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MODIFICATION OF THE "HANGING DROP" METHOD FOR CULTURING REAGGREGATES OF DISSOCIATED CELLS AND EXPLANTS OF EMBRYONIC LUNG

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Of the few methods of obtaining reaggregates of dissociated embryonic tissue cells the "hanging drop" method, in the modern form suggested by Steinberg [6], is attractive because it reproduces the natural quality of the reaggregation process, which takes place spontaneously. Other methods are based on artificial approximation of cells by agitating the suspension in special shakers [5] or by centrifugation [7]. Another advantage of the "hanging drop" method is that the process of cell aggregation can be kept under constant observation without being disturbed. A disadvantage of the method is the comparatively small volume of nutrient medium (1 drop = 0.05 ml), as a result of which the nutrients in the system are quickly exhausted. As the writers showed previously [2] the process of organotypical aggregation consists of several successive stages, so that the suspension of chicken embryonic lung cells must be cultured for at least 3-4 days without a change of nutrient medium.

The object of this investigation was to modify the "hanging drop" method by increasing the volume of nutrient medium and to compare the results of aggregation of dissociated mouse and chicken embryonic lung cells when the classical method and the suggested modification were used. The possibility of applying the modified "hanging drop" method to culture pieces of embryonic tissue also was investigated.

EXPERIMENTAL METHOD

Lungs of 10-day Brown Leghorn chick enbryos and 17-day A and C57BL mouse embryos were used. Embryonic lungs were dissociated into cells by Moscona's method [4]. The finely shredded pieces of embryonic lung, measuring 1-2 mm in diameter, were incubated at 37°C in calcium-free Tyrode solution and in a 0.25% solution of trypsin, after which the dissociating solutions were replaced by "growth" nutrient medium consisting of the following components: Eagle's medium (100%), glutamine (1%), inactivated bovine serum (10%), and chick embryonic extract prepared from 10-day embryos in Eagle's medium in the ratio of 1:1 and added to the medium after centrifugation. The chick embryonic lungs were kept in the dissociating solutions for 15-20 min and the mouse embryonic lungs for 25-30 min, for the latter are much harder to dissociate into cells. Undissociated cell complexes were removed by centrifugation and the completeness of their sedimentation was verified under the microscope. The resulting cell suspension was adjusted to a concentration of $2 \cdot 10^6$-$6 \cdot 10^6$ cells/ml medium and 1 drop was applied to siliconized coverslips. These were inverted with the drop beneath and placed on glass rings glued with epoxide glue to the bottom of a petri dish. Besides the classical "hanging drop" method as described above, cells also were cultured by a modified method in specially designed vessels, in which the conditions for cell culture on the surface of tension of the drop were similar to those in the "hanging drop" method. The vessels were

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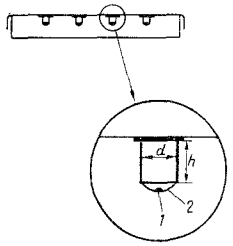

Fig. 1. Vessel for cell culture by modified "hanging drop" method. d) Diameter of culture tube (4 mm); h) height of culture tube (5 mm). 1) Aggregate or explant; 2) nutrient medium (culture surface).

small segments of tubes, 4 mm in diameter at the base, glued with epoxide glue to the inner surface of a petri dish. The height of the tubes was 5 mm (Fig. 1).

The cell suspension was poured into the tubes, after which the lid was inverted and placed over the base of the petri dish. Under these circumstances the nutrient medium formed a drop on the free ends of the tubes, the volume of which was roughly 3 times greater than that of the ordinary "hanging" drops formed on coverslips: 0.15 and 0.05 ml of medium, respectively. The dissociated cells settled on the surface of tension of the drop, thus using it as a maintenance substrate. Besides cell suspensions small fragments (0.3-0.5 mm in diameter) of mouse embryonic lungs also were cultured in these vessels.

Culture was carried out at 37°C in an atmosphere of air with the addition of 5% CO_2 for 7 days. The nutrient medium was changed on the 4th-5th days of the experiment. Aggregates and explants for histological analysis were embedded in paraffin wax; serial sections were stained with hematoxylin and eosin and their morphology and viability were studied.

EXPERIMENTAL RESULTS

A comparative study of the results of culture of a suspension of dissociated cells in a "hanging drop" by the classical method showed that under these conditions organotypical aggregation took place only in a suspension of chick embryonic lung cells. Aggregates after 4-7 days were spherical formations consisting of cavities of different sizes, lined with cubical epithelium and surrounded by loose interstitial tissue. In some cavities desquamated degenerating cells could be seen. On the whole the morphological picture of sections of the aggregates was similar to that of intact lung tissue (Fig. 2). Under the same conditions dissociated mouse embryonic lung cells did not form these organotypical structures. Only occassionally, in the early periods of culture (1 day), was the initial stage of aggregation observed in the form of the appearance of flat, irregular groups of cells on the surface of tension of the drop, as was described in detail previously by the writers in experiments with dissociated chick embryonic lung cells [2]. However, by contrast with the latter, mouse lung cells died under these conditions after 2-3 days of culture.

Organotypical aggregation of dissociated mouse embryonic lung cells could be obtained only by culturing the suspension in modified "hanging drops" formed in the vessels described above (Fig. 1). The morphological picture of the aggregates on the 4th-7th days of culture was similar to that of aggregates of embryonic rabbit lung cells at the same time of the experiment. However, by contrast with the latter, as a rule marked trophic disturbances developed in the center, leading to the formation of central necroses. Similar central necroses developed at this time also in explants of intact mouse embryonic lung tissue when

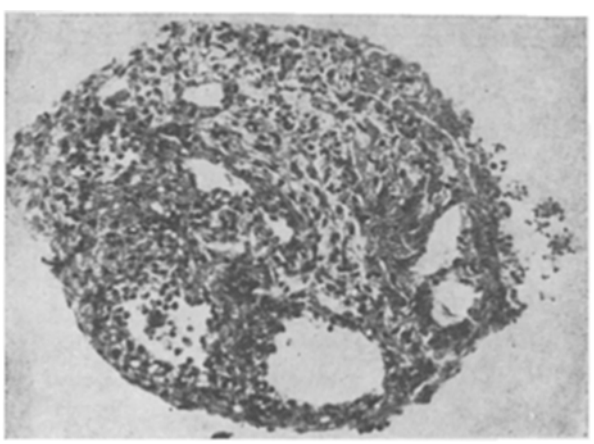

Fig. 2. General appearance of organotypical aggregate (section) obtained from dissociated chick embryonic lung cells. Cavities lined with epithelium, similar to respiratory passages, can be seen. Magnification 125 ×.

cultured by the modified "hanging drop" method. Development of central necroses also is characteristic of explants of embryonic lungs [1] and other tissues [3] during organotypical culture on the surface of a solid porous substrate, and it is evidently a form of adaptation of the tissue to the conditions of organotypical growth *in vitro*. It can be concluded from these results that mouse embryonic lung cells evidently possess more stringent growth requirements than chick embryonic lung cells.

The experimental results showed that the suggested modification of the "hanging drop" method has advantages over the ordinary classical method. The comparatively large volume of nutrient medium in the modified "hanging drop" method means that it can be used not only to obtain organotypical aggregates of dissociated cells, but also for the culture of embryonic tissue fragments. A comparative study of the morphogenesis and survival of embryonic tissues and organotypical aggregates under "hanging drop" conditions and on a solid porous substrate may be useful when studying the role of the support in tissue morphogenesis. Finally, in the writers' opinion, this method offers good prospects for the study of confrontation of embryonic organs and tissues.

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