Preovulatory and Postovulatory Progestins in Monkey Ovary and Ovarian Vein Blood.* (28334)

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The physiology of reproduction in the rhesus monkey has received much study, but as yet little is known about the cyclic patterns of ovarian progestin release. Recent reports on subprimates indicate that several different progestational steroids are present in ovarian tissue(1,2). In the rabbit the principal ovarian progestin is 20 α-hydroxypregn-4-en-3-one (20 α-ol) and immediately following intravenous gonadotropin considerable amounts of this steroid are released into the ovarian vein blood(2,3).

This paper gives evidence that the monkey ovary contains and releases progesterone, 17 α-hydroxyprogesterone (17 α-OH) and 20 α-ol under varying conditions of gonadotropic stimulation, both in presence and absence of corpora lutea. Results also indicate that the monkey ovary behaves similarly to the rabbit ovary in response to acutely administered gonadotropin.

Material and methods. The progestin content of ovarian venous blood plasma and ovarian tissue was measured in 10 sexually mature rhesus monkeys (8 *Macaca mulatta,* 4-6 kg and 2 *Macaca irus,* 2-3 kg). Ovarian veins were cannulated under pentobarbital anesthesia (30 mg/kg, IP) using polyethylene tubing (od. 0.05″). Blood was collected in heparinized, 15 ml graduated centrifuge tubes for consecutive periods ranging between 1-4 hours with blood flow rates of 0.5 to 1.0 ml/min. The plasma was immediately separated from the cells by centrifuging (1500 RPM, 10 min) and saved for assay. The cells were washed once with saline and the wash was added to the plasma. Washed cells were resuspended in physiological saline and returned to the animal through a leg vein cannula. Following blood collections the cannulated ovary was removed, weighed and homogenized with water in a glass tissue grinder. Plasma and tissue were extracted overnight at 4°C in 3 vols of ether and washed twice the next morning with additional 50 ml of ether using a separatory funnel. Ether fractions were combined, washed with successive 10 ml amounts of 0.1 N NaOH until the base layer was clear, then washed × 2 with 10 ml water, and evaporated to about 5 ml in a round bottom distilling flask (mantle temperature <70°C). The remaining ether was evaporated under nitrogen and the residue was partitioned × 2 between 1 ml N-heptane and 1 ml abs methanol. The methanol was removed under nitrogen and the residue was redissolved in 0.1 ml ethyl acetate and chromatogrammed in a descending system on Whatman #1 22× 7″ paper strips. Strips were equilibrated overnight at room temperature in a closed tank containing 500 ml stationary phase (methanol:water:: 4:1). The mobile phase (N-heptane:ethyl acetate::5:2) was added in the morning. Following development (3 hours), strips were dried and purple fluorescent spots located with a Mineralight UV lamp. For quantitation, strips were sprayed twice with 2,4-dinitrophenylhydrazine solution (200 mg in 700 ml 1% HCl in ethanol) dried, and washed immediately in warm tap water. Phenylhydrazone areas were located against known steroid standards, cut out, minced with scissors and eluted twice with ethanol (4 ml overnight, 2 ml 1 hour). Ethanol washes were combined and color was developed by adding 0.2 ml 0.1 N NaOH just before quantitation in a colorimeter at 540 mμ. Each sample was corrected against its own background blank of the same surface area, cut from a vertically adjacent region of the chromatogram and treated in the same manner.

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TABLE I. Progestins in Ovarian Effluent and Ovarian Tissue.

Exp	Preparation schedule: Chronic treatment	Acute treatment	Ovary wt (mg)	Progestin: Ovarian effluent (μg/gl/hr) Prog.	17 α-OH	20 α-ol	Ovarian tissue (μg/100 mg) Prog.	20 α-ol
1	None	HCG* 250 U IV 1 hr†		0	0	0		
2	"	None	186 (2)‡	0	0	0	0	0
3	"	"	351 (2)	0	0	0	0	0
4	HMG§ 8 mg × 2, 5 days 4 mg × 2, 2 days, with HCG 1500 U × 2, last 3 days	"	473 (1)‡	0	0	0	0	0
5	FSH‖ 2 mg × 2, 5 days	"	1452 (2)				0	0
6¶	PMS** 500 U × 2, 13 days, with HCG 1000 U × 2, last 6 days	"	720 (2)				0	0
7	PMS 125 U × 1, 8 days, with HCG 1666 U × 1, last 3 days	HCG 250 U IV 1 hr		0	0	3.4		
8	FSH 4 mg × 2, 10 days FSH 2 mg × 2, 3 days, with HCG 1000 U × 2, last 3 days	HCG 2000 U IV 1 hr	1674 (2)	0	0	3.5	0	3.7
9¶	a. FSH 2 mg × 2, 18 days, with HCG 1000 U × 2, last 5 days	None	810 (1)				0.86	0
	b. *Idem*	HCG 10,000 IV	660 (1)	16.8	3.5	3.2	1.52	0
10	FSH 4 mg × 2, 9 days, with HCG 2000 U × 2, last 3 days	None	1214 (1)				0.78	0
11	a. FSH 4 mg × 2, 7 days, with HCG 1000 U × 2, last 2 days	None		7.2	11.0	trace		
	b. *Idem*	HCG 2000 U IV	1256 (1)	11.1	13.0	6.1	0	0
12	PMS 125 U × 2, 12 days HCG 1000 U × 2, 12 days, with FSH 4 mg × 2, last 6 days	HCG 2000 U IV 2 hr†		29.0	20.0	9.2		
13	FSH 2 mg × 2, 12 days, with HCG 1000 U × 2, last 7 days	HCG 1000 U IP 2 hr		21.1	16.1	37.2		

* Human chorionic gonadotropin.
† Prior to start of blood collection.
‡ (2) both ovaries, (1) one ovary.
§ Human menopausal gonadotropin.
‖ Follicle stimulating hormone.
¶ *M. irus.*
** Pregnant mare's serum gonadotropin.

Prior to progestin assays, some of the monkeys were prepared with subcutaneous injections of gonadotropins according to the schedules suggested by van Wagenen and Simpson(4,5,6). The gonadotropins used for preparation were pregnant mare's serum (PMS),‡ porcine follicle stimulating hormone (FSH),§ human postmenopausal urinary gonadotropin (HMG)‖ and human chorionic gonadotropin (HCG).¶

Results. No progestin was detectable in either ovaries or ovarian effluent of three untreated monkeys either at mid-cycle or at random. The ovaries of these animals were small, pale and showed no evidence of activity (Table I, Exp. 1, 2, 3). Negative results were also obtained in 3 other monkeys which had received inadequate gonadotropic preparation, although ovaries were larger and more vascular, with definite evidence of follicular development (Exp. 4, 5, 6).

In 4 experiments involving preparation

‡ "Equinex", Ayerst.

§ Courtesy of J. D. Fisher, Armour Pharmaceutical Co.

‖ Courtesy of Dr. G. A. Overbeek, N. V. Organon, Co.

¶ "Follutein", Squibb.

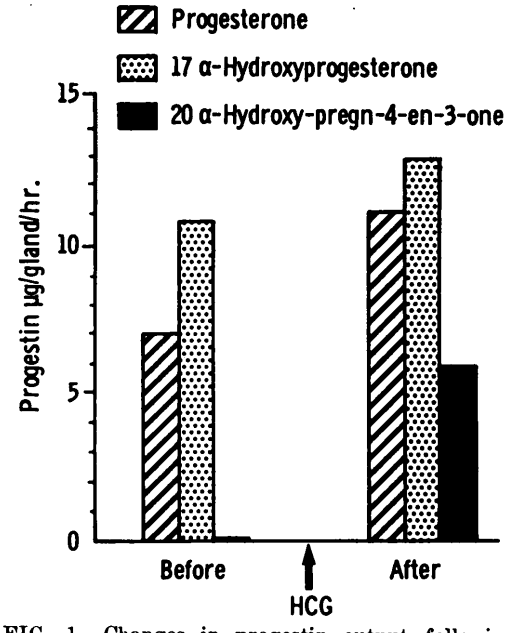

FIG. 1. Changes in progestin output following IV injection of 2000 U HCG. Blood was collected consecutively for 2 hr before and 2 hr after acute HCG. This monkey had been chronically prepared with FSH and HCG (Exp 11).

with PMS or FSH supplemented by HCG, usually given during the first half of the menstrual cycle, there were definite reddening and swelling of the sexual skin, desquamation of the vaginal mucosa and enlarged ovaries with bulging but unruptured follicles (Exp. 7, 8, 9, 10). Within 1-2 hours after acute injection of HCG, *preovulatory* progestin was measurable in ovarian vein blood plasma as 20 α-ol (Exp. 7, 8) and as progesterone, 17 α-OH and 20 α-ol (Exp. 9). In Experiment 9, where the first ovary (a) was removed just prior to injection of 10,000 U of HCG and the second ovary (b) was removed 3 hours later, the second ovary showed a 2-fold increase in progesterone content.

In 3 monkeys, chronic preparation with gonadotropin induced ovulation as well as sexual skin changes and vaginal desquamation, and ovaries showed recently formed corpora lutea (Exp. 11, 12, 13). The ovarian vein blood in all cases contained progesterone, 17 α-OH and 20 α-ol in varying proportions. In Experiment 11, the immediate response to gonadotropin was tested by giving a single intravenous injection of 2000 U of HCG between a 2 hour (a) and a second 2 hour (b) blood collection period. This produced a rise in ovarian output of progesterone and 17 α-OH coupled with a substantial increase in 20 α-ol (Fig. 1).

Discussion. The principal ovarian progestins found in the adult monkey were progesterone, 17 α-OH and 20 α-ol. These steroids are present in human ovarian tissue(7,8,9) and have progestational activity(9). No progestin was detectable in monkeys prior to adequate gonadotropic preparation. However, when the preparation schedules recommended by van Wagenen and Simpson were followed(4,5,6), one or all 3 of these progestins were measurable in both ovarian effluent and tissue. The highest total levels of progestin were associated with the presence of corpora lutea but progestin was also found in ovarian blood and tissue prior to ovulation. Our results substantiate the findings of Forbes *et al.*(10); namely, that cyclic changes of progestational activity in peripheral blood of monkeys occur before as well as after ovulation. However, the physicochemical methods used in our studies, contrasted to their bioassay techniques, allow chromatographic separation of the progestins released.

Human chorionic gonadotropin (HCG) appears to be the major component of both the acute and chronic preparation schedules in the monkey. In addition, our findings indicate that the primate ovary can respond rapidly to this gonadotropin of human origin with an increased synthesis and release of progestational steroids. Ovarian response to gonadotropin in the monkey, a cyclic mammal, therefore appears to be similar to that of the rabbit, a non-cyclic reflex ovulator.

Summary. 1) Progesterone, 17 α-hydroxyprogesterone and 20 α-hydroxy-pregn-4-en-3-one were measurable in ovarian plasma or ovarian tissue following gonadotropic stimulation in 2 members of the Genus Lyssodes, *M. mulatta* and *M. irus*. 2) The presence of these progestins was not dependent upon the occurrence of ruptured follicles or recently formed corpora lutea. 3) Shortly after intravenous administration of human chorionic

gonadotropin, the synthesis and release of these steroids were measurably increased.

1. Short, R. V., *J. Endocrin.*, 1961, v22, 153.
2. Hilliard, J., Archibald, D., Sawyer, C. H., *Endocrinology*, 1963, v72, 59.
3. Simmer, H., Hilliard, J., Archibald, D., *ibid.*, 1963, v72, 67.
4. van Wagenen, G., Simpson, M. E., *Rev. Suisse de Zool.*, 1957, v64, 807.
5. Simpson, M. E., van Wagenen, G., *Fertil. and Steril.*, 1958, v9, 386.
6. ———, *ibid.*, 1962, v13, 140.
7. Zander, J., *Nature*, 1954, v174, 406.
8. ———, *J. Biol. Chem.*, 1958, v232, 117.
9. Zander, J., Forbes, T. R., v. Münstermann, A. M., Neher, R., *J. Clin. Endocrinol. & Metabol.*, 1958, v18, 337.
10. Forbes, T. R., Hooker, C. W., Pfeiffer, C. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 177.

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A Method for Tissue Culture of *Hydra* Cells.* (28335)

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Hydra is one of the simplest of the multicellular animals. It has also some characteristics of plants. *Hydra* provides, therefore, a unique material for cellular investigation. However, the presence of the cnidoblasts and nematocysts in *Hydra* has complicated the study of functional properties of constituent cells. A method to isolate the *Hydra* cell *in vitro* is highly desirable, and would be useful in studying effects of immediate environments, division, and differentiation. The culture of coelenterate tissue *in vitro* has not been successful until recently in the instance of anemone *Anthopleura elegantissima*(1). In the case of *Hydra,* Papenfuss and Bokenham (2) had studied the ectoderm and endoderm cultured independently in filtered pond water, and reported that the tissues always disintegrated. No information on tissue culture of fresh water *Hydra* cells in nutritive media has appeared in the literature. This communication describes a method for cultivation of *Hydra* cells *in vitro*.

Materials and methods. A preliminary study of the cell culture of *Hydra littoralis*§ revealed the presence of severe contamination by bacteria-like microorganisms, which could not be eliminated by carefully improving the aseptic techniques.‖ Our further studies showed that these contaminating bodies were, in fact, an intracellular parasite to the *Hydra,* the Microsporadia, previously tentatively identified as a species of *Plistophora*(3). One source of the infection was traced invariably to the artemia used for feeding the *Hydra*. Although Microsporadia-infected *Hydra* continued to bud and regenerated normally and showed no significant physiological or morphological changes, the spores of the Microsporadia rapidly germinated, multiplied and destroyed the host cells when the latter were cultured *in vitro*. Consequently it was imperative that the spores of the parasite be removed from the *Hydra* cells. Fumidil B was employed as this preparation has been demonstrated to be effective for this purpose(3). To prevent reinfection, subsequently, prior to the preparation of the *Hydra* cell suspension, the *Hydra* were cultivated without feeding for 2 days in the sterilized culture solution(4) containing 0.125 g neomycin, 0.125 g streptomycin, 0.200 g

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