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CHAPTER 1

Some Cell Culture Procedures in Diagnostic Medical Virology

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During the last 20 years cell culture has become an invaluable tool in diagnostic medical virology. Cultured cells had been used previously for the detection of virus but it was not until type 2 poliovirus was grown in cultures of nonneural tissue from human embryos that the technique began to receive due recognition. An easily observed destructive effect or cytopathogenic effect (CPE) was produced in the cells by the virus and this effect could be prevented by type-specific immune sera providing a convenient neutralization test. Many viruses other than poliovirus could be detected and neutralized using this technique and a large number of previously unknown viruses was isolated. There have been continuous developments in cell culture and with each new technique comes the possibility of detecting the more elusive viruses. As a result of this, there are now many different methods of cell culture used in diagnostic virology and it is impossible to describe all of these here. I will attempt to give representative examples and references with the accent more on different methods than on preparation of cultures; such technical details will be found elsewhere in this book. The main uses of cell culture in diagnostic medical virology are to demonstrate the presence of virus and to detect humoral antibodies in patients’ sera. The production of antigens will not be discussed.

MONOLAYER CULTURES

General Method of Preparation. Culture of cells in monolayers is the most common method used in diagnostic virology laboratories. Cell suspensions are prepared from tissue or from stock cultures by using one of the dispersing agents. The suspension is then diluted in growth medium to contain from 50,000 to 200,000 cells per milliliter and dispensed into suitable containers; these may be large bottles for stock cultures, test tubes for roller culture, Petri

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1 J. F. Enders, T. H. Weller, and F. C. Robbins, Science 109, 85 (1949).
2 F. C. Robbins, J. F. Enders, and T. H. Weller, Proc. Soc. Exp. Biol. Med. 75, 378 (1950).
dishes, or coverslips in Leighton tubes. Growth medium usually consists of a synthetic medium such as 199 or Eagle's Basal Medium with added 10% calf serum. The serum may be varied in type or amount according to the virus or cells being cultured. After about 3 days the cultures are changed to maintenance medium which may contain 2% serum or be serum-free. Antibiotics are usually included. The optimum temperature for growth is 37°C, but after inoculation a lower temperature may be more satisfactory. Incubation may be stationary or in a roller drum. Containers which are not stoppered, e.g., Petri dishes, must be incubated in an atmosphere containing 5% \( \text{CO}_2 \) in air.

Types of monolayer cultures may be divided into (a) primary or secondary, (b) continuous, and (c) semicontinuous.

**Primary or Secondary Cultures.** These cells retain their diploid chromosome number, a factor which makes them particularly susceptible to certain viruses. Their disadvantage lies in the fact that they must be prepared from fresh tissue which may vary in susceptibility. Kidney of various species is particularly suitable and monkey kidney is used routinely in most diagnostic laboratories. Simian viruses may be present in monkey kidney and apart from herpesvirus simiae, which may be a hazard to personnel, SV5 and SV40 may interfere with isolation of other viral agents. Human amnion and human thyroid also provide satisfactory cell cultures although amnion cultures require more care in preparation. Monkey kidney cultures are satisfactory for the isolation of the myxoviruses and the enteroviruses except coxsackie A. Human amnion cells support the multiplication of measles and enteroviruses including some types of coxsackie A.

**Continuous Cell Cultures.** The discovery of a cell line, e.g., HeLa cells, which could be subcultured indefinitely was of considerable importance. These cells were isolated from an epidermoid carcinoma of the cervix\(^3\) and were found to support the growth of a large number of viruses. Since then, other cell lines, HEp-2 (human epithelium), have been isolated and it has also been possible to transform normal cells, e.g., BHK 21 (baby hamster kidney) and RK 13 (rabbit kidney). The Bristol strain of HeLa cells is particularly useful for the isolation of respiratory syncytial virus, and RK 13 cells produce a recognizable CPE when infected with rubella virus. Adenoviruses and herpes simplex produce a characteristic CPE in HeLa and HEp-2 cells.

Although the advantages of continuous cell cultures seemed so considerable that they might replace primary and secondary cultures, this has not been the case. During transformation of the cells the chromosome number becomes abnormal and the susceptibility to certain viruses is lowered.

**Semicontinuous Cell Cultures.** The ideal cell system for a diagnostic virology laboratory would be a continuous line which retained its diploid chromosome number. An attempt to attain this ideal system was made by subculturing

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\(^3\) G. O. Gey, W. D. Coffmann, and M. T. Kubicek, *Cancer Res.* **12**, 264 (1952).
cells from human embryonic tissue. The cells, of the fibroblast type, remained diploid and could be subcultured up to fifty times. Such cultures can be preserved by freezing and thus theoretically provide a constant supply of cells.

These semicontinuous cells may be as susceptible to certain viruses as primary or secondary cells but this is not always the case. They provide a particularly satisfactory host system for cytomegalovirus, varicella zoster, herpes simplex, and certain rhinoviruses.

**Routine Isolation of Viruses**

It would be impossible in a routine laboratory to inoculate each specimen into every type of cell culture. A routine procedure is therefore employed and the specimen is inoculated into three types of cells which will support the growth of as many viruses as possible. For this purpose the cells are usually grown in stoppered test tubes which may or may not be “rolled.” Rotation only seems to be essential for the isolation of certain rhinoviruses which also require a lower temperature (33°C) and a lower pH (6.8). A typical routine method for virus isolation may include the inoculation of: (a) secondary monkey kidney or human amnion cells, and (b) HeLa or Hep-2 cells. If the presence of rhinoviruses, cytomegaloviruses, or varicella zoster is suspected, human embryo fibroblasts should also be inoculated; rubella virus can be isolated in RK 13 cells while respiratory syncytial virus requires the more susceptible Bristol strain of HeLa cells.

Two tubes of each type of culture are inoculated with a small amount of the specimen (0.2 ml) and the tubes are then incubated. They are examined microscopically using a low-power objective every 2 days for 10 days or longer depending on the virus. If there is no evidence of virus after this time the cells may be inoculated into fresh cultures (blind passage).

**Detection of Virus in Cell Cultures**

*Degenerative Changes (CPE) in the Cells.* Different virus groups produce characteristic changes in the monolayers which can be seen microscopically, e.g., adenoviruses produce a clumping effect while enteroviruses produce rounding and shrinking of the cells. The CPE and the type of cells in which it is seen help to diagnose the virus. Identification is carried out by neutralization test in which the infected fluid is treated with known antisera; these virus–serum mixtures are inoculated into cell cultures and after incubation the cells are examined to determine which antiserum has neutralized the effect of the virus. In the case of large groups of viruses such as the echoviruses it is usual to combine several antisera in pools.

The CPE of a virus may be made more visible by the staining of cells, e.g.,

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1. Hayflick and P. S. Moorehead, *Exp. Cell Res.* 25, 585 (1961); cf. Sections II and IV, this volume.
for identification of respiratory syncytial virus a 1:8000 dilution of neutral red is added to the inoculated cultures.

**Hemadsorption Test.** This technique has been found useful for the detection of certain viruses, such as parainfluenza, which do not always give a definite CPE in tissue culture.\(^5\) The test is performed by adding 0.2 ml of 0.4% guinea pig or human “O” erythrocytes to roller tube cultures which have been incubated for 5 to 7 days following inoculation; uninoculated controls must be included. The tubes are incubated at 4°C for 20 minutes and then examined microscopically. A positive test is indicated by the adherence of red cells to the cultures. A hemadsorption inhibition test is then carried out by inoculating infected fluid into more cultures and incubating them until the hemadsorption test is positive. The medium is then replaced with fresh medium containing specific antisera and the tubes are incubated at 37°C for 1 hour. The fluid is removed and a hemadsorption test is carried out. Inhibition of hemadsorption by a particular antiserum indicates the identity of the virus.

**Fluorescent Antibody Technique.** Virus may be detected in cell cultures by this method before CPE occurs, thus providing a quick specific diagnostic test. The method has been successfully used for the diagnosis of respiratory syncytial virus infections.\(^6\) The tube cultures are inoculated and incubated in the usual way. For fluorescent antibody examination the cells are scraped off the tubes and transferred to slides. After fixing they are then stained by the indirect method with rabbit antiserum, e.g., against respiratory syncytial virus, followed by fluorescein-conjugated anti-rabbit globulin. The virus can easily be detected within the cells when the preparations are examined in the ultraviolet microscope.

**Interference Test.** This test is not used routinely but may be useful to detect the presence of viruses which cannot be demonstrated by conventional methods. For example some rhinoviruses and rubella virus were first isolated by this technique.\(^7,8\) African green monkey kidney cell cultures were originally used for rubella. Throat washings or other suitable specimens are inoculated into each of two tube cultures and after 1 hour at room temperature the inoculum is removed and replaced by nutrient medium. Following incubation for 7 days, one of each pair of cultures is tested by challenge with 1000 TCID\(_{50}\) of echovirus type 11; control cultures are also inoculated and all cultures are incubated. When the control cultures show an advanced CPE, fluids are collected from control and test cultures and assayed for hemagglutination. Reduction of the hemagglutination titer in the test culture indicates the presence of a virus in the initial inoculum.

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\(^5\) R. M. Chanock, R. H. Parrott, K. Cook, B. E. Andrewes, J. A. Bell, T. Reichelderfer, A. Z. Kapikian, F. M. Mastrota, and R. J. Huebner, *N. Engl. J. Med.* 258, 207 (1958).

\(^6\) P. S. Gardner and J. McQuillin, *Brit. Med. J.* 3, 340 (1968).

\(^7\) G. Hitchcock and D. A. J. Tyrrell, *Lancet* i, 237 (1960).

\(^8\) E. L. Buescher, P. D. Parkman, M. S. Artenstein, and S. B. Halstead, *Fed. Proc.* 21, 466 (1962).
Mixed Culture Technique (Cocultivation)

This method has proved of value when a viral agent is difficult to isolate from the original tissue, e.g., isolation of measles from a brain biopsy.\(^9\)

Primary cultures are prepared from such tissues by standard methods and allowed to grow into monolayers. They are then trypsinized and suspended in growth medium. One volume of this suspension is then combined with two volumes of HeLa cells and this mixture is cultured (see monolayer cultures). It is possible that a CPE will develop in the HeLa cells although no CPE was seen in the primary culture of the tissue.

Organ Culture

 Cultures of chopped tissues have been used for many years to support the growth of viruses but the techniques were not readily applicable to diagnostic virology. However, a simple method of cultivating fragments of ciliated epithelium was described\(^10\) which resulted in the isolation of new rhinoviruses and a new member of the coronavirus group.\(^11\)

For the isolation of respiratory viruses, tracheas of 14- to 22-week-old human embryos are chopped into fragments; these are planted with the ciliated surface uppermost on the scratched surface of a plastic Petri dish and medium (e.g., Medium 199) is added. After approximately 2 days the cultures are inoculated by dropping a small amount of the specimen onto the fragments and incubating.

Virus may be detected in the following ways: (a) stopping of ciliary activity which may take 7–10 days, (b) fragments may be rubbed gently in distilled water and the suspension examined by negative contrast in the electron microscope, (c) the medium may be collected, sedimented in the ultracentrifuge, and examined in the electron microscope, or (d) the tissue may be examined for pathological changes.

A recent report describes the use of human embryo liver cultures for experiments with Australia antigen.\(^12\) The antigen was detected by complement fixation test and by means of electron microscopy after freezing and thawing of the cells.

Detection of Viral Antibodies

Immunofluorescence Studies

1. Monolayer cultures. Cells may be cultured on coverslips, in Leighton tubes, or in Petri dishes. A suspension of cells is prepared in growth medium and

\(^9\) L. Horta-Barbosa, D. A. Fuccillo, and J. L. Sever, Nature (London) 221, 974 (1969).
\(^10\) B. Hoorn, Acta Oto-Laryngol. Suppl. 188, 138 (1964).
\(^11\) D. A. J. Tyrrell and M. L. Bynoe, Brit. Med. J. 1, 1467 (1965).
\(^12\) A. J. Zuckerman, P. M. Baines, and J. D. Almeida, Nature (London) 236, 78 (1972).
added in a concentration which will produce a monolayer. If Petri dishes are used it is helpful to make the coverslips adhere to the glass with a small spot of wax or silicone grease. Petri dishes may need to be incubated in an atmosphere of 5% CO₂ in air but the use of HEPES buffer will overcome this. The method has been found particularly useful in the diagnosis of rubella to detect rubella-specific IgM antibody. Coverslip cultures of BHK 21 cells are prepared and inoculated with rubella virus. After incubation the cultures are fixed in acetone and can be preserved at −20°C in the presence of silica gel. Uninfected cultures are also necessary. To detect rubella-specific IgM in human serum, dilutions of the serum are overlaid on the rubella-infected coverslip cultures. After incubation for 30 minutes followed by a thorough washing the coverslips are treated with fluorescein-labeled anti-human IgM. Specific fluorescence indicates the presence of rubella-specific IgM antibody due to recent infection.

2. Suspended cell cultures. Indirect immunofluorescence is used routinely to measure antibodies to EB virus. Coverslip cultures cannot be prepared in the usual way since EB cells are cultured in suspension and do not attach to the surface of a glass vessel. Cell smears are therefore prepared. After centrifugation of the cultures the supernatant fluid is removed and the cells are resuspended in the remaining fluid. A small drop of this suspension is placed on a coverslip, evenly distributed over the surface, and allowed to dry thoroughly at 37°C. The coverslips are then fixed. Using the indirect method the coverslip preparations are exposed to diluted test serum (1:8) for 1 hour at 37°C. After washing, fluorescein isothiocyanate-labeled serum prepared to the appropriate globulin is added and left for a further incubation period; the coverslips are then washed and mounted. Specific fluorescence in the cells indicates EB antibody and the test can be made quantitative by using dilutions of serum.

Plaque Inhibition Test. The original demonstration that poliovirus could produce plaques in monolayer cultures of monkey kidney cells has led to the demonstration of plaque production by most viruses. Cell monolayers are prepared in flat bottles or Petri dishes. The growth medium is removed and the cells are inoculated with virus. After a suitable time for adsorption (1 hour) the virus is removed and the monolayers are overlaid with growth medium containing agar (1.5%); neutral red may be incorporated in the medium. An accurate measurement of the number of plaque-forming units may be calculated after incubation. The neutralizing capacity of serum may be measured by mixing the serum with a known amount of virus and comparing the number of plaques produced by the mixture with the number produced by virus without serum. This method is sensitive and provides a more accurate neutralization test for enteroviruses than tube cultures but it is more cumbersome and is therefore not used widely in routine laboratories. The plaque test has also been used to differentiate between wild and vaccine strains of poliovirus.

13 M. Haire and D. S. M. Hadden, J. Med. Microbiol. 5, 237 (1972).
14 G. Henle and W. Henle, J. Bacteriol. 91, 1248 (1966).
15 R. Dulbecco and M. Vogt, J. Exp. Med. 99, 167 (1954).
16 G. D. Hsiung and J. L. Melnick, Virology 1, 533 (1955).
Metabolic Inhibition Test. This test depends on the ability to use the metabolism of cell cultures as an indication of virus growth. Acid is produced by actively growing cultures but if the metabolism is inhibited by the presence of virus the pH will be different; such differences can be measured if an indicator is incorporated in the medium. Neutralization tests can therefore be carried out using this principle, i.e., if virus is neutralized by antiserum and inoculated into cell cultures the cells will continue to metabolize whereas if there is no neutralization metabolism will be reduced. This test can be carried out in tubes, macroplates or microplates. Rigid disposable plastic plates have proved satisfactory. Serum dilutions in 0.025-ml amounts can be prepared in the plates and an equal quantity of virus added. After incubation of the serum-virus mixtures for 1 hour a suitable dilution of cells in 0.1 ml medium is added. The plates are then sealed with transparent tape and incubated. If phenol red is incorporated in the medium, neutralization will be indicated by a yellow color; the virus control will be pink. Time of incubation will vary for different viruses and modifications such as the addition of extra glucose and/or magnesium chloride have been made. The test has been used for coxsackie B viruses and rhinoviruses and has proved valuable to distinguish between wild and vaccine strains of poliovirus. It is economical on reagents and space.

17 F. L. Shand, J. Med. Lab. Technol. 18, 75 (1961).

CHAPTER 2
Diagnostic Use of Cell Cultures Initiated from Amniocentesis

Carlo Valenti

The prenatal detection of genetic disorders, by the analysis of cultivated fetal cells obtained by amniocentesis during the second trimester, has recently added a new useful and very promising dimension to genetic counseling, once based on empirical statistical data.1-3 The indications to genetic diagnostic

1 A. Dorfman (ed.), "Antenatal Diagnosis." The University of Chicago Press, Chicago, Illinois, 1972.
2 A. Milunsky, J. W. Littlefield, J. N. Kaufer, E. H. Kolodny, V. E. Shih, and L. Atkins, N. Engl. J. Med. 283, 1370, 1441, 1498 (1970).
3 C. Valenti, Ric. Clini. Lab. 1, 443 (1971).