Establishment of a Cell Line (XTC-2) from the South African Clawed Toad, *Xenopus laevis*

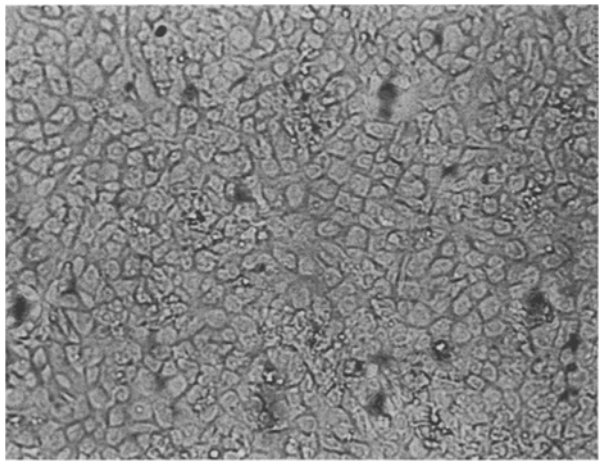
The culture of cells from poikilothermic animals offers a unique opportunity to study the growth of homeothermic vertebrate viruses (e.g. arboviruses) in host cells phylogenetically far removed from the normal hosts and at temperatures below those they are subjected to in their normal hosts[1]. Several cell lines have been established from *Rana* spp. (WOLF and QUIMBY[2], RAFFERTY[3,4]) and from adult tissues of *Xenopus laevis* (RAFFERTY[4]). Further cell lines from this species were established by ARTHUR and BALLS[5], but details are yet to be published. We describe below the establishment of an additional cell line from *Xenopus laevis* with a view to its potential application in studies on arbovirus multiplication in cells in vitro.

A *Xenopus laevis* tadpole/toad, with 4 legs and half digested tail was used for setting up the culture. It was surface-sterilized by leaving overnight in 100 ml sterile distilled water containing 1000 units of penicillin, 1 mg streptomycin and 5 µg fungizone per ml, and 0.1 ml of anti-PPLO agent (Grand Island Biological Company, New York) in a sterile beaker covered with aluminium foil. The following day the tadpole/toad was washed 3 times in sterile distilled water and twice in phosphate buffered saline (PBS). The intestines, eyes, skin and tail were then removed. The carcass was cut up and washed 3 times in a Ca and Mg free trypsin diluent[6], and the pieces put in 8 ml of 0.25% trypsin solution (Difco, 1:250) and stirred on a magnetic stirrer at room temperature for 10 min and the supernate discarded. A further 8 ml of trypsin solution was added and stirring continued for another 10 min. The supernate, after mixing with a pasteur pipette, was removed to a container. A total of 32 ml of trypsin solution was used in this way, and the cell suspensions from each trypsinisation 'run' were pooled and kept at 4°C until digestion of the *Xenopus* pieces was complete. The suspension was filtered through sterile gauze to remove the larger tissue clumps and centrifuged at 800 rpm for 8 min. The cells were washed in 6 ml of PBS and respun. They were then suspended in 4 ml of medium in a 1 oz medical flat and incubated at 28°C ± 1°C.

The medium based on that of AUCLAIR[7] has the following composition: NCTC 109 (Wellcome Research Laboratories, Beckenham) 100 ml; glass distilled water 50 ml; lactalbumin hydrolysate (Difco) 50 mg; glutamine 5% (Difco) 0.6 ml; $NaHCO_3$ (saturated solution) 2 ml. The pH is adjusted to 7.5 with 2% KOH, and the medium sterilized by passing it through a sintered glass filter. The medium is completed by the addition of heat-inactivated foetal calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland) to give a final concentration of 10% of serum, and antibiotics (1000 units penicillin and 1 mg streptomycin per ml) just before use.

The culture was set up on 13 March 1969, and 4 days later the surface of the bottle was covered with clear fibroblast-type cells. The cells were washed with medium to remove any debris and 4 ml of fresh medium put into the bottle. On the 6th day of culture when the bottle was covered with a complete layer of fibroblast-type cells with occasional small islets of epithelial cells, the first subculture was carried out. The cells were washed twice with trypsin diluent containing antibiotics and then 1 ml of 0.05% pronase (Calbiochem, grade B) in trypsin diluent was put in the bottle, left in contact with the cells for 1 min, removed and the film of pronase left for 1 further min. 8 ml of medium were then put in and the cells removed from the glass by scraping with a sterile rubber-covered glass rod (rubber policeman). After suspending the cells in the medium by pipetting, they were seeded into two 1 oz bottles. 5 subsequent subcultures were carried out at intervals of 2, 3, 4, 6 and 7 days during which the degree of division was increased from 1:2 to 1:5, and the volume in each bottle reduced to 3 ml. The cells at this stage were still fibroblast-type with occasional patches of epithelial-type cells. Further subcultures were carried out at weekly intervals with a subdivision of 1:3 or 1:4. At the 8th subculture the total time for the pronase to act was reduced to 1 min. In subsequent subcultures, the use of pronase was completely omitted and the cells released from the glass surface by scraping down with a rubber policeman. By the 10th subculture the culture consisted mainly of large epithelial-type cells and some of the bottles were transfered to an incubator at 24° ± 1°C. By June 1969 when the cells in this series were at the 13th subculture the temperature of this incubator was reduced to 22°C and the cultures have been maintained at this temperature since. The line has subsequently been readapted to 28°C and currently it is being maintained in parallel at 22°C and 28°C.

The cells are subcultured once a week, usually by 1:2 to 1:10 division. They were taken through 19 subcultures in medium without antibiotics to detect the presence of latent bacterial contamination. The cultures remained healthy. The cells can be grown in petri dishes in open gas exchange with the atmosphere, despite the initial increase in pH. The cells are now at the 171st subculture level. During the early stages of growth or when a confluent monolayer has not formed, the cells appear spindle shaped, but the closely packed cells of a confluent monolayer present an epithelial-type morphology (Figure). There is a 7-to 8-fold increase in cell number during 5 days of growth. After about a week's growth, rounded



Xenopus laevis cell line (XTC-2). 74th subculture. 10 days after seeding at 22°C. Live. × 75.

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floating cells are seen, probably due to overgrowth. Further overgrowth results in retraction and rolling up of the cell sheet. The majority of the cells have the diploid ($2n = 36$) number of chromosomes. They have been stored in 10% dimethyl sulphoxide in medium in liquid nitrogen (–196°C) and successfully resuscitated. Studies on the infection of the cells with arboviruses are in progress.

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Résumé. On décrit une méthode pour l'établissement d'une culture de cellules du crapaud a griffes sudafricain, *Xenopus laevis*, afin de l'infecter avec des arbovirus. Les cellules (XTC-2) ont subi une culture continue pendant 3 ans. Elles ont un nombre diploïde de chromosomes. Elles furent cultivées aux températures de 22°C et de 28°C dans un milieu de culture dilué de NCTC 109 avec 10% de sérum de veau foetal.

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Extended Survival of the Chick Embryo in vitro

Recent work has suggested that warm-blooded central nervous tissues, cultured in isolation under in vitro conditions, preserve many of the functional properties responsible for normal prenatal behavior patterns in situ[1]. It would be desirable to compare this development with that of similar preparations cultured in a more natural environment. Chorio-allantoic transplantation[2] offers optimal conditions, at least for avian tissues, but since its use in ovo would place severe restrictions upon physiological experimentation it was decided to adapt this method for use in vitro. Techniques for culturing chick embryos in vitro with preservation of the extra-embryonic circulation are in wide use now, using egg albumin as a source of nutrients, but survival has been for only a few days[3,4]. Because methods which utilize the yolk together with the albumin offer much improved survival[5,6] we chose these as the starting point for devising a suitable long-term culture system.

Fig. 1. Photograph of a cultured embryo, explanted after 2 days of incubation in ovo, which attained normal stage 41 after development for 15 days in vitro.

Methods. Fertilized white leghorn chicken eggs were incubated in ovo for 2 days at 38–39°C and about 65% relative humidity. This pre-incubation prior to transferring the embryos to in vitro conditions (using sterile precautions) improved the percentage of preparations attaining advanced stages of development. Specially designed dishes were used for this study, of such shape and size that the egg contents largely filled the vessel, with the yolk sinking down to occupy the bottom portion. The outward tapering walls of the dish ensured that the albumin formed a pool which submerged the germinal area, and that a large area ($\pm$ 8 cm in diameter) would be available for gas exchange at the upper surface. These geometric considerations are apparently critical for survival of these embryos within impermeable containers (R. DEHAAN, personal communication). The preparation was covered with a sterile plastic petri-dish and placed inside a polyethylene bag, which was then closed off tightly. All cultures were transferred to an incubator for premature babies, maintaining the same temperature and humidity as during the initial incubation in ovo, and in which a specially constructed rack enabled easy daily individual examination.

Results. A first wave of mortalities struck at stage 25 and lasted through stage 30[7], claiming almost 50% of the cultures. Of 37 cases followed thereafter, only 1 death occurred before stage 34, but 21 preparations were then lost between stages 35 and 38. The remaining 15 all attained embryonic stage 40, and 5 of these survived into stage 41 (Figure 1). The earlier fatalities were correlated with the degree of yolk leakage but the final wave of deaths affected many cases where the yolk sac appeared intact and the albumin transparent. The most clearly relevant differences from the natural in ovo situation

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