9. E. V. Jensen et al., Adv. Exp. Med. Biol., 36, 60 (1973).
10. S. G. Korenman and B. R. Rao, Proc. Nat. Acad. Sci. USA, 61, 1028 (1968).
11. S. G. Korenman, J. Clin. Endocrinol., 28, 127 (1968).
12. O. H. Lowry et al., J. Biol. Chem., 193, 256 (1951).
13. J. Mester, D. M. Robertson, et al., Biochem. J., 120, 831 (1970).

CULTIVATION OF MOUSE EMBRYONIC LIVER EPITHELIUM

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Epithelial cells of mouse embryonic liver, cultivated in vitro, communicated with each other by highly permeable intercellular contacts, they synthesized α-fetoprotein, and they possessed the property of contact inhibition of phagocytosis. The cultivation technique is described.

KEY WORDS: *Mouse embryonic liver culture; α-fetoprotein; phagocytosis.*

Liver tissue is a favorite object with which to study cellular differentiation and processes of induction and carcinogenesis. A cell system cultivated in vitro, is the optimal model for the study of these processes. Nevertheless, many investigations of this type are carried out in vivo, because of the difficulty of keeping normal epithelium for a long time in vitro. Investigations have recently been published in which epithelial cells have been grown by different methods [8-10, 12, 14].

A method of cultivating mouse embryonic liver epithelium is described in this paper, some characteristics of the resulting culture are given, and the method of identification of epithelial cells of the hepatic parenchyma within the culture is detailed.

EXPERIMENTAL METHOD

The method is a variant of that developed by Éraizer et al. for a cultivation of human embryonic liver [8]. As the method of identification of the epithelial cells the immunohistochemical determination of α-fetoprotein (α-FP) was used, because the production of this substance is known to be a specific function of the hepatic epithelium [1]. Cultures of primary embryonic fibroblast-like cells and primary cultures of mouse embryonic liver were used. The cultures were grown on Eagle's medium with lactalbumin hydrolysate (1:1) and 10% bovine serum. To the medium for cultivating epithelial cells 10% calf embryonic serum and 0.4% glucose were added. To inhibit growth of fibroblast-like cells the medium on which liver cells were seeded contained prednisolone (Gedeon Richter, Hungary) in a concentration of 75 μg/ml. On the day after seeding the medium was changed for one not containing prednisolone. Noninbred mouse embryos aged 13-14 days were used. Cells were grown on coverslips placed in plastic dishes (Linbro, USA).

The α-FP content in the medium was determined by the standard method of precipitation in agar, using a test system [5]. To determine the location of α-FP in the cells the indirect immunofluorescence method [14] was used. Monospecific rabbit antibodies against mouse α-FP, isolated with the aid of immunosorbents, and anti-rabbit donkey serum labeled with fluorescein isothiocyanate, prepared by the Department of Luminescent Sera, N. F. Gamaleya Institute of Epidemiology and Microbiology, were used. The living cultures were fixed by various methods: the standard method of fixation in a mixture of ethanol with glacial acetic

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acid (99:1) in the cold for 15, 30, and 60 min and fixation with cold acetone for 1 h. The cultures were incubated for 30 min at room temperature with antibodies against α-FP, washed for 10 min with physiological saline, and then incubated again for 30 min with labeled serum.

To study the intercellular contacts, fluorescein (mol. wt. 332, microelectrophoresis through a glass microelectrode) was injected into individual cells and the spread of the dye into neighboring cells was recorded in the dark field of a luminescence microscope.

To study phagocytosis, 0.01-0.03% of a suspension of carmine was added to the medium. The unphagocytosed carmine was carefully washed away after 5 and 24 h and the cultures were fixed.

EXPERIMENTAL RESULTS

The most important factors for cultivation of mouse liver epithelial cells are the age of the embryos and the seeding density. Optimal growth was observed when the liver from mouse embryos aged 13-14 days were used. If liver cells from older embryos (15-18 days) were seeded growth was chiefly macrophagal. With a low seeding density the cultures did not form layers and the separate islands of cells died in the course of time. Too high a seeding density led to separation of the cells from the glass. The density obtained by seeding liver cells from six or seven embryos per dish with an area of 8.7 cm^2 proved to be optimal. The volume of medium was 1.2-1.5 ml per dish. On the second to third day of cultivation, such cultures formed a layer of cells. The best layer was obtained by mixing bovine serum with calf embryonic serum.

Several types of cells could be seen on the preparations: firmly packed islands of epithelial cells, sometimes joined into a single sheet, stratified in places, and with single cells of macrophage type which never formed a sheet lying among the islands. Sometimes long fibroblast-like cells could be observed between the islands of epithelial cells and inside the sheet. A culture contained many mitotically dividing cells. One feature distinguishing the epithelium from fibroblast-like cells was inhibition of phagocytosis in the central zones of the dense epithelial sheets [2, 4]. The marginal cells of the sheet, which had a free edge, were actively phagocytic. In the present experiments the following features were observed after incubation for 5-24 h with carmine and subsequent rinsing. In the separate epithelial islands the peripheral cells contained much larger quantities of carmine than the central cells (Fig. 1). In the continuous sheets most cells contained no carmine, but in single cells and groups of cells inside the sheets, accumulation of carmine could be observed. Groups of cells phagocytosing carmine could evidently be either peripheral cells of confluent islands or a collection of fibroblast-like cells between the epithelial islands. All cells of macrophagal type also phagocytosed carmine actively. On insertion of the electrode containing fluorescein into one of the cells of an epithelial island, passage of the dye into neighboring epithelial cells could be observed in the dark field. Each cell stained five to 10 neighboring cells. Liver cells in culture in these experiments were thus found to be connected by highly permeable intercellular contacts, like normal liver cells [11]. All the liver cultures actively synthesized α-FP. In medium collected 24-48 h after the previous change of medium α-FP was always found by the precipitation in agar method without the need to concentrate the medium. The location of α-FP in the cells was studied in cultures fixed on the 2nd-4th day of cultivation. Of all the methods of fixation used the best proved to be fixation with acetone in the cold for 1 h. To differentiate the specific location of α-FP within the cells from its passive accumulation from the medium together with other serum proteins, fixed cultures were treated with antibodies against bovine serum, the concentration of which in the medium was fairly high. As a rule bovine serum was found in round cells which which were often found on the top of the sheet (Fig. 2). These were evidently dying cells. Such cells could easily be recognized in preparations treated with antibodies against α-FP. Fluorescence of this sort was disregarded. In cultures treated with antibodies against α-FP and neutralized with homologous antigen, specific fluorescence was absent. The general intensity of fluorescence was sharply increased in fixed cultures treated with monospecific antibodies against α-FP. Fluorescent cells formed large zones inside the sheet of cells (Fig. 3). Stratified zones of the cultures were particularly brightly fluorescent. Completely dark places could also be seen at the same time, and when examined in phase contrast these were found to be a cell monolayer. Fluorescent cells varied in size from tiny, closely packed, brightly fluorescent cells to large cells with large nuclei but with weak fluorescense. Single cells of macrophage type possessed the background level of fluorescence. In

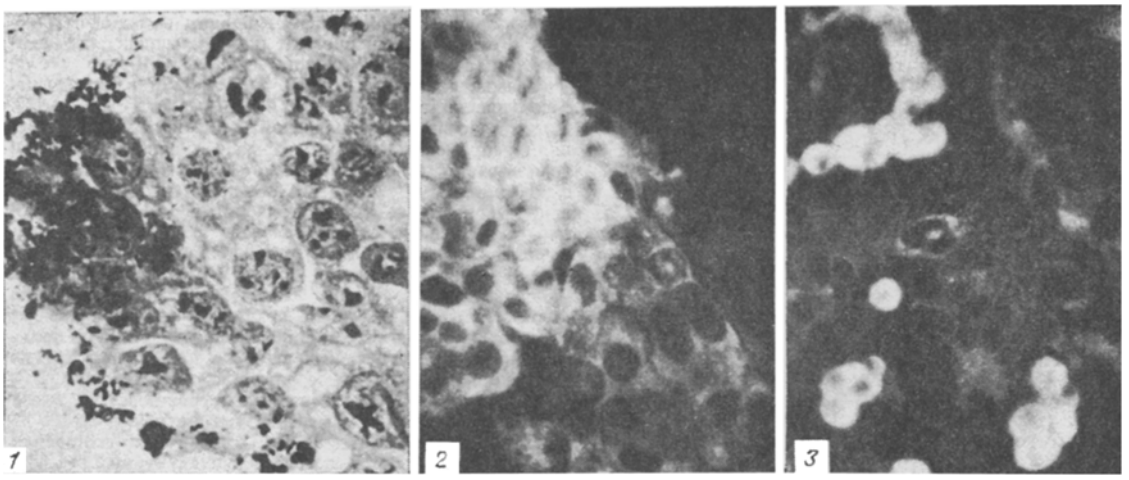

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Phagocytosis of carmine by epithelial islet: border cells of sheet are actively phagocytosing carmine. Hematoxylin. Magnification 40×10×1.25.

Fig. 2. Incubation of culture with antibodies against α-FP: cells containing α-FP fluoresce. Magnification 40×8.

Fig. 3. Incubation of culture with antibodies against bovine serum: dying cells fluoresce. Magnification 40×8.

some cases bands of fibroblast-like cells could be observed at the border of the sheet and inside it. Specific fluorescence was absent in such cells.

Connective-tissue cells in vivo are known to abstract α-FP from the surrounding medium [7]. It was therefore interesting to study whether α-FP could accumulate in fibroblast-like cells cultivated simultaneously with the hepatic epithelium. For this purpose a culture of fibroblast-like cells was kept for 4 days in a vessel containing a culture of liver cells. The cultures were fixed and treated with antibodies against α-FP. Despite growth in the medium containing α-FP, no specific fluorescence was found in the culture of fibroblast cells grown together with hepatic epithelium.

Since α-FP is a specific protein synthesized by embryonic liver, its presence in the cell could serve as a criterion that it belonged to the hepatic epithelium. However, absence of fluorescence does not always mean that a particular cell is not epithelial. For instance, cells of the bile duct epithelium in vivo have no specific fluorescence when treated with antibodies against α-FP [6]. Further, according to the observations of Sell et al., the synthesis of α-FP and its secretion into the medium depend on the phase of the mitotic cycle at which the hepatocyte is found.

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LITERATURE CITED

1. G. I. Abelev, Adv. Cancer Res., 14, 295 (1971).
2. Yu. M. Vasil'ev, I. M. Gel'fand, L. V. Domnina, et al., Proc. Nat. Acad. Sci. USA, 75, 719 (1975).
3. O. I. Epifanova and V. V. Terskikh, The Method of Autoradiography in the Study of Cell Cycles [in Russian], Moscow (1969).
4. O. S. Zakharova and L. V. Domnina, Tsitologiya, 17, 84 (1976).
5. N. I. Khramkova, in: Immunochemical Analysis (ed. by L. A. Zil'ber) [in Russian], Moscow (1968), pp. 143-164.

6. N. V. Éngel'gardt, L. Ya. Shipova, A. I. Gusev, et al., Byull. Éksp. Biol. Med., No. 12, 62 (1969).
7. N. V. Éngel'gardt, A. I. Gusev, L. Ya. Shipova, et al., Internat. J. Cancer, 7, 198 (1971).
8. T. L. Éraizer, K. N. Grinberg, and S. G. Vorsanova, Byull. Éksp. Biol. Med., No. 5, 123 (1975).
9. D. M. Bissel, L. E. Hammaker, and U. A. Meyer, J. Cell Biol., 59, 722 (1973).
10. H. L. Leffert and D. Paul, J. Cell Biol., 52, 559 (1972).
11. R. D. Penn, J. Cell Biol., 29, 171 (1966).
12. C. I. Slack and J. E. Palmer, Exp. Cell. Res., 55, 416 (1969).
13. B. Weinstein, J. M. Orenstein, R. Gebert, et al., Cancer Res., 35, 253 (1975).
14. T. H. Weller and A. H. Coons, Proc. Soc. Exp. Biol., 86, 789 (1954).
15. G. M. Williams, E. K. Weisburger, and J. H. Weisburger, Exp. Cell Res., 69, 106 (1971).

ISOLATION AND CHARACTERISTICS OF A CONTINUOUS CELL CULTURE FROM HUMAN SPLEEN

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A continuous line of human spleen cells was obtained. Important factors in the isolation of this strain were seeding a large number of cells and prolonged incubation of the culture; these two factors created conditions for gradual modification of cellular metabolism, without which the cells could not have adapted themselves to prolonged culture. A continuous culture could not be obtained from one colony and a "shaking" method had therefore to be used. Cultures were obtained only after the addition of 1% phytohemagglutinin to the nutrient medium.

KEY WORDS: *Spleen — continuous culture; "shaking" method; phytohemagglutinin.*

How to increase the resistance of the body is one of the most important problems in the general and infectious pathology of man and animals. Resistance of the body to pathological agents of various sorts is largely determined by the state of the reticuloendothelial system. The method of growing cells *in vitro* offers wide opportunities for the study of the mechanisms of antiviral immunity, cell resistance, and so on.

The object of this investigation was to obtain a continuous cell line from adult human spleen.

EXPERIMENTAL METHOD

The spleen was removed at operation from a patient with lymphogranulomatosis. The tissue was trypsinized by the usual method 1-2 h after the operation. The cell concentration was adjusted to 2,000,000/ml and the suspension transferred to 1-liter flasks. Altogether 14 such flasks were seeded. The primary culture medium (No. 1) consisted of 20% calf serum, 30% lactalbumin hydrolysate, and 50% Eagle's medium for diploid cells. The sera were inactivated. The medium for further growth of the culture (No. 2) consisted of 30% conditioned medium (medium No. 1 in which the cells had been grown for 3 days), 10% calf serum, 10% nor-

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