synthesis during conjugation in other ciliates is only available from the detailed studies by Sapra and Ammermann with Stylonychia[12,13]. These investigators measured the incorporation of RNA precursors into RNA of conjugating Stylonychia as well as RNA inhibition by Actinomycin D.

They concluded that: a) RNA synthesis during the first 5 h after pair formation was substantially lower in comparison to their vegetative state. b) 1 h later, i.e. between 5–6 h since pairing, they noticed a marked increase in RNA synthesis. c) 6 h since pairing and thereafter, little, if any, RNA synthesis could be observed. In addition the inhibition of RNA synthesis by Actinomycin D during 5–6 h since initial pairing, resulted in the blockage of further developmental processes in conjugation.

In the ciliates Stylonychia and Tetrahymena, a short duration of about 1 h of critical mRNA synthesis seems to govern the continuation of sexual reproduction. However this critical mRNA synthesis in both ciliates occurs at different periods of the conjugation process. In Tetrahymena such mRNA synthesis occurs during costimulation, i.e. before pairing; in Stylonychia it occurs after about 5 h postpairing. However these different timings of long acting mRNAs do not cause a principle difference in their mode of action.

In Stynlonychia as well as Tetrahymena, it seems that the 'sequence of events dependent upon presynthesized messengers include, in a strict chronological order, meiosis of the micronucleus, formation exchange, and fertilization of the gametic nuclei, and differentiation of the new macronucleus from the zygote'[13].

As previously mentioned, little has been published on RNA synthesis in other conjugating ciliates. Rao[14] and Berger[15] concluded that RNA synthesis occurs continuously during the course of macronuclear development in conjugating Euplotes and Paramecium, respectively. However Nobili[16] presumed that 'synthesis of probably specific RNA occurs any time sexually mature paramecia become reactive'. The fact that Tetrahymena cells appear to overcome the inhibitory effects of cordycepin after several hours following experimental treatment (figure 3) is mirrored by their reaction to certain antimitotic drugs. Such a phenomenon was shown by Wunderlich and Peyk[17] while testing the effect of colchicine and colcemid on vegetative division of Tetrahymena, and by Roberts and Orias[18] by testing protein synthesis inhibitors.

The systems involved in overcoming such inhibitory effects are as yet little understood. Perhaps Tetrahymena possesses an enzyme system capable of decomposition of these drugs, including cordycepin.

12 G. R. Sapra and D. Ammermann, Exp. Cell Res. *78*, 168 (1973).
13 G. R. Sapra and D. Ammermann, Dev. Biol. *36*, 105 (1974).
14 M. V. N. Rao, Exp. Cell Res. *49*, 411 (1968).
15 J. D. Berger, Ph. D. thesis, Univ. of Indiana (1969).
16 R. Nobili, J. Protozool. *10* (suppl.), 24 (1963).
17 F. Wunderlich and D. Peyk, Exp. Cell Res. *57*, 142 (1969).
18 C. T. Roberts and E. Orias, J. Cell Biol. *62*, 707 (1974).

Leucocyte invasion of the vaginal epithelium in the absence of bacteria in mice

T. R. Koiter[1] and P. van der Schoot[2]

Department of Endocrinology, Growth and Reproduction, Medical Faculty, Erasmus University, P. O. Box 1738, Rotterdam (The Netherlands), 15 November 1976

Summary. Influx of leucocytes in the vagina at metoestrus occurs in germfree mice and also in sterile isotransplants of vaginal tissue in conventional mice. In contrast to the situation in rats, bacteria thus do not seem to be required for the production of postovulatory leucotactic stimuli in the vagina.

Influx of leucocytes into the vaginal epithelium and lumen is a wellknown characteristic of the early postovulatory period (metoestrus) in small rodents. In rats, this influx appears to depend on the presence of bacteria in the vaginal lumen[3,4]. No data are available on the microbial-leucocytic relationship at metoestrus in the other rodents. However, the occurrence of a leucocytic vaginal exudate in germfree mice has been reported after repeated injections of progesterone[5]. This suggests that, in mice, leucocytic influx of the vagina at metoestrus does not depend on the presence of microorganisms in the vagina and that, thus, species differences exist in the mechanism underlying influx of leucocytes into the vaginal epithelium after ovulation. The data on mice, however, were obtained exclusively after injection of progesterone, and not during the normal ovarian cycle. We therefore examined the possible relationship between bacteria and leucocytes with the same methods as used in rats[3,4].

Materials and methods. Experiment 1. The numbers of aerobic and facultative anaerobic bacteria were determined in Swiss (9 animals) and ND_2/Rij (8 animals) cyclic mice. Vaginas with the cervix still attached were removed and placed in bottles with 10 ml saline and 3 g glassbeads (⌀ 3 mm) which were sterilized before use. The bacteria in the vaginas were suspended by shaking the bottles on a whirlmixer for 2 min. From the suspensions, 1:10 dilution series were made in nutrient broth, which were incubated for 48 h at 37 °C. The stages of the ovarian cycle, at which the vaginas were obtained, were approximated by examining the contents of the Fallopian tubes microscopically (fresh oocytes with surrounding cumulus-cells: day of ovulation; old oocytes without surrounding cumulus-cells: day after ovulation; no oocytes but clearly visible corpora lutea in the ovaries: all other stages of the ovarian cycle).

1 Present address: Department of Experimental Endocrinology, University of Groningen, Bloemsingel 1, Groningen, The Netherlands.

2 Acknowledgments. Data with germfree mice could be obtained through the generous cooperation of Prof. Dr F. Wensinck and Dr M. P. Hazenberg (Dept. Medical Microbiology, Erasmus University), C. Lekkerkerker and J. ten Veen (TNO, Zeist, The Netherlands). The skillfull cooperation of Miss P. D. M. van der Vaart and Mrs A. Verschoor-Burggraaf is gratefully acknowledged.

3 P. van der Schoot, J. Reprod. Fert. *45*, 61 (1975).
4 T. R. Koiter, M. P. Hazenberg and P. van der Schoot, J. expl. Zool., (in press).
5 D. L. Beaver, Am. J. Path. *37*, 769 (1960).

Experiment 2. A comparison was made of the vaginal smear cycles of conventional and germfree mice from 3 strains: Swiss (8 germfree mice; 10 conventional), ND_2/Rij (3 germfree mice; 9 conventional), and DBA (5 germfree; 17 conventional). Smears were taken daily over 2 subsequent weeks (5 times a week); autopsy was done on the day of the last smear between 11.00 and 16.00 h to study ovarian and vaginal histology. Germfree animals were tested for germfree condition at autopsy by culturing faeces on blood agar plates. For histological study, tissues were fixed in Bouin's solution and embedded in paraffin wax; 10 μm sections were stained with haematoxylin and eosin.

Experiment 3. Vaginal graft histology and in situ vaginal histology were compared in 9 C_3H-mice. For grafting, pieces of vaginal tissue (2–4 mm^2; 2 pieces per recipient) were obtained from isogeneic prepuberal donors which still had a closed, sterile vagina; the grafts were placed under the left renal capsule and left there for 3 weeks. After 3 weeks vaginal graft histology, in situ vaginal histology, and ovarian histology were analyzed.

Results. Experiment 1. There were 10^5–10^7 bacteria in the vagina of 4 out of 5 mice examined on the day of ovulation; in the 5th animal, no bacteria were found. In 8 out of 12 mice examined during the dioestrous period, there were 10^2 or less bacteria; in the remaining 4, there were 10^3 (1 mouse), 10^4 (1 mouse) and 10^5 (2 mice). Gram-stained vaginal smears revealed the presence of many bacteria on days with vaginal cornification: the bacteria often adhered in large numbers to cornified cells.

Experiment 2. There were no differences between the vaginal smear patterns obtained from the 3 strains of germfree mice. All mice showed cyclic changes in vaginal smear morphology characterized by the regular occurrence of leucocytes. All animals showed at least once a sequence of 2 successive days with cornified vaginal smears followed by a day with a leucocytic smear and no cornified cells. This sequence represents in conventional animals the sequence of oestrous, metoestrous-1, and metoestrous-2 smears[6]. Metoestrous-2 smears from germfree mice contained less leucocytes than those from conventional mice. Ovarian histology of germfree animals revealed that 5 animals had been killed in the afternoon of the day of ovulation (oestrus), 3 on the first day after ovulation (metoestrus-1) and 8 during the dioestrous period. No differences in vaginal histology were noticed

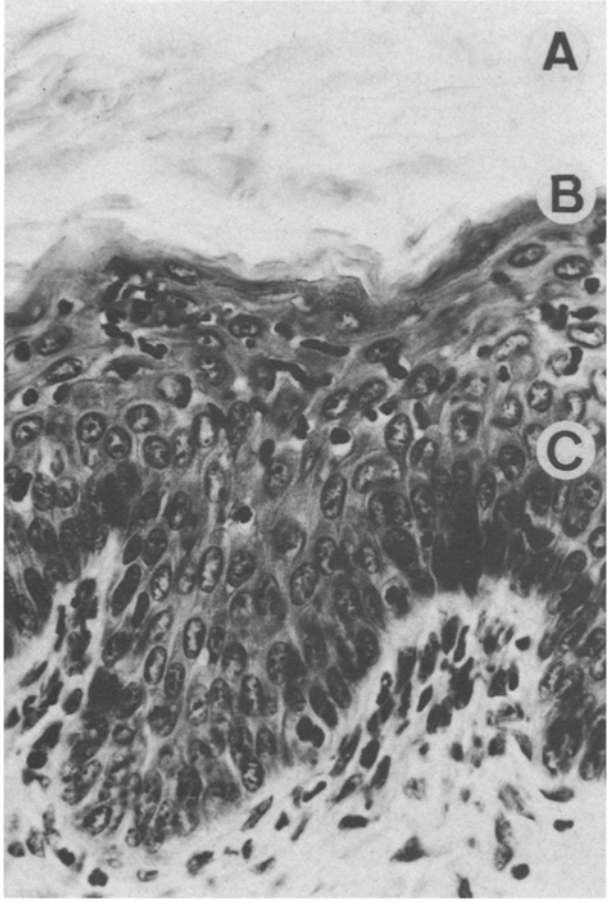

Fig. 1. Vaginal epithelial histology in germfree mice on the day after ovulation (metoestrus-1; Allen[6]). The epithelial surface consists of continuous layers of densely packed cornified cells (B); such cells are also present throughout the vaginal lumen (A) and are obtained in vaginal smears at that stage of the cycle. Leucocytes have penetrated the vaginal epithelium and are present in the layers of nucleated epithelial cells just below the cornified cells (C). This picture is not different from that obtained in conventional mice[6]. ×350.

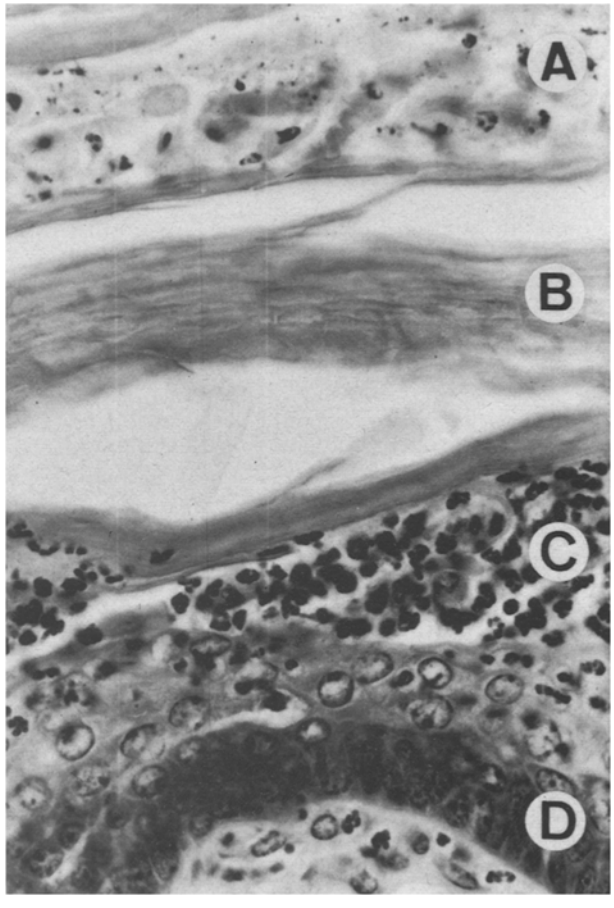

Fig. 2. Vaginal graft histology at metoestrus-2. The vaginal graft had been removed on the 2nd day after ovulation; the vagina in situ showed extensive leucocyte invasion. During the period preceding autopsy, there had been the shedding of nucleated cells (A) from the vaginal graft epithelium (D) followed by the shedding of cornified cells (B) and again nucleated cells (C). The last cells are surrounded by large numbers of leucocytes which can also be seen penetrating the vaginal epithelium. The small black dots present in the 'old' layers of nucleated cells (A) probably represent the remnants of disintegrated leucocytes. In rats, leucocytes are never observed in grafted vaginal epithelium. ×370.

between germfree and conventional animals (figure 1). Experiment 3. Vaginal graft histology revealed cyclic changes in the appearance of cells which had been shed from the vaginal epithelial surface at various stages of the ovarian cycle. Massive penetration of leucocytes was observed in 4 grafts of 2 animals autopsied at metoestrus-2 (figure 2). Early signs of leucocytic influx were visible in the epithelium of two vaginal grafts in 1 animal sacrified at metoestrus-1. Signs of leucocytic remnants were present in the shedded cell layers of all vaginal transplants obtained during other stages of the ovarian cycle.

Discussion. The present experiments confirm cyclic changes in bacterial numbers during the ovarian cycle in the vagina of female mice[7-9]. In accordance with data in rats, bacterial numbers are low during the dioestrous period, and, generally, high when the vaginal epithelium shows maximal development around the time of ovulation. It was not clear whether the bacteria had already disappeared at metoestrus-1 when leucocytes were present in the upper layers of the vaginal epithelium but not yet in the vaginal lumen, or whether disappearance of bacteria coincided with the appearance of leucocytes in the vagina lumen at metoestrus-2.

Daily vaginal smears from germfree mice revealed cyclic changes in the vaginal smear contents. Leucocytic smears occurred regularly in all 3 strains of mice. In the germfree animals, leucocytes appeared in the vaginal epithelium at the same stage of the cycle (metoestrus-1; figure 1) in the upper epithelial layers as in conventional animals[6,10]; the crowding of leucocytes in the epithelium at that time did not seem to be different from that in conventional animals. Leucocytes were present in large numbers in the vaginal epithelium, lumen, and smear at metoestrus-2. It thus seems clear that influx of leucocytes in the postovulatory period in mice occurs in the absence of leucotactic stimuli from bacterial origin. It is difficult to conclude from the lower number of leucocytes in vaginal smears from germfree animals that the leucocytic response in germfree mice is quantitatively different from that in conventional mice. The technique of making smears from germfree animals in their special homecages is different from that of making smears from conventional animals in open cages.

The morphology of sterile transplanted vaginal tissue in cyclic mice revealed large numbers of leucocytes penetrating into the epithelium at metoestrus-1 and metoestrus-2 but not during the period with epithelial keratinization. Such leucocytic response was never seen in similar studies with rats[3]. This finding confirms the above conclusion of leucocytic influx into the vaginal epithelium in the absence of leucotactic stimuli of bacterial origin. It is concluded from the occurrence of leucocytes in the transplants that leucotactic stimuli arise in the epithelium itself. This conclusion is further supported by comparison of the histology of leucocytic influx in rats and mice. In rats leucocytes only penetrate the vaginal epithelium after it has lost its covering layers of cornified cells about 12 h after ovulation[4]. In mice, however, leucocytes start to penetrate in large numbers into the vaginal epithelium when still covered by a thick densely packed layer of cornified cells[6,10] (figure 1). Since cornified cells by themselves are not leucotactic[11] and, indeed, aggregation of leucocytes onto cornified cells is never observed, one would assume that the layers of disintegrating nucleated cells beneath the cornified cells start the formation of leucotactic material in the period after ovulation. It is wellknown that dying cells can produce leucotactic substances during autolysis[12].

6 E. Allen, Am. J. Anat. *30*, 297 (1922).
7 N. Takasugi and H. A. Bern, Proc. Soc. exp. Biol. Med. *109*, 622 (1962).
8 G. J. Fruhman, Proc. Soc. exp. Biol. Med. *122*, 493 (1966).
9 N. Lavenda, I. Y. Mahmoud and J. D. Leith, J. Reprod. Fert. *18*, 171 (1969).
10 A. S. Bingel and N. B. Schwartz, J. Reprod. Fert. *19*, 215 (1969).
11 H. Burrows, J. Path. Bact. *41*, 43 (1935).
12 M. Bessis, Antibiotics Chemother. *19*, 369 (1974).

On the importance of thiols and disulphides and the antiviral action of dichloropyrimidines[1]

R. Pompei, M. A. Marcialis, O. Flore, M. E. Marongiu and A. Garzia

Institute of Microbiology II, University of Cagliari, via G. T. Porcell 12, Cagliari (Italia), 21 February 1977

Summary. Free SH or SS groups of infected cells are not involved in the antiviral action of dichloropyrimidines.

Dichloropyrimidines inhibit the growth of Polio 1, Vaccinia and Herpes simplex virus in cell cultures[2,3]. Considering that the inhibitory effect is antagonized by the combined addition of cysteine (or cystine) plus glutamine to the culture medium[4] and, in addition, that several thiols and disulphides enhance virus growth[5], the hypothesis might be advanced according to which dichloropyrimidines react with either SH or SS groups needed for virus growth, thus impairing production of infectious particles. To shed some light on that question, it has been deemed useful to establish whether sulphidryl reagents, known to react strongly with thiols, have an antiviral effect or potentiate that of dichloropyrimidines and, on the other hand, to determine if and which thiols and disulphides antagonize dichloropyrimidine inhibition of virus growth.

Material and methods. Cysteine and cystine, cysteamine and cystamine, mercaptopropionylglycine, glutathione SH and SS, N-ethylmaleimide, parachloromercuribenzoic acid (CMB), and $CuCl_2$ were furnished by Fluka, Buchs SG, Switzerland, as well as 2-amino-4,6-dichloropyrimidine (ADCP), which was the only dichloropyrimidine tested. Ethacrynic acid was obtained as aqueous extract from tablets of Reomax (Bioindustria, Italy). Virus strains (kindly provided by the National Institutes of Health, Bethesda, Md., USA) were Polio 1, Vesicular stomatitis, Encephalomyocarditis, Newcastle disease, Vaccinia and Herpes simplex 1 virus. Experiments were

1 This work has been supported by a grant of the Consiglio Nazionale delle Ricerche, Roma (Italy).
2 P. La Colla, M. A. Marcialis, O. Flore, A. Firinu, A. Garzia and B. Loddo, Chemotherapy *6*, 295 (1976).
3 M. A. Marcialis, O. Flore, A. Firinu, P. La Colla, A. Garzia and B. Loddo, Experientia *30*, 1272 (1974).
4 M. A. Marcialis, M. L. Schivo, A. Atzeni, A. Garzia and B. Loddo, Experientia *29*, 1559 (1973).
5 M. A. Marcialis, O. Flore, M. E. Marongiu, R. Pompei, P. La Colla and B. Loddo, Experientia, submitted for publication.