tive; supersonic lysate (slightly opalescent) was more active; and supersonic extract (water-clear), which resulted from lysis of very few cells, was most lethal to chlortetracycline-treated mice. This inverse relation between cell rupture and extract potency may indicate that the extractable factor is liberated from the surface of *C. albicans* cells. Surface origin is compatible also with the activity of heat-killed cells.

Summary. Supersonic vibration of viable *C. albicans* cells released a substance lethal to mice treated with chlortetracycline. In the absence of antibiotic the cell-free preparation was inactive. The combination of supersonic extract with chlortetracycline was highly lethal to mice, that with oxytetracycline was slightly active, while that with chloramphenicol or streptomycin was inactive. Evidence was presented for the combined toxicity of viable *C. albicans* cells and chlortetracycline to diverse animal species.

1. Henrici, A. T., *J. Bact.*, 1940, v39, 113.
2. Winner, H. I., *J. Path. and Bact.*, 1956, v71, 234.
3. Seligmann, E., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 481.
4. Roth, F. J., Jr., Eylar, O. R., and Syverton, J. T., *Bact. Proc.*, 1954, 87.
5. Winter, W. D., Jr., and Foley, G. E., *J. Inf. Dis.*, 1956, v98, 150.
6. Lipnick, M. J., Kligman, A. M., and Strauss, R., *J. Invest. Derm.*, 1952, v18, 247.
7. Huppert, M., MacPherson, D. A., and Cazin, J., *J. Bact.*, 1953, v65, 171.
8. Sieburth, J. M., and Roth, F. J., Jr., *ibid.*, 1954, v67, 460.
9. Salvin, S. B., *J. Immunol.*, 1952, v69, 89.

Received September 4, 1956. P.S.E.B.M., 1957, v94.

Continuous Cultivation of Epithelial Cell Strain (FL) from Human Amniotic Membrane.* (23003)

JØRGEN FOGH AND ROSEMARY O. LUND. (Introduced by W. M. Stanley)
Virus Laboratory, University of California, Berkeley.

Several investigators have succeeded in establishing epithelial-like cell lines from normal human tissues. Chang developed subcultures of cells from conjunctiva, liver, appendix and kidney(1), Perry *et al.* cultivated a cell line derived from normal human skin (2) and Jordan carried human nasal cells in continuous culture(3). Primary cultivation of cells from human amniotic membranes in tissue cultures has been reported(4). Further work in this and other laboratories has shown amnion cultures to be suitable for studies of several viruses(5-7) and as a source of normal human tissue for research on cancer problems. This stimulated attempts to develop amnion cells in subculture. We report here successful continuous cultivation of a strain (FL strain) of human epithelial-like cells derived from normal amniotic membrane. They grew as good confluent monolayer cultures of clear flat cells on the glass surface in medium without embryo extract. Their growth rate was high and they were cultivated in media containing human serum as well as several animal sera. For tissue culture work in which use of first generation material is not essential, the use of this strain of cells in serial cultivation offers several practical advantages, namely elimination of difficulties involved in collecting fresh material and a long trypsinization procedure. Susceptibility of this cell strain to polio and APC viruses will be reported later(8).

Material and methods. Placental tissue† was handled as aseptically as possible at the delivery room. Disinfectants of "surface active" type were completely avoided during de-

* Aided by Am. Cancer Soc. Institutional Grant and Natl. Foundation for Infantile Paralysis.

† We wish to express our appreciation to Alta Bates Hospital, Berkeley, California, for providing placenta material.

livery. The whole placenta was placed in large sterile beaker and sent to this laboratory within a few hours. *Media and sera.* The following substances were used: 1. Trypsin (Difco, 1:250) at concentration of 1:400 in Tyrode's solution. 2. Versene (Disodium versenate, Versenes, Inc.) at concentration of 1:5000 in calcium and magnesium free phosphate buffer solution. 3. Lactalbumin hydrolysate (Nutritional Biochemicals). 4. Yeast extract (Difco). 5. Bovine albumin powder V (Armour). LY-medium is our designation for a medium consisting of Earle's balanced salt solution with 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 0.4% dextrose. Pooled human serum consisting of 16 pools from 8 donors and 7 pools from 30 donors has been used. The serum was not heat inactivated but filtered once through Selas filter (0.03 porosity) after mixing with basic medium. *Animal sera.* Ox and lamb sera were collected from slaughter houses as pools from 12 and 6 animals, respectively. Swine and horse sera were obtained from individual animals. In our work 2 pools of ox serum, 1 pool of lamb serum and serum from 1 swine and 3 horses were used. All animal sera were filtered once through Selas filter, heated at 56°C for one hour, and refiltered in basic medium before use.

Technic for studies of cell multiplication. The cultures were propagated in Povitsky bottles in LY-medium with 20% human serum for various lengths of time. Medium was removed and versene added to the bottle. After incubation at 37°C for several minutes the cells were completely removed from glass surface. Cells were sedimented from suspension in clinical centrifuge and then resuspended in small known volume of same medium. Cell concentration was determined by hemocytometer. The suspension was diluted to approximately 50,000 cells/ml and dispensed in test tubes, 1 ml/tube. Tubes were incubated at 37°C without fluid change. At 24-hour intervals during the following 7 days, 5 tubes/day were treated with versene in the same way and the average number of cells/tube calculated from hemocytometer counts. *Cultivation of cell strain.* The cells from which this strain was developed originated from a placenta delivered from healthy woman, Mar. 13, 1956. The amniotic membrane was stripped, stored overnight in 0.25% trypsin in Tyrode's solution, and processed the following day. After treating the tissue with trypsin in Tyrode's solution with magnetic stirring, a cell pack of 2.7 ml was obtained by light centrifugation. The cells were dispensed in different kinds of containers and media for various purposes. Cultures from which the FL strain originated grew in 50 mm Petri dishes in medium consisting of 20% ox serum and Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate. Each dish had been seeded with amount equivalent to 1/30 ml of cell pack, and in the following days incubated at 37°C in 4% CO_2 atmosphere. The preparations did not grow out to confluent cultures. On the 12th day, cells were transferred to test tubes with rubber stoppers. They were first treated with 3 ml versene at 37°C for ½ hour. At this time 1.5 ml trypsin was added/plate. After 15 to 20 minutes the cells were released from glass surface as single cells. Cells were sedimented from suspension by centrifugation, resuspended in LY-medium with 20% human serum and finally dispensed in a few tubes at very high concentration. At the next 2 weekly transfers the yield of cells obtained from enzymatic digestion decreased, and the cells were seeded in fewer tubes each time, until at 3rd transfer only one tube was seeded. After 3rd transfer the majority of cells had become vacuolated and enlarged. The medium was changed to 20% human serum in Earle's balanced salt solution with 0.5% lactalbumin hydrolysate and the cultures kept on this medium in the following transfers. From the 3rd to 6th transfer covering a period of 2 months, growth in the tube was patchy. The culture area was not increased until cells in 6th transfer started to grow more rapidly. At this stage the medium was changed to 20% human serum in LY-medium. The cells were now transferred to 2 tubes in which they grew out to confluency, and were then transferred to 4 tubes 3 days later. Since then the cells have been dividing

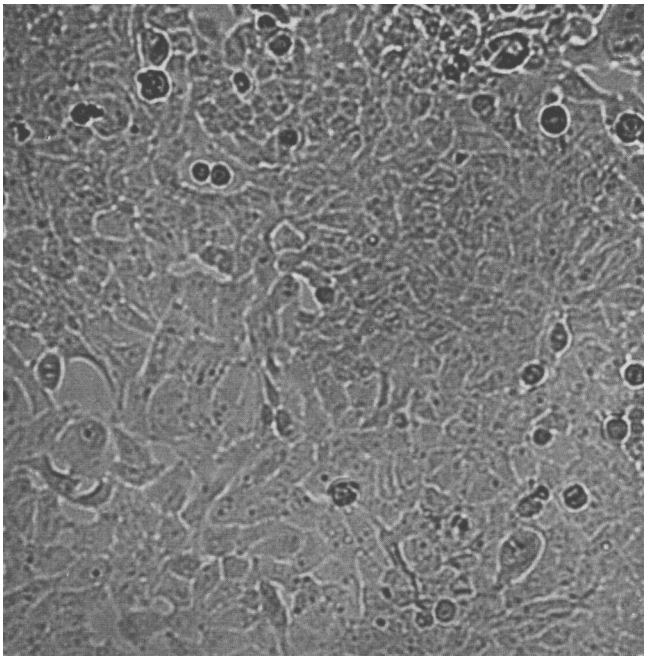

FIG. 1. Culture of FL cells in the 14. transfer. Grown on LY-medium with 20% human serum (100X).

rapidly. These FL cells have been cultivated continuously for 8 months, are now in their 30th passage and show an 8-10 fold cell increase within 7 days. Up to 10th transfer the procedure applied, necessary for complete removal of cells from glass surface, was the combined treatment of cultures with trypsin and versene. Since the 10th transfer all cells

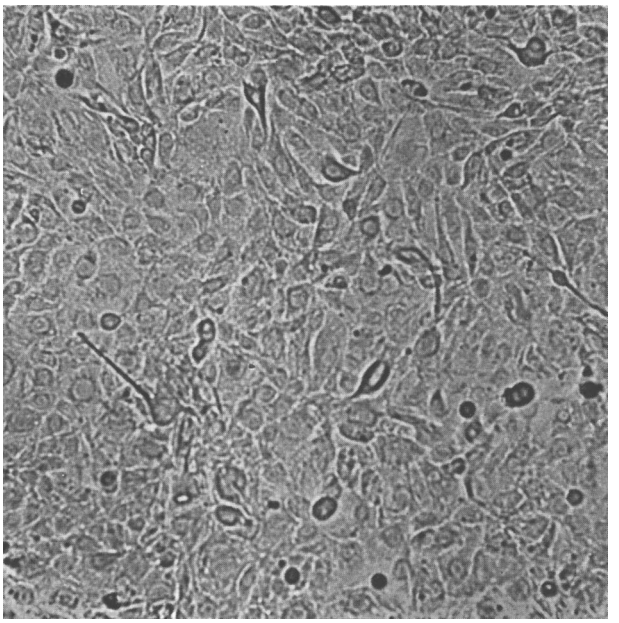

FIG. 2. Culture of primary amnion cells. Grown on LY-medium with 20% human serum (100X).

responded to treatment with versene alone and the trypsin was omitted subsequently. During this entire period of continuous cultivation, even at the time when growth was patchy, the cells kept their epithelial-like characteristics. During the stage when vacuolization and enlargement occurred, the cells became only slightly larger than originally. A fibroblastic-like stage was never observed.

Results. The serially propagated strain of amnion cells is shown in Fig. 1 as it appeared in the 14th transfer. It is being cultivated in tubes, plates and bottles of different sizes and forms uniform monolayer cultures of clear, flat, epithelial-like cells which in gross appearance are similar to cultures of cells directly isolated from an amniotic membrane (Fig. 2). Many mitotic cell forms were observed indicating high dividing activity of this cell strain.

FL cells on different media. Media containing human serum. Since June 14, 1956 FL cells have been grown in LY-medium plus 20% pooled human serum. At time of this writing (11/15/1956) they are in their 30th transfer. They were routinely subcultivated in this medium once a week with fluid change at 4-5 day. Test tubes, serum dilution bottles, 50 mm Petri dishes and Povitsky bottles were seeded respectively with 50,000, 1 million, 700,000 and 3 million cells and cultures were confluent within a week. Of 23 serum pools only one has shown a "toxic" effect on this cell strain and this appeared as a significant decrease in growth rate. This effect was not noticed on strains of HeLa cells cultured under identical conditions (Fig. 3). Attempts to reduce the amount of serum have so far resulted in slower growing cultures after a number of transfers. In LY-medium with 10% human serum cultures have been transferred 8 times within 2½ months with a dilution of 1:8 at the last few transfers, but the cells were somewhat granular and odd cell shapes were observed. Media containing 20% and 10% human serum in mixtures of bovine amniotic fluid and LY-medium have been tried and good cell growth has been observed for several passages.

Media containing animal serum. Cells

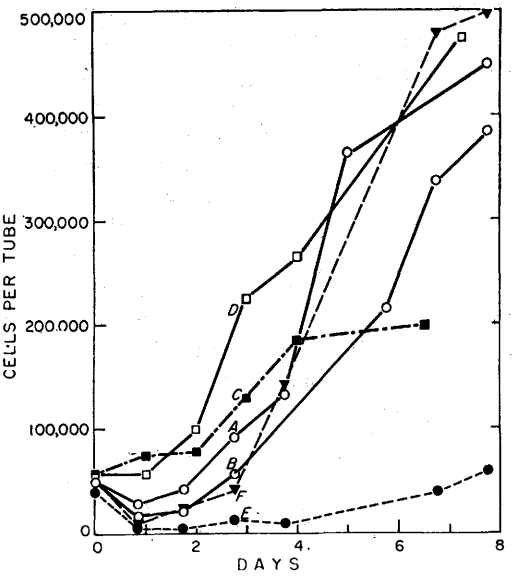

FIG. 3. Multiplication of FL and HeLa cells in LY-medium with 20% human serum. Curves A and B represent 2 different experiments in which FL cells from 7-day-old cultures were propagated in medium with serum from 2 different pools. C and D represent cells transferred from 3-day-old cultures and seeded in 1 and 2 ml respectively of conditioned medium. Curve E represents the effect of a "toxic" batch of human serum on multiplication of FL cells, while F represents growth of HeLa cells in the identical medium.

from 15th transfer in medium containing human serum were subcultured in media with animal sera. Sera from swine, lamb, horse and ox were tried in concentration of 20% in combination with different media. In the LY-medium all cells were kept alive and dividing for 6 transfers over 2½ months. At this stage however cultures grown on swine and lamb serum were eventually discarded, since they had become very granular and stopped dividing. Cells grown on ox and horse serum appeared to be healthy and they are at the present stage in their 11th transfer over a total period of 4½ months since cultivation in animal serum was started. In the last 3 passages the LY-medium has been modified by increasing dextrose concentration to 0.6% and sodium bicarbonate concentrations to 0.22%. Under these conditions the increase/week was 6-8 fold and cultures consisted of clear, normal looking cells. Cells grown in 10% ox serum in LY-medium gave the same appearance as those in 20% ox

serum in LY-medium but growth was slower (dilution 1:5 after 1 month). In LY-medium containing 0.5% bovine albumin powder and 5% ox serum, the cells were slow growing, but healthy. In media without serum, cells did not appear to divide. Cultures grown in test tubes in medium containing serum were washed several times to remove the serum. Several media without serum were tested for their ability to keep the cell sheet intact. In medium 199 and the LY-medium the sheet began to disintegrate within 5-7 days. Addition of a small amount of rabbit or chicken serum (2-4%) extended this period approximately 1-2 days.

Multiplication studies. The increase in cell concentration with time for FL cells under different conditions is shown in Fig. 3. The points represent number of cells seeded/tube at time 0 and average number of cells/tube counted in 5 tubes after various times of incubation.

When cultures were transferred at 7 day intervals the number decreased from 50,000 to 20,000 after the first day. Soon after the cells began to multiply and exceeded the initial count within 2 to 2½ days. From there on the cells multiplied rapidly with a generation time of 30 to 36 hours. At the 8th day the number reached was 400.000 to 450.000 cells/tube. Expressed as proliferation index (10), *i.e.* number of cells at end of 7 days divided by number of cells inoculated, a value of approximately 8 was obtained under these conditions. To avoid the initial decrease in number of cells, experiments were performed with cells transferred from young cultures and seeded in conditioned medium, *i.e.* medium in which they had already been growing for 3 days. By use of this technic there was no initial loss and cells multiplied rapidly, reaching a concentration of 200.000 and 475,000/tube at the 7th day. Apparently 1 ml of medium does not contain sufficient nutrient to support a high level of cell multiplication.

One pool of serum out of 23 showed toxic effect on FL cells although this serum did not affect growth of HeLa cells. During multiplication studies the pH of medium in which cells grew was measured simultaneously with each cell counting. The cells were normally seeded in medium of approximately pH 7.5; one day later the pH increased and rapid division of cells did not occur until the pH of medium by cell metabolism had been readjusted to 7.5°. It appears from a few experiments that it is possible to shorten this lag period by seeding the cells in a medium at lower pH. In this way an 18 fold increase in cell concentration has been obtained within a week using 3 day old cells seeded in 2 ml conditioned medium with pH of 7.0 and a fluid change after 3 days.

Discussion. The method of cultivation described differs from previous published methods in that this cell line was developed by trypsinization of the primary source and cultivated in a medium without embryo extract. Starting with a high number of cells the population diminished considerably during the first stages. Accordingly the area of growth on the glass surface was deliberately diminished so that the number of cells/area and / volume of culture fluid was still high. It seems likely that this method, in accordance with the hypothesis of Earle(9) helped the cells to adjust to continuous growth on a glass surface.

The appearance of cells has at all stages been epithelial-like. They did not grow out of predominantly fibroblastic cultures as was the case with the line of nasal cells described by Jordan(3).

The FL cell strain which has now been in large scale production for 5 months appears to be easily maintained in stock and divides rapidly enough to provide adequate amounts of cultures for production, diagnostic, or research purposes. Our main experience so far concerns cultures grown in medium containing human serum but good evidence has been obtained that this cell line can be carried on animal serum.

Multiplication studies of FL cells have revealed growth rates of the same order of magnitude as recently found for Chang's cells (10) although the conditions of cultivation are different. In our experience, the difficulties in obtaining growth promoting human

sera as described by Chang(1,11) has not been a problem. We have found that only one of 23 human serum pools was toxic to FL cells.

In preliminary experiments with FL and HeLa cells the FL cells have not produced tumors in treated rats using a technic‡ in which HeLa cells produced significant tumors. More work is needed however before a conclusion can be established.

Summary. A strain of human cells derived from a normal amniotic membrane has been cultivated in serial passage for 8 months in 30 transfers. This cell line was developed by trypsinization of the primary source and cultivated in a medium without embryo extract. The appearance of the cell has been epithelial-like at all stages of cultivation. Growth characteristics in media containing human serum or animal sera are reported. The cell strain has been grown in large scale.

‡ Huebner, R. J., personal communication.

1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

2. Perry, V. P., Evans, V. J., Earle, W. R., Hyatt, G. W., and Bedell, W. C., *Am. J. Hyg.,* 1956, v63, 52.

3. Jordan, W. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 867.

4. Zitcer, E. M., Fogh, J., and Dunnebacke, T. H., *Science,* 1955, v122, 30.

5. Fogh, J., and Lund, R. O., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 80.

6. Weinstein, H. J., Alexander, C., Yoshihara, G. M., and Kirby, W. M. M., *ibid.,* 1956, v92, 535.

7. Dunnebacke, T. H., *Virology,* 1956, v2, 811.

8. Fogh, J., and Lund, R. O., PROC. SOC. EXP. BIOL. AND MED.,

9. Sanford, K. K., Earle, W. R., and Likely, G. D., *J. Nat. Cancer Inst.,* 1948, v9, 229.

10. McAllister, R. M., and Coriell, L. L., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 389.

11. Chang, R. S., *Trans. N. Y. Acad. Sci.,* 1955, v17, 584.

Received December 19, 1956. P.S.E.B.M., 1957, v94.

Estrogen Antagonisms: Inhibition of Estrone-Induced Uterine Growth by Testosterone Propionate, Progesterone and 17-ethyl-19-nortestosterone. (23004)

RICHARD A. EDGREN AND DAVID W. CALHOUN
Division of Biological Research, G. D. Searle and Co., Chicago, Ill.

Recent literature has demonstrated a considerable interest in substances which antagonize the action of estrogenic compounds. Androgens have long been known to inhibit, at least in part, the effects of estrogens on various target organs(1). Progesterone, corticoids and various estrogenic substances also have been implicated (see 2 for review of literature). The reports generally leave much to be desired from a comparative standpoint as end points used vary from laboratory to laboratory and few workers have attempted more than qualitative analyses; thus adequate potency evaluations are difficult. The present communication discusses an estrogen-antagonistic action of 17-ethyl-19-nortestosterone (Nilevar) and compares activity of this substance to activities of testosterone propionate and progesterone, using estrogen-induced uterine growth as an index of activity.

Materials and methods. The estrone-stimulated uterine growth of intact, immature mice was employed for purposes of assay following essentially the procedure of Rubin, *et al.*(3). Our modification of this test has already been described(4). The response index was weight of uterus in mg. Five experiments were carried out using each of the 3 substances: progesterone, testosterone propionate and 17-ethyl-19-nortestosterone. A dose of 0.3 μg of estrone was employed as standard uterine growth-stimulator, against which the test compounds were assayed. In each experiment groups of 8-10 mice were treated with the estrone standard alone and estrone in combination with a series of doses