

BRIEF COMMUNICATION

**DISTURBANCE OF OESTROUS CYCLES IN RATS
FOLLOWING ADMINISTRATION OF FREUND'S
COMPLETE ADJUVANT**

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Summary. Female rats injected with Freund's complete adjuvant in the hind limb footpads had abnormal oestrous cycles characterized by prolonged leucocytic phases. This was observed within 2 days in some animals given adjuvant on the first day of di-oestrus. About one-fourth of the animals with abnormal cycles had the same number of continuous L-days as animals made pseudopregnant by electrical stimulation (11 to 16 days). Although electrical stimulation of the cervix induced pseudopregnancy optimally when applied at pro-oestrus, adjuvant induced pseudopregnancy equally well whether injected at pro-oestrus or di-oestrus.

Control experiments, conducted during attempts to determine whether decidual tissue has organ-specific antigens, revealed that Freund's complete adjuvant, injected without any uterine or decidual tissue, modified the rat's normal oestrous cycle.

This report draws attention to the fact that the use of adjuvant may throw new light on certain aspects of reproduction. In addition, this information may be useful to investigators of rat arthritic disease (Stoerk, Bielinski & Budzilovich, 1954; Flax & Waksman, 1963; Pearson & Wood, 1964; Formanek, Rosak & Steffen, 1964).

Three series of experiments were done; data for the first two preliminary series are given in the text, those for the third appear in Table 1. Adult females of the Biodynamics strain (BI, 150 to 218 g) and of the Carworth Farms strain (CFN, 190 to 291 g) were used. They were maintained in air-conditioned quarters with a 10 to 14 hr light cycle. Vaginal smears were examined daily at 08.00 to 09.00 hours for at least two normal oestrous cycles before treatment and continued throughout the experiment. The normal cycle of the BI strain is 4 days: 1 day of pro-oestrus, 1 of oestrus, and 2 of di-oestrus (O, Co, L₁, L₂); that of the CFN strain is 5 days (O, Co₁, Co₂, L₁, L₂). The pseudopregnant rat of both strains has a leucocytic phase of 11 to 16 days.

In the first series of experiments, rats of the BI strain were given saline extracts

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of pseudopregnant uterine or decidual tissue, in adjuvant into the hind limb footpads (0.1 ml in each pad). Two millilitres of the supernatant of tissue extract (1.5 mg wet wt in 10 ml saline) was emulsified with 5 ml adjuvant.

Nine of twelve females given decidual extract in adjuvant and three of six given decidual-free uterine extract in adjuvant showed oestrous cycles markedly disturbed by prolonged L-phases. This disturbance was not seen in seven females given decidual tissue extract in adjuvant into the dorsal skin.

Rats of Series 2 (also of the BI strain) received tissue-free adjuvant, or Freund's incomplete adjuvant (lacking only mycobacteria) diluted with an equal volume of saline during different phases of the oestrous cycle. Of seven

TABLE 1
EFFECT OF FREUND'S COMPLETE ADJUVANT AND MYCOBACTERIA ON THE OESTROUS CYCLE OF RATS

Substance injected*	Injection site	Rat strain†	Injected at oestrus or L-phase‡	No. in group	Pseudo-pregnant response	Decidual cell reaction	Oestrous cycle disturbed§
Complete adjuvant emulsified with saline	Hind pads	BI	O	12	3	2	9
	Hind pads	BI	L ₁	12	4	4	8
	Hind pads	CFN	L ₁	16	3	2	10
	Front pads	CFN	L ₁	16	0	0	4
	Dorsal skin	CFN	L ₁	16	0	0	0
Mycobacterium butyricum in saline	Hind pads	BI	O	12	0	0	3
	Hind pads	BI	L ₁	24	1	0	4
	Hind pads	CFN	L ₁	24	0	0	0
	Front pads	CFN	L ₁	8	0	0	0

* Each rat injected at two sites, 0.1 ml/site; 50 µg of *M. butyricum*/site except for twelve animals of BI strain injected on L₁ that received 200 µg/site.

† Biodynamics departmental (BI) and Carworth Farm, (CFN) strains.

‡ Between 10.00 and 15.00 hours.

§ Observed as extra days of L-phase, cornified phase or both.

females given adjuvant into the hind limb footpads on Day 0, five showed prolonged L-phases, three of these lasting for 11 to 16 days.

Twenty-eight of thirty-seven rats or 75% injected into one footpad with adjuvant on L₁ and into the other pad 8 to 11 days later showed abnormal cycles. In each, the L-phase persisted for 5 or more days; three had 27, 32 and 39 L-days. In some of these animals the prolonged L-phase was observable within 2 days of receipt of adjuvant, in others the effect was delayed for one complete cycle or more, and abnormal cycles were occasionally seen to alternate with normal ones.

The effectiveness of the dead mycobacteria in the adjuvant in inducing the altered cycles was revealed by the observation that only two of eighteen females injected into the hind limb footpads with incomplete adjuvant showed any alteration.

To test luteal function in some animals during periods of prolonged L-phase, decidualization of the uterus was induced. This was accomplished on L₄ by traumatizing the uterine horns with an intraluminally inserted needle. Deciduo-mata were looked for 5 days later.

It is of interest that among fifteen adjuvant-treated animals with cycles

disturbed by L-periods lasting longer than 10 days, eleven had decidual reactions. In none of six animals in which the L-phases were only of 5 to 10 days duration were deciduomata found.

In experiments of Series 3 the investigation was concerned with the effect of adjuvant with regard to site and time of injection, as well as to rat strain differences and to the use of mycobacteria alone. Mycobacteria were injected as saline suspensions (50 μg in 0.1 ml except where otherwise described, see Table 1).

In confirmation of the preliminary experiments, the majority of rats (nine of twelve) of the Biodynamics strain injected with adjuvant into footpads on the day of oestrus showed cycle abnormalities; three had L-phases characteristic of pseudopregnancy and in two of these decidual cell reactions were observed. Of twelve injected on L₁, eight showed cycle alterations; four had L-periods lasting for 11 to 16 days and deciduomata developed in all four. The results seen among sixteen CFN strain rats injected on L₁ were hardly different; ten had altered cycles, three were of pseudopregnancy duration and of these two showed decidual responses.

When deposited in sites other than footpads, the effect of adjuvant was much less. Thus, only four of sixteen CFN animals receiving adjuvant into front pads showed disturbed cycles and all sixteen CFN rats injected with adjuvant into the dorsal skin remained normal.

Experiments of Series 2 had revealed that adjuvant without mycobacteria had little effect on the cycles. In Series 3, mycobacteria alone, i.e. without mineral oil and emulsifier, also had little effect. Only three of twelve BI rats receiving the bacteria into hind pads on Day 0 showed interference with their cycles. Of twenty-four injected with mycobacteria at L-phase, four had erratic cycles and only one of these was pseudopregnant. (Twelve animals of this group of twenty-four received 200 μg of *M. butyricum*/footpad, but the incidence of disturbance of the cycle was not increased.) None of CFN rats given 50 μg of *M. butyricum*/hind footpad and none of eight given the same quantity/front footpad at L-phase showed cycle abnormalities. Thus, adjuvant is superior to either incomplete adjuvant or the mycobacteria alone in producing alterations in the oestrous cycle and the most effective site is the hind footpad.

The most marked change brought about by adjuvant is a persistence of leucocytes in the vaginal smear and less frequently there is prolongation of the cornified phase. Among animals with disturbed cycles 25% had L-phases equal to those of rats in pseudopregnancy. As the physiological state of pseudopregnancy can be induced by a variety of stimuli (Lataste, 1887; Long & Evans 1922; Shelesnyak, 1931; Swingle, Seay, Perlmutter, Collins, Barlow & Fedor, 1951; and Psychoyos, 1959) it is reasonable to assume that the pseudopregnancy induced by adjuvant is also stress-induced. However, the production of cycle irregularities in rats that were not pseudopregnant still requires explanation.

An immunological mechanism might provide an alternate explanation for the prolonged appearance of leucocytes in vaginal smears. This is suggested because leucocytes have been found in the vagina during the normal oestrous cycles of germ-free rats (J. R. Battisto and S. Scher, unpublished results).

If the leucocytes are present in response to altered autologous tissue antigens and not to bacterial flora, then adjuvant would serve to heighten the immunological activity of the leucocytes and prolong their appearance in vaginal contents. Because the 2- or 4-day interval within which the cycle alterations can be observed is too short an interval for full development of hypersensitivity *de novo*, the sensitivity for autologous antigens would have to be present in some animals before receipt of adjuvant.

Waksman & Wennerstein (1963) and Pearson & Wood (1964) have provided evidence that induction of arthritis and of other lesions in rats by adjuvant is brought about by initiating a delayed-type hypersensitivity. Though the responsible antigen of the rat was not identified in their studies, the earliest symptoms they observed followed a full immunological period (10 to 14 days).

The optimal time during an oestrous cycle for inducing pseudopregnancy by adjuvant was found to be different from the best time for inducing pseudopregnancy by other means. For instance, electrical stimulation of the cervix induces pseudopregnancy best when applied at pro-oestrus whereas adjuvant was effective whether given at pro-oestrus or di-oestrus.

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