Tissue Culture in the Laboratory Diagnosis of Viral Infections

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ABSTRACT

Schmidt, Nathalie J.: Tissue culture in the laboratory diagnosis of viral infections. *Am. J. Clin. Pathol.* 57: 820-828, 1972. The use of both rhesus monkey kidney and human fetal diploid cell cultures for attempts to isolate virus from human clinical specimens recovered approximately 30% more isolates from fecal, throat, skin lesion, and tissue specimens than were recovered in a single cell culture system. The sensitivities of the two cell types for isolation of viruses in the major groups infecting man are compared. The use of both BS-C-1 and RK-13 cells yielded approximately 25% more rubella virus isolates than were recovered in a single cell system. Procedures such as the establishment of cell cultures from tissues suspected of harboring viruses, cocultivation of cells from biopsy or autopsy specimens with other cell types, and the use of organ cultures, have in certain situations resulted in greater sensitivity for recovery of viruses. Micro cell culture tests have been devised for virus identification and antibody assays; these are far more economical than conventional tests in terms of reagents, space requirements, and personnel time. Methods have been developed for the production of higher-titered viral serologic antigens in cell culture systems.

IN THE PAST TWO DECADES the use of *in vitro* cell cultures in diagnostic virology has resulted in the discovery of almost 200 viruses which infect man. Cell cultures have also provided a practical and economical host system which, in many instances, may be substituted for animals or embryonated eggs in viral isolation attempts, neutralization tests, or preparation of serologic antigens.

Cell culture media, suspensions of viable cells, and prepared cell cultures are now available commercially for laboratories

lacking facilities for preparation of such materials, and satisfactory antisera for identification of viral isolates are gradually becoming available from commercial sources. The development of disposable cell culture vessels in glass or plastic has also facilitated viral diagnostic studies.

Systematic comparisons of the sensitivities of various cell culture systems for isolation or propagation of viruses made in recent years have indicated the relative usefulness of various cell types for diagnostic virology, and have guided in the selection of the most reliable and sensitive host cell systems for diagnostic purposes.

Certain modifications of cell culture techniques, such as the establishment of cell cultures from tissues suspected of harboring

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viruses, the use of mixed cell cultures for isolation of viruses from biopsy or autopsy specimens, and the use of simple organ cultures have, in certain instances, resulted in greater sensitivity for virus recovery.

Micro cell culture systems have been developed for use in virus neutralization tests, and these have resulted in great savings in terms of reagents, space requirements, and personnel time.

Some of the conditions have been defined for the production of higher-titered serologic antigens in cell culture systems, and thus more sensitive and specific diagnostic antigens have become available.

Cell Cultures Employed in Diagnostic Virology

Primary cell cultures are initiated from tissues taken directly from the organism; the cells may be subcultivated once, giving rise to secondary cultures in which the cells are morphologically similar to those in primary cultures, and similar in viral susceptibilities. Further subcultivation may be difficult to achieve, and it may result in conversion of the cells to the heteroploid state, which is frequently accompanied by decreased susceptibility to certain viruses.

Under special conditions of cultivation, diploid cell lines may be established which maintain their characteristic chromosomal configuration through a number of serial passages.¹⁴ However, the cells do not have the property of indefinite serial cultivation, but tend to decline at the fortieth to fiftieth passage. Diploid cell lines are generally similar to primary cultures in their viral susceptibilities, but since lines may vary in their susceptibility to viruses, those intended for use in viral isolation attempts should first be checked for sensitivity by testing for susceptibility to some of the more fastidious respiratory and enteric viruses. Diploid cell lines are more readily available to the diagnostic laboratory than

are primary cell cultures, and they may be stored in the frozen state.

Continuous cell lines have become heteroploid on serial passage; they can be subcultivated indefinitely, and many lines have been carried through hundreds of passages. Although continuous lines have a narrower range of viral susceptibilities than do primary or diploid cell cultures, viruses can generally be adapted to growth in continuous lines. The cells multiply rapidly and cultures attain high cell populations, thus providing a useful source of cells for neutralizing antibody assays and for production of serologic antigens.

Comparative Sensitivities of Certain Cell Culture Systems for Isolation of Human Viruses

Primary or secondary rhesus or grivet monkey cell cultures have been found to be among the most sensitive host systems available for isolation of a wide variety of human enteric and respiratory viruses, but the fact that the cells are generally contaminated with simian viruses presents a serious drawback to their use. These contaminants may produce a cytopathic effect or a hemadsorption reaction which masks the presence of viruses recovered from the human clinical specimens, or rare contaminants such as *Herpesvirus simiae* or the Marburg agent may be extremely hazardous to laboratory workers handling the cultures. Diploid or continuous cell lines established from simian kidneys have been somewhat less sensitive than primary or secondary cells for isolation of human viruses.^{8, 11, 36}

Human fetal diploid lung and kidney cell lines were found to be far more sensitive than primary or secondary monkey kidney cells for isolation of rhinoviruses,^{12, 43} a group of more than 90 distinct virus types which produce common colds or minor upper respiratory tract infections,

Table 1. Isolation of Viruses from Clinical Specimens in Primary or Secondary Rhesus Monkey Kidney (MK) Cells and in Human Fetal Diploid (HFD) Lung or Kidney Cells (1966-1970)

	Type of Specimen						Total
	Fecal	Throat	Spinal Fluid	Skin Lesion	Urine	Autopsy Tissue	
Number of specimens examined	7,108	3,138	1,373	316	178	810	12,923
Number of isolates recovered							
Adenoviruses	106	127	—	—	—	—	233
Enteroviruses	1,210	259	116	1	—	10	1,596
Rhinoviruses	—	77	—	—	—	—	77
Influenza viruses	—	128	—	—	—	12	140
Parainfluenza viruses	—	181	—	—	—	2	183
Mumps virus	—	25	10	—	—	—	35
Respiratory syncytial virus	—	79	—	—	—	—	79
Herpes simplex viruses	—	96	2	88	—	14	200
Varicella-zoster virus	—	—	—	13	—	3	16
Cytomegalovirus	—	8	—	—	16	7	31
Vaccinia virus	—	3	—	14	—	—	17
Reoviruses	6	—	—	—	—	—	6
Isolates recovered in MK and/or HFD	1,322	983	128	116	16	48	2,613
Isolation rate (%)	19	31	9	37	9	6	20
Isolates recovered in MK	919	708	81	50	0	24	1,782
Isolation rate (%)	13	23	6	16	0	3	14
Isolates recovered in HFD	1,027	556	83	113	16	25	1,820
Isolation rate (%)	14	18	6	36	9	3	14

and early studies indicated that human fetal diploid cell lines were highly sensitive for propagation and isolation of other human viruses as well.^{7, 13, 31}

For the past 8 years, clinical specimens examined in this laboratory for the presence of virus have been inoculated into both primary or secondary rhesus monkey kidney (MK) cells and human fetal diploid (HFD) cells (either lung or kidney, depending upon the type of specimen). Rubella virus isolation attempts are performed in the BS-C-1 line of grivet monkey kidney cells and the RK-13 line of rabbit kidney cells,³⁹ since rhesus MK and HFD cells do not show clear-cut interference or a cytopathic effect when infected with this virus. It has been found that the parallel use of two cell culture systems contributes an appreciable number of viral isolates over those recovered in a single cell culture system.

Table 1 compares the viral isolation rates from various clinical materials in both MK and HFD cells with those in each individual cell culture system for the 5-year period from 1966 through 1970. Figure 1 compares the sensitivity of MK cells with that of HFD cells for isolation of viruses in each major group. Data on rubella virus isolations are presented separately in Table 2, since different cell culture systems were used. It is evident in Table 1 that the use of both cell culture systems increased the viral isolation rates from each type of clinical specimen, with the exception of urine specimens, from which only cytomegalovirus was recovered, and only in HFD cells.

In Figure 1 it is evident that MK cells were somewhat more sensitive than HFD cells for isolation of poliovirus strains; these were vaccine strains recovered from individuals with recent histories of oral immunization. The two cell culture systems

FIG. 1. Comparative sensitivities of rhesus monkey kidney cell cultures and human fetal diploid cell cultures for isolation of certain viruses infecting man.

were similar in sensitivity for isolation of group A coxsackieviruses; these were predominately types A9 and A16. For isolation of group B coxsackieviruses, however, MK cells were much more sensitive than HFD cells. On the other hand, HFD cells were more sensitive than MK cells for recovery of echoviruses. Overall, 72% of the enterovirus isolates were recovered in MK, and 77% in HFD cells. All of the throat specimens were inoculated into HFD lung cells, and the other specimens into HFD kidney cells. A direct comparison of the sensitivities of HFD lung and kidney cells for isolation of viruses was not made, but the fact that 77% of the 1,337 enteroviruses isolated from stool specimens, spinal fluid specimens, or tissues were recovered in HFD kidney cells and 76% of the 259 enteroviruses isolated from throat washings were recovered in HFD lung cells suggests that

the lung and kidney cells were similar in susceptibility to the human enteroviruses.

Figure 1 shows that the herpesviruses infecting man are rarely recovered in MK cells, with the exception of genital strains (type 2 viruses) of *Herpesvirus hominis*. This herpesvirus type is also known to have greater pathogenicity than type 1 (non-genital) strains for other laboratory hosts such as embryonated eggs, mice, and rabbits.^{20, 23} *Herpesvirus hominis* and varicella-zoster viruses were recovered in either HFD lung or kidney cells, but isolation attempts for cytomegaloviruses were performed, and virus was recovered in HFD lung cells only.

Comparing the sensitivities of MK and HFD cells for isolation of respiratory viruses and mumps virus, it is evident in Figure 1 that the influenza and parainfluenza viruses were recovered almost exclu-

Table 2. Comparative Sensitivities of BS-C-1 Grivet Monkey Kidney Cells and RK-13 Rabbit Kidney Cells for Isolation of Rubella Virus (1966-1970)

Type of Specimen	Number of Isolation Attempts	Number of Isolates Recovered	Number of Rubella Isolates Recovered in				
			Both Cell Systems	BS-C-1 Only	RK-13 Only	BS-C-1	RK-13
Nasopharynx or throat	521	102	54	28	20	82	74
Tissues	118	32	13	1	18	14	31
TOTAL	639	134	67	29	38	96	105

sively in MK cells. All of the mumps virus strains were isolated in MK, but a few were also recovered in HFD cells. The two cell culture systems were similar in sensitivity for isolation of respiratory syncytial virus. HFD lung cells were markedly more sensitive than MK cells for isolation of rhinoviruses; the only rhinovirus types recovered in MK were types 1a, 1b, and 14, but 41 different immunotypes were recovered in HFD lung cells.

HFD cells were more sensitive than MK cells for recovery of adenoviruses. Seventy-nine per cent of the 106 adenoviruses isolated from fecal specimens were recovered in HFD cells, and 82% of the 127 isolated from throat specimens were recovered in HFD lung cells, suggesting that these two types of HFD cells were also similar in sensitivity for isolation of adenoviruses. The MK and HFD cell culture systems were similar in sensitivity for isolation of vaccinia virus. The few reovirus isolations were made in MK cells only.

Table 2 shows that the BS-C-1 and RK-13 cell lines were roughly comparable overall in sensitivity for isolation of rubella virus, but the use of both cell culture systems contributed additional isolates to those recovered in a single system.

Modified Cell Culture Procedures Employed to Increase Sensitivity for Isolation and Subpassage of Certain Viruses

The importance of using viable, infected cells for recovery and subpassage of certain

human viruses has become apparent over the years in which cell culture host systems have been employed in diagnostic virology. It was early recognized that varicella-zoster (V-Z) virus could be subpassaged only through the use of viable, infected cells as an inoculum,⁴⁵ since infectious virus is spread by cell-to-cell transfer rather than through infected culture fluids, and it was found that trypsin-dispersed cells were more effective in transferring infectivity than were cells dispersed by mechanical means.⁴²

Certain viruses which are not so closely cell-associated as V-Z virus are also subpassaged most effectively through the use of trypsin-dispersed, infected cells. Thus, cytomegalovirus is best propagated by mixing trypsin-dispersed, infected cells with trypsinized uninfected human fibroblast cells and planting.² It has been shown that hamster-adapted measles virus may be isolated in cell cultures from trypsinized, infected brain tissue, but not from homogenates of the same tissue.^{4, 40} In this laboratory it has been found that, at low passage levels, measles and adenovirus isolates are subcultivated most effectively through the use of trypsin-dispersed, infected cells as an inoculum rather than culture fluids or cells scraped from infected cultures.

The superiority of viable cells over tissue homogenates for recovery of certain viruses from autopsy or biopsy tissues has been illustrated in studies comparing virus recovery in cell cultures established from

human organs obtained at autopsy or by biopsy with recovery in the usual laboratory cell culture systems inoculated with suspensions of the same organs. In attempting to isolate viruses from kidneys and other organs of children with congenital malformations, Benyesh-Melnick and colleagues³ recovered nine isolates in cell cultures prepared from the organs themselves, but only two of these viruses were also recovered in cell cultures inoculated with suspensions of the organs. Similarly, Rawls and associates²⁴ recovered rubella virus from cultured cells of certain organs, but not from suspensions of the organs. The greater success of viral isolation through culturing cells from tissue specimens may be due to the need for cell-to-cell transfer of virus from viable cells, as in the case of V-Z virus, or it is possible that homogenization of organs or tissues releases viral inhibitory substances. Inhibitors for measles virus have been demonstrated in extracts of rat brain,⁴ and inhibitors of dengue virus infectivity have been detected in certain organs of monkeys.²¹

The cultivation of brain cells from patients with subacute sclerosing panencephalitis (SSPE) together with HeLa cells¹⁵ or BS-C-1 grivet monkey kidney cells²² has permitted the recovery of infectious measles virus from the cells, and thus has furnished direct evidence of the etiologic role of measles virus in this clinical entity. Cultures of the brain cells alone showed the presence of measles antigen by fluorescent antibody staining, and the presence of syncytia, but infectious virus was not recoverable. Apparently the HeLa or BS-C-1 cells contributed some factor essential to the production of complete, infectious virus which was lacking or ineffective in the brain cell cultures. Cocultivation technics will probably receive wide use in future efforts to recover viruses in central nervous system diseases and chronic diseases suspected to have a viral etiology.

The cultivation of tissue explants in a simple, chemically-defined medium without serum or plasma tends to restrict the outgrowth of undifferentiated cells and permits the *in vitro* cultivation of tissues in which the various constituents retain their normal relationship to each other and maintain their normal function. The use of such "organ" cultures of ciliated nasal or tracheal epithelium from human embryos for virus isolation attempts has led to the recognition of new human respiratory viruses which cannot be isolated in undifferentiated monolayer cell cultures.^{18, 44} These are members of the human coronavirus group,⁵ and they are becoming increasingly implicated as important etiologic agents of acute upper respiratory tract illness.

It is possible that the increased use of organ cultures for virus isolation attempts may lead to the discovery of additional, heretofore unrecognized, viruses which infect man.

Identification of Viruses Recovered in Cell Culture Systems

Based upon the type of clinical specimen, the disease suspected, the type of cytopathic effect seen in inoculated cell cultures, or the demonstration of hemadsorption or interference, a suitable procedure is selected for identification of viruses isolated in cell culture systems.²⁸

Fluorescent antibody staining of infected cell culture material provides a rapid means of identification and differentiation between herpes simplex, varicella-zoster, and vaccinia viruses,³⁸ and also for the identification of rubella³⁹ and respiratory syncytial²⁷ viruses.

The use of the "intersecting serum pool" scheme³⁰ for virus neutralization tests has greatly facilitated identification of isolates in the large adenovirus, enterovirus, and rhinovirus groups, which consist of many different immunologic types. Antisera to the various immunotypes within each

group are pooled so that each antiserum is incorporated into two different pools, and identification is made by demonstrating neutralization of the isolate by the two pools sharing a common, type-specific immune serum. Antisera for as many as 36 immunotypes can be incorporated into only 12 pools, and antisera for 49 viral types, into 14 pools.

In some instances influenza virus isolates possess sufficiently high hemagglutinating titers to permit identification by *in vitro* hemagglutination-inhibition (HI) tests on the MK cell culture fluid. If titers are not sufficiently high for HI tests, hemadsorbing isolates may be identified by neutralization or hemadsorption inhibition tests.

Viruses replicating in organ cultures generally do not produce an overt effect, and their presence is usually detected by means of electron microscopy.

Micro Technics for Viral Neutralization Tests

In the past few years microneutralization tests utilizing cell cultures in microtiter plates have been developed for a variety of viruses. These tests may be used for identification of viral isolates, for serodiagnosis of viral infections, or for evaluation of viral vaccines. The micro tests require about one-tenth of the amounts of cells and medium used for equivalent tests in tube cultures, and they require far less incubation space. In addition to the economy of reagents and space, the tests are easier to perform and read than tests which involve the handling of individual tube cultures.

Microneutralization tests may be read by microscopic observation of the inhibition of a viral cytopathic effect,^{16,17,25} or a metabolic inhibition system may be used,²⁹ in which case the tests are read macroscopically using colorimetric endpoints. Microneutralization tests utilizing hemadsorption as an indicator of unneutralized virus have

been devised for assay of antibodies to certain myxoviruses.³⁵

The successful adaptation of micromethods for virus neutralization tests has been made possible largely through the development of disposable microtiter plates (with either flat-bottomed or "U"-shaped cups) from plastics which are nontoxic and consistently permit cellular growth. For a number of years the development and large-scale use of microneutralization tests was hampered by inconsistencies in the ability of disposable plastic plates to support the growth of cells in culture. In recent years, however, suitable microtiter plates have become available from several sources.

Production of Viral Serologic Antigens in Cell Cultures

It has been found that the highest-titered viral hemagglutinating (HA) or complement-fixing (CF) antigens are produced by infecting large populations of host cells. Continuous passage cell lines which multiply rapidly and attain high cell populations in culture generally give rise to more potent antigens than do primary or diploid cultures containing fewer cells. The HeLa, KB, and HEp-2 continuous lines of human epithelial cells have been employed for many years in the production of serologic antigens. The BHK-21 continuous line of baby hamster kidney cells¹⁹ has found wide use in recent years in the production of serologic antigens for a variety of viruses such as rubella,^{10, 32, 41} certain arboviruses,³⁴ rabies virus,⁹ herpes simplex, vaccinia, and reoviruses.³⁴

In addition to the use of rapidly-multiplying cell lines, mechanical devices such as suspension cultures and roller bottle cultures may also be employed to obtain large host cell populations for production of viral antigens.

It has been shown that much of the viral antigen produced in cell cultures remains associated with the host cells rather than

being released into the culture medium, and high-titered serologic antigens have been produced by suspending the cellular phase of virus-infected cultures in a small volume of fluid and then releasing the viral antigens by sonication or by freezing and thawing the cells.^{1, 26, 34, 37} For some viruses, extraction of the infected cells with alkaline buffers is more effective than mechanical disruption for release of antigen.^{32, 34} In the case of rubella virus, CF antigens prepared by alkaline buffer extraction of infected cells have been shown to be more sensitive for detection of antibody than are antigens produced by mechanical disruption of the cells or by concentration of the fluid phase of the culture.^{9, 33}

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