the preimplantation period in pregnant rabbits. In addition to uteroglobin, which comprises about 35% of the proteins in blastoceolic fluid (13), there is a pregnancy specific post-albumin, prealbumin, and several β-glycoproteins (24, 25). Serum proteins such as albumin, transferrin, IGG, and α- and β-macroglobulin have also been identified in the uterine lumen (26). "Nonspecific" inhibition or interference of the binding of ^{125}I-hCG to its receptor by serum proteins *in vitro* has been observed in this laboratory and has been discussed in considerable detail by Lee and Ryan (27).

Summary. No significant differences in progesterone concentrations in serum were observed between pregnant and pseudopregnant rabbits at any time prior to implantation. There is a factor present in blastoceolic fluid which reduces the binding of ^{125}I-hCG to receptor sites on luteal membranes, but this factor is also present in uterine flushings obtained from pregnant and pseudopregnant rabbits and has an estimated molecular weight of greater than 150,000. When rabbit blastocysts collected 120 hr after insemination are incubated *in vitro* for 24 hr with ^{3}H-amino acids, no large molecular weight proteins can be detected in blastoceolic fluid or incubation medium. These data suggest that the preimplantation rabbit blastocyst does not secrete a gonadotropic factor similar to hCG or LH.

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