Collagenase in Equine Cell Culture Preparation

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Equine kidney cells disaggregated by treatment with 0.01% collagenase were used in the preparation of primary monolayer cell cultures. The primary cells could be stored for long periods in liquid nitrogen and subsequently subcultivated. These techniques provided a long-term supply of equine kidney cells, free of apparent contamination, from the kidneys of a single fetus.

Viruses are usually best isolated from infected animals in primary or low-passage diploid cells from the natural host of the virus. The literature on equine viruses indicates that often this procedure was not followed in experimental studies, but that nonequine cell culture systems were used. The main reason for this is that equine cells are not easily cultured in the laboratory by routine methods.

Except in certain parts of France and Belgium, horses are generally not slaughtered at a young age for meat, unlike other domestic animals, and a reasonably priced and regular supply of fresh healthy equine organs for cell culturing is rarely available. But horses are castrated at a young age, and horse testis cultures could be used for studies of equine viruses. Also, fresh organs could be obtained from young healthy horses sacrificed because of irreparable accidental traumatism, or foals could be specifically bought as organ donors. These possibilities suffer from a major handicap, however. Latent infections with equine herpesvirus type 2 (EHV-2) are widespread in horse populations, especially in young horses. These latent infections become unmasked in cell cultures, quite often after several subpassages of cells or virus material. As suggested by Studdert (21), this problem can be avoided by using fetal equine organs, since EHV-2 does not seem to pass the placental barrier of the mare.

A little-known difficulty in the culturing of equine cells is a peculiar reaction of horse tissues to digestion by trypsin. It is a common observation that a mucoid material is liberated from mammalian cells by trypsin treatment (23), but equine tissues produce so much of this that it is impossible to sediment the isolated cells by centrifugation. Dilution of the trypsin with saline may lower the viscosity enough for depositing the cells in the centrifuge, but manipulations are increased and cell yields are low. Substitution of

collagenase as the cell-dispersing agent solves the problem, since this proteolytic enzyme acts essentially on the intercellular matrix and less on the cell membrane.

Collagenase has been extensively used by biologists, embryologists and histologists, but seldom by virologists. Technical manuals on virology generally ignore it, or at best mention its applicability without giving the practical details (12); thus, cell dispersion with collagenase is not yet a standard virological procedure. Yet collagenase is commercially available at a reasonable price and in adequate purity; it is easy to use and gives excellent results.

Collagenase or clostridiopeptidases A (EC 3.4.24.3) are enzymes that hydrolyze native collagen at or near physiological pH. They are found in animal tissues and in cultures of several bacterial species. The commercial product is a bacterial exoenzyme obtained from filtrates of *Clostridium histolyticum* broth cultures by precipitation with ammonium sulfate and dried by lyophilization. Crude preparations contain several physiochemical forms of collagenases, besides small amounts of other proteases and peptidases. The bacterial enzyme is active at pH 6 to 8, the optimum depending on the buffer in the solution (10). Tris(hydroxymethyl)amino-methane, phosphate, borate, and Veronal buffers have an optimum at pH 7.4 to 7.6.

Lyophilized collagenase can be stored at 4°C for years without loss of activity. In solution, stability depends on the type of buffer, pH, concentration, and temperature. Under laboratory, i.e., physiological conditions, collagenase is remarkably stable. Collagenase solutions may be kept frozen for several months without significant loss, and at 4°C for at least a month. Even at room temperature or at 37°C, the enzymatic activity lasts for several days (10). For longer periods at ambient temperature, Mandl (10) found better preservation in water than in phos-

phate buffer. This observation, although irrelevant for the short digestion time used for cell dispersal, seems to have been the motive of some to chose GKN as solvent for the enzyme, a solution which contains NaCl, KCl, and glucose at concentrations of Hanks balanced salt solution (1, 6, 15). Collagenase in this solution may give acceptable digestion of tissues, but the diluent is not recommended, since it lacks calcium ions which are required for both the binding of the enzyme to the substrate and for full enzymatic effectiveness (10, 11, 19). Calcium ions also assure the stability of the enzymatic function. As a consequence, calcium-chelating agents such as ethylenediaminetetraacetate (EDTA) are incompatible with collagenase. Magnesium ions cannot substitute for calcium ions (20). It is better and simpler to dissolve collagenase in standard tissue culture solutions (5, 7, 14, 22).

Enzymologists measure the activity of collagenase in units, but these are determined by various methods; thus, the unit values of commercial preparations have only a relative meaning. The tissue culture literature gives the collagenase concentrations usually in terms of the dry weight of collagenase per volume of solvent. Data vary from 0.4% (5, 14) to 2% (4, 7, 8), 0.01% (1, 6, 15) and 0.00025% (22).

The basic principle, manipulations and equipment for tissue trypsinization apply equally to cell dispersion with collagenase. Since detailed trypsinization techniques have been described (12, 18, 24) and virus laboratories have developed their preferred variations of the technique, only an outline of our procedure is given here, emphasizing the essentials and pointing out the critical steps. The following solutions are used:

(i) Eagle minimum essential medium (3) with Hanks balanced salt solution (HMEM) or with Earle's balanced salt solution (EMEM) are obtained from commercial sources. The dry powders are dissolved according to the manufacturer's instructions. (ii) Growth medium is HMEM with 10% newborn or fetal bovine serum and the usual antibiotics (pH 7.4). (iii) Maintenance medium is EMEM with 2 to 5% fetal bovine serum and antibiotics, adjusted to pH 7.6 with a 7.5% NaHCO_3 solution. (iv) Collagenase (type I; Sigma Chemical Co., St. Louis, Mo.) is stored at -20°C as a 0.1% solution in HMEM, sterilized by passage through a $0.45\text{-}\mu\text{m}$ membrane filter. The working solution of 0.01% is a 1:10 dilution of the stock solution in HMEM (pH 7.4) and is brought to 35 to 37°C in a water bath before use. (v) Washing fluid is Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (2) or phosphate-buffered saline. The omission of the divalent ions is irrelevant for the

collagenase procedure. It is chosen for convenience, since the solution is already available in most laboratories for use in trypsin treatment of cells. (vi) Trypsin-EDTA solution is used for subculturing monolayers and contains (in grams per liter): NaCl, 8.00; KCl, 0.40; dextrose, 1.00; NaHCO_3 , 0.58; trypsin (Difco 1.250), 0.50; EDTA (disodium salt), 0.20. It is sterilized by filtration and stored frozen (9). (vii) Cell-freezing medium (16) is HMEM with 15% fetal bovine serum and 10% dimethyl sulfoxide. The dimethyl sulfoxide is sterilized by autoclaving.

Organs or tissues for culturing are taken aseptically from mid- to end- term equine fetuses or 1-day-old foals, and are processed within 2 h after the death of the donor animal. Dissecting, mincing, and washing in phosphate-buffered saline are done by standard procedures. Collagenase digestion is carried out in an indented flask on a magnetic stirrer. The first 10-min digest is discarded, then the fluid digestion phase is collected every 30 min until enough cells are obtained. The cells are deposited by slow centrifugation ($800 \times g$ for 5 min) and kept in growth medium until the end of this process. Cell release is very rapid with collagenase, and 50 g of tissue stirred in 100 ml of collagenase usually yields 5 to 6 ml of cell deposit. Two or three 30-min digestions will provide sufficient cells for about 100 Blake bottles (growth surface, 75 by 200 mm). The cell harvest is filtered through four layers of sterile gauze, then suspended in growth medium at a concentration of 1 ml of cell deposit in 500 ml of medium. Blake bottles are seeded with 50 to 60 ml of this suspension or tubes with 1 ml. Monolayer cell sheets are formed in 3 to 4 days with fetal or neonatal kidneys, and in 7 days with cells from older foal or adult kidneys.

For a continuing supply of cultivatable equine cells, the monolayers in Blake bottles are detached from the glass and stored in liquid nitrogen. Cell detachment, however, is not possible with collagenase; the cultured cells round up but do not come off the glass. Instead, the standard trypsin-EDTA formula (9) can be used, in which equine cells react like other cultured mammalian cells. The cells of one Blake bottle separated from the trypsin-EDTA solution by slow centrifugation, are resuspended in 2 ml of freezing medium and slowly frozen in plastic freezer vials, first at 4°C for 1 h or more, then for at least 24 h in a styrofoam-insulated container at -70°C to assure slow heat withdrawal; for prolonged storage, the vials are placed in liquid nitrogen. Frozen cells are revived by rapid thawing of the vial in a 37°C water bath, dilution of the vial's content in 50 to 60 ml of growth medium, and cultured in a Blake bottle. The medium is

changed after overnight incubation at 37°C, and cultures are ready for inoculation or subpassage in about 4 days. The first outgrowth of cells stored in the frozen state is usually irregular and contains clumps and debris, but uniform and clean cultures are obtained upon subpassage. Cells from one Blake bottle can be suspended in 100 to 120 ml of growth medium, and this suspension can be seeded in 1-ml volumes into tubes or 5-ml volumes in 60-mm dishes. Tube cultures can be kept for at least 2 weeks; over acidification is avoided with a 3- to 4-day feeding schedule.

The described method has been used in this laboratory for over 2 years with satisfactory results. Cell stocks frozen for 2 years are still viable. The collagenase treatment has been applied with equal success to avian and other mammalian tissues, and the pessimistic evaluation of the enzyme by Rinaldini (17) has not been realized. On the contrary, for a number of experimental situations, collagenase surpasses trypsin as a cell-dispersing agent and should be added to the virology methodology.

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LITERATURE CITED

1. Cursiefen, D., and H. Becht. 1975. In vitro cultivation of cells from the chorioallantoic membrane of chick embryos. *Med. Microbiol. Immunol.* **161**:3-10.
2. Dulbecco, R., and M. Vogt. 1954. Plaque formation and pure lines of poliomyelitis viruses. *J. Exp. Med.* **99**:167-182.
3. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* **130**:432-437.
4. Hilfer, S. R., and J. M. Brown. 1971. Collagenase, its effectiveness as a dispersing agent for embryonic chick thyroid and heart. *Exp. Cell Res.* **65**:246-249.
5. Hilwig, I., S. Schuster, W. Heptner, and E. von Wasielewski. 1968. Über das Wachstum der Pankreaszellen von Säugetieren als Monolayer Cultures. *Zschr. Zellforsch. Mikrosk. Anat.* **90**:333-346.
6. Hinz, R. W., and J. T. Syverton. 1959. Mammalian cell cultures for study of influenza virus. I. Preparation of monolayer cultures with collagenase. *Proc. Soc. Exp. Biol. Med.* **101**:19-22.
7. Kodoma, H., G. Sato, and S. Miura. 1975. Avian encephalomyelitis virus in chicken pancreatic cell cultures. *Avian Dis.* **19**:556-565.
8. Lasfargues, E. Y. 1957. Cultivation and behaviour in vitro of the normal mammary epithelium of the adult mouse. *Anat. Rec.* **127**:117-125.
9. Madin, S. H., and N. B. Darby, Jr. 1958. Established kidney cell lines of normal adult bovine and ovine origin. *Proc. Soc. Exp. Biol. Med.* **98**:574-576.
10. Mandl, I. 1961. Collagenases and elastases. *Adv. Enzymol.* **23**:163-263.
11. Mandl, I. 1972. *Collagenase*. Gordon and Breach Science Publishers, New York.
12. Mayr, A., P. A. Bachmann, B. Bibrack, and G. Wittmann. 1974. *Virologische Arbeitsmethoden*, vol. I. VEB Gustav Fischer Verlag, Jena.
13. Moscona, A., O. A. Trowell, and E. N. Willmer. 1965. Preparation of cell suspensions, p. 49-60. In E. N. Willmer (ed.), *Cells and tissues in culture. Methods, biology and physiology*, vol. I. Academic Press, Inc., New York.
14. Moskalewski, S. 1965. Isolation and culture of the islets of Langerhans of the guinea pig. *Gen. Comp. Endocrinol.* **5**:342-352.
15. Pollard, M., and T. J. Starr. 1960. Propagation of duck hepatitis virus in tissue cultures prepared with collagenase. *J. Bacteriol.* **80**:571-572.
16. Porterfield, J. S., and J. M. Ashwood-Smith. 1962. Preservation of cells in tissue culture by glycerol and dimethyl-sulphoxide. *Nature (London)* **193**:548-550.
17. Rinaldini, L. M. J. 1958. The isolation of living cells from animal tissues. *Int. Rev. Cytol.* **7**:587-657.
18. Schmidt, N. J. 1969. Tissue culture techniques for diagnostic virology, p. 79-178. In E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic procedures for viral and rickettsial infections*, 4th ed. American Public Health Association, Inc. New York.
19. Seifter, S., and P. M. Gallop. 1962. Collagenase from *Clostridium histolyticum*. *Methods Enzymol.* **5**:659-665.
20. Seifter, S., and E. Harper. 1970. Collagenase. *Methods Enzymol.* **19**:613-635.
21. Studdert, M. J. 1974. Comparative aspects of equine herpesviruses. *Cornell Vet.* **64**:94-122.
22. Tong, W. 1974. The isolation and culture of thyroid cells. *Methods Enzymol.* **32**:745-758.
23. Weiss, L. 1961. Sialic acid as a structural component of some mammalian tissue cell surfaces. *Nature (London)* **191**:1108-1109.
24. Youngner, J. S. 1954. Monolayer tissue cultures. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. *Proc. Soc. Exp. Biol. Med.* **85**:202-205.