Development in vitro of mouse embryos from the two-cell egg stage to the early somite stage

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SUMMARY

About 1–3% of mouse blastocysts, which had initially been cultured from the two-cell stage in chemically defined medium or about 3–5% of blastocysts which were explanted from the uterus, developed to the early somite stage when cultured in vitro on collagen. Two-cell eggs were initially cultivated in chemically defined medium to the blastocyst stage. Blastocysts were then transferred to Eagle's minimal essential medium (MEM) plus 10% heat-inactivated calf serum. Two barriers to further development were overcome. First, the formation of endoderm and ectoderm from the inner cell mass immediately after attachment to collagen. Second, formation of the embryo proper from the embryonic region. Both barriers were overcome by using heat-inactivated human cord serum after the blastocysts hatched from the zona pellucida and attached to collagen. After attachment, embryos were cultured in MEM plus 20% heat-inactivated human cord serum which was changed daily until early somite stages. Apparently normal healthy development in vitro occurred, as judged by light and electron microscopic examination.

INTRODUCTION

Previous work has shown that mouse blastocysts attached to reconstituted rat tail collagen can develop in vitro to the early somite stage (Hsu, 1973). Two barriers were overcome which permitted development to this stage. The first (New, 1971), formation of egg cylinders (embryonic and extra-embryonic regions) from the inner cell mass, was overcome by incubating the embryos in heat-inactivated foetal calf serum (Hsu, 1971, 1972). The second, development to the early somite stage from egg cylinders, was overcome by incubating the embryos in heat-inactivated human cord serum (Hsu, 1973). This method involved two types of serum depending upon the stage of development. Recently it was found that both barriers could be overcome by human cord serum. This simplified

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Kirby (1965) observed that morulae obtained from the oviduct gave rise only to extra-embryonic membranes when grafted to extra-uterine sites. He concluded that a uterine factor was necessary for further embryonic development. Stevens (1968) found that a small proportion of two-cell (but not one-cell) strain 129 eggs developed embryonic and extra-embryonic derivatives when grafted to adult testes. The results presented here of culturing two-cell eggs also indicate that the ‘uterine factor’ postulated by Kirby is not always necessary for the development of embryos.

MATERIALS AND METHODS

A. Preparation of rat tail collagen

The protocol used is a slight modification of that of Ehrmann & Gey (1956). Collagen was prepared under sterile conditions from tails of 3-month-old McCollum strain rats. Tails were removed, scrubbed thoroughly with soap and water, and soaked in a large Petri dish containing gauze saturated with 95% ethanol. Tendons were recovered by successively fracturing the tail (beginning at the tip) with 2 large Kelly clamps and freed by shearing and pulling motions. The long tendons from each tail segment were cut and placed in a Petri dish full of distilled water. The tendons were teased into finer filaments with two pairs of fine forceps. All the tendons from one tail were then transferred to a 250 ml centrifuge bottle containing 150 ml of 1:1000, acetic acid:water, and stored for 24 h at 4 °C. Dissolved collagen was separated from the swollen fibers by centrifuging them for about an hour at 2300 rev/min and removing the clear supernatant. The extraction was repeated 3-4 times at 24-hour intervals. The supernatant was replaced each time with only half its volume of the acetic acid solution. Pooled and clarified collagen extracts were frozen at −20 °C in 50 ml aliquots. When required, the aliquots were thawed and dialyzed in cellulose tubing with several changes of demineralized water for 48 h at 4 °C. One ml of the collagen solution was used to coat a 35 mm diameter plastic culture dish. A drop of NH₄OH was put on the cover of the culture dish at room temperature for 15 min so that the ammonia vapor would neutralize the collagen and cause it to gel. Residual NH₄OH was removed by rinsing the dishes several times in sterile water. The dishes were stored with a layer of water over the collagen at 37 °C in a humidified incubator until used. The collagen-coated dishes were equilibrated with the culture medium a few hours before use.

B. Culture media and paraffin oil

Standard egg-culture media (Biggers, Whitten & Whittingham, 1971, in Methods in Mammalian Embryology, p. 101, table 6-5) was used to cultivate
Development of mouse embryos in vitro

Development of mouse embryos in vitro 237
two-cell eggs to the stage of blastocyst. Then blastocysts were cultured in Eagle's minimum essential medium (MEM, Microbiological Associates Inc.) supplemented with antibiotics and 1 mM pyruvate.

Paraffin oil was purchased from the Fisher Scientific Co. (white, light, laboratory grade, Saybolt viscosity 125/135). 50 ml of paraffin oil was saturated with 5 ml of chemically defined medium by bubbling with the gas mixture of 5% CO₂, 5% O₂ and 90% N₂ for 30 min. This gave a fine emulsion at room temperature, which was stored overnight. The next day the oil-medium mixture was centrifuged and the oil layer saturated with medium was removed and stored at 4 °C for use during the ensuing week.

The stock solution of chemically defined medium was prepared from doubly distilled water. Chemically defined medium was made from the stock solutions in each experiment and gassed for 30 min with a mixture of 5% CO₂, 5% O₂ and 90% N₂.

C. Culture from two-cell eggs

Random-bred Albino Swiss mice, CF 1, from Carworth Farm, New York, U.S.A., were used.

Two-cell mouse eggs were collected and prepared according to established procedures (Brinster, 1963; Biggers, Whitten & Whittingham, 1971; Whitten, 1971). Female mice were superovulated by injecting 5 i.u. of pregnant mare serum gonadotrophin followed 48 h later by 5 i.u. of human chorionic gonadotrophin (HCG). These mice were caged overnight with males of proved fertility. Insemination was verified the next morning by the presence of a copulatory plug in the vagina. Two-cell eggs were collected by flushing the oviducts of the inseminated females with chemically defined medium (Biggers, Whitten & Whittingham, 1971) approximately 48 h after HCG injection. Groups of fifteen to thirty eggs were grown in a drop of the same medium under 2 ml of paraffin oil in a 35 mm diameter plastic culture dish. These culture dishes were incubated at 37 °C in a bacteriological anaerobic jar, as recommended by Whitten (1971), in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. After 3 days the developing blastocysts were transferred to MEM supplemented with 10% heat-inactivated calf serum (Grand Island Biological Company, New York). MEM was added, drop by drop to avoid rapid change of culture media, to the chemically defined medium containing the eggs under paraffin oil. About 40 blastocysts were transferred to a collagen-coated culture dish which contained 2.5 ml of MEM plus 10% heat-inactivated calf serum.

D. Culture from blastocysts

Blastocysts were flushed from the uterine horns with MEM plus 10% heat-inactivated calf serum approximately 81 h after injection of HCG. Blastocysts from 15 to 20 mice were collected and pooled in an embryological watch-
glass and then transferred to fresh medium. Groups of 30–50 embryos were placed in 35 mm diameter plastic culture dishes coated with reconstituted rat tail collagen. The blastocysts were incubated initially with 2.5 ml of MEM supplemented with 10% heat-inactivated calf serum in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

On the second day of incubation, when the blastocysts attached to the collagen, 10% heat-inactivated human cord serum was added. From the third day of incubation until the early somite stage, which occurred after 9 days from blastocyst, the medium of MEM plus 20% heat-inactivated human cord serum was changed every day. The importance of changing medium every day has been previously described (Hsu, 1973). Human cord serum was obtained from placenta at the delivery room and stored at −60 °C after separation by centrifugation.

It may also be important to warm the MEM medium to 37 °C in a water bath before use.

Embryos which had developed in vitro were fixed in Vandegrift’s solution, serially sectioned at 7 μm and stained with hematoxylin and eosin.

Electron microscopy. Embryos were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% buffered osmium tetroxide, dehydrated in
RESULTS

After three days of incubation in chemically defined medium, more than 95% of the two-cell eggs developed to the blastocyst stage. The blastocysts were transferred to MEM plus 10% heat-inactivated calf serum, where they began to shed the zona pellucida. Soon the denuded blastocysts lay flat on the surface of the collagen, and the trophoblast invaded the underlying collagen leaving the rounded inner cell mass protruding from the surface.
The invading trophoblast underwent giant-cell transformation, while the inner cell mass grew rapidly to form egg-cylinders. The egg-cylinder grew out from the original inner cell mass which was fixed by trophoblastic cells to the collagen. The elongated egg-cylinder consisted proximally of the extra-embryonic region and distally of the embryonic region. The embryonic region of the egg-cylinder developed into the embryo proper, while the extra-embryonic region gave rise to yolk sac which partially enveloped it. Histological sections of the egg-cylinders which developed in vitro have been compared with those developed in vivo (Hsu, 1972).

One of the embryos which developed in vitro is shown in Fig. 3A and histological sections of embryos grown in vitro are shown in Figs. 1, 2, 3B and 4.

Histologically the embryo shown in Fig. 3B was equivalent to about 8½ days of gestation. It appeared to be composed of normal healthy tissues. The neural tube was wavy, but otherwise well formed, with paired somites alongside. The walls of the fore and hind gut were thickened and formed deep pockets.

The amnion, yolk sac, and allantois had normal relationships with embryonic parts (Figs. 1, 2 and 3B).

The trophoblast invaded the underlying collagen and only the inner cell mass developed into embryos and yolk sac. It had appeared formerly that trophoblastic giant cells, Reichert's membrane and parietal endoderm were absent.
Development of mouse embryos in vitro

Fig. 4. Region of attachment to collagen of an embryo cultured from blastocyst for 9 days on collagen. Note trophoblastic giant cell (tgc), and distal endoderm (de) cells surrounding a clump of Reichert's membrane material (rm) secreted by them. \( \times 300 \).

(Hsu, 1972). However, more favorable histological material prepared at the Jackson Laboratory, Bar Harbor, Maine, showed that the region of ectoplacental cone which attached to the collagen contained trophoblastic giant cells, a residue of Reichert's membrane secreted in solid clumps and thick sheets, and parietal endoderm as shown in Fig. 4. Reichert's membrane and the parietal endoderm developed only in the ectoplacental cone region where the collagen attached, and not around the yolk sac and embryo.

The visceral endoderm of the yolk sac was fully differentiated with many microvilli protruding from the entire surface of the cells (Fig. 5). The microvilli are probably both absorptive and secretory and necessary for the growth of egg-cylinders.

Further evidence for apparently normal development is demonstrated in the developing heart (Fig. 6). Myofibrils appear normal in longitudinal cross-section and characteristic pools of glycogen can be discerned.

More than 95 % of the two-cell egg developed into blastocysts and attached to the collagen by this method, of which about 50 % of blastocysts differentiated endoderm, 10 % formed egg-cylinders or yolk sac without containing embryo proper, and only 1–3 % of the original two-cell egg developed embryos.
Fig. 5. An electron micrograph of yolk sac showing microvilli from endoderm. The electron micrographs of Fig. 5 and Fig. 6 were made at the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland. $\times 8000$. 
Fig. 6. Heart cells containing myofibrils (m). Note the pools of B-glycogen particles (gl), characteristic of cardiac tissues. Section stained with lead citrate (x 11000). Inset: cross-section of myofibril demonstrating hexagonal array of thick and thin filaments. x 60 000.
Fig. 7. Increased volume of inner cell mass when human cord serum is used for cultivation for six days from the stage of blastocyst. No embryos developed beyond this stage. Trophoblastic giant cells are on one side (lower left). \( \times 300 \).

**DISCUSSION**

Cleavage of mouse eggs has been accomplished *in vitro* and the resulting blastocysts developed into normal progeny when transplanted into foster mothers. A small proportion of two-cell eggs of strain 129 mice developed intra- and extra-embryonic derivatives when they were ectopically transplanted to adult testis (Stevens, 1968). Results presented here show that blastocysts from the uterus and those cultivated *in vitro* from two-cell eggs have similar developmental potentialities.

Human cord serum has been used after the blastocysts erupt from the zona pellucida. Human cord serum before that stage increased the volume of the inner cell mass after attachment to the collagen, but no further embryo development was observed beyond this stage (Fig. 7). Several lots of human cord serum from different individuals were used with consistent results. This may indicate
Development of mouse embryos in vitro

that human cord serum is beneficial to mouse embryos only after a specific period of development and not before. This coincides with the time when estrogen and progesterone are essential for specific stages of implantation and subsequent development of mouse embryos.

Although the hormonal activity of foetal calf serum and human cord serum are not well understood, steroid hormones may stimulate target cells to release factor(s) which are similar to those in the embryonic milieu.

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