A dioestrous increase in thymocyte proliferation during the oestrous cycle

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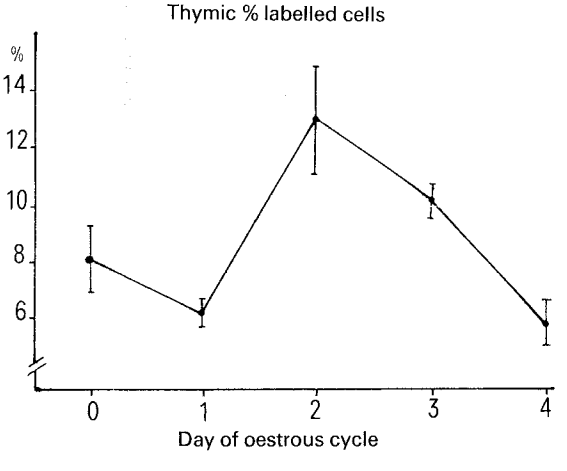
Summary. Coitus, which precedes internal fertilisation, is a unique physiological event which allows motile allogeneic spermatozoa to enter the female host and invade her tissues. The cyclic cellular proliferation observed in the thymus of the female rat may be an important preparation of her immune system for this event.
Key words. Oestrous cycle; thymus; thymocyte proliferation; rat.

The immunological component of intrauterine gestation is initiated by the first coital act. Spermatozoa are antigenic[1] and are capable of eliciting both humoral[2] and cell-mediated[3] immune responses. Indeed the first coital act generates cellular changes in the uterine regional lymph nodes consistent with an immune response[4] and such changes are again evident after implantation[5]. The various mechanisms proposed to explain the survival of the foetal allograft include a non-specific reduction of maternal immunity during gestation[6,7] mediated by blocking[8] or enhancing[9] factors or by alteration of hormonal status[10,11]. Significant changes in steroid hormone secretion do occur in association with pregnancy and these hormones are known to affect not only the female reproductive tract but also lymphocytes and lymphoid tissues[12,13]. This examination of the lymphoid tissues of an inbred rat strain during the oestrous cycle demonstrated a cyclic proliferative response in the thymus which may be an important preparation of the female's immune system for coitus.

Materials and methods. Thirty-two AO ($RT1^u$/AgB2) sexually mature virgin female rats were used in this investigation. All animals were bred from departmental stock maintained in the Manchester University Medical School Animal Unit on a standard diet and with a 12-h light cycle. At the time of sacrifice the rats had a body weight of 150 g or more and were between 13 and 15 weeks of age in order to minimise age related changes in the lymphoid tissues. In the sexually mature rat the various stages of the oestrous cycle can be determined by low-power (× 40) microscopic observation of the unstained smear taken daily with a wire loop from the lower vagina[14]. Although the rat cycle is subject to individual variations, the oestrous phase recurs every 4–5 days in the absence of mating[15]. In the present study vaginal smears were prepared from the rats at 10.00 h each day. The oestrous phase animals, designated group 0, were sacrificed at approximately 11.00 h on the day oestrus was identified in the vaginal smear. The remaining groups, designated 1–4, were sacrificed at 24-h intervals after the vaginal smear had confirmed the appropriate phase of the cycle. All groups contained at least 6 animals. On the day of sacrifice each animal was anaesthetised with ether and subsequently given an i.p. injection of 12 mg of veterinary nembutal ('Sagatal': May & Baker Ltd., Dagenham). The weight of the animal was recorded and after exsanguination the uterus, thymus, uterine regional lymph nodes and popliteal lymph nodes were removed and cleaned. The uterus was opened along its length and weighed, while the lymphoid tissues were weighed and placed in 199 tissue culture medium (Wellcome Reagents Ltd., Beckenham) adjusted to pH 7.2. A cell suspension from each of the lymphoid tissues was prepared by repeated squashing. Each cell suspension was subsequently filtered through a stainless steel mesh (144 holes/cm^2) and made up to a final volume of 10 ml for the thymus and 5 ml for each lymph node group. 1 ml was taken from each of these final volumes and used for the cell counts, done with a Coulter ZB counting instrument.

After removal of aliquots for the cell counts 10 µl of [6-^{3}H] thymidine (Amersham International plc, Aylesbury) at an activity of 100 µCi/ml were added for each 1 ml of cell suspension. After mixing, the suspensions were incubated in a water bath at 37 °C for 1 h with occasional shaking. The suspensions were then centrifuged at 2000 rpm (700 g) for 10 min and the resultant pellets resuspended in phosphate-buffered saline at a concentration of 5×10^6 cells/ml. Previous work has shown that at this dilution the cells require no further washing. This cell concentration is also optimal for preparing smears in the Shandon-Elliot cytocentrifuge. After radiolabelling six smears were prepared from each lymphocyte suspension. The smears were prepared on slides previously coated with gelatin/chrome alum[16] to prevent peeling of the nuclear emulsion used in the autoradiography. The smears were dried in air and fixed in methanol, dipped in diluted Ilford K2 nuclear emulsion (Ilford Ltd., Knutsford) and stored at 4 °C in the dark for three weeks. After this period, when a trial slide showed clear labelling, the remaining slides from that tissue were developed in Kodak D19 developer, fixed in acid-hardening fixer, stained with haematoxylin and eosin and mounted. A non-random strip counting method was used to assay, at × 400 magnification, the number of labelled and unlabelled cells and thus the percentage of cells labelled. Only cells in the DNA-generative 'S' phase of mitosis incorporate thymidine[17] and, assuming a random distribution of mitotic phases, a constant proportion of the actively proliferating cells, approaching 50%[18] will be in the 'S' phase. The consistency and low standard errors of the results obtained from each group in this study support this assumption. Consequently, it seemed reasonable to use the figures for percentage labelling as a measure of proliferative activity; not an absolute measure, but an accurate reflection of the relative differences between groups.



Mean values and standard errors for the intrathymic levels of cellular proliferation, expressed as percentages, in 13–15-week-old AO virgin rats during their oestrous cycle.

		Day of oestrous cycle				
		0	1	2	3	4
Body weight	g	173.33	179.33	196.00	184.67	173.67
		+/− 3.19	6.07	2.90	6.20	4.88
Uterine weight	mg	325.20	265.08	275.54	417.33	398.80
		+/− 27.15	4.46	11.12	12.96	14.28
Thymus weight	mg	292.50	252.18	282.27	268.00	227.17
		+/− 14.14	18.93	18.70	11.81	18.04
Cell content	$\times 10^6$	893.27	779.17	759.69	785.05	682.57
		+/− 53.50	89.23	75.14	64.01	70.72
Cell density	$\times 10^6$/100 mg tissue	308.30	304.32	269.66	291.93	299.02
		+/− 22.59	13.13	18.59	17.69	14.78
Labelled cells	%	8.23	6.27	13.17	10.50	6.24
		+/− 1.14	0.43	1.84	0.56	0.78
URLN weight	mg	22.67	34.57	30.79	26.05	30.48
		+/− 3.63	2.49	5.98	2.99	1.52
Cell content	$\times 10^6$	8.99	14.21	12.71	10.18	7.46
		+/− 2.72	1.87	2.49	2.02	0.68
Cell density	$\times 10^6$/100 mg tissue	35.75	40.53	41.57	37.40	24.53
		+/− 6.15	3.19	4.89	3.45	2.11
Labelled cells	%	1.47	0.93	2.06	1.01	0.88
		+/− 0.29	0.12	0.59	0.22	0.16
PLN weight	mg	7.75	10.73	9.30	9.27	8.93
		+/− 0.63	0.67	0.47	0.75	0.43
Cell content	$\times 10^6$	3.49	4.33	3.18	3.44	2.86
		+/− 0.59	0.48	0.46	0.19	0.39
Cell density	$\times 10^6$/100 mg tissue	44.63	40.39	34.82	37.84	31.59
		+/− 6.76	3.45	5.25	2.30	3.43
Labelled cells	%	2.20	1.20	2.36	1.34	1.49
		+/− 0.64	0.31	0.91	0.38	0.53

Means of results +/− SE; URLN, Uterine regional lymph modes; PLN, Popliteal lymph nodes.

Results. Each rat used in this study yielded figures for body weight and tissue weights, both absolute and relative to body weight, for the uterus, thymus, uterine regional lymph nodes and popliteal lymph nodes. Each lymphoid tissue provided figures for the total lymphocyte content and density, and the percentage of labelled cells. Statistical analysis of these data was by analysis of variance[19]. In presenting the results differences between groups were considered to be significant if p was 0.05 or less. These results do not include the relative tissue weights since these values and their statistical analyses presented the same patterns of response as did the absolute tissue weights. The results are shown in the table in which the numerical values of the group means are given with their standard errors. The body weight on day 2 was significantly greater than those recorded during the oestrous phase (day 0) and on days 1 and 4. The weight of the uterus fell significantly below oestrous weight on days 1 and 2 and rose significantly above these values on days 3 and 4. No significant differences were seen in any of the observations made from the lymph nodes. The only significant differences observed in the results obtained from the thymus concerned the percentages of labelled cells: the value on day 2 was significantly greater than on days 0, 1 and 4, while that on day 3 was significantly greater than those on days 1 and 4.

Discussion. In general terms body weight is the sum of body fat and lean body mass. Lean body mass, however, is itself a composite concept including as it does total body water, skeletal muscle mass, bone and viscera. Observations of body weight which include no measure of the individual components are therefore of limited value. Nevertheless, a number of factors are known to influence body weight and the most important of these are endocrine and genetic factors[20] as well as age and diet. Since the animals used in this investigation were from our own long-established inbred strain, fed a standard diet in identical environmental circumstances and were all between 13 and 15 weeks of age, the most likely cause of the transient mid-cycle weight gain is hormonal. The cyclic fall in uterine weight between sequential oestrous phases is hormonally dependent[21] and has been observed previously[22,23].

Although no significant changes occurred in thymic weight, cell content or cell density during the oestrous cycle, thymocyte proliferation increased significantly as shown in the figure. In the absence of any significant change in the other thymic observations this 'burst' of thymocyte proliferation during dioestrus was probably associated with increased T cell output. The mid-cycle increase in thymocyte proliferation is almost certainly caused by ovarian hormones. Unless there is a significant delay between increased hormone secretion and increased thymocyte proliferation this increased mitotic activity within the thymus must be oestrogen dependent since oestrogen levels begin to rise during early dioestrus while progesterone levels remain very low until midway through prooestrus[24]. Oestrogen stimulates endometrial mitotic activity[25] and the presence of thymic oestrogen receptors[26] suggests a comparable intrathymic role. Since it has recently been shown[27] that it is the thymic epithelium which binds oestrogen at levels comparable with uterine tissue, perhaps its effect within the thymus is mediated by thymic hormones.

The thymus generates T cells independently of the ebb and flow of antigenic stimuli[28] and they recirculate via blood and lymph through the secondary lymphoid tissues. The cumulative mass of secondary lymphoid tissue and its widespread distribution in lymph nodes, spleen, tonsil, appendix and mucous membranes suggest that a dioestrous increase in thymocyte proliferation is unlikely to be associated with any significant change in any one group of lymph nodes, and none was observed in either the uterine regional or popliteal lymph nodes. The steady state of intranodal proliferation during the oestrous cycle was not unexpected since increased lymphocyte proliferation in secondary lymphoid tissues occurs directly or indirectly as the result of antigenic challenge[28]. Since mating occurs only in the oestrous phase of the cycle the mid-cycle increase in thymocyte proliferation may be interpreted as a preparation for a unique physiological event which allows motile allogeneic spermatozoa to enter the female host and invade her tissues[29]. It would therefore not seem unreasonable to propose that the ovarian hormones which prepare the female reproductive tract for

pregnancy also prepare the host for the allogeneic challenge of mating. Such a relationship between the ovary and thymus would add further support to the concept of a thymic-ovarian interaction[30].

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Lunar soil and Zagami meteorite inhibit biosynthesis of itoic acid, a siderophore

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Summary. Lunar soil, Zagami meteorite, and Gorda Ridge ocean basalt inhibited the synthesis of iotic acid and coproporphyrin III in iron-deficient *Bacillus subtilis* cultures. Synthetic ilmenite did not. The results indicated that the three natural samples served as sources of iron for the organism, but the ilmenite did not.
Key words. Siderophore; *Bacillus subtilis;* itoic acid; porphyrin; lunar soil; meteorite; ilmenite.

Since Fe(III) is extremely insoluble in water, almost all microorganisms, especially under iron-deficient growth conditions, excrete iron-binding compounds known as siderophores that chelate iron, and transport the bound iron into the cells[1,2]. *Bacillus subtilis,* for instance, when grown in an iron-deficient growth medium[3] (FeDGM), excretes 2,3-dihydroxybenzoylglycine (itoic acid), the first chemically identified siderophore[4], and coproporphyrin III[5] into the culture medium. Excretion of these two compounds is extremely sensitive to iron concentration in the growth medium, and stops as iron concentration rises[4-7]. The present study was undertaken to test whether extraterrestrial minerals and terrestrial minerals of a similar elemental composition serve as sources of iron for the organism and inhibit thereby the excretion of these compounds.

Materials and methods. Samples of lunar soil (10084,151), Zagami meteorite, Gorda Ridge ocean basalt, Quebec ilmenite, and synthetic ilmenite were provided by the Solar System Exploration Division of the Johnson Space Center, Houston, Texas. These mineral samples (10 mg) were autoclaved in 250-ml flasks containing 50 ml FeDGM. The flasks were inoculated with five drops of a freshly grown *Bacillus subtilis* (ATCC 15933) in FeDGM, and incubated for three days at 30 °C in a gyratory shaker at about 50 cycles/min. The cells were removed by centrifugation at 10,000 g for 10 min, and the absorbance of each supernatant, diluted five-fold, was read at 320 nm and 405 nm for the measurement of itoic acid and corproporphyrin III, respectively[4,8]. All flasks containing FeDGM were previously rinsed thoroughly with 6 N HCl in order to remove any possible iron contamination on the inner wall of the flasks.

Results and discussion. A typical spectrum of the supernatant of the 3-day-old *Bacillus subtilis* culture grown in FeDGM showed two peaks which were not observed with the addition of iron (fig.). The peak near 320 nm was indicative of itoic acid and another at 405 nm of coproporphyrin III. The lunar

Effect of extraterrestrial and terrestrial minerals on biosynthesis of itoic acid and coproporphyrin III by *Bacillus subtilis*. Each number represents absorbance of the 3-day-old culture supernatants at the approximate absorption maxima of itoic acid (320 nm) and coproporphyrin III (405 nm). The numbers without parentheses are the mean absorbance of four determinations, and those in parentheses the ranges. The addition of the minerals was at 200 mg/l of FeDGM. The minerals were virtually insoluble, and stayed as solids at the bottom of the culture flasks.

Minerals added to FeDGM	Absorbance of 3-day-old culture supernatants at 320 nm	405 nm
None	5.00 (5.32 –4.70)	1.07 (1.23 –0.925)
Lunar soil	0.661 (0.690–0.625)	0.216 (0.225–0.210)
Zagami meteorite	0.584 (0.615–0.555)	0.196 (0.205–0.190)
Gorda Ridge basalt	0.663 (0.680–0.655)	0.215 (0.225–0.200)
Quebec ilmenite	1.14 (1.24 –1.05)	0.615 (0.830–0.500)
Synthetic ilmenite	3.17 (3.53 –2.71)	1.58 (2.09 –1.13)