Lazare, R., Urbanska, A., Life Sciences, 1963, v3, 193.
14. Muscholl, E., Maitre, L., Experientia, 1963, v19, 658.
15. Goldberg, L. I., DaCosta, F. M., Ozaki, M., Nature, 1960, v188, 502.
16. Stone, C. A., Ross, C. A., Wenger, H. C., Ludden, C. T., Blessing, J. A., Totaro, J. A., Porter, C. A., J. Pharmacol. Exp. Therap., 1962, v136, 80.

Received December 2, 1964. P.S.E.B.M., 1965, v118.

Mouse Leukocytes in Culture.* (30026)

H. G. WILLARD, I. B. H. HOPPE† AND P. NETTESHEIM‡ (Introduced by L. H. Smith)
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

In vitro culture of most mammalian cells has long been hampered by an inability to provide the proper conditions for maintenance of normal and functional activity. Recently, the technique developed by Moorhead *et al*(1) has allowed the continual maintenance of a sufficiently high metabolic state in human leukocytes so that mitosis, a normal cellular activity, could occur.

The culture of mouse leukocytes is especially appealing, since it has been shown(2, 3) that peripheral blood contains stem cells capable of promoting recovery of lethally irradiated rodents. Successful *in vitro* cultivation of these cells might lead to development of different stem cell lines devoted to formation of different, specific types of blood cells. Also, karyotype analysis of mouse leukocyte chromosomes without sacrifice of the donor would be of great advantage to mouse geneticists.

Leukocyte culture has been carried out with some success in a variety of animals, including the monkey(4); hamster(5); ox (6); snake(7); chicken(8); and rat(9). Nichols and Levan(10) have successfully applied this method to various laboratory mammals, including rat, guinea pig, rabbit, and mouse. Although human, rat, and guinea pig leukocytes have been successfully cultivated in our laboratory, and mitotic figures have been obtained from fresh mouse spleen and marrow, only sporadic success has been achieved with mouse peripheral leukocytes. The present study was undertaken to investigate culture conditions necessary for maintenance and growth of these cells.

Materials and methods. Male and female hybrid mice (C3H/Anf Cum × C57BL/Cum)F_1 were principally used in this study, although a few individuals of the BALB/c and C3H strains were also tested. Ages ranged from 2 weeks to 1½ years. Mice were narcotized with 1:10 nembutal or with ether, and were bled by cardiac or tail puncture into a syringe or tube rinsed with heparin or EDTA (1% in 0.7% NaCl). For some cultures, blood from individual mice was used; for others, the blood from a number of mice was pooled in a chilled tube.

Various agents were used for the separation of leukocytes. These included 3% dextran 1:1 (mol wt 275,000); 6% dextran (mol wt 75,000); 4% fibrinogen; and buffy coats obtained by centrifugation in a refrigerated centrifuge at speeds from 200-2000 rpm for 10-30 minutes. Heparinized whole blood was also tried. Separation at room temperature generally yielded a cleaner preparation of leukocytes.

A wide variety of culture conditions was used: maintenance of the cultures in a 5% CO_2 pH-regulated incubator; regulation of pH during culture from 5.8 to 7.6; varying erythrocyte concentration from 50,000 to 2.5 million per culture, and varying leukocyte concentration from 500,000 to 10 million per ml of culture medium; incubation with and without commercial Difco phytohaemagglutinin-M (PHA); use of a PHA prepared in our laboratory from cranberry beans; and

* Sponsored by U. S. Atomic Energy Commission under contract with Union Carbide Corp.

† I. Medizinische Universitätsklinik, Berlin-Charlottenburg, Germany.

‡ Visiting investigator from Univ. of Freiburg, Germany.

cultures with and without penicillin and streptomycin.

Various culture media were used: TC 199; TC 109; CMRL 1066; Hanks' BSS; Tyrode's; Puck's growth medium; and Puck's fibroblastic medium. Sera from a variety of animals were used: fetal bovine, calf, agamma calf, rabbit, horse, guinea pig, human AB, isologous mouse, and chicken—at concentrations from 10-40%. Various concentrations of colchicine (10^{-3} to 10^{-8} M) were checked for mitotic arrest, as well as length of incubation with colchicine.

The following culture temperatures were used: 23, 26, 37, 39-40°C. Culture volume was varied from 2 ml to 15 ml, and cultures were maintained in 80-ml screw-cap prescription bottles; screw-cap tubes; 50-ml Erlenmeyer flasks; and 15 × 125 mm test tubes. The gas phase above the cells was varied: air; 5% CO_2 in air; 100% oxygen (used for 1 hour at beginning of culture, intermittently throughout the culture period, and for 6 hours). Suspensions were also cultured in darkness by means of black-taped bottles. Leukocyte cultures were normally prepared by use of TC 199, 25% calf serum, PHA, and antibiotics. To test for mitotic activity, colchicine was added at a final concentration of 10^{-5} M and cells were subjected to hypotonic treatment and fixation for spread metaphase preparations. Usually, a single variable was studied at a time.

To test for DNA synthesis, one set of cultures was incubated with tritiated thymidine *in vitro* (1 μc/ml) for 30 minutes and then smeared for autoradiograms. Mouse leukocytes were also cultured for 3 or 4 days in diffusion chambers implanted into the peritoneal cavity of isologous mice as described by Capalbo *et al*(11). Leukocytes were suspended in 0.2 ml of TC 199 with added 1% isologous mouse serum and antibiotics. One hour before removing the chambers from the peritoneal cavity of the recipients, a pulse injection of tritiated thymidine was given intraperitoneally.

Results. In the first phase of our work, 3-4-day mouse leukocyte cultures were terminated after incubation with colchicine and subjected to hypotonic treatment. Mitotic figures were rarely seen in these preparations although our human leukocyte cultures were routinely successful. Since this method of analysis seemed to be of little value in indicating the source of our difficulties, another endpoint was selected. To establish whether or not mouse leukocytes were undergoing the same morphologic changes ordinarily associated with PHA-induced human leukocyte transformation, 1-ml samples of the cultures were removed daily after gentle agitation, centrifuged at 1000 rpm for 10 minutes, and brush smears were made for Giemsa staining. It became apparent that the mouse leukocytes were not holding up well under any of the culture parameters studied, and were not transforming in response to PHA.

In a typical culture, using 3% dextran separation and 75% TC199 with 25% fetal calf serum, the total cell number decreased to about 44% of its original value within 48 hours, then dropped to nearly 0% within 72 hours. As in human leukocyte cultures(12), mouse polymorphonuclear leukocytes disappeared rapidly. By 24 hours, eosinophils and neutrophils represented less than 2% of the total cell population and by 48 hours, less than 1%. Occasional neutrophils with pyknotic nuclei could still be identified. Some eosinophils persisted as morphologically unchanged cells through the second day of culture. Monocytes and lymphocytes persisted through 2 days of culture with little apparent morphological change. By day 3, intact cells could still be seen in wet preparations, but the cells were too fragile to make good smears for staining and counting. The occasional intact cell was a swollen lymphocyte. Little evidence was found of the basophilic blast-like cell typically found in human leukocyte cultures. Cells incubated with tritiated thymidine *in vitro* showed no labeling during culture.

Attempts to culture leukemoid leukocytes from mice were also unsuccessful, although leukemoid leukocytes will incorporate H^3-thymidine *in vitro,* and can effect erythropoiesis in irradiated mice(13).

In an attempt to demonstrate that peripheral leukocytes capable of mitosis are actually present in the mouse, leukocytes were cultured for 3-4 days in diffusion chambers *in vivo* in isologous hosts. In the first experi-

ment, cells were cultured without PHA, and were then carried through the routine chromosome analysis procedure. Approximately 1% of the unstimulated cells was in mitosis indicating that some peripheral mouse leukocytes are capable of division. Brush smears were also made for staining and differential counting (3000 cells were scored). Cells were classified as large or small mononuclears. The former group includes large lymphocytes, monocytes, macrophages, and a group of cells (blast or "transformed") characterized by a deeply basophilic, vacuolated cytoplasm and a nucleus somewhat smaller than that of the large lymphocyte. The differential counts showed that approximately 82% of the cells in the chambers were small mononuclears. In a second experiment, mouse leukocytes were isolated in the presence of PHA before being placed in the chambers, and the host mice were injected daily intraperitoneally with 0.2 ml of PHA. On the third and fourth days of culture, these mice were injected with ^{3}H-thymidine, and smears of the pooled cells were made for autoradiograms and differential counting. Results showed that approximately one-third of the large cells and less than a tenth of the small cells were labeled. Comparison of data from the 2 experiments indicated that the relative percentage of large cells from cultures containing PHA was approximately 3 times that of large cells from cultures not containing PHA, although the absolute cell number was about the same. Polymorphonuclear leukocytes were in various stages of disintegration and were not counted.

Attempts to grow mouse leukocytes *in vivo* in sterile dialysis bags placed in peritoneal cavities of isologous hosts were unsuccessful, although human leukocytes grown by this method in a mouse showed considerable mitotic stimulation, even without exposure to PHA.

Discussion. Attempts to establish proper metabolic conditions for mouse leukocytes *in vitro* in this laboratory have been unsuccessful to date. There seems to be very little effect on mouse leukocyte cultures by such different parameters as pH; temperature; type or concentration of media, sera, or antibiotics; or method of separation. Inability to maintain even a small population of cells beyond a few days would indicate that culture conditions are at fault. Since the mouse has a faster rate of metabolism than most laboratory mammals, its leukocytes may be insufficiently supported by all culture conditions investigated to date, although other hematopoietic tissues of the mouse have been maintained in culture over varying periods(14, 15,16).

If the leukocyte capable of mitosis is derived from lymphocytes as first postulated by Maximow(17) and as indicated by others (12,18), it might be expected that mouse blood would provide a large number of dividing cells because of the high percentage of lymphocytes in peripheral blood. In our diffusion chamber studies, there was an increase in number of large mononuclear cells (presumably at the expense of small cells) in response to PHA. These large cells were able to incorporate tritiated thymidine, and presumably many of them would have divided although no attempt was made to investigate the mitotic potential. It may be concluded that although the *in vivo* culture conditions are quite favorable for maintenance and growth of mouse leukocytes, the poor preservation of these cells *in vitro* would preclude any cell proliferation.

Summary. Efforts to establish a routine method for *in vitro* culture of mouse peripheral blood leukocytes are described. These cells generally failed to respond mitotically to PHA under a wide variety of culture conditions. In diffusion chambers placed in the peritoneal cavity of mice, however, proliferative activity of leukocytes was observed. Failure to obtain successful *in vitro* cultures therefore is probably attributable to culture conditions unfavorable for support of mouse leukocytes.

1. Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., Hungerford, D. A., Exp. Cell Res., 1960, v20, 613.

2. Brecher, G., Cronkite, E. P., Proc. Soc. Exp. Biol. and Med., 1951, v77, 292.

3. Goodman, J. W., Hodgson, G. S., Blood, 1962, v19, 702.

4. Becak, W., Andreade, J. D., Becak, M. L., Am. Nat., 1961, v95, 383.

5. Galton, Michael, Holt, Sarah F., Proc. Soc. Exp.

Biol. and Med., 1963, v114, 218.
6. Biggers, J. D., McFeely, R. A., Nature, 1963, v199, 718.
7. Becak, W., Becak, M. L., Nazareth, H. R. S., Am. Nat., 1963, v97, 253.
8. Newcomer, Earl H., Donnelly, Grace M., Stain Technol., 1963, v38, 54.
9. Dowd, G., Dunn, K., Moloney, W. C., Blood, 1964, v23, 564.
10. Nichols, W. W., Levan, A., ibid., 1962, v20, 106.
11. Capalbo, E., Albright, J. F., Bennett, W. E., J. Immunol., 1964, v92, 243.
12. MacKinney, A. A., Jr., Stohlman, F., Jr., Brecher, G., Blood, 1962, v19, 349.
13. Smith, L. H., Popp, R. A., St. Amand, W., Proc. Soc. Exp. Biol. and Med., 1960, v103, 232.
14. Fell, Honor B., Hughes, A. F., Quart. J. Microscop. Sci., 1959, v90, 355.
15. Billen, Daniel, J. Nat. Cancer Inst., 1959, v23, 1389.
16. Ginsburg, Haim, Sachs, Leo, ibid., 1963, v31, 1.
17. Maximow, A., Arch. Path., 1927, v4, 557.
18. Marshall, W. H., Roberts, K. B., Quart. J. Exp. Physiol., 1963, v48, 146.

Received December 7, 1964. P.S.E.B.M., 1965, v118.

In vitro Synthesis of Steroids by Experimentally Induced Cystic Ovaries.* (30027)

GLORIA V. CALLARD AND JAMES H. LEATHEM
Bureau of Biological Research, Rutgers, The State University, New Brunswick, N. J.

Spontaneous ovarian cysts in women(1) and domestic animals(2,3) are associated with symptoms of endocrine dysfunction which have been attributed to abnormal ovarian and adrenal steroid secretion. *In vivo* and *in vitro* analyses of human(4) and bovine(3,5) cystic ovarian tissue suggested an increased steroidogenesis but an inability to convert C_{19} intermediates to estrogens with resultant accumulation of androgens.

Ovaries containing cystic follicles can be induced by administration of human chorionic gonadotropin to hypothyroid rats(6). Once formed, experimental ovarian cysts do not regress despite correction of the hypothyroid state, thus providing a means of studying a type of cystic ovarian disease under controlled experimental conditions. An attempt was made to analyze *in vitro* steroid production of cystic ovaries experimentally induced in rats.

Materials and methods. Female Sprague-Dawley rats weighing 60-65 g were used. Ovarian cysts were induced by adding 0.04% propylthiouracil (PTU) to the drinking water for 30 days and injecting 10 i.u. of human chorionic gonadotropin (HCG) subcutaneously daily for the last 20 days. Euthyroid rats received a similar treatment with HCG but did not develop cystic ovaries. Uninjected hypothyroid and euthyroid rats served as controls.

One ovary from each animal was incubated with ^{3}H-pregn-5-ene-3β-ol-20-one (1c/mM) while the other was incubated with ^{3}H-androst-4-ene-3,17-dione (3c/mM). To each 800 mg of sliced ovarian tissue in a 50 ml Erlenmeyer flask was added 10 ml Krebs-Hensleit buffer (pH 7.4) with 200 mg per 100 ml glucose and labelled precursor (1.11×10^6 dpm) in 0.1 ml propylene glycol. Flasks were incubated in an atmosphere of 95% oxygen and 5% carbon dioxide at 37°C with constant shaking for a total of 12 hours with a change of medium and addition of fresh precursor each 4 hours. Media from all flasks of one group were pooled, extracted and separated into nonphenolic, aqueous, and alkaline fractions(7).

Chromatography was carried out on Whatman No. 1 paper in the following systems: (A) petroleum ether 5/methanol 4:water 1; (B5) benzene 10/methanol 5:water 5(8); benzene 1:hexane 1/formamide(9); (E4) isooctane 500/t-butanol 225:methanol 225: water 50; (E2B) isooctane 500/t-butanol 250: water 450(10).

* With the assistance of an NSF Graduate Fellowship (GVC) and Grant 2G835, Nat. Inst. of General Med. Sciences, USPHS.