New Medium and Conditions for the Culture of Monolayers of Agallian Leafhopper Cells Used in Studies of Certain Plant Viruses

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ABSTRACT

Established cell monolayers of *Agallia constricta*, an insect vector of certain plant viruses, were used to determine what concentrations of the medium components used by Chiu and Black were optimal for growth. Optimal pH and osmotic pressure were also determined. As a result, a new medium was prepared in which the cells grew more rapidly, were more susceptible to inoculation, and yielded more virus.

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The study of certain plant viruses that multiply in their insect vectors has been facilitated by bioassay of the viruses on tissue cultures of vector cells (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14). Extracts containing most such viruses cannot be inoculated directly to plants. One of the few that can be so inoculated gives about 5,000 times more lesions on vector cell monolayers than it does on leaves, on the basis of the number of cells exposed to inoculation (6); it gives countable lesions in two days on monolayers and in two wk on leaves. The technique may also prove to be advantageous for the study of mycoplasma-like and rickettsia-like pathogens causing disease in plants.

Research with this technique has been retarded because of the difficulty of isolating and growing cells of leafhoppers and aphids in tissue culture. Chiu and Black (2) succeeded in establishing several agallian leafhopper cell lines in culture using a modification of the medium developed by Schneider (16) for the maintenance of *Drosophila* cells. Subsequently the medium of Chiu and Black was used to establish cell lines from other leafhopper species (15, 17). Nevertheless, in our laboratory, with the medium of Chiu and Black, we experienced some difficulties during the subculture of agallian leafhopper cells that induced us to make a major effort to improve the medium.

Extensive tests resulted in radical changes in the medium. The best growth of the agallian cell line, AC20, was obtained at 28 C, a pH of 6.43, an osmotic pressure of 360 mosM and component concns per liter of $CaCl_2 \cdot 2H_2O$, 0.36 g; $MgSO_4 \cdot 7H_2O$, 1.2 g; KH_2PO_4, 0.27 g; KCl, 1.6 g; NaCl, 1.0 g; $NaHCO_3$, 0.9 g; dextrose, 9.0 g; lactalbumin hydrolysate, 10.0 g; yeast hydrolysate, 8.0 g; histidine-HCl (monohydrate) 4.8 g; histidine (free base), 3.45 g; and 100 ml of fetal bovine serum (heat-treated at 56 C for 30 min). The new medium is strongly buffered at the optimum pH by the histidines, even at lower concns than those above, but the histidine concns given, produced better growth than higher or lower concns at the same pH. Different commercial batches of certain components such as fetal bovine serum, lactalbumin hydrolysate and histidine were sometimes unsatisfactory and new batches always should be tested for their suitability before being used. With different batches of components the ratio of the two histidines may need to be slightly changed to get the same pH. Sometimes the pH becomes slightly more alkaline in storage; it may be adjusted to the correct pH with small volumes of 2.0 N HCl and, if necessary, refiltered.

Two different antibiotic formulas have been used satisfactorily with 1 liter of this medium: (i) penicillin G, 100,000 units; streptomycin, 100 mg; and neomycin, 50 mg; and (ii) Gentamicin, 50 mg. The first antibiotic formulation is much more economical and is the only one we have used extensively, but the second one has proved very useful for certain purposes; e.g., the elimination of certain contaminations from cell lines when used at a concn of 200 mg per liter (H. T. Hsu, *personal communication*).

Various methods of preparing the medium may result in the formation of irreversible precipitates. To prevent this the $NaHCO_3$, KH_2PO_4, and the histidines were first dissolved one after another in somewhat less than half the total volume of glass-distilled water. A second vessel containing about 300 ml of water for each liter of medium was prepared and to it $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KCl, NaCl, dextrose, antibiotics, yeast hydrolysate, fetal bovine serum, and lactalbumin hydrolysate, were added in that order and incorporated in the solution. After mixing the two solutions and adjusting to final volume with water, the medium was sterilized by Millipore filtration. The medium deteriorates if stored at 25 C but can be stored at 4 C for at least 6 mo without detectable ill effects.

In previous work a concn of 0.05% trypsin in Rinaldini's solution at pH 7.5 was used for harvesting cells to be transferred (2). An improved transfer procedure using papain was developed. At the time of subculture, the insect cells were washed with a solution each liter of which contained: KCl, 3.2 g; NaCl, 2.0 g; K_2HPO_4, 0.69 g; dextrose, 24.36 g; disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA), 0.38 g; cysteine, 0.88 g; histidine-HCl (monohydrate), 2.39 g; histidine (free base), 5.21 g and the same concn of antibiotics as the medium. The solution had a pH of 6.4 and an osmolarity of 360 mosM. After the monolayers had been washed, the cells were detached by treating for about 5 min with the same solution containing twice-crystallized papain (currently available at about $15 per 100 mg) at a concn of 0.01 mg per ml. The activity of this papain solution was still satisfactory after storage for 1 mo at 4 C. The mild action of papain, an enzyme that can be used to detach cells at the optimal pH for the growth of

most cultured insect cells, may make it very useful in work with other insect tissue cultures.

After confluence into a monolayer the cells in a flask were counted (12), detached by treatment with the papain solution and about 2×10^6 of the harvested cells were transferred to each new flask in 4 ml of fresh medium. After one and three days the average number of cells in the flask was about 2.4×10^6 and 4.2×10^6, respectively. Between 24 and 48 h, 48 and 72 h, and 24 and 72 h at 28 C the average doubling times were about 38 h, 106 h, and 55 h, respectively. When AC20 cells were in good condition, subcultures were commonly made by transferring cells from one flask to two every 3 days.

In comparison with the medium of Chiu and Black (2) who reported a doubling time of about 86 h at 27 C the new medium resulted not only in shorter doubling times, but noticeably more cells in mitosis, an improved cell appearance, and a reduction or virtual elimination of cell debris; furthermore, in the new medium the cells were more susceptible to virus inoculation and yielded more virus. The improved growth was consistent during more than 40 subcultures.

The new medium and conditions have also improved the growth of a cell line of *Agalliopsis novella* (Say) established by Windsor (17) and when adjusted to pH 6.30, has improved the growth of cell lines of *Aceratagallia sanguinolenta* (Provancher).

Our studies were facilitated by the use of a cell-counting technique devised for the phase contrast microscope (12). This counting technique has since been improved for the AC20 cell system so that the nuclear count can be converted directly to number of cells per flask by the use of a conversion table that takes into account the changing area of the nuclei during the active period of growth.

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