THE *IN VITRO* CULTURE OF EMBRYONIC CHICKEN HEART CELLS

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In the early 1900's, Burrows [1] and Carrel [2] reported the serial cultivation of cells derived from an adult chicken heart. Since that time, numerous laboratories have experienced difficulties in long-term avian cell culture. After many unsuccessful attempts, with several modifications in technic, embryonic chick heart cells were established and maintained in this laboratory as monolayer cultures. Two such strains were originated independently; they are designated as CH_{11} and CH_{16} respectively, and are in the 41st and 28th subculture at the time of this writing.

MATERIALS AND METHODS

Twelve six-day embryonic chick hearts were removed and washed in Eagle's medium containing penicillin, streptomycin, and 15 per cent *unfiltered* bovine serum (serum prepared under sterile conditions without filtration by Hyland Laboratories, Los Angeles, Calif., and retested for sterility before use). Hereinafter, this mixture is referred to as nutrient medium. After washing, the intact hearts were suspended in 25 ml 0.25 per cent trypsin solution dissolved in calcium and magnesium free salt solution (1 liter 20 × solution: 0.024 g phenol red, 160 g NaCl, 8 g KCl, 0.9 g Na_2HPO_4, 0.6 g KH_2PO_4, to which 0.1 per cent methylcellulose was added. The hearts were agitated in a Bellco spinner flask (Bellco Glass Company, Vineland, N.J.) on a magnetic stirrer for 20 min at 37°C. The resulting suspension was centrifuged at 300 *g* for 5 min, decanted, and the packed cell sediment was resuspended in 15 ml nutrient medium. Aliquot parts of this suspension were used to inoculate three 32-oz bottles which contained 45 ml nutrient medium. These were incubated at 37°C. Cell counts were not performed prior to inoculation. Various lengths of agitation and various concentrations of trypsin were tried and the above conditions were found to be optimal. In controlled experiments, isolation of cells was not achieved if the methylcellulose was omitted in the trypsinization procedure. Media in which the unfiltered fetal bovine serum was substituted by filtered fetal bovine serum, made

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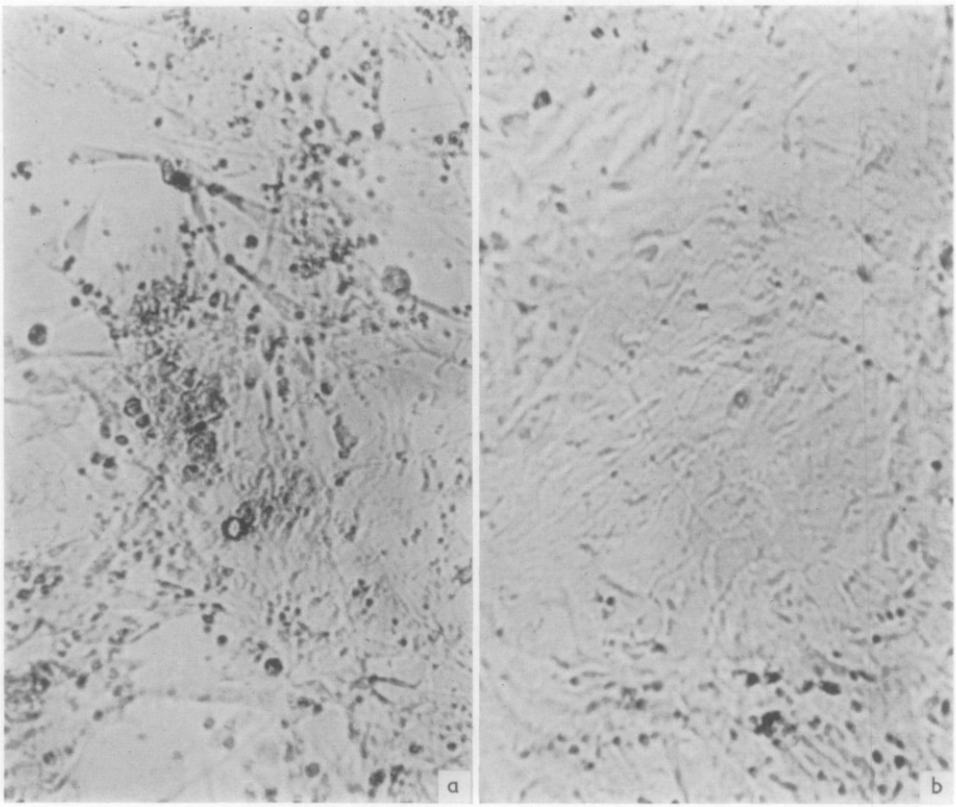

Fig. 1.—(*a*) Unwashed fibroblast monolayer culture of CH_{11} cell strain (× 200). (*b*) CH_{11} cell strain after washing with trypsin and methylcellulose (× 200).

primary isolation of chick heart cells impossible. It also impaired the propagation of cells already isolated.

Cell propagation was improved by gently washing the cells with 10 ml trypsin solution containing methylcellulose the second day after transfer. The trypsin solution was very gently agitated over the cells for about 30 sec and poured off. This resulted in a clean clear sheet of fibroblasts that multiplied rapidly (Fig. 1).

Subcultures were performed approximately twice a week and occasionally three times per week. Our method of subculture utilized part of the supernatant (conditioned) media of the propagating cell line. This medium was decanted into a sterile container, the cells were trypsinized approximately 2–4 min, and the cell suspension was centrifuged for 5 min at 300 *g*. The trypsin was decanted and 15 ml of the conditioned medium was added to the packed cell sediment. The packed cells were then washed and resuspended and 15 ml nutrient medium was added. Aliquots were placed in 2 clean 32-oz bottles and the volume was brought to 50 ml with nutrient medium.

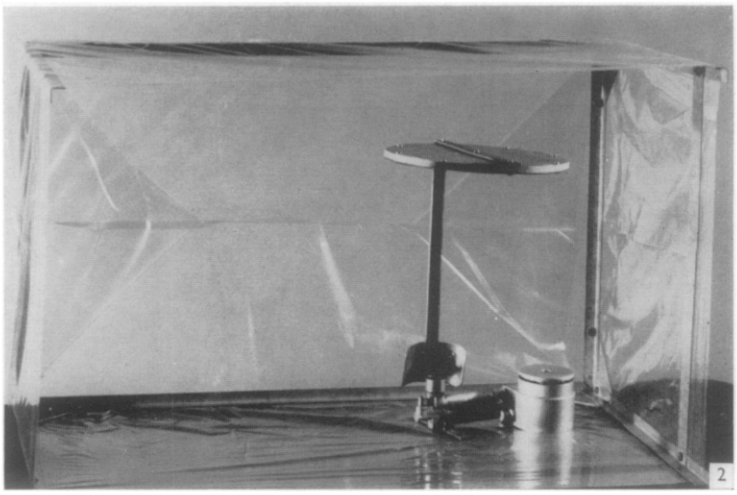

Fig. 2.—Disposable hood illustrating plastic cover, metal frame and shield for the microburner.

Only when the cells were handled in this manner were the subcultures predictably successful. If the cells were divided equally into three bottles at subculture, growth was poor or failed completely. Therefore, the approximate number of generations for these strains is roughly equal to the number of subcultures. For histologic examinations, cells were grown in tubes from which they were later removed by the collodion method [5].

In order to avoid cross-contamination of cell lines, a disposable hood was constructed. It consisted of a metal frame, 1/2″ galvanized angle iron, covered with a light gauge disposable plastic such as used in the dry cleaning industry (A. J. M. Enterprises, Inc., Detroit, Mich.). The dimensions of the frame are 15 in $\times$15 in $\times$25 in. The frame can be easily sterilized either by autoclaving or by washing with alcohol or antiseptic solution. The open side of the plastic cover faces the technician. A shield had to be devised for the burner in order to protect the plastic cover. Fig. 2 illustrates the disposable plastic cover, shield and metal frame. Since we began using this hood, cross-contamination of cell lines has been eliminated as demonstrated by chromosome monitoring. It is now used routinely for all cell lines.

These cell strains have been frozen, stored at -170°C, thawed and replanted several times according to the method of Stulberg, *et al.* [14]. Consistent recovery has been obtained.

Strain CH_{11} was isolated on May 7, 1963, and passed through 14 subcultures (63 da). A substrain was then frozen for 269 days, thawed, and carried to the 41st subculture (97 da). Strain CH_{16} was isolated on May 20, 1964, and cultured continuously for 28 passages (116 da).

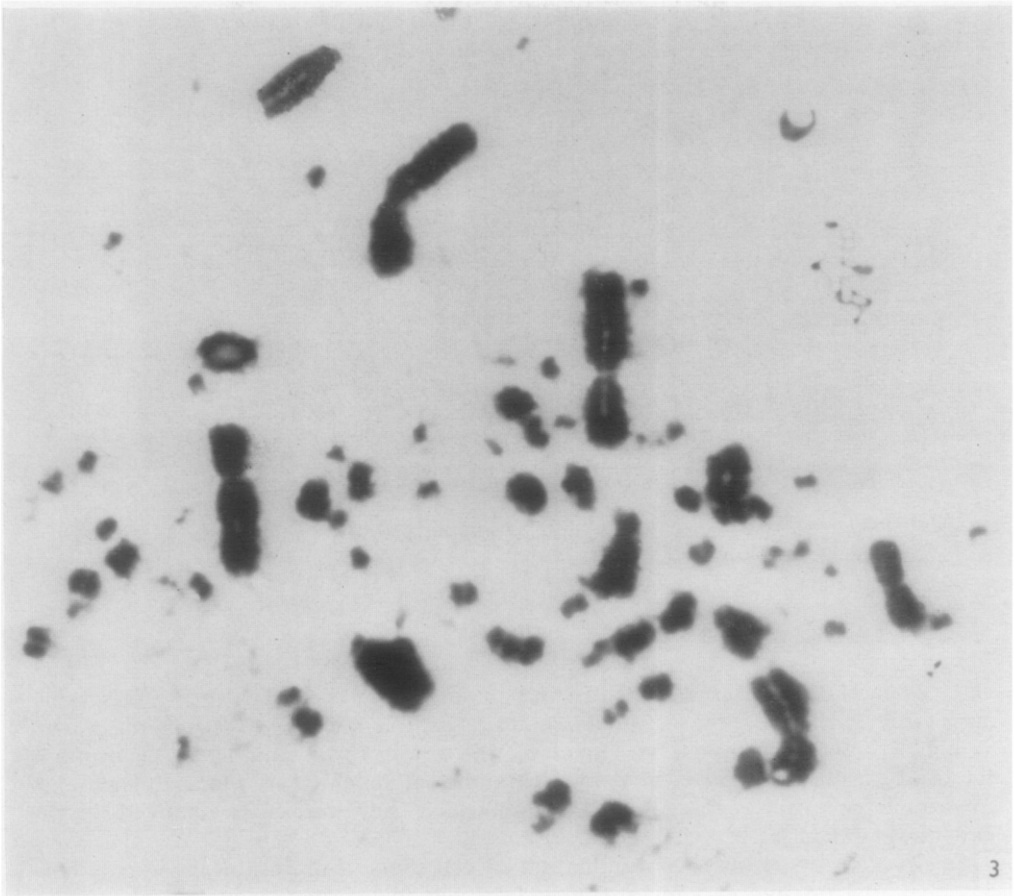

Fig. 3.—Photomicrograph of dividing fibroblast of CH_{11} cell strain at the 15th subculture. Note macro- and microchromosomes.

RESULTS

The cells grew into a complete sheet of heavily granulated fibroblasts. They were transferred successfully by trypsinization and, during this procedure, it was noted that the volume of packed cells obtained from each bottle appeared small in relation to the amount of growth which had occurred. This was due to the large number of clear fibroblasts which were still attached to the glass in each bottle after trypsinization. When nutrient medium was added to these cells, they formed a complete sheet in about 1 week.

The strains which developed were of avian origin as attested by chromosome studies. These studies were made on strain CH_{11} at the 6th, 8th, 13th, 15th, and 18th subcultures and on strain CH_{16} at the 8th subculture. In each case, the dividing metaphase cells revealed both macro and microchromosomes which is typical of the karyotype of *Gallus domesticus* [11]. The chromosomes are depicted in Fig. 3. Although accurate counts were not obtained, most of the cells contained 10 to 14 macrochromosomes and 100 to 200 microchromosomes.

Pulsation of the cells was observed for 21 days and after 2 transfers in strain CH_{11}. The pulsations were 120 beats per min which corresponds with the pulse rate of adult chicken hearts *in vivo*. The contractions were synchronous. They were rhythmic and regular for the first 9 days. Then they became irregular and only some of the cells pulsated until finally no pulsations were recorded after the 21st day. Pulsation of chick heart cells *in vitro* have been reported by several authors [8, 13].

DISCUSSION

Prior to this report, only a few avian cell cultures have been successfully established. Carrel and Ebeling's chick embryo fibroblast line [3] was successfully maintained for 34 years. Prier and Sullivan [12] described a strain established from 45 pooled chick embryo hearts which was fibroblastic in appearance during the first five subcultures but then transformed into an epithelioid strain. The latter strain supported the growth of polio virus while the original fibroblasts did not. They could not rule out contamination with a mammalian cell line. In 1963, Hisuchi [7] reported an established line derived from a whole chicken embryo. Their report, however, does not include either chromosome identification or mixed agglutination tests for species specificity. Melendez and Hanson [9] attempted to establish cell strains from 99 trypsinized embryonic chick hearts. Of these, 97 did not pass the 10th subculture, one was lost at the 13th transfer, and one has survived 28 passages. They were able to freeze, thaw and recover this latter strain and it was determined to have an apparent diploid complement of *Gallus domesticus* chromosomes.

One of the basic problems we encountered in our laboratory was cross-contamination of the chick heart cell strains with mammalian cells. Contamination with human cell lines was demonstrated using the mixed agglutination tests of Franks *et al.* [6]. This contamination occurred despite the fact that the avian strains were handled by technicians who did not work

with mammalian cells and the media and trypsin were specially marked. Coriell [4] demonstrated that from an artificially produced aerosol spray of cells, viable cells could be recovered. Apparently, under ordinary laboratory conditions, aerosol cell sprays might occur and be responsible for the cross-contamination. This problem led to the development of the disposable hood.

Merchant [10] has shown that methylcellulose increases the number of viable cells in harvesting and transplanting cells. It supposedly acts by protecting the cell surface and by influencing the aggregation of cells. Since no growth was obtained with filtered fetal bovine serum, it is apparent that one or more components of the serum are removed by the filtration or that the concentration of some factor is being reduced. The filtered serum did not produce growth in any concentration. Further studies are anticipated to determine the nature of these components.

SUMMARY

Modifications of mammalian cell culture technic have resulted in the successful development of embryonic chicken heart cell strains. These include (1) the addition of methylcellulose to the trypsin solution, (2) *unfiltered* fetal bovine serum in the nutrient medium and (3) the use of a disposable plastic hood to prevent cross-contamination with other cell lines. These cells have been successfully subcultured 41 times and recovered after storage at −170°C.

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