LONG-TERM CULTURE OF ORGANOTYPICAL AGGREGATES OBTAINED FROM DISSOCIATED EMBRYONIC MOUSE LUNGS

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The mechanism of reconstruction and development of organotypical structures from dissociated embryonic cells during hanging drop culture by Steinberg's method [6] and in our own modification [3] was studied previously. It was shown that such organotypical aggregates are completely formed after 4-5 days.

The object of the present investigation was to determine whether such aggregates are capable of further growth and differentiation. Since the aggregates formed in a hanging drop died fairly quickly (on the 8th-10th day), the first task was to select optimal conditions for longer survival of the aggregates *in vitro*.

For explanted fragments of organs to maintain their viability and to preserve their organotypical structure for a long time it is common knowledge that they must be cultured at the boundary between the gaseous and liquid phases (nutrient medium), by the use of a porous supporting substrate. This is the basis for all modern modifications of the organ culture method [4, 5], which the writers used successfully in the past for long-term culture of various embryonic tissues and, in particular, lungs [1, 2]. With this in mind, we used this method to culture aggregates formed from dissociated embryonic mouse lungs and to study their morphogenesis.

EXPERIMENTAL METHOD

Lungs from 17-day embryos of line A mice were cut into small pieces and incubated in solutions of Versene and 0.25% trypsin, 15 min in each, at 37°C. They were then washed in nutrient growth medium and gently pipeted several times. The resulting suspension of single, chiefly mesenchymal cells and undissociated epithelial complexes was separated by sedimentation of the latter by the action of gravity for 2-3 min. The supernatant was centrifuged at 1500 rpm for 20 sec to remove the small epithelial complexes from the suspension of single cells. The concentration of single cells was adjusted to 5 million/ml medium. The volume of nutrient medium in which the epithelial complexes were contained was made equal to the volume of medium containing the suspension of single cells; they were then pooled in the proportion of 1:2, respectively. This mixture was poured into special vessels for obtaining aggregates and cultured in a hanging drop for 4 days by the method described previously. The aggregates thus formed were carefully removed from the hanging drop and planted out on millipore filters, supported by tantalum platform grids in deep watch glasses, into which the nutrient medium was poured so that its level came up to the undersurface of the filter. The fragments were cultured at 37°C in an atmosphere of air with a constant supply of 5% CO_2 The nutrient medium was changed every 3-4 days. The aggregates were studied on the 4th, 7th, 14th, and 21st days of culture after appropriate histological treatment. The 4-day aggregates were fixed in Bouin's fluid and the rest in 70% ethanol; they were embedded in paraffin wax and serial sections (4 μ) or total preparations were obtained and stained with hematoxylin and eosin.

To select optimal conditions for growth of the aggregates the following brands of millipore filters were tested: Aufs (pore diameter 0.6-0.9 μ), Hawp (0.65 μ), Synpore (0.23 μ), Hawp (0.45 μ), for use with media of the following composition: 1) 71.5% of medium 199, 25% in-

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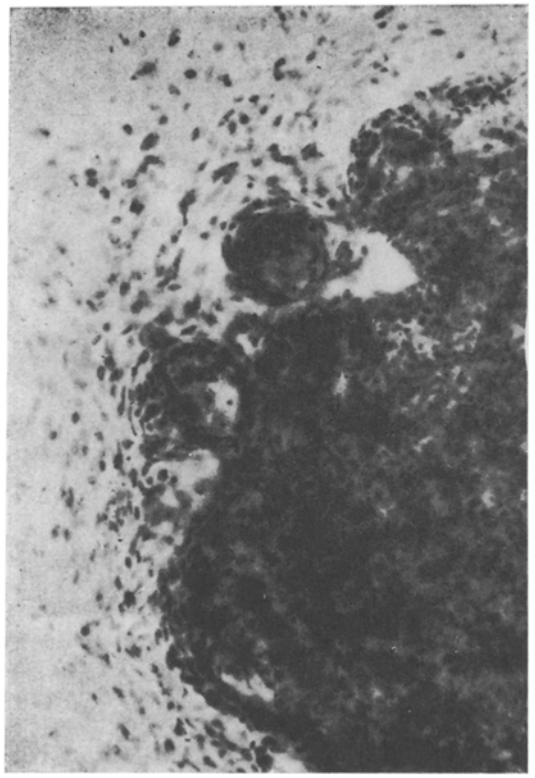

Fig. 1

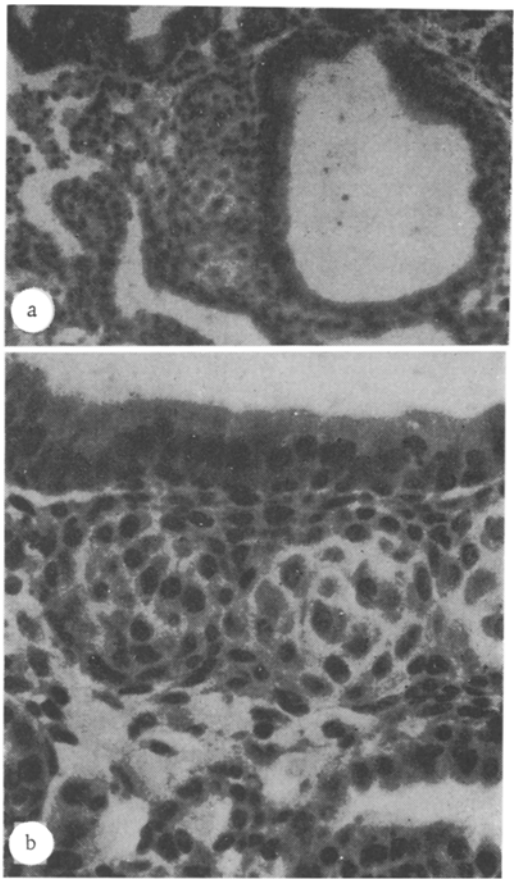

Fig. 2

Fig. 1. Zone of growth around aggregate. Fibroblast-like cells and epithelial buds; 14th day of culture, 200 ×.

Fig. 2. Characteristic organotypical structures in aggregate. Rudiments of embryonic cartilage adjacent to bronchial cavity lined with cylindrical epithelium. Magnification: a) 200, b) 500 ×.

activated bovine serum, 2.5% chick embryonic extract, 1% of 40% glucose solution; and 2) 69% of medium 199, 20% inactivated bovine serum, 10% chick embryonic extract, 1% of 40% glucose, 20 mg glutamine/100 ml medium, and 15 ml of 5% ascorbic acid solution/100 ml medium. The embryonic extract was prepared from 11-day chick embryos, the homogenate was diluted 1:1 with Hanks' solution, and centrifuged (2000 rpm) for 30 min; the resultant cell-free supernatant was added to the nutrient media in the above quantities.

EXPERIMENTAL RESULTS

Aggregated formed on the 4th day of culture were small spherical formations the tissue of which consisted of small cavities lined with cubical epithelium and (sometimes) with long spindle-shaped cells. As a rule these cavities were located at the periphery of the aggregate, and its central part consisted most frequently of an amorphous mass of round cells. In some cavities lined with cubical epithelium and resembling the lumen of bronchioles, desquamated degenerating cells were seen. Most aggregates were surrounded by a capsule of fibroblast-like cells. When these aggregates were planted out on millipore filters, they spread rapidly over the surface during the first 3 days. Migration of fibroblast-like cells and the formation of a zone of growth around the aggregates took place at the same time. This zone of growth was most strongly developed when the aggregates were cultured on Aufs filters (pore diameter between 0.6 and 0.9 μ). It consisted mainly of long fibroblast-like cells and a few epithelial bud-shaped outgrowths (Fig. 1). In the course of culture changes in the morphology of the aggregates were observed. Large cavities lined with high cylindrical epithelium and with basally situated nuclei appeared in them. Often these cavities were surrounded by rudiments of embryonic cartilage (Fig. 2), which was never found when the ag-

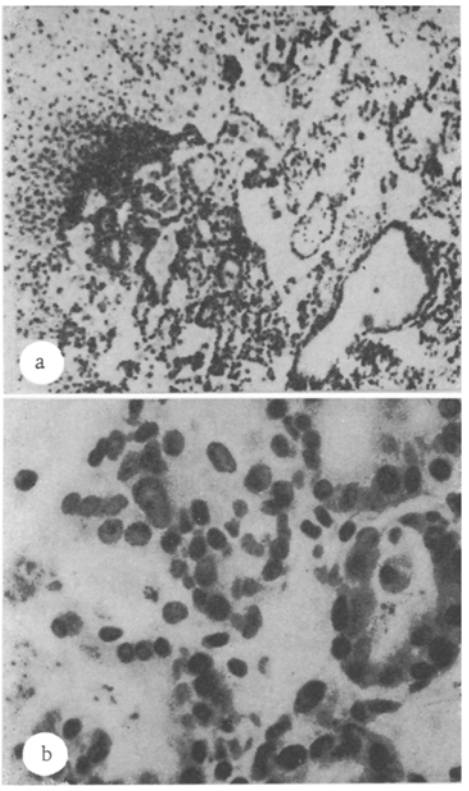

Fig. 3. General appearance of aggregate (a), structures resembling alveoli (b), 21st day of culture. Magnification: a) 80, b) 500 ×.

gregates were cultured in a hanging drop. Examination of serial sections showed complex ramification of these structures, similar to the embryonic bronchi of intact lungs developing in organ cultures. Most of the rest of the aggregate consisted of epithelial rosettes and numerous small cavities lined with cubical epithelium. Starting with the 14th day, the epithelium lining them became much flatter and formed structures resembling alveoli, in which desquamated degenerating cells often accumulated. However, even on the 21st day of culture no degenerative changes could be observed in the epithelium lining the organotypical structures described above (Fig. 3). With an increase in the duration of culture the quantity of mesenchyme in the aggregates was appreciably reduced. By the 21st day they consisted of a few elongated cells which were adjacent to the epithelial lining of the cavities, and also of developing rudiments of cartilage. Many mesenchymal fibroblast-like cells grew into the pores of the filter, to form a unique type of support beneath the aggregate.

When aggregates were cultured on Hawp and Synpore filters (pore diameter 0.45 and 0.23 μ, respectively) the zone of growth was quite narrow, sometimes occupying only a small area, and as a rule it consisted of fibroblast-like cells only. Starting from the 7th day, marked degenerative changes appeared in the aggregates, and by the 14th day extensive areas of central necrosis developed. Culture of aggregates on the different nutrient media had no significant effect on their survival.

It was thus possible to choose the best conditions for long-term culture of organotypical aggregates. Millipore filters with a pore diameter from 0.6 to 0.9 μ proved to be most suitable. Medium 1 (see Experimental Method), which was the one we usually used for organ cultures of embryonic lungs in our previous experiments, can be used as the nutrient medium [1, 2, 4]. Judging from the absence of degenerative changes in the aggregates on the 21st day of culture, this is not the upper limit and could be prolonged if necessary. However, even during this period of culture, the aggregates were shown to be capable of further growth and organotypical differentiation. The method of organotypical aggregation of dissociated embryonic lung cells, as developed previously [2], in conjunction with the method of long-term culture of aggregates suggested in this paper, provide a convenient system with which to study

the morphogenetic potential of embryonic tissues and their interaction during embryogenesis under normal conditions and in the presence of certain pathological processes, such as transplacental carcinogenesis.

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ERRATA

BBM-10, October, 1981, V. V. Zakusov et al. p. 1369. The first sentence of the section Experimental Results should read "EA in the course of 20-40 min caused an increase in LP to the noxious thermal stimulus on average by 99 ± 4% (from 13.6 ± 0.6 to 27.0 ± 1.1 sec) and inhibition of the second positive component of EP on average by 43 ± 3% from the initial."