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Pregnancy Block Elicited by Urinary Proteins of Male Mice

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ABSTRACT

Pregnant mice exposed to male urinary proteins isolated by salting out of excreted urine, bladder urine, or urine from castrated and testosterone-treated males blocked pregnancy in 3–4 days after treatment. Also homogenates of preputial glands evoked the Bruce effect. Females exposed to urinary proteins had shortened oestrous cycle. The results suggest that male mice produce only one pheromone which can evoke different effects depending on a physiological state of the female.

INTRODUCTION

It is known that hormonal responses of female mice to male pheromones include acceleration of puberty (Vandenbergh, 1967), shortening of the oestrous cycle (Whitten, 1956) and blocking of early stages of pregnancy (Bruce, 1959). Several studies demonstrated that urine is the main source of male pheromones, and the production of these biologically active substances is under permanent control of androgens (Parkes and Bruce, 1961; Dominic, 1965; Bronson and Whitten, 1968; Vandenbergh, 1969).

The urine of male mice contains a high level of proteins. Major urinary protein complex depends on androgen hormones and occurs in mature males and androgen-treated females (Rümke et al., 1964; Finlayson et al., 1965).

Present investigations were designed to study the effect of male urine non-dialyzable fraction rich in proteins on the early stages of pregnancy, course of the oestrous cycle and time of puberty of female mice.

MATERIALS AND METHODS

Animals used for the experiments were maintained in polyethylene cages, lighting schedule 14:10 (lights on 7 am to 9 pm), and fed a standard pelleted diet and water.

Urine, preputual glands and blood were obtained from adult, sexually experienced animals. External urine was collected from 25 Outbred males (stock maintained in our Department by random mating), 20 C57 BL/kw males, 20 Outbred females and 10 castrated and testosterone-treated Outbred males, by holding animals over test tubes with a gentle massage of the abdominal wall. Forty-seven Outbred males were ether anesthetized and urine withdrawn from the bladder into a syringe. Preputial glands were removed from Outbred males and immediately homogenized with 1 ml physiological saline. Blood for testing serum proteins was obtained from 20 Outbred males by a puncture of the jugular vein.

The urine was dialyzed for 24 h at 4°C in a Visking Tubing 8/32 (The Scientific Instrument Centre, London) and the non-dialyzable fraction was salted out with solid ammonium sulphate at 80 percent saturation. The precipitate was centrifuged, dissolved in distilled water to the original volume and dialyzed overnight against phosphate-buffered physiological saline.

The concentration of protein was estimated by Lowry et al. (1951) method. All samples were kept frozen at −20°C until used.

Test for the Pregnancy Block

For testing the biological activity of urinary proteins CBA females were used. They were mated to CBA males and the morning when the vaginal plug was found was assumed as the first day of pregnancy. On the second day the female was transferred either to an empty cage, or to a cage separated by a net and containing either an Outbred male or C57 male. Females kept in separate cages in a male-free room were treated with a solution of proteins, or homogenate of preputial glands, during second and third days of pregnancy. The solution was given in drops on the oro-nasal groove 7 times during the second day and 3 times during third day. The total volume per one female was 0.5–0.7 ml of the solution. Every morning the vaginal smears were obtained and the first day when the smear contained only squamous epithelial cells and the vagina was characteristically softened was regarded as the oestrus. The appearance of blood in the vaginal smear, usually between 10th and 12th day, was considered as pregnancy.

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TABLE 1. Pregnancy blocks of CBA females after exposure to urinary proteins or preputial glands of males.

Treatment	No. of blocked/ no. of treated	Pregnancy block (%)
Control (not exposed)	2/36	6*
Exposed to males:		
Outbred	16/20	80
C57 BL/kw	5/20	25
Exposed to urinary proteins:		
From outbred males	9/10	90
From outbred males (dilution 1:10)	3/11	27
From C57 BL/kw males	6/13	46
From bladder of outbred males	8/11	72
From castrated outbred males	0/10	0
From castrated outbred males + testosterone	7/10	70
From outbred females	0/8	0
Exposed to saline homogenate of the preputial gland	9/10	90
Exposed to serum proteins of outbred males	0/6	0

*Pseudopregnancy.

Test for the Whitten Effect

Thirty mature, nulliparous KE females were divided into three experimental groups. Two females were kept in one cage. They were either isolated from the male, or kept with an Outbred male behind the net. Females separated from males, as well as females treated with urinary proteins, were kept in a male-free room. The solution of urinary proteins was applied locally in the area of the external nares twice a day: at 9 a.m. and 9 p.m. For each application approximately 0.05 ml solution was used. The course of the oestrous cycle was determined by vaginal smears obtained every morning.

Test for Accelerating Puberty

The time of vagina opening and the first oestrus were estimated in Outbred females. The newborn females were kept with the mother in litters consisting of six individuals until the end of the experiment. Mothers with young females were reared in cages either without male, or with the father, or without male but the young females were treated with urinary proteins. The solution was applied on the oro-nasal groove twice a day (at 9 a.m. and 9 p.m.) starting from 21st day of life. Every morning the vagina was checked and after its opening the smear was obtained.

The data were analyzed by using the heterogeneity Ch-square or Student's *t* test (Zar, 1974).

RESULTS

Table 1 summarizes the results obtained after exposure of pregnant females to several sources of pheromones. CBA females kept in the cages with Outbred males blocked pregnancy, and a new oestrus was observed on the second to fourth day after exposure. C57 males did not evoke significant effect on pregnancy.

Pregnancy blocks were observed after treatment with the proteins salted out from the external collected urine of males, or proteins obtained after the bladder puncture. Treatment with proteins obtained from urine of castrated males, or from urine of females was completely ineffective. When castrated males were injected with testosterone their urinary proteins provoked block of pregnancy. Saline homogenate of preputial glands evoked a similar effect as the urinary proteins of mature males. Only urinary proteins of females or castrated males were unable to produce the Bruce effect ($P<0.05$, by heterogeneity Ch-square).

Concentration of proteins in urine was estimated (Fig. 1) and significant differences between experimental groups were found. Mature males produced and excreted with urine more proteins than castrated males or females. After administration of testosterone to castrated males an increased level of proteins was found in urine ($P<0.01$).

In the second set of experiments KE females showed a shortened length of the oestrous cycle after contact with proteins salted out from the urine of outbred males (Table 2). The length of the cycle after treatment with urinary proteins

TABLE 2. The effect of urinary proteins or male presence on the length of oestrous cycle in KE females.

Treatment	Total no. of cycles	Cycle length (days) (mean ± SEM)
Nontreated	25	6.2 ± 0.04
Urinary proteins	27	4.3 ± 0.34[a]
Outbred males	25	4.4 ± 0.52[a]

[a]P<0.001 versus nontreated.

was similar to the period of oestrous cycle of females reared with the Outbred male behind the net. In both cases the oestrous stages occurred significantly more frequently than in nontreated females (P<0.001).

As shown by the results presented in Table 3 urinary proteins of Outbred males influenced sexual maturation of females. In Outbred females the vagina opening was observed earlier after contact with urinary proteins than in females treated with physiological saline, although the differences were not significant (P>0.05). Urinary proteins accelerated the appearance of the first oestrus and in females treated with these proteins typical cornified cells in vaginal smears were found earlier than in the control group (P<0.01).

DISCUSSION

As demonstrated previously by Vandenbergh and coworkers (1975) exposure of immature female mice to urinary proteins of male mice advances the onset of puberty. The results of the present study show that proteins salted out from male urine evoke a typical Bruce effect and the females exposed to urinary proteins

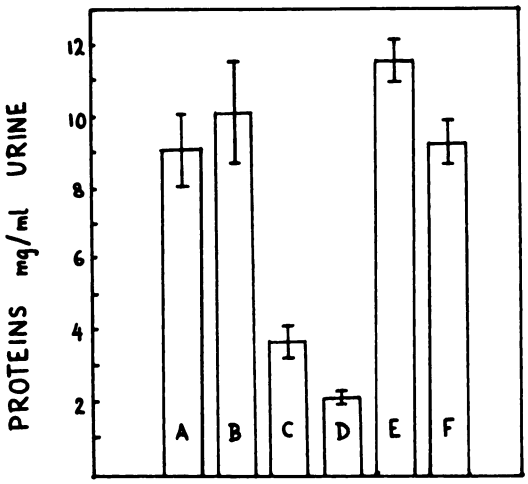

FIG. 1. Protein level in external urine (fraction salted out with ammonium sulphate): A—C57 males, B—Outbred males, C—Outbred females, D—castrated Outbred males, E—castrated, testosterone treated Outbred males, F—bladder urine of Outbred males. Bars represent mean values and the vertical line—standard error of the mean.

blocked pregnancy. The effect was similar to the reaction elicited by the presence of nonstud males in the cage. The frequencies of implantation failure after exposure to C57 BL/kw males and Outbred males correlate with the results obtained after treatment with urinary proteins. The concentration of a biologically active substance in tested solution is important: urinary proteins of Outbred males diluted 1:10 were less effective (Table 1). Following this observation it can be suggested that although C57 BL/kw males produce the pheromone its concentration in urine is much lower in comparison with Outbred males. This is in agreement with previous observations of Hoppe (1975).

TABLE 3. Time of the vagina opening and first oestrus in outbred females exposed to urinary proteins.

Treatment	Vagina opening		First oestrus	
	Body weight	Day of life	Body weight	Day of life
	(mean ± SE)			
Physiol. saline (18)	13.5 ± 0.3	25.0 ± 0.5	20.1 ± 0.4	33.4 ± 1.1[a]
Urinary proteins (24)	12.7 ± 0.2	22.9 ± 0.2	18.8 ± 0.3	29.9 ± 0.4[a]
With father (15)	13.0 ± 0.4	23.3 ± 0.4	19.5 ± 0.2	31.2 ± 0.7

Number of tested females in parentheses.

[a]P<0.01.

The small amounts of proteins obtained by salting out of female urine, or of urine of castrated males, did not show any biological activity. Injection of testosterone to the castrated males stimulated excretion of proteins and restored ability to the inhibition of implantation. A similar correlation between the puberty accelerating pheromone, testosterone treatment and urinary protein level was considered by Lombardi et al. (1976).

The high level of proteins in urine of male mice is connected with the presence of a sex-dependent protein (Finlayson et al., 1965). However, when this protein was separated on a Sephadex G-50 column it did not evoke the Bruce effect. Hence it can be concluded that the sex-dependent protein is not related directly to the pregnancy blocking pheromone. On the other hand it is possible that the Bruce effect is evoked by a substance similar to or identical with a peptide with puberty accelerating activity (Vandenbergh et al., 1976).

The bladder urine was proved to be as effective in shortening the oestrous cycle as external urine (Bronson and Whitten, 1968). The females blocked pregnancy equally after contact with proteins salted out from bladder urine or from external urine. These results support the hypothesis (Bronson, 1971) that the same pheromone is responsible for the Bruce and Whitten effects.

Preputial glands are known to be the source of pheromones: the sex-attractant pheromone (Bronson and Caroom, 1971) and oestrus inductor pheromone (Chipman and Albrecht, 1974) were localized in these glands. The results presented here suggest that the preputial gland contains also the pregnancy blocking pheromone.

It is possible that several organs are involved in the synthesis of pheromones. As suggested by Hoppe (1975) the kidney may play an important role in the production of biologically active substances. Formation of pheromones in kidney or preputial glands may explain pregnancy blocks after exposure to the homogenate of preputial glands or proteins from bladder urine.

The chemical structure of olfactory stimulant in mice is unknown, but this androgen-dependent substance may be excreted with urine in a protein-bound form, as already suggested by the experiments of Vandenbergh and coworkers (1975, 1976).

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