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Physical and Chemical Methods for Enhancing Rapid Detection of Viruses and Other Agents

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INTRODUCTION

The foundation of diagnostic virology is the detection of viruses or their components. However, it is not uncommon for viral diagnosis to depend on detecting immunoglobulin G or M antibodies to specific viral proteins. Viruses such as Epstein-Barr virus, hepatitis A, B, C, D, and E viruses, human immunodeficiency virus (HIV), rotaviruses, Norwalk agent, arboviruses, enteric adenoviruses, papillomaviruses, and parvoviruses cannot be or are not easily isolated in cell cultures by viral diagnostic laboratories. Thus, viral diagnostic laboratories must either depend on direct tests for antigen or nucleic acid detection for making a diagnosis or test for antibody to the agent.

For viral diagnosis, isolation is sensitive because a positive result can be obtained with a single infectious virion. In addition, viral isolation permits the detection of unexpected viruses, new viruses, or multiple viruses in a specimen. Two major limitations of viral isolation are the long time required to isolate some viruses and the inability of some viruses to be grown in vitro. Since viral isolation is important for viral diagnosis, methods to speed or enhance viral isolation and detection can be helpful. Therefore, the essence of this review is a focus on physical and chemical factors that aid and enhance the replication and detection of viruses in cell cultures. This information is provided with the hope that it will stimulate new avenues of research for the rapid isolation, replication, and detection of viruses in cell cultures.

ROLLING AND ROCKING OF CELLS

The roller culture method for propagating eukaryotic cells was introduced by Gey in 1933 (83). In 1954, Earle et al. (54) observed that increasing the constant rate of roller tube rotation from the usual 0.1 to 10 rpm led to 80% or more mouse L-cell growth than in nonrolled cultures. The optimal rolling rate for cell growth was found to be between 2.5 and 20 rpm (54). Cultures not rolled and those rolled at 0.1 rpm had similar growth rates after 2 weeks of incubation (55). It was not until the third week of culture rotation that differences in cell growth in rolled and nonrolled cultures were observed. However, cell growth was found to be significantly better for cultures rolled at 10 rpm than for nonrolled cultures after 1, 2, and 3 weeks of incubation. Rolling conditions corresponding to a circumferential flow of medium of 34.6 to 138.2 cm/min was better than 1 cm/min, which was the flow rate used for cultures rotated at the lower velocity. Thus, studies by Earle et al. (55) demonstrated that the dynamics of cell growth with roller cultures are complex, and need to be carefully controlled, and that rolling cultures at higher speeds (10 rpm) can enhance cell growth over that obtained with stationary cultures or with cultures rolled at lower speeds (0.1 rpm).

We have observed that rolling primate epithelial cell cultures at 96 rpm (1.9 × *g*) for 4 to 70 h significantly increases the production of cellular RNA over that in nonrolled cultures (277). Rolling cultures for 23 or 46 h at 2 rpm also results in the incorporation of significantly more ^{3}H-uridine into host cell RNA than into control RNA. Because of new cellular RNA synthesis in rolled cultures, viral replication also may be stimulated in rolled or centrifuged cultures.

Earle et al. (54) demonstrated that mouse L cells proliferated well in suspension with agitation. Zimmerman et al. (346) reported that human endothelial cells are activated or stimulated to release cyclo-oxygenase metabolites after rocking for 5 min (50 tilts per min; angle of tilt, 30°). Shear stress also stimulates certain cells. The shear stress on endothelial cells lining the cardiovascular system has been estimated to be as much as 30 to 100 dynes/cm^3 (47). Mechanical stress can stimulate aortic endothelial cells to proliferate (279). It is now possible to grow cells in a mechanically active environment, and the degree of cyclic and mechanical tension or compression on cells can be controlled (15, 25, 334). The role that shear stress may have in cell function and viral replication has not been established. The effect of rolling or rocking on cellular physiology and gene expression also has not been thoroughly investigated. Most cell culture studies generally ignore the importance of motion on cells. Rolling and rocking of cells may activate or stimulate cells to produce more virus or antigens. Preliminary studies from our laboratory have demonstrated that cells rolled at 96 rpm for 8 h before being infected with herpes simplex virus (HSV) and then not rolled after infection produce more virus than cells not rolled before infection. The viral yield for cultures rolled and then infected was two- to threefold more than that for cultures not rolled and infected, $P < 0.05$ (277).

CENTRIFUGATION OF CELLS

In 1910, Conklin reported the effects of centrifugation (600 × *g* for several minutes) on the development of freshwater

gastropods (35). Both normal and abnormal maturation of gastropod eggs was observed. The detrimental effects of centrifugation on eggs increased from the time of the first maturation to that of the first cleavage. Eggs centrifuged during the maturation division usually developed normally. In 1932, Harvey and Marsland (113) found that amoebas could survive centrifugation at 12,500 × *g*. Using rat carcinoma and rat embryonic tissue, Guyer and Claus (112) showed that centrifugation of tissue for 20 min at 400,000 × *g* before implantation of the tissue into rats made no perceptible changes in the growth of implants compared with control (noncentrifuged) implants. Macdougald et al. (168) reported in 1937 that embryonic chicken heart tissue had normal growth and mitosis after centrifugation at 400,000 × *g* for 30 min. Dornfeld in 1936 and 1937 (49, 50) centrifuged rat adrenal organs at 400,000 × *g* for 30 min. After centrifugation, pieces of adrenal organs were autotransplanted into adrenalectomized rats (50). The adrenal cortical cells were able to reconstitute, regenerate, and provide normal physiological functions so that rats receiving the ultracentrifuged implants survived, while adrenalectomized rats without implants died.

Ascaris eggs can tolerate high *g* forces (1,000 × *g*) for 10 to 15 days (248). Under these conditions, 70 to 80% of the ascaris eggs developed normally and within the same time as control eggs. However, at 1,500 × *g* normal egg development was altered.

Centrifugation has been reported to have an adverse effect on in vitro limb chondrogenesis (53). Mouse limb buds from 11.5- to 13.5-day embryos were significantly depressed in development after centrifugation at 2.6 × *g* for up to 6 days. The teratogenic effect of centrifugation on limb buds was assumed to be due to changes in tension and/or pressure on cells accompanied by alterations in cell morphometry and membranes. Centrifugation seemed to affect limb development when applied during the chondrogenic period of limb growth.

Gey et al. (85) found that HeLa cells, human fibrosarcoma cells, rat fibroblasts, and fibrosarcoma cells would tolerate centrifugation at 111,000 × *g* for 3 h. Burkitt lymphoma cells had no loss in viability when centrifuged at 42,200 × *g* for 90 min at 0°C (311). In 1971, Litwin (165) reported that human diploid fibroblasts centrifuged at 120 × *g* before being seeded at each passage had more cell growth and greater longevity than cells not centrifuged. For centrifuged cultures, fibroblast longevity increased more than 10 cell divisions over that of noncentrifuged cultures. However, the increases in cell growth and longevity for centrifuged fibroblasts could have been due to differences in medium conditions used for the centrifuged versus the noncentrifuged cultures. Cultures that were not centrifuged had their medium changed at 6 or 20 h after being planted, while centrifuged cultures did not have a medium change.

In 1971, Pollard (222) found that DNA and RNA syntheses in bacterial cells suspended in an isodense medium was not affected by centrifugation at 50,000 × *g* for 30 to 110 min. Similarly, protein synthesis was normal after 80 min of centrifugation at 50,000 × *g*.

Tschopp and Cogoli (300) subjected five different cells (HeLa cells, chicken embryo fibroblasts, rat sarcoma cells, Friend leukemia virus-transformed cells, and human lymphocytes) to centrifugation at 10 to 40 × *g* for up to 4 days and found that cell proliferation was enhanced. Of the five cells studied, only lymphocytes did not survive a 3-day exposure to centrifugation greater than 20 × *g*. The increase in cell proliferation was due to the *g* effect and not to an increase in hydrostatic pressure generated by centrifugation. Similar studies with centrifugation and cell proliferation were carried out by Kumei et al. (148). These investigators found that centrifugation at 35 × *g* for up to 4 days caused a significant proliferation of HeLa cells. Centrifugation significantly reduced the HeLa cell generation time by several hours, and there was a concomitant enhancement in c-*myc* gene expression.

The centrifugation of many different cells at *g* forces of 1,000 to 100,000 × *g* for several hours generally does not have a deleterious effect on their viability or function. Studies to determine how different *g* forces and other forms of motion may affect gene function and other cellular processes are needed. Since centrifugation appears to activate gene expression and stimulates cell proliferation, studies to standardize the effect of centrifugation on cells are also needed. The centrifugation of cells may be useful for the activation of cells for viral diagnosis and could make them more susceptible to viral infection and more sensitive to viral replication. The effect that centrifugation has on cells used for viral isolations is not known. A general review of the literature indicates that little information on the effects of rolling and centrifugation on cell and gene functions is available.

To summarize, the effect of rolling, rocking, or centrifugation on cells is to mechanically "stress" them. Such stress may be beneficial for viral replication. Cells stressed by different forms of motion may have more cell proliferation, decreased cell generation times, activated genes, altered cell metabolism, and increased cell longevity. A summary of representative motion studies on cells, tissue, organs, and organisms is presented in Table 1.

EFFECT OF MOTION ON PROPAGATION OF VIRUSES AND OTHER AGENTS

Low-Speed Rolling

Until 1988, cultures used for viral diagnosis generally were rolled at 0.2 to 2 rpm. Since 1988, several reports using conditions for rotating cell cultures at 96 (1.9 × *g*) or 383 (30.2 × *g*) rpm have been published (127, 129, 179, 276). For this review, ≤2 rpm is considered low-speed rolling because historically these speeds generally were the only ones used, while ≥20 rpm is considered high-speed rolling. The effect of low-speed rolling on different virus-host cell systems is discussed below.

Vaccinia virus. The first report on rolling (0.1 rpm) for viral replication was published in 1940 by Feller et al. (67). Vaccinia virus was found in roller cultures of chicken embryonic tissue for up to 9 weeks after inoculation. Using rabbit mononuclear cells in roller cultures, Florman and Enders (68) found that vaccinia virus could be cultivated and detected for 2 to 3 weeks after inoculation of cultures. In 1988, it was reported (129) that viral yields for vaccinia virus vectors carrying rotavirus gene inserts were sevenfold greater for cultures rolled at 2.0 rpm than for cultures not rolled. The production of foci by vaccinia virus vectors in cultures rolled at 2.0 rpm was also significantly enhanced compared with that of stationary cultures (129).

Enteroviruses. In 1952, Robbins et al. (235) demonstrated that poliovirus in roller cultures (0.2 to 0.3 rpm) could be grown to titers comparable to or higher than those obtained in suspended cultures. Melnick and Riordan also found that poliovirus often grew to higher titers in rolled cultures (186). Working with poliovirus and Sindbis virus, Frothingham (73)

TABLE 1. Summary of representative motion studies on cells and organisms

Yr(s)	Cells, organ, or organism	Motion or g force ($\times g$)	Centrifugation time	Outcome	Reference(s)
1910	Gastropod eggs	600	2–240 min	Normal and abnormal maturation	35
1932	Amoeba	12,500	NI[a]	Cell survival	113
1936	Embryonic and malignant tissue	400,000	20 min	Typical growth	112
1936, 1937	Adrenal organ	400,000	30 min	Normal cell function	49, 50
1937	Embryonic chicken heart tissue	400,000	30 min	Normal growth and mitosis	168
1954	HeLa and other cells	111,000	180 min	Typical growth	85
1954	L cells	<1–20 rpm	7–21 days	Increased growth	55
1968	Human lymphoid	42,000	90 min	No loss in visibility at 0°C	311
1971	Human fibroblasts	120–200	20–30 min at every passage	Increased growth and longevity	165
1971	Bacteria	50,000	>90 min	Normal DNA, RNA, and protein syntheses	222
1972	Nematode eggs	1,000	10–15 days	Normal development	248
1983	Mouse embryos	2.6	6 days	Suppressed development	53
1983	HeLa and other cells	10–40	1–6 days	Increased growth	300
1985	Human endothelial cells	Rocking	5 min	Cell activation and stimulation	346
1989	HeLa	18–70	4 days	Increased growth	148

[a] NI, not indicated.

found that rolling at 0.3 rpm enhanced the production of cythpathic effects (CPE) by poliovirus in young primary human amnion cell cultures and that CPE was more pronounced in rolled cultures inoculated with low concentrations of some polioviruses. However, no effect of rolling on CPE production could be demonstrated with Sindbis virus-infected amnion cells.

In 1962, Mufson et al. (200) reported that HEp-2 cultures rolled at 0.2 rpm were more sensitive for coxsackievirus A-21 isolation than nonrolled cultures. Upon reinoculation of 49 positive specimens, they found that all 49 were CPE positive in rolled cultures, while only 38 (78%) were CPE positive in stationary cultures. Of 49 rolled specimens, 43 (88%) were positive by 4 days, while none of the stationary specimens were positive at day 4 and only 6 of 49 (12%) were positive by day 10. Upon blind passage of the 11 CPE-negative stationary cultures, 5 became positive in roller cultures, and 4 of the 5 were positive when passed into cultures that were not rolled.

The amount of coxsackievirus A-21 in clinical specimens was also determined by infectivity titrations with both rolled and nonrolled cultures of HEp-2 cells and human embryonic kidney (HEK) cells (200). Titers of virus in rolled HEp-2 cells were generally 10- to 1,000-fold greater than in stationary cultures, and in all cases the final titer occurred sooner in rolled cultures. With HEK cells, no consistent trend favoring rolled versus nonrolled conditions was observed. However, with either condition, maximal viral titers were demonstrated more rapidly with HEK cells than with rolled HEp-2 cells. These studies demonstrate that the host cell and rolling conditions can influence the time of CPE appearance and the degree of CPE.

The work of Frothingham (73) with poliovirus and Sindbis virus and the development of CPE in old versus young cultures also emphasize the role of host cells in CPE production. Frothingham found that the cytopathic response of primary human amnion cell cultures to poliovirus type 2 was absent in stationary cultures infected at 5 to 10 days of age. However, when stationary cultures were infected at 30 or more days of age, complete or maximal CPE occurred. Young, primary amnion cultures that were rolled and infected with poliovirus developed 1+ CPE. The enhancing effect of rolling on poliovirus CPE was greatest in cultures incubated for 11 to 21 days before being infected. With Sindbis virus, 28-day-old stationary amnion cultures had more viral CPE (4+) than 7-day-old cultures (1+ CPE). Rolling did not enhance Sindbis virus CPE. Therefore, marked differences among viruses, host cells, and rolling exist. Possibly, rolling may enhance cell aging.

Other roller culture studies by Mufson et al. (200) suggested that at low multiplicities of infection equivalent or more coxsackievirus was produced in rolled HEp-2 cultures; however, no statistical comparisons were made. Also, the effect of rotation on the earlier development of CPE was more marked when small quantities of virus were used to initiate infection. Thus, with small amounts of virus in an inoculum, stationary cultures took longer to develop CPE. With larger viral inocula, no differences were seen.

Roller culture studies carried out in our laboratory with coxsackievirus A-21 in HeLa cells gave results similar to those reported by Mufson et al. (200). HeLa cell cultures infected with coxsackievirus A-21 and rolled at 2 rpm produced 10- to 600-fold more virus than stationary cultures (126).

Rhinoviruses. Using the roller culture technique (0.2 rpm), Andrewes et al. (9) were able for the first time to serially propagate a common cold virus in human lung cell cultures. Tyrrell and Parsons (302) increased the amount of CPE produced by common cold viruses in cell cultures by using secondary rather than primary monkey kidney cell cultures, 0.03% rather than 0.1% bicarbonate, rolled rather than stationary cultures, and incubation at 33°C rather than 36°C.

In 1959, Price et al. (224) reported that isolation and replication of rhinoviruses in roller cultures gave more consistent results than isolation in stationary cultures. The growth rates of rhinoviruses JH and 2060 were compared in rolled and nonrolled monkey kidney cell cultures by Holper et al. (121). Higher concentrations of infectious virus were

obtained in rolled (10^5 to 10^6 50% tissue culture infective doses per ml) than in stationary ($10^{3.0}$ to $10^{4.0}$ 50% tissue culture infective doses per ml) cultures. When stationary cultures were infected with very small amounts of rhinovirus, only a slight amount of new virus was produced (<10-fold). However, rolled cultures infected with the same small amounts of rhinovirus often produced 1,000-fold more new virus.

With primary monkey kidney cells, Mogabgab and Homes (195) found that rhinovirus infectivity titers were higher in rolled than in nonrolled cultures. In addition, rolled secondary monkey kidney cell cultures developed maximal CPE in one-half the time of nonrolled secondary cultures; however, viral yields were no different in secondary cell cultures that were rolled and those that were not rolled.

Reoviruses. In 1962, Lerner et al. (159) reported that the CPE for all three types of reovirus was markedly increased in rhesus monkey kidney cell cultures rolled at 0.2 rpm. Furthermore, CPE in rolled cultures appeared 2 to 3 days earlier than in stationary cultures for all three reoviruses. In addition, hemagglutinin (HA) and infectivity titers were higher in rolled cultures, and HA activity was detected sooner in rolled cultures.

Parainfluenza viruses. Parainfluenza virus type 4 is difficult to isolate from clinical specimens. In primary monkey kidney cells, this virus grows slowly and produces weak hemadsorption patterns. Canchola et al. (27) found that the sensitivity for detecting parainfluenza virus type 4 in primary monkey kidney cell cultures generally was increased 10-fold in rolled cultures.

Rotaviruses. The enhancement of calf rotavirus CPE by rolling infected bovine kidney cell cultures has been reported by Kurogi et al. (149). McNulty et al. (183) noted that stationary cultures infected with a calf rotavirus had less than 50% of the CPE than was observed in rolled infected cultures. Also, the onset of CPE was delayed for 24 to 48 h in stationary cultures. No differences in viral titers were detected between rolled and stationary cultures. The absence of rotavirus CPE in stationary but not rolled cultures has been reported by another investigator (147).

In 1979, Koves (147) isolated bovine rotavirus from feces by using calf kidney roller cultures but was unable to isolate virus from the same samples in stationary cultures. Similar results with porcine rotaviruses were reported by Fukusho et al. (75). Porcine rotavirus was consistently isolated in MA-104 roller cultures after a combination of trypsin treatment of the inoculum and incorporation of trypsin into the maintenance medium. Stationary cultures treated with trypsin failed to give similar results. It was also found that rolling cultures and the incorporation of trypsin into the maintenance medium were necessary for the successful titration of a porcine rotavirus. Furthermore, rolling alone or trypsin treatment alone was not satisfactory for the continued passage of a porcine rotavirus.

Human rotaviruses were isolated by using roller cultures and trypsin treatment (114, 245, 303, 339). Ward et al. (312) found that human rotaviruses could not be isolated in flask cultures that were rocked. However, three of four of the same fecal specimens were positive when the roller culture method was used. Some human rotaviruses have been isolated without using roller cultures (4), and such isolates may be different from those isolated in roller cultures.

Terrett and Saif adapted a porcine group C rotavirus to serial propagation in primary porcine kidney roller cultures (292). Saif and coworkers were unsuccessful in adapting group C rotaviruses to propagate in stationary cultures of continuous MA-104 cells or primary porcine kidney cells (241, 293). Roller MA-104 cultures consistently had approximately 20-fold more rotavirus-infected cells than did stationary cultures (241).

HSV. Using both laboratory strains and clinical specimens, Mavromoustakis et al. (178, 179) found that both fibroblast and epithelial cell cultures rolled at 2 rpm produced significantly more HSV foci than stationary cultures. Furthermore, the viral yield from rolled cultures was 2.9- to 8-fold greater than that from control cultures. The time to maximal CPE was also 24 h longer for nonrolled cultures (179). When low multiplicities of infection of a laboratory strain of HSV were used, 2 (5%) of 37 of the nonrolled cultures were positive at 96 h, while 18 (45%) of 40 of the rolled cultures were positive ($P < 0.01$) (179).

Other herpesviruses. King and Goyal (142) studied the effect of rolling at 0.2 rpm on the isolation and cultivation of pseudorabies (PR), infectious bovine rhinotracheitis, and equine rhinopneumonitis viruses. Within a few days after inoculation, rolled porcine kidney cell cultures for PR virus had more advanced CPE than stationary cultures, and rolled cultures inoculated with low concentrations of virus were CPE positive while stationary cultures were negative. Similar results were obtained with equine rhinopneumonitis virus. CPE for infectious bovine rhinotracheitis virus in bovine turbinate cells also occurred sooner in rolled cultures than in nonrolled cultures. When clinical specimens known to be positive for PR virus were tested by the roller method, rolled cultures had enhanced CPE and more dilutions were positive (142).

RSV. Rolling respiratory syncytial virus (RSV)-infected HEp-2 cell cultures at 2.0 rpm resulted in the production of significantly more viral antigens than in cultures that were not rolled (276). The mean optical densities obtained were 1.48 ± 0.08 with antigens from four rolled cultures and 0.21 ± 0.17 with antigens from four nonrolled cultures, $P < 0.05$.

Other agents. In 1939, Gey and Bang (84) used the roller culture technique and human fibroblasts to propagate *Chlamydia trachomatis*, the agent causing lymphogranuloma venereum. In 1954, Weiss and Huang (317) used shaker and roller cultures (0.2 rpm) to propagate both feline and murine pneumonitis agents in chicken embryo cultures. In 1954, these agents were considered to be viruses, but today they are classified as chlamydiae. Cultures agitated (54 movements per min at 1.5 in. [ca. 3.8 cm]) had significantly more infected cells (310 ± 73) than cultures not agitated (100 ± 18).

High-Speed Rolling and Orbital Motion

Vaccinia virus. The amount of CPE produced by vaccinia virus in GM-70 cell cultures rolled at 96 rpm was significantly enhanced compared with that produced by cultures not rolled (129). In addition, cultures infected with a low multiplicity of infection and rolled at 96 rpm developed maximal CPE 4 days earlier. The numbers of foci were 46.3 ± 8.4 for virus-infected cultures rolled at 96 rpm and 16.3 ± 6.5 for cultures not rolled. The foci in cultures rolled at 96 rpm were also significantly larger. The mean focus diameters in cultures at 0 and 96 rpm were 0.16 ± 0.03 and 0.28 ± 0.05 mm, respectively. Compared with stationary cultures, cultures rolled at 96 rpm had a 75-fold geometric mean increase in viral yield. The enhancement in viral yield between rolling at 96 rpm and stationary conditions ranged from 10- to 400-fold.

HSV. When HSV-infected BGM-70 cell cultures were rolled for 24 h at 96 rpm and then assayed for virus, the viral

yield was 7.3-fold higher than that in nonrolled cultures, $P < 0.01$ (179). Of 37 cultures infected with a low multiplicity of infection of HSV and rolled at 96 rpm, 33 (89%) were CPE positive at 96 h postinoculation, while only 2 (5%) of 37 were CPE positive in the nonrolled cultures ($P < 0.01$). HSV-infected cultures rolled at 96 rpm produced maximal CPE 2 days sooner than cultures that were not rolled (179).

CPE produced by HSV in BGM-70 cell cultures rolled at 96 rpm were compared with CPE produced in shell vial cultures that were centrifuged at 700 × *g* for 40 min (127). At 24 h postinfection, 42 (53%) of the rolled cultures were CPE positive, while only 16 (21%) of the shell vials were CPE positive, $P < 0.01$. This information suggests that cultures continuously rolled at 96 rpm may be better for CPE detection than shell vial cultures. When low-titered HSV specimens were inoculated into both roller tubes and shell vials, 39 (95%) of 41 were immunoperoxidase positive in high-speed roller tubes at 64 h postinoculation, while only 24 (58%) of 41 were immunoperoxidase positive in shell vials, $P < 0.01$ (127). The mean number of foci in cultures rolled at 96 rpm was 14-fold greater than that in shell vial cultures. Additional studies with HSV have demonstrated that continuous rolling of BGM-70 cultures at 383 rpm (30.2 × *g*) for 4 days enhanced HSV yields 53-fold over that of cultures incubated without motion (126). Furthermore, shell vial cultures that were not centrifuged but were incubated for 16 h on an orbital shaker at 90 rpm had significantly more HSV foci than shell vials handled by a conventional shell vial method (126). These results suggest that continuous orbital motion or rolling of cultures at high speeds may be useful for detecting HSV and also should be considered for the detection of other viruses.

CMV. Roller culture studies with human cytomegalovirus (CMV) have resulted in enhanced viral CPE. More human foreskin fibroblast cell cultures were CPE positive for CMV when rolled continuously at 96 rpm, and the amount of CPE was significantly enhanced ($P < 0.05$; Student's *t* test) as determined by a mean CPE score (126). The mean CPE for rolled cultures was 3.0 ± 0.4 versus 1.4 ± 0.5 for cultures not rolled ($n = 27$ cultures).

Enteroviruses. Roller culture studies carried out in our laboratory at high speeds (96 rpm) with coxsackievirus A-21 grown in HeLa cells gave results similar to those reported for low speeds by Mufson et al. (200). Specifically, HeLa cell cultures were infected with coxsackievirus A-21 and then incubated at 37°C either without motion or with rolling at 96 rpm. Cultures rolled at 96 rpm produced 1,500- to 2,500-fold more virus than stationary cultures (126). When stationary cultures infected 2 days previously with coxsackievirus A-21 were transferred to a roller apparatus and rolled at 96 rpm, maximal (100%) CPE occurred after 2 days of rolling. Stationary cultures developed only 10 to 25% CPE during the same 4-day time interval.

RSV. The effect of continuous orbital motion and motion at 96 rpm on RSV growth in HEp-2 cell cultures was examined by Sturgill and Hughes (276). Cultures infected with low concentrations of RSV and continuously rolled at 96 rpm produced significantly more viral antigens as detected by enzyme immunoassay than cultures incubated without motion. The mean optical densities obtained with antigen from cultures rolled at 96 rpm, from cultures incubated on an orbital shaker (150 rpm), and from stationary cultures were 1.78 ± 0.22, 1.25 ± 0.8, and 0.21 ± 0.17, respectively. CPE for RSV also were enhanced in rolled cultures when compared with stationary cultures. Of 47 cultures rolled at 96 rpm, 20 (42.6%) had a 3+ or greater CPE rating, while only a single stationary culture reached a 3+ CPE rating. The mean CPE for cultures rolled at 96 rpm was 2.1 versus 0.9 for nonrolled cultures, $P < 0.05$. At 96 rpm, 9 (41%) of 22 of the RSV isolates were detected 1 to 2 days sooner, while only 1 (4%) of 22 was detected sooner in nonrolled cultures. These studies indicate that cultures used for RSV isolation should be continuously rolled at 96 rpm or rolled at some optimal speed which still needs to be determined.

Data from studies evaluating the effect of rolling on virus-infected cells are summarized in Table 2. It is clear that motion in the form of continuous high-speed rolling at 96 rpm can have a significant impact on the detection of some viruses in cell cultures. Rolling at 96 rpm is superior to rolling at 0.2 or 2.0 rpm for viral replication and CPE production for certain viruses (179, 276). In summary, continuous rolling or motion can (i) enhance CPE; (ii) result in more specimens being CPE positive; (iii) lead to an earlier appearance of CPE; (iv) result in maximal CPE occurring sooner (2 to 4 days earlier); (v) result in higher viral titers and greater viral yields (10- to 1,000-fold increase); and (vi) enhance the infection process by allowing for more virus-cell interaction and more cell-to-cell spread of virus.

Centrifugation

Centrifugation to enhance viral detection is used on a routine basis in diagnostic virology laboratories. Smith, Gleaves, Espy, Paya, and coworkers were instrumental in introducing centrifugation and the shell vial assay for the routine detection of many different viruses in a diagnostic laboratory (58, 61, 88, 96, 97, 99, 174, 175, 214, 215, 263, 265–267). Other terms used to describe this centrifugation process and viral detection include the following: (i) enzyme-linked immunosorbent assay (ELISA) spin amplification technique, or ELISA-SAT (189, 313); (ii) precytopathic method (135); (iii) early antigen assay (153); (iv) centrifugation culture (2, 37, 56, 87, 90, 91, 94); (v) spin-amplified rapid assay, or SARA (11); (vi) spin amplified (72, 207, 208); (vii) centrifugation-mediated adsorption (208); (viii) spin-amplified tissue culture, SATC or SATC-EIA (48); (ix) spin-amplified culture with immunofluorescent visualization, or SAC/IF (19); and (x) double-label shell vial test, or DLST (309).

Culture vessels used for centrifugation studies have been shell vials or flat-bottom tubes; 24-, 48-, and 96-well plates; flasks; and roller tubes. One problem that has been reported for shell vials when used in a diagnostic setting is the cross-contamination of specimens because of aerosols generated by opening shell vial caps (242). Information about centrifugation used for specific viruses is provided below and in Table 3.

Eastern equine encephalitis virus. In 1954, Gey et al. (85) reported the effect of centrifuging Eastern equine encephalitis virus onto HeLa cells. Centrifugation was for 1 h at >100,000 × *g*. Centrifuged cultures had more cell death after 2 days than occurred in noncentrifuged roller cultures infected with the same dilutions of virus.

Vaccinia virus. The use of centrifugation with vaccinia virus was reported in 1960 (261). Centrifugation (8,000 × *g*) was used to bring suspensions of L cells and vaccinia virus together on an agar surface. With centrifugation, attachment of virus to L cells was 100 times faster, and 60% viral adsorption occurred within 4.5 min.

Myxoma virus. The first centrifugation report of shell vial cultures and the immune detection of viruses was in 1962

TABLE 2. Summary of rolling studies used for the growth of viruses

Yr(s)	Virus or agent	rpm	Outcome	Selected reference(s)
1940	Vaccinia virus	0.1	Long-term virus production	67
1952, 1959	Poliovirus	0.2 to 0.3	Excellent viral yields, higher titers, enhanced CPE	73, 235
1953, 1959, 1960, 1961	Rhinovirus	0.2	First propagation of virus, higher yields and titers, maximal CPE sooner	9, 121, 195, 224
1954	Feline pneumonitis	Shaking (54/min)	Enhanced infection	317
1962	Reovirus	0.2	Enhanced CPE, earlier detection of CPE, higher titers	159
1962	Coxsackievirus A-21	0.2	Enhanced CPE, earlier detection of CPE, higher titers, greater sensitivity	200
1964	Parainfluenza virus		10-fold increase in sensitivity	27
1976, 1977, 1979, 1981, 1984, 1988	Rotavirus	0.2–0.4	Enhanced CPE, earlier detection of CPE, more sensitive	75, 147, 149, 183, 241, 312
1987	PR	0.2	Enhanced CPE, greater sensitivity	142
1987	Infectious bovine rhinotracheitis virus	0.2	Earlier detection of CPE	142
1987	Equine rhinotracheitis virus	0.2	Greater sensitivity	142
1988	HSV	2.0	More foci, greater yields, maximal CPE sooner, greater sensitivity	178, 179
1988	Vaccinia virus	96.0	More CPE and foci, maximal CPE sooner, greater yields	129
1989	RSV	2.0 150 (orbital)	More antigens, enhanced CPE	276
1988, 1989	HSV	96.0 90 (orbital)	Greater yields, maximal CPE sooner, greater sensitivity	127, 179
1991	CMV	96.0	Enhanced CPE	126
1991	Enterovirus	96.0	Greater yields and more CPE	126

(211). Padgett and Walker used centrifugation at 1,270 × *g* for 10 to 20 min to promote the adsorption of myxoma virus onto preformed rabbit kidney cell monolayers. After centrifugation, the cultures were incubated for 8 h, and the cells on round cover glasses were fixed and stained with fluorescein-conjugated antimyxoma serum. Padgett and Walker (211) also observed that centrifugation of myxoma virus resulted in a 50% increase in the detection of viral plaques.

CMV and sand rat herpesvirus. In 1968, Osborn and Walker (209) used 1-oz. (ca. 29.6-ml) bottle cultures of mouse embryo cells for centrifugal studies with murine CMV (MCMV). They found that MCMV produced 10- to 100-fold more plaques when viral inocula were centrifuged at 1,900 × *g* for 30 min onto cells. No enhancement in plaque formation was seen when monolayers were centrifuged at 1,900 × *g* for 30 min prior to adding virus for a plaque assay. In the same study, HSV and PR virus had only a threefold enhancement in infectivity after centrifugal inoculation of virus onto mouse and chicken embryo cell cultures (209).

In 1976, Hudson et al. (124) confirmed and expanded the earlier centrifugal enhancement studies of Osborn and Walker (209). Hudson and coworkers found that centrifugal enhancement of MCMV was not because of the genetic heterogeneity of MCMV. In addition, the source of virus, the state of viral purity, and the source or type of cells infected did not alter centrifugal enhancement. Furthermore, centrifugal enhancement was seen with human CMV strain AD169. Centrifugal enhancement of MCMV compared with that of HSV was not due to a major increase in MCMV penetration into cells by the centrifugal process. Hudson et al. (124) suggested that the centrifugal field may exert some beneficial physiological or biochemical effect on host cells, making them more permissive for CMV infection and replication.

By using synchronous cultures, Hudson (123) found that centrifugal enhancement for MCMV was not dependent on any particular cell cycle phase. Extrinsic factors such as dibutyryl cyclic AMP, dibutyryl cyclic GMP, cortisol, imidazole, cytochalasin B, $CaCl_2$, and DEAE-dextran, which affect cell metabolism, gene regulation, cell growth, or morphology, did not alter the centrifugal enhancement effect. Virus could be left in contact with cells for 8 h prior to centrifugation, and enhancement would still occur. According to Hudson (123), centrifugation may influence viral gene regulation and force certain viruses into a more productive infection.

Additional centrifugal studies by Hudson (123) demonstrated that another herpesvirus, the sand rat herpesvirus, was enhanced 18- to 35-fold in mouse, rat, and human cell lines after centrifugation at 1,000 × *g* for 30 min. In contrast, HSV type 1, bovine herpesvirus type 1, Sindbis virus, infectious hematopoietic virus, and infectious pancreatic virus did not show an enhancement in infectivity similar to that of MCMV or sand rat herpesvirus. Thus, some viruses appear to be affected more than others by centrifugation.

Smith, Gleaves, Paya, Espy, and colleagues have demonstrated the rapid detection of human CMV by using human fibroblast cell cultures in shell vials that were centrifuged and later immune stained with monoclonal antibodies to early viral antigens (59, 66, 86, 87, 90–94, 96–98, 135, 175, 213–216, 263, 267). These investigators showed that CMV could be detected within hours by these methods in contrast to detection by CPE, which took days to weeks. Additional centrifugation studies dealing with CMV have been pub-

TABLE 3. Summary of centrifugation studies used for the growth of infectious agents

Yr(s)	Virus or agent	*g* force (× *g*)	Centrifugation time (min)	Centrifugation outcome	Selected reference(s)
1954	Eastern equine encephalitis virus	>100,000	60	Enhanced cell death	85
1960	Vaccinia virus	8,000	NI[a]	Increased rate (100-fold) of viral attachment	261
1960, 1963, 1974, 1990	Rickettsiae and chlamydia	1,600–15,000	60	10- to 10,000-fold enhancement in infectivity, rapid detection, greater sensitivity	40, 103, 104, 229, 316
1962	Myxoma virus	1,900	15–30	Enhanced plaque production	211
1968	MCMV	1,900	30	10- to 100-fold more plaques	209
	HSV and PR virus	1,900	30	3-fold enhancement in infectivity	209
1975, 1977, 1984, 1989	Rotaviruses	600–3,000	60–120	>1,000-fold increase in infected cells; noncentrifuged cultures negative	14, 24, 29, 164
1976	MCMV	800	30	15- to 85-fold enhancement in PFU	124
	HSV			3- to 5-fold enhancement in PFU	
1978, 1980	HSV	1,100	10	10-fold enhancement in infectivity	290, 291
		28,000–45,000	90–138	100-fold enhancement in infectivity	
1980	HSV	28,000	138	Enhanced infectivity at 4°C	291
1981, 1985, 1986, 1989	HSV	15,000	60	10- to 100-fold increase in sensitivity, rapid detection	41, 42, 309, 310
1984–1992	CMV	170–5,300	15–90	Rapid detection, 2- to 20-fold enhancement in foci or nuclei, more CPE, shorter mean CPE time, more positive cultures, variable sensitivity	1, 2, 7, 11, 21, 26, 30, 37, 45, 56, 59, 62, 66, 70, 79, 81, 86, 87, 92–94, 97, 118, 119, 123, 132–135, 141, 153, 157, 158, 160, 163, 166, 169, 173, 175, 181, 182, 190, 191, 198, 199, 208, 213, 215, 220, 223, 227, 228, 233, 237, 253, 263, 267, 268, 282, 284, 297, 298, 315, 318, 321, 322, 324, 325, 327, 330, 333, 337, 338
1984, 1989	Adenoviruses	15,000	60	No enhancement in isolation at 72 h, rapid detection	43, 309
1985–1992	HSV	700–4,000	15–45	Rapid detection, more CPE, more antigen, variable sensitivity	22, 42, 48, 60, 63, 72, 89, 95, 99, 123, 130, 161, 167, 187, 207, 218, 225, 244, 254, 301, 313, 323, 328, 329, 331, 345
1986–1992	Influenza viruses	700–1,000	10–60	Specimens positive earlier, higher HA titers, more rapid detection, more plaques, sometimes more sensitive	17, 61, 111, 162, 177, 193, 226, 255, 256, 274, 314, 326
1987–1992	Adenovirus	87–700	30–60	5-fold enhancement in no. of foci, more rapid, sometimes more sensitive	58, 170, 177, 226, 332
1988	MCMV and other viruses	1,000	30	Variable enhancement in infectivity	123
1988, 1989	VZV	700–1,000	45–60	More sensitive and rapid for detection	88, 250, 319
1988	Calicivirus	1,200	30	Enhanced infectivity	69
1989	HIV	2,500	30–120	10-fold enhancement in infectivity	221
	HHV-6[b]			100-fold enhancement in infectivity	
1989	Bluetongue virus	200	30	10- to 20-fold more plaques	280
1990	BK virus[c]	700	45	4-fold more foci, rapid detection	174
1990–1992	RSV	700–1,000	60	2- to 40-fold increase in sensitivity with reference viruses, more positive cultures, variable sensitivity with clinical isolates, rapid detection	138, 177, 188, 226, 238, 264, 314
1991	Measles virus	700	60	Rapid detection	194
1992	Parainfluenza viruses	700	30	Rapid detection	177, 226

[a] NI, not indicated.
[b] HHV-6, human herpesvirus type 6.
[c] BK virus, polyomavirus BK.

lished (1–3, 7, 11, 21, 26, 30, 37, 45, 56, 70, 79, 81, 115, 118, 119, 132–134, 136, 141, 153, 157, 158, 160, 163, 166, 169, 173, 181, 182, 190, 191, 197–199, 208, 220, 223, 227, 228, 233, 237, 253, 268, 273, 282, 284, 297, 298, 315, 318, 321, 322, 324, 325, 327, 330, 333, 337, 338). Several review articles have reported the use of centrifugation for CMV diagnosis (106, 107, 265, 266). The infectivity of human CMV was shown to be enhanced 8% by centrifugation of standard tube cultures at 2,000 × *g* for 1 h (cited in Miller et al. [190]). The rapid detection of CMV by immune staining of cell cultures not centrifuged has been reported, and CMV can be detected directly in leukocytes and lavage specimens, etc. (21, 80–82, 92, 108, 175, 233, 234, 271, 272, 281, 304–306, 337, 338). Since replacing centrifugal enhancement with nonmechanical treatments such as the use of mitogens has not been possible, CMV should be assayed and detected with *g* forces near 1,000 × *g*.

Rotaviruses. In the viral diagnostic laboratory, group A rotavirus infections are rapidly diagnosed by the detection of antigen in stool specimens. Rotaviruses can also be detected by the immune staining of centrifuged cultures. For example, in 1975, Banatvala et al. (14) found that six of seven electron microscopy-positive human rotavirus specimens were indirect immunofluorescence assay positive in centrifuged pig kidney cell cultures. Cells were grown on 12-mm-diameter coverslips in flat-bottom vials (13 by 94 mm). Inoculated cultures were centrifuged at 3,000 × *g* for 2 h and then stained 48 h later for viral antigens. Noncentrifuged cultures were negative for rotavirus antigens. Thus, the study by Banatvala et al. (14) was the second documented report involving the immune detection of a virus following the centrifugation of infected cell cultures. The first centrifugation report was in 1962 by Padgett and Walker (211) for the immune detection of myxoma virus.

In 1977, Bryden et al. (24) confirmed the findings of Banatvala et al. and were able to detect human rotaviruses at 18 h postinfection in three different cell lines after centrifugation at 1,200 × *g* for 70 to 75 min. For this study, primary and secondary HEK cells, primary calf kidney cells, and a monkey kidney cell line (LLC-MK2) were used. Rotaviruses were detected in 31 of 84 specimens by the immune staining of centrifuged cultures. By electron microscopy, 35 of 84 specimens were positive. All cell cultures were comparable in their sensitivity for the detection of virus. In addition, the number of infected cells increased more than 1,000-fold in centrifuged cultures.

Cevenini et al. (29) demonstrated that human rotaviruses could be detected rapidly in cell cultures after centrifugation at 1,200 × *g* for 1 h and immune staining 24 h later. Of 30 fecal samples positive for rotavirus by electron microscopy, 28 (93%) were positive in centrifuged cell cultures.

Lipson and Zelinsky-Papez (164) found that centrifugation was necessary for the immune detection of human rotaviruses in cell cultures. Repeated attempts to detect rotavirus without centrifugation were unsuccessful. Agliano et al. (4) have reported that some human rotaviruses can be isolated without using centrifugation. Centrifugation may be useful in isolating non-group A rotaviruses and other enteric viruses that grow poorly in conventional culture systems.

HSV and PR virus. In 1968, Osborn and Walker (209) reported that HSV and PR virus had a threefold enhancement in infectivity after the centrifugal inoculation of mouse and chicken embryo bottle cultures at 1,900 × *g* for 30 min. Tenser showed in 1978 that centrifugal inoculation of Vero and rabbit kidney cell tube cultures (1,100 × *g* for 10 min) and the ultracentrifugal inoculation of tube cultures (28,000 to 95,000 × *g* for 90 to 138 min) resulted in nearly 10- and 100-fold increases in HSV infectivity, respectively, over that of cultures not centrifuged (290). For these studies, virus was detected by CPE. As in the studies of Osborn and Walker (209), cultures centrifuged before infection with HSV displayed no enhancement in infectivity. A later HSV study by Tenser and Dunstan (291) confirmed Tenser's earlier work, and they postulated that centrifugal enhancement may involve a greater penetration of virus into cells. Osborn and Walker (209) attributed increased MCMV infectivity after centrifugation to enhanced adsorption of virus onto cell monolayers. In another study, viral adsorption with myxoma virus increased after centrifugation of inocula onto cells (211).

The effect of high-speed centrifugation (15,000 × *g* for 60 min) on the isolation of HSV in HEp-2 cells from clinical and laboratory specimens was studied by Darougar et al. (41). Centrifugation significantly increased isolation rates over those of a noncentrifugal method (41). The number of CPE-positive clinical specimens in flat-bottom cultures that were centrifuged was 74 (46%) of 160 specimens versus 56 (35%) of 160 specimens for control cultures. When clinical and laboratory specimens of HSV were diluted and then reisolated, the centrifugal method was more sensitive at all dilutions tested (41). When 20 specimens were tested by endpoint titrations, the centrifugal method was 10-fold more sensitive for 15 specimens and 100-fold more sensitive for 5 specimens. There was no difference in time of appearance of HSV CPE between the centrifuged and noncentrifuged cultures.

Other high-speed centrifugation studies (15,000 × *g* for 60 min) for HSV detection were reported by Darougar et al. (42) and Walpita and Darougar (309). Both of these studies used HEp-2 cells in flat-bottom tubes or shell vials and immune staining after 48 h for HSV detection. The centrifugation method was more sensitive than the noncentrifugation method in one study (42) but not the other (309). These differences were attributed to variations in sample size, with the latter study using a smaller number of specimens.

The first reports of the rapid detection of HSV using centrifuged cultures with immune staining were by Gleaves et al. (99), and Walpita et al. (310). Since then, many other studies that involve centrifugation or the immune staining of centrifuged cultures for the rapid detection of HSV have been published (19, 22, 42, 48, 60, 63, 72, 89, 95, 100, 130, 161, 167, 189, 207, 218, 225, 244, 254, 301, 309, 313, 323, 328, 329, 331, 336, 345).

Salmon et al. (244) found no increase in sensitivity for HSV detection after immunoperoxidase staining of centrifuged mink lung shell vial cultures versus immune staining of noncentrifuged cultures. Peterson et al. (218) and others (254, 328, 329) have reported that shell vials or 24-well plates that were centrifuged and then stained 16 to 24 h postinoculation were less sensitive for HSV detection than conventional cell culture methods. In the study by Peterson et al. (218), the sensitivity of the shell vial method was dependent on the staining procedure, with an indirect polyclonal antibody staining method being better than staining with a direct monoclonal antibody. Of 82 specimens positive by conventional culture methods, the shell vial formats detected 68 with MRC-5 cells and direct monoclonal antibody staining, 74 with human lung (MRC-5) cells and indirect polyclonal antibody staining, 64 with primary rabbit kidney (PRK) cells and monoclonal antibody staining, and 77 with rabbit kidney cells and polyclonal antibody staining. Peterson et al. (218) reported sensitivities of 82 to 96% for HSV detection in

centrifuged cultures, while Woods and Mills reported sensitivities of 70 and 71% (328, 329). The studies by Woods and Mills (328, 329) involved the direct fluorescent monoclonal antibody staining of MRC-5 or A549 cells, with some cultures being treated with dexamethasone (DEX). Seal et al. (254) reported a sensitivity of 66.2% for their direct fluorescent-antibody method with shell vials. It is important to note that the PRK cell cultures used by Seal et al. (254) were older than those used by other investigators.

For the HSV studies reviewed above, shell vials or plates were used. When conventional PRK culture tubes were centrifuged at 750 × *g* for 40 min, Oefinger et al. (207) found that the sensitivity for HSV isolation was enhanced, CPE developed more rapidly, CPE was greater, and ELISA antigen levels were higher. Specifically, 51 (40.2%) of 127 positive cultures processed by centrifugation either had CPE present or developed it more rapidly than cultures not centrifuged. The number of HSV-positive specimens from 864 specimens after centrifugation was 127 versus 112 (88.2%) for noncentrifuged tube cultures.

HSV has been detected in centrifuged cultures by ELISA or DNA probes (48, 60, 72, 130, 189, 313, 331). An ELISA-SAT with PRK and MRC-5 cells had a sensitivity and specificity equal to or better than those of a conventional cell culture technique, and all results were objectively determined after 2 days, whereas only 69% of the specimens were CPE positive after 2 days (189). This study included 300 specimens and 84 were HSV positive, with 82 being positive by ELISA-SAT (97.6% sensitivity) versus 80 being positive (95.2% sensitivity) by cell culture. Both methods were 100% specific. When a rapid absorbent matrix pad and ELISA testing were used with centrifuged A549 cell cultures for HSV detection, the sensitivity at 16 to 20 h was only 82.5% of that of conventional cell culture isolation (130). When DEX-treated PRK and MRC-5 cells were used in a 36-h ELISA-SAT, the sensitivities ranged from 88.8 to 97.3% (313). The sensitivity and specificity of a DNA probe (Enzo Biochemicals, Inc.) for detecting HSV in centrifuged MRC-5 cell cultures were 98 and 99%, respectively (60). Using a different probe (Ortho Diagnostics Systems, Inc.) and different cells and conditions, Woods and Yam (331) reported a sensitivity of only 82% for the probe used with centrifuged cultures. The DNA probe also was found to be more sensitive than staining with monoclonal antibodies following centrifugal inoculation of cultures. They recommended that centrifugal inoculation of A549 cells grown in 24-well plates followed by overnight incubation and nucleic acid hybridization should not replace conventional cell cultures for the detection of HSV in clinical specimens. With another probe (Diagnostic Hybrids, Inc.), the overall sensitivity for detecting HSV in centrifuged cultures was 97.8% (131 of 134 specimens), but the sensitivity was only 89.9% (26 of 29) for specimens from asymptomatic patients (72).

Using a mouse model, Boerman et al. (19) found that the polymerase chain reaction detected more HSV type-1 in cerebrospinal fluid than did a centrifugal culture method. The polymerase chain reaction method may be essential for samples such as cerebrospinal fluid and cervical specimens that often have little virus to be amplified by the centrifugal culture method.

HSV can also be rapidly detected in noncentrifuged cultures by immune staining (65, 116, 128, 137, 152, 180, 187, 192, 202, 203, 219, 239, 243, 257, 344). Most studies that use commercial immunoperoxidase staining kits and noncentrifuged cell cultures have found immunoperoxidase staining at ≤48 h to be less sensitive than conventional cultures for detecting HSV (65, 116, 128, 239, 243, 257). The sensitivity for detecting HSV in noncentrifuged cultures by immune staining has been variable and has ranged from 48 to 100% (65, 187, 192, 202, 203, 243). The differences in sensitivity reported for the detection of HSV by immune staining of noncentrifuged and centrifuged cultures can be attributed to differences in host cells, type of specimen, incubation temperatures, reagents, and other conditions used for testing. It is important to note that Landry et al. and Zhao and coworkers (152, 344) have reported that, when highly sensitive cell cultures such as guinea pig embryo cells, mink lung, or PRK cells are used for detecting low concentrations of HSV, CPE detection is comparable in sensitivity and rapidity to viral antigen or DNA detection in less sensitive cell cultures, such as MRC-5 or Vero cells.

Adenoviruses. In 1984, Darougar et al. (43) centrifuged 33 clinical and 39 laboratory adenovirus samples at 15,000 × *g* for 1 h onto HEp-2 cells that were growing on 13-mm-diameter coverslips. After incubation for 48 h, the cells were fixed and immune stained with group-specific horse adenovirus antiserum for viral antigens. The centrifugation method did not improve the isolation rate of adenoviruses from 72 samples. Similar results were reported by Walpita and Darougar (309), but in their hands, centrifugation and immune staining led to a more rapid detection of adenoviruses. August and Warford (12) reported that adenoviruses can be detected rapidly in noncentrifuged shell vial cultures by immune staining with a monoclonal antibody. Immune staining at 72 h postinoculation detected 86.7% of the adenovirus cell culture isolates.

Espy et al. (58) found that adenoviruses could be detected more quickly in HEp-2 cells by a centrifugation method than by a conventional culture method. Ninety-seven percent of the adenovirus strains were detected within 48 h after immune staining with a group-reactive monoclonal antibody, while it took an average of 4 days for CPE development. For the maximal detection of adenovirus foci, 30 min of centrifugation at 700 × *g* was required. Numbers of foci were not enhanced by centrifugation for 45 or 60 min. However, the number of adenovirus foci was significantly enhanced at 700 × *g* compared with the numbers produced after centrifugation at 87.5, 175, or 350 × *g* (58). Adenovirus-infected cultures centrifuged at ≤700 × *g* for 30 to 60 min had a fivefold increase in foci compared with noncentrifuged cultures.

Centrifugation and immune staining studies for the detection of adenoviruses were also carried out by Woods et al. (332), Mahafzah and Landry (170), Matthey et al. (177), and Rabalais et al. (226). Mahafzah and Landry found that with one 50% tissue culture infective dose, positive cells were not detected until day 5 postinfection. At day 5 postinfection, 100% of the 35 reinoculated clinical specimens were positive in shell vials, while 91% of the specimens were CPE positive in HEK cultures, 43% were CPE positive in A549 cultures, 26% were CPE positive in MRC-5 cultures, and 11% were CPE positive in HEp-2 cultures. The shell vial method was thus more sensitive at 5 days than conventional culture systems except for HEK cultures ($P < 0.0001$). However, on day 2 postinfection, the shell vial method was significantly more sensitive than CPE detection in HEK cultures.

The studies described above indicate that the immune staining of centrifuged cultures is more rapid and possibly more sensitive than conventional culture methods for the detection of adenoviruses. It would be interesting to use immune staining to compare adenovirus-infected monolayers from shell vials, conventional cultures, and cultures

rolled continuously at high speeds to see whether differences in numbers of foci and sensitivity could be detected.

Influenza viruses. Espy et al. (61) were the first to report the use of centrifuged shell vial cultures and immune staining for the rapid detection of influenza viruses. These investigators used primary rhesus monkey kidney cells and a mixture of monoclonal antibodies for the detection of influenza A and B viruses. Centrifugation for 30 min at 700 × *g* resulted in significantly more foci compared with control cultures. Centrifugation at 700 × *g* for 45 or 60 min did not yield greater numbers of foci when compared with foci produced at 700 × *g* for 30 min. At 48 h postinoculation, 27 (60%) of the isolates were positive by the shell vial method. Conventional cell cultures detected 45 influenza viruses, with a mean detection time of 4 days. Similar results with centrifuged Madden-Darby canine kidney (MDCK) cell cultures and influenza virus detection were reported by Guenthner and Linnemann (111). However, Stokes et al. (274) and Bartholoma and Forbes (17) reported sensitivities of 84 to 91% for centrifuged influenza cultures stained at 16 to 18 h postinfection. The increase in sensitivity for these later studies could be explained by differences in reagents, incubation conditions used during centrifugation, and cell and medium conditions. For example, Stokes et al. (274) reported using trypsin with their MDCK cells, while Guenthner and Linnemann did not (111).

Using MDCK cells in shell vials for influenza virus isolation, Ling and Doraisingham (162) found that, generally, specimens became hemagglutination positive earlier and peak HA titers were higher in centrifuged cultures than in conventional tube cultures. In addition, more clinical specimens were HA positive in centrifuged cultures than in noncentrifuged cultures (24 [15.2%] versus 6 [3.8%]; $P < 0.005$). Similar results were reported by Rabalais et al. (226) for the detection of influenza B virus. They found that more specimens were indirect immunofluorescence assay positive in centrifuged cultures than in noncentrifuged cultures.

Centrifuged 24-well plate MDCK cell cultures were used by Woods and Johnson (326) for the immune detection of influenza A viruses. They found no significant difference in the detection of influenza A viruses in centrifuged versus noncentrifuged cultures, $P > 0.05$. Of 234 specimens, influenza A virus was detected in 23, with 16 (70%) being recovered by conventional cell culture and 21 (91%) being recovered by centrifugation. Compared with the conventional cell culture method, the centrifugation and immune staining method was more rapid for detecting influenza A viruses. CPE was observed in 13 (81%) of the 16 cell culture-positive specimens after an average of 6 days, while all 21 centrifuged cultures were positive at 40 h by immune staining. It would be of interest to determine whether hemadsorption would be as effective as immune staining in detecting myxovirus and paramyxoviruses in centrifuged cultures.

Similar centrifugation studies for influenza virus detection with MDCK cells in 24-well plates were carried out by Mills et al. (193). Centrifugation did not yield a greater number of positive specimens. Of 451 clinical specimens, 28 (6.2%) were positive for influenza A virus in conventional culture tubes, while 23 (5.1%) were detected in centrifuged cultures at 40 h postinfection. Influenza B virus was recovered from 35 (7.8%) of the specimens by conventional culture isolation, while 30 (6.6%) were positive in centrifuged cultures. Immune staining of centrifuged cultures after 2 days was more rapid, while 4 to 5 days were required for CPE development (193). A similar time reduction for detecting influenza viruses has been reported by other investigators (177). However, the rapid detection of influenza virus in noncentrifuged cell cultures by immune staining has been reported by Evans and Olson (64) and Swensen and Kaplan (283). In the last studies, the sensitivities for detecting influenza virus at 24 h postinoculation in noncentrifuged cultures were 82 and 86%, respectively.

Seno et al. (255, 256) found that centrifugation of MDCK plate cultures significantly increased the number of isolates, increased the number of influenza virus plaques by 2.9-fold, and also caused a 10-fold increase in the HA activity of 76% of the isolates. Centrifugation for 60 min at 700 × *g* resulted in the most number of plaques. For the studies by Seno et al. (255, 256), viruses in centrifuged cultures were detected by hemagglutination and not by immune staining, and control plate cultures were not centrifuged.

Waris et al. (314) used centrifuged MDCK cells and tested 23 known influenza virus-positive specimens that had been stored frozen. Upon retesting, 22 (96%) were positive after 2 days by immune staining of centrifuged plate cultures; 4 (17%) were positive by CPE in conventional cultures, and 10 were antigen positive, for a total of 14 (61%). It is clear from all of these studies that further investigations to help resolve the differences in sensitivity reported for influenza virus detection in centrifuged and noncentrifuged cultures are necessary. What is needed are standard conditions with the only variable being motion or centrifugation.

VZV. The immune detection of varicella-zoster virus (VZV) in cellular smears of vesicular lesions is more sensitive than viral isolation in conventional cell cultures and is the method of choice for the laboratory diagnosis of VZV infection (51, 88, 231, 250, 251). Recently, centrifuged cultures and immune staining have been used for the rapid diagnosis of VZV infections (88, 250, 265, 319).

Data from a study by Gleaves et al. (88) showed that the immune staining of centrifuged cultures at 48 h was more sensitive than conventional cell cultures for the detection of VZV. Of 39 specimens positive for VZV, 36 (92%) were positive by direct fluorescent-antibody staining of specimen smears, 23 (59%) were positive at 24 h in centrifuged cultures, and 25 (64%) were positive in conventional cell cultures. At 48 h, 31 specimens (79%) were positive in centrifuged cultures. For centrifuged and conventional cultures, MRC-5 cells and human foreskin fibroblasts, respectively, were used. Additional studies with the same cells, same passage levels, and same cell densities for both centrifuged and noncentrifuged cultures need to be done. It is clear from this study, however, that the immune staining of centrifuged cultures was more rapid than a conventional culture method for detecting VZV (48 h for staining versus a mean of 12.5 days for CPE). Other studies by West et al. (319) and Schirm et al. (250) support the work of Gleaves et al. (88), indicating that immune staining of centrifuged cultures is more rapid and can be more sensitive for VZV detection.

Calicivirus. A porcine calicivirus-like virus was adapted to serial propagation in primary porcine kidney cell cultures (69). For viral propagation, the best results were obtained when six-well culture plates were centrifuged at 1,200 × *g* for 30 min at room temperature. No infected cells were detected at any of seven serial passages when roller cultures were used.

Bluetongue virus. Sundin and Mecham (280) found that the centrifugation of baby hamster kidney cells at 200 × *g* for 30 min with an inoculum of bluetongue virus enhanced viral replication 10- to 20-fold. Centrifugation also enhanced the

detection of virus from blood samples. Virus was detected earlier and longer after infection when serially collected blood samples were centrifuged onto cell monolayers. In addition, viral titers at the peak of viremia were approximately fourfold greater in centrifuged samples.

HIV. Four HIV-1 strains, centrifuged with either fresh peripheral blood lymphocytes or T-cell lines, yielded a 10-fold enhancement in infectivity compared with noncentrifuged cultures (221). The cell-virus mixtures were centrifuged at 2,500 × *g* for 30, 60, 90, or 120 min. Centrifugation for 60 min was significantly better than centrifugation for 30 min, and centrifugation for 120 min did not significantly increase the infectivity titer.

Human herpesvirus-6. Centrifugation of peripheral blood lymphocytes has been used for the growth of human herpesvirus-6 (221). The titer of human herpesvirus-6 in peripheral blood lymphocytes increased approximately 100-fold after centrifugation at 2,500 × *g* for 60 min.

RSV. Centrifugation of cell cultures has been used for the isolation and detection of RSV (138, 177, 188, 226, 238, 264, 314). Waris et al. (314) found that the immune staining of centrifuged cultures detected more RSV in four cell lines than were detected by CPE alone or by CPE and immune staining of conventional cultures. Johnston and Siegel (138) evaluated two nonculture methods (direct immunofluorescence and enzyme immunoassay) and two culture methods (centrifuged cultures and conventional cultures) for the detection of RSV in clinical specimens. Of 410 specimens, 175 (42.6%) were positive for RSV. Twenty-one (12%) of the 175 RSV-positive specimens were detected by only one method, while 43 (25%) were positive by two methods. Enzyme immunoassay detected 153 (87%) of the 175 RSV-positive specimens, direct fluorescent-antibody assay identified 146 (83%), shell vial culture detected 127 (73%), and conventional culture detected 70 (40%). The conventional culture method detected an additional 19 viruses other than RSV. Information from this study suggests that the shell vial method was better than a conventional culture method for detecting RSV. However, no statistical analyses were reported.

Smith et al. (264) reported no differences in sensitivity for detecting RSV in 50 stored positive clinical specimens by a shell vial method and immune staining versus a conventional culture method. RSV was recovered in 46 shell vial cultures compared with 45 conventional tube cultures. However, the shell vial method detected RSV in 4 and 11% of the specimens determined previously to be negative by direct fluorescent-antibody assay and enzyme immunoassay, respectively. The shell vial method also was more rapid than the conventional cell culture method for RSV detection. By 16 h, 43 (93%) of the shell vials were positive. With conventional cell culture tubes, CPE was detected from 2 to 8 days postinoculation (mean, 4.5 days). Shell vials were negative for CPE after 3 days of incubation. From these reports, it is clear that additional and carefully controlled comparative studies with RSV and motion are needed. It also appears that shell vials may not be an ideal system for the development of certain viral CPE. We have observed a reduction in viral CPE in shell vials compared with roller cultures for HSV (127).

Polyomavirus. Marshall et al. (174) have reported that BK virus, a human polyomavirus, can be detected rapidly by using a shell vial culture method. HEK cells were found to be more sensitive than MRC-5 or rhabdomyosarcoma cells for detecting BK virus. Since BK virus grows slowly in conventional cultures, the shell vial method should be useful for following BK virus infections in immunosuppressed patients.

Measles and parainfluenza viruses. Minnich et al. (194) found that measles virus could be detected efficiently in A549 cell cultures with a spin amplification assay. Results were generally available after 1 to 2 days with centrifuged cultures versus 5 to 10 days with conventional cultures. Parainfluenza viruses also have been detected by the shell vial method (177, 226).

Other agents. In 1960, Weiss and Dressler (316) found that centrifugation at 1,600 to 1,800 × *g* for 1 h at 20°C enhanced the infection of chicken embryo cells or human skin cells with *Rickettsia prowazekii*, *Coxiella burnetii*, *Chlamydia psittaci*, and *Chlamydia trachomatis* by 10- to 10,000-fold over that obtained with stationary cultures. The oscillation "shaking" of cultures at 80 cycles per min for 1 h at room temperature did not enhance infection with *Chlamydia psittaci*.

In 1960, Gordon et al. (104) used centrifugation to aid in isolation of *Chlamydia trachomatis* in chick cells grown on 12-mm-diameter coverslips. In 1963, Gordon et al. (103) demonstrated that *Chlamydia trachomatis* could rapidly be detected at 48 h in McCoy cell cultures that had been centrifuged. In contrast, yolk sac cultures were not positive until day 10 after inoculation with the same material. Other studies by Gordon et al. (102) showed that centrifugation of specimens at 1,800 × *g* for 55 min was fourfold more sensitive for the detection of chlamydiae than the yolk sac culture method.

In 1974, Darougar et al. (40) investigated the effects of temperature and *g* force on the isolation and cultivation of *Chlamydia trachomatis* in irradiated McCoy cells. They found that centrifugation of specimens (1,800 × *g* for 60 min) at 35°C was fourfold better than centrifugation of specimens at 18°C ($P < 0.02$). In addition, the isolation of chlamydiae without the centrifugation of specimens was compared with centrifugation at either 2,500 or 15,000 × *g*. Of 141 specimens, 57 (40%) were positive after centrifugation at 15,000 × *g* and 46 (33%) were positive after centrifugation at 2,500 × *g*, $P < 0.05$. Only three of the specimens (2%) were positive without centrifugation. In addition, the number of inclusions was 3.6-fold greater for specimens centrifuged at 15,000 × *g* than for specimens centrifuged at 2,500 × *g*. Centrifugation of specimens at 15,000 × *g* for 1 h was also better than centrifugation at 15,000 × *g* for 15 or 30 min. Thus, for the isolation of chlamydiae, centrifugation at 15,000 × *g* for 1 h at 35°C was superior to any centrifugation at a lesser *g* force for a shorter period.

Centrifuged human embryonic lung shell vial cultures have been used for the routine isolation of *Rickettsia conorii* from ticks and blood (57, 172, 217). In addition, *Coxiella burnetii*, the agent causing Q fever, has been readily isolated and rapidly detected by using centrifuged human embryonic lung cell cultures (229).

The effects of high- and low-speed centrifugations on different virus-host cell systems is shown in Table 3. It is clear from Table 3 that centrifugation can be very beneficial for enhancing viral infections. In addition, centrifugation of specimens with cell cultures has become practical and routine for most, if not all, viral diagnostic laboratories. The enhancement of viral infectivity by motion (rolling or centrifugation) should be used as an amplification step in conjunction with various diagnostic probes.

To summarize, it is important to emphasize that centrifugation studies need to be carefully evaluated because appropriate controls have not always been used. It is not uncom-

mon to find different conditions in the same study. For example, different cells, different cell numbers, varying cell confluency, different culture vessels, different incubation temperatures and times, different detection methods, and different specimen volumes have been used in the same study of both centrifuged and noncentrifuged cultures. Furthermore, for viral detection the comparison of immune staining of centrifuged cultures with CPE in conventional cultures is biased and favors immune detection. The immune staining of cultures should be more rapid for viral detection than detection by CPE. The best comparison would be the immune staining of antigens in both centrifuged and noncentrifuged cultures. When this comparison is made, the true value of centrifugation for viral detection can be determined. Finally, the centrifugation of viruses and other infectious agents with cells can (i) enhance infection and cell killing by 3- to 10,000-fold (24, 123, 209, 221, 280, 290); (ii) result in significantly more viral plaques or foci by 2- to 100-fold (58, 209, 211, 255); (iii) increase viral isolation or detection rates for some viruses (14, 41, 164, 207); (iv) be essential for isolating certain viruses (14, 164); and (v) reduce viral detection times.

EFFECTS OF CHEMICALS, HORMONES, HEAT SHOCK, ENZYMES, pH, O_2, CELL PHYSIOLOGY, AND IONS ON THE PROPAGATION OF VIRUSES

Chemicals (DMSO and NaB)

The treatment of virus-infected cells with dimethyl sulfoxide (DMSO) can enhance viral transformation (143), increase plaque numbers and plaque size (33), increase the number of cells producing antigens (318, 320), and increase viral yields (252, 285). Both viral RNA and viral DNA yields can be increased by DMSO treatment of infected cells, and sodium butyrate (NaB) can enhance DNA viral yields (288). The importance of DMSO and NaB in a diagnostic setting needs further evaluation because DMSO and NaB can modulate gene expression. Specific information on the DMSO and NaB treatment of infected cells is provided below.

Picornaviruses. The effect of DMSO on poliovirus infectivity titers was studied by Amstey and Parkman (8). No enhancement in viral infectivity was detected when 10-fold serial dilutions of virus in 20 or 40% DMSO were used. However, poliovirus RNA infectivity was increased 3 $\log_{10}$ 50% tissue culture infective doses per mg of RNA when 40% DMSO was used during a 20-min RNA absorption period. The enhancement in RNA infectivity was observed for concentrations of DMSO ranging from 5 to 80%. When RNA infectivity with DMSO was compared with RNA infectivity with 1.0 M $MgSO_4$, titers 100- to 1,000-fold greater were obtained with DMSO.

Papovaviruses. Transformation of BHK-21/C13 cells by polyomavirus was significantly enhanced by treatment with 1% DMSO (143). Enhanced transformation with DMSO can occur by treating cells as late as 120 h after infection with virus. Plaque formation by simian virus 40 is also enhanced by using DMSO (33). Plaque numbers were increased three- to fourfold when DMSO was used to treat simian virus 40-infected cells. The final concentrations of DMSO in the agar overlays that gave the best plaque results ranged from 0.1 to 1.0%. In addition to increasing the number of plaques, DMSO treatment of simian virus 40-infected cells resulted in larger plaques.

CMV. The number of rhabdomyosarcoma cells producing CMV increased almost 100-fold following treatment with DMSO (285). The amount of virus produced in DMSO-treated confluent cultures was approximately 10,000-fold greater than that in control cultures. The treatment of subconfluent cultures with 1% DMSO resulted in eightfold more CMV being produced. In contrast, DMSO treatment of confluent or subconfluent HSV-infected cells did not result in increased yields of HSV (285). Furthermore, similar amounts of HSV were produced in both confluent and subconfluent cultures that were not treated with DMSO.

For CMV, DMSO treatment of confluent cells did not significantly affect the number of cells synthesizing immediate early or early antigens (285). On the other hand, late antigen production was significantly increased in confluent cultures. Pretreating cells with DMSO for 48 to 120 h before infecting the cells with CMV resulted in more cells producing virus. These results indicate that DMSO treatment acts on the cell and not on the virus. DMSO enhancement of CMV production probably does not involve events associated with viral penetration or uncoating but may involve events associated with a host function(s).

West et al. (318) and Li and Fong (160) have used DMSO with centrifuged cultures for the detection of CMV. West et al. (318) found that commercially prepared MRC-5 cells grown in shell vials and pretreated with 1% DMSO for 24 h were more sensitive than nontreated cells for the detection of CMV (strain AD169). DMSO was most effective when added after the centrifugation step. Earlier and more extensive CPE was also observed when conventional tube cultures of MRC-5 cells were pretreated for 24 h with 1% DMSO. The average number of days for CPE development with DMSO-treated cultures was 6.1 versus 11.8 for nontreated cultures. In addition to DMSO, chemicals such as NaB have been used to activate, stimulate, or induce herpesviruses, particularly CMV (288).

HSV. Treatment of HSV-infected MRC-5 cells with DMSO resulted in a 2.5- to 4.3-fold increase in immunofluorescent foci when compared with control cultures (320). When clinical specimens and the shell vial method were used, the positive rate for DMSO-treated cultures was 14 to 33% higher than for nontreated cultures.

Hepatitis B virus. The addition of 1.5% DMSO to hepatocyte culture medium prolonged cell survival and improved functional stability (110). Consequently, secretion of hepatitis virus e antigen was greater in DMSO-treated cells, and more viral DNA was detected (109, 110).

Myxoviruses. The infectivity of fowl plaque virus was increased 80-fold when 4% DMSO was added to culture medium immediately after infection of cells (252). Hemagglutinating activity and neuraminidase activity were increased 6- and 2.5-fold, respectively, by the same DMSO treatment. When 4% DMSO was added immediately after infection and removed 6 h later, viral yields were normal. DMSO did not stimulate viral protein synthesis. Reassortant viruses, which were restricted for growth or the production of functional HA, were able to replicate and produce functional HA in cells treated with DMSO. In the absence of DMSO, the reassortant viruses replicated poorly and produced few, if any, functional HA molecules. These studies support the hypothesis that DMSO helps to improve the assembly of HA and neuraminidase envelope components and the maturation of viral particles. Thus, DMSO can increase infectivity and viral yields and facilitate viral assembly.

In the viral diagnostic laboratory, cultures used for viral isolation are often confluent and may be less susceptible for certain viral isolations. Confluent cells are used because

cells may be purchased that way or are cultured too long before being used. Using compounds such as DMSO or NaB to increase cell permissiveness should aid in the rapid detection of viruses. Additional studies involving DMSO, NaB, and motion for the isolation of viruses in different cells is warranted.

To summarize, DMSO treatment of certain virus-infected cells has been reported to result in (i) enhanced viral transformation; (ii) increased viral yields, from 80- to 10,000-fold (252, 285); (iii) increased plaque numbers and plaque size and more extensive CPE; (iv) increased nucleic acid infectivity; (v) increased cell survival; (vi) earlier CPE (by nearly 50%); and (vii) more positive viral cultures.

Hormones

Hormones such as DEX have been reported to increase viral yields, cause viral plaques to be larger and to appear earlier, result in the earlier appearance of CPE and more extensive CPE, and lead to more positive viral cultures (36, 196, 212, 298, 313, 318, 335). Hormones can modulate gene expression. The use of hormone-treated cell cultures in the viral diagnostic laboratory has been reported previously (62, 298, 313, 318, 320, 321, 329, 332). Additional studies with hormones in different virus-host systems are needed to better evaluate the use of hormones in a diagnostic setting. Specific information about the effects of hormones on the viral infection of cells in vitro is provided below.

Retroviruses. Paran et al. (212) and Wu et al. (335) showed that DEX at a concentration of 10^{-6} M stimulated the production of type C retroviruses. Wu et al. (335) suggested that the effect of DEX on type C virus production involved posttranscriptional virus functions. Hormones can also enhance retrovirus transformation of cells (247).

Polyomavirus. Morhenn et al. (196) reported that 3T3 cells and mouse embryo fibroblasts treated continuously for 7 to 10 days with physiological doses of corticosteroids (0.1 and 1.0 μM DEX or cortisol) produced at least 10-fold more polyomavirus plaques than nontreated cultures. In addition, viral plaques were larger and appeared earlier in treated cultures.

CMV. When fibroblast cultures were treated with 10^{-5} to 10^{-8} M DEX for 24 h before infection, CMV yields were enhanced 2.5- to 14.3-fold over those in nontreated cultures (286). Plaque numbers also were 5- to 11-fold greater, and plaques appeared sooner and were larger in treated cultures. One-step growth studies of CMV in hormone-treated and nontreated cultures revealed that the viral eclipse period was shortened by 1 day in DEX-treated cultures. Additional studies have demonstrated that CMV in human epithelial cells can be enhanced by DEX (287).

Centrifugal and conventional culture studies with DEX carried out by Thiele and Woods (298) demonstrated that DEX treatment can enhance the detection of CMV under certain conditions. At a concentration of 10^{-5} M DEX, the sensitivity for detecting CMV by a conventional cell culture method was increased. Of 251 specimens, 46 were positive for CMV, with 39 (85%) being positive in conventional cultures treated with DEX, while only 30 (65%) were positive in nontreated cultures ($P < 0.01$). In a centrifugal assay with 24-well plates, 42 specimens (91%) were positive in nontreated cultures. DEX used with centrifuged cultures did not significantly increase the detection of CMV, but it did increase the number of infected cells per coverslip.

Enhancement of plaque production by CMV AD169 was observed after both pre- and post-diethylstilbestrol treatment (10^{-5} to 10^{-7} M) of Flow 5000 cells (230). However, viral titers were not increased.

Forbes et al. (71) found that treatment of fibroblasts with 10^{-5} M hydrocortisone had a stimulatory effect on CMV antigen and virus production in shell vials. For example, at 4 h after viral infection, the production of immediate early proteins in cells pretreated for 24 h with hydrocortisone was enhanced 11- to 12-fold compared with that in nontreated control cultures. With few exceptions, the hormones human chorionic gonadotropin, progesterone, and estradiol, when used at physiological concentrations, had suppressive effects on CMV infections (71). Characterization of hormonal and other factors that can regulate cellular development and metabolic processes for CMV and other viruses may be useful for enhanced viral growth and detection in the diagnostic setting.

HSV. A ≥10-fold increase in HSV yield was found by Costa et al. (36) for cultures treated with DEX or cortisol. When DEX at concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M and cortisol at 10^{-6} M were incorporated into overlay medium, a two- to fivefold increase in HSV plaque number occurred. With 3T3 cells, steroids increased viral yields and plaquing efficiency, while in Swiss mouse fibroblasts no increase in viral yields or plaquing efficiency was observed. Differences in viral release between syncytium-forming and non-syncytium-forming viruses also were detected. Thus, the hormonal effect on HSV replication was dependent on the cell type and the viral strains used. Not all hormone-treated cell cultures led to enhanced viral production or viral release. In addition to increasing HSV yields, hormonal treatment with diethylstilbistrol can lead to enhanced HSV biochemical transformation of mouse cells (230).

DEX can also enhance the detection of HSV in clinical specimens (329). Conventional A549 cell cultures treated with DEX resulted in significantly more specimens being positive for HSV at 24 h (46 versus 27 specimens) and 48 h (72 versus 59 specimens). There was no significant difference between DEX-treated and nontreated A549 cells for the detection of HSV in centrifuged cultures (329).

Adenoviruses. Woods et al. (332) found that DEX did not enhance the detection of adenoviruses in A549 cells. Furthermore, DEX significantly lengthened the detection time of adenovirus CPE, and CPE was often stronger in nontreated cultures.

It is clear from the studies described above that hormonal treatment of different virus-host systems can have a variable effect on viral growth and CPE. Hormones can induce heat shock proteins (139, 307), and heat shock can enhance viral replication (76, 342). Maybe the mechanism of hormone-enhanced viral replication is via heat shock proteins. Additional experiments to confirm and expand the above studies could help with the growth and detection of viruses. However, such studies must carefully control host cell and serum conditions. Previously, little attention has been focused on these variables.

To summarize, the treatment of certain cells with some hormones may (i) enhance viral yields at least 5- to 100-fold (36, 71, 286); (ii) enhance plaque formation at least 11-fold (196, 286); (iii) enhance plaque size (196); (iv) result in earlier plaque formation (196); (v) result in earlier CPE and more CPE; (vi) shorten the viral eclipse period (286); and (vii) increase the sensitivity for viral detection (298).

Hormones, DMSO, and Calcium

The treatment of cell cultures with hormones, DMSO, and/or calcium can enhance the detection of certain viruses (160, 318, 320–322). The beneficial effects of these agents can depend on culture confluency. Since many virology laboratories use older cell cultures or commercial diploid cell cultures that have reached confluency, the treatment of cultures with these agents has been advocated (321). The effects of these agents on viral propagation are reviewed below.

CMV. Espy et al. (62) report that treatment of MRC-5 cells in shell vials with 1% DMSO and 10^{-5} M DEX for 24 h before CMV infection and for 16 h after infection did not result in an increase in sensitivity or an increase in the number of fluorescent foci in treated cultures. Different results were obtained by West et al. (318) when they treated centrifuged and conventional cultures with the same compounds. They used commercial shell vials with confluent MRC-5 monolayers and conventional MRC-5 tube cultures and found that treatment of shell vial cultures with either 1% DMSO or 10^{-5} M DEX before and after CMV inoculation increased the number of fluorescence-positive cells by three- to sixfold over control cultures. The greatest enhancement occurred with cells pretreated with DEX and then treated with both DMSO and DEX. With conventional tube cultures, pre- and posttreatment with DMSO or DEX resulted in a more rapid appearance of and more extensive CPE. When clinical specimens were inoculated into DMSO-, DEX-, or DEX and DMSO-treated shell vial cultures, there was a 22 to 32% increase in positive cultures compared with control cultures. The best results were obtained when cultures were pretreated with DEX before infection and then also treated with DEX plus DMSO after infection. Treatment of CMV-infected cultures with the mRNA inhibitor cordycepin indicated that enhancement of CMV by DEX was probably due to cellular mRNAs or their protein product(s). Alternatively, enhancement by DEX could be due to an increase in viral mRNA production. No mechanism for enhancement of CMV by DMSO was postulated by West et al. (318). The differences between the studies of Espy et al. (62) and West et al. (318) may be attributed to such variables as source of serum, cells, time of infection after seeding of cells, cell passage level, and cell density.

West and Baker (321) found that 100-fold more CMV (strain AD169) was produced in cultures treated with DEX-DMSO-Ca than in nontreated cultures. When low-titered clinical specimens were retested, 11 (27.5%) of all positive specimens were found only in shell vials treated with DEX-DMSO-Ca. With freshly tested specimens, the DEX-DMSO-Ca group accounted for 20% of the positive cultures. In addition, cultures treated only with 2 mM calcium had more foci than nontreated cultures. However, enhancement was variable and less pronounced with different cells obtained from other suppliers (322). It is important to note that cells obtained from the same or a different supplier can vary in their sensitivity for isolating viruses. Cells of the same type may not always have equal sensitivities for isolating viruses. Because of different cell passage levels and selection events, cells of the same type can vary.

HSV. Warford et al. (313) conducted studies of HSV detection by CPE in MRC-5 cells pretreated with DEX-DMSO versus nontreated MRC-5 cells. HSV CPE was recognized 1 to 3 days sooner in 8.1 to 35.9% of the DEX-DMSO-treated cultures. Greater CPE scores also were noted for 12.8 to 19.1% of the treated cultures compared with 3.2 to 5.1% of the nontreated cultures (313).

RSV. For RSV, calcium is required for cell fusion and syncytium formation (260). In calcium-free cultures, CPE for RSV is not produced. It has also been reported that glutamine is essential for RSV cell fusion and giant cell formation (171). The supplementation of serum-free cultures or other cultures with calcium or with calcium and hormones may be beneficial for isolating and detecting certain viruses.

Heat Shock

In a wide variety of cell types, genetic regulatory mechanisms are altered after rapid exposure to a superoptimal temperature (heat shock). Heat shock results in the formation of a restricted set of polypeptides called heat shock proteins or chaperones. Treatments other than heat, such as with hormones, amino acid analogs, chelating drugs, or arsenite, can induce the heat shock proteins (117, 139, 289, 307). Viruses also can induce heat shock proteins (34, 78, 140, 204, 205). Heat shock can enhance viral replication (76, 342). The effects of heat shock on viral replication are not well understood. Possibly, heat shock or other cell treatments such as rolling or centrifugation activate cells to make them more susceptible to viral infection and replication. The utility of heat shock alone and in combination with other treatments for viral isolation and replication in a diagnostic setting remains to be determined. The association of hormones and heat shock treatment for viral replication needs additional investigation. The effect of heat shock on in vitro viral infections is reviewed below.

CMV. The treatment of fibroblasts at 44°C for 10 min before CMV infection or heat treatment at 24 h post-CMV infection resulted in an approximately 10-fold increase in CMV yield compared with that in control cultures (343). Heat shock of CMV-infected cultures also reduced the eclipse period and enhanced CMV antigen production in nonpermissive cells (343).

Epstein-Barr virus. Heat shock has been reported to enhance Epstein-Barr virus antigen production in Raji and P3HR1 cells (342). Raji cells that are Epstein-Barr virus nonproducer cells were induced to express early antigen(s) after heat shock, while the heat shock of P3HR1 producer cells resulted in more viral capsid antigen-positive cells.

HIV. Heat shock enhanced HIV type 1 replication in peripheral blood lymphocyte cultures (76, 232). Enhanced HIV infection occurred with 35 of 36 positive clinical samples cocultivated with heat-shocked cells. Heat-shocked cultures were positive 3 to 14 days before control cultures, and p24 antigen levels were two to three times higher in heat-shocked cultures.

In summary, the heat shocking of virus-infected cells or cells to be infected can (i) increase antigen and viral yields 10-fold, (ii) reduce the viral eclipse period, (iii) induce antigen expression in nonproducer cells, and (iv) reduce viral detection times.

Enzymes

Proteolytic cleavage of certain viral enveloped glycoproteins and proteolytic enhancement of viral propagation have been reported for several RNA viruses (28, 74, 122, 131, 145, 154, 155, 185, 201, 249, 262, 278). Proteolytic cleavage plays an important role in the function of viral glycoproteins, often resulting in cell fusion activity that results in the initiation and spread of viral infections.

In the diagnostic laboratory, the use of enzymes for enhancing viral infectivity or CPE is not a routine procedure. Since some wild-type viral isolates may grow poorly in the absence of exogenous proteolytic enzymes, enzymes may be warranted for primary viral isolations. Information on the effect of enzymes on CPE, plaque formation, viral propagation, and activation of important RNA viruses is reviewed below.

Influenza viruses. For influenza A viruses, there are currently 14 HA and 9 neuraminidase subtypes. Cleavage of the HA polypeptide into HA1 and HA2 is a requirement for infectivity (145, 154). Cleavage of HA results in the generation of a hydrophobic amino terminus of HA2 that mediates fusion between the viral envelope and the plasma membrane. In most cell cultures, the HA of virulent influenza viruses is cleaved to HA1 and HA2 in the absence of exogenous trypsin. For some influenza viruses, the HA is not cleaved unless trypsin is added to the culture medium. Thus, to aid in the isolation of certain influenza viruses, exogenous trypsin is added to the culture medium.

Rotaviruses and reoviruses. Some viruses, such as rotaviruses, can be present in vast quantities in clinical specimens and can be rapidly detected without using culture methods. However, for growing these viruses, proteolytic enzymes are often required. The use of trypsin or other proteolytic enzymes has been shown to aid or enhance the growth of rotaviruses and reoviruses in cell cultures (6, 13, 20, 31, 32, 105, 144, 150, 184, 241, 246, 269, 270, 292, 294–296). Babiuk et al. (13) observed that, if bovine rotaviruses were not passaged in the presence of trypsin, infectivity was gradually lost. Almeida et al. (6) found that yields of bovine rotavirus were increased up to 3 $\log_{10}$ when trypsin was incorporated into cell culture medium. The proteolytic enzymes trypsin and elastase have been shown to enhance the growth of simian, porcine, and bovine rotaviruses (105). For multiple cycles of human rotavirus replication, the presence of trypsin in the medium was required (144). However, Agliano et al. (4) reported the isolation and growth of human rotaviruses without the use of trypsin. These authors suggested that the isolated viruses may be different from previously recovered human rotaviruses because growth-enhancing techniques were not used.

Coronaviruses. Coronaviruses are often difficult to culture and are not routinely isolated by viral diagnostic laboratories. Proteolytic enzymes enhance the replication and cell fusion of some coronaviruses (44, 77, 120, 146, 240, 275, 278, 299, 340). The production of coronavirus CPE in the form of polykaryons or plaque formation appears to depend on the presence of proteolytic enzymes (18, 120, 210, 275, 299, 339). Additional studies with different host cells, enzymes, and motion for the isolation of coronaviruses are worth pursuing.

Astroviruses. Astroviruses have been propagated in cells when trypsin has been incorporated into cell culture medium (156). Titers of astrovirus grown in HEK cultures with trypsin were 2 to 3 $\log_{10}$ greater than titers in cultures without trypsin, and in the absence of trypsin, little astrovirus was released from cells. Trypsin was also necessary for plaque formation by astroviruses (125).

Caliciviruses. Trypsin in tissue culture medium appears to be required for the replication of certain animal and human caliciviruses (38, 39). Inclusion of trypsin in cell culture medium is important for good viral yields (246).

pH and Oxygen

A critical factor for viral replication or development of viral CPE by certain viruses is the pH of the cell culture medium. Lancz and Bradstreet (151) demonstrated that an overlay medium of pH 6.3 essentially inhibited plaque formation by HSV but not replication. At pH 6.6, there was a 50 to 70% decrease in HSV plaque number compared with plaque formation at pH 7.0. Roizman has shown that the optimal extracellular pH for the replication of three strains of HSV was between 6.5 and 7.3 (236). For CMV, Zerbini et al. (341) found that at pH 5.8, 6.0, and 6.2 viral replication was partially inhibited. Late CMV antigen production occurred only in cultures maintained at pH 6.5 or higher. Maximal viral yields were obtained at pH 6.8, while no infectious virus was produced in cultures maintained at pH 6.2 or lower.

At a pH $\geq$7.4, virtually no CPE or viral yields were detected for a canine calicivirus grown in dog kidney cells (246). A pH below 7.0 resulted in extensive CPE and high yields of virus. For a porcine calicivirus, more infected cells (30%) were detected at pH 7.4, while <1% of the cells were antigen positive at pH 6.8 (69). For a coronavirus, little CPE and small syncytia were produced at pH 6.0, while cultures at pH 8.0 had large syncytia and monolayers were mostly destroyed (5). In addition, maximal virus-induced cell fusion occurred at this pH.

Other enveloped viruses such as La Crosse virus (101) and viruses causing hemorrhagic fevers with renal syndrome (10) cause cell-to-cell fusion and giant polykaryons under acidic conditions of pHs between 5.1 and 6.5. For some bunyaviruses, cell fusion occurs within 15 min after the pH has been lowered to 4.9 to 6.3, and maximal cell fusion can be obtained within 1 h (10).

In the viral diagnostic laboratory, it is difficult to vary the pH of the culture medium to optimize viral replication or CPE production because many specimens must be handled and different host cells are used to isolate a variety of viruses that may be present in a clinical specimen. However, the information given above demonstrates that pH can have a significant influence on viral replication and CPE production. For viruses that generally do not produce typical CPE, changing the pH when cultures are hemadsorbed may be useful for detecting additional viruses.

Oxygen is essential for viral replication (16, 52). The host cell is also an important variable in the oxygen requirement for viral growth (16). The oxygen requirements for different viruses and even closely related viruses can vary (16, 52). The use of rolled cultures may increase the oxygen available to cells and help promote viral growth.

Cell Physiology and Ions

The physiological state of a cell can affect the replication of a virus, and viral replication in vitro is affected by culture conditions. For example, confluent cultures have a lower susceptibility to CMV infection than subconfluent cultures (46, 285). DeMarchi and Kaplan (46) found that subconfluent cultures infected with CMV yielded 25- to 140-fold more infectious virus than cells in confluent cultures. Many cells in confluent cultures did not support CMV replication. Furthermore, cells in subconfluent cultures that were treated with 5-fluorouracil, an inhibitor of DNA synthesis, produced 700- to 800-fold less CMV than nontreated subconfluent cultures. Nontreated cultures and cultures treated with 5-fluorouracil and then infected with HSV produced similar

amounts of virus. These results demonstrate that the replication of CMV is more dependent on host cellular function(s) than is that of HSV. In addition, Tanaka et al. (285) found that subconfluent cultures produced significantly more CMV (4.1×10^5 PFU/10^6 cells) than confluent cultures (4.2×10^1 PFU/10^6 cells). CMV replication was also inhibited or eliminated in serum-starved cells (46). Thus, CMV does not grow well in contact-inhibited cells or in cells in which DNA synthesis has been inhibited or down regulated, indicating that proper cell physiology is important for viral isolation and detection.

Another approach for improving viral yields and increasing infection is to further augment culture conditions. For example, increasing the ionic strength of the cell culture medium can enhance the propagation of poliovirus (308), dengue virus (176), and measles virus (23). For measles virus, supplementation of the culture medium with 25 to 50 mM $MgSO_4$ provided a consistent stimulatory effect. The ion or salt enhancement of measles virus was in the range of 4- to 100-fold for stationary cultures, 4- to 200-fold for microcarrier cultures, and 2- to 20-fold for roller cultures. Measles virus titers were higher and plaques were larger in treated than in control cultures. With other virus-host systems, such as rotaviruses and CMV, calcium plays an important role in viral replication and assembly (206, 258, 259). Previously, the importance of calcium for viral CPE and replication was noted for RSV (171).

CONCLUSION

Physical, chemical, and heat treatments of cells can be beneficial for enhancing viral replication and yields. The effect of rolling, rocking, or centrifugation of cells is to physically stress them. Cells stressed by different forms of motion may have more cell proliferation, decreased cell generation times, activated genes, and altered cell metabolism. Cells stressed by motion can be used for the isolation of viruses from clinical specimens. For some viruses, plaque and CPE formation, antigen production, and viral yields and titers can be significantly enhanced in rolled cultures compared with stationary cultures. In addition, rolling infected cultures at 96 rpm can be superior to rolling at ≤ 2 rpm for viral replication and the production of CPE for certain viruses.

The centrifugation of clinical specimens with cell cultures can be beneficial for enhancing viral infections. After centrifugation, virus-infected cultures have been detected by immune staining, by ELISA, or with nucleic acid probes. The immune staining of centrifuged cultures has been shown to be more rapid than CPE for the detection of viruses. However, comparing the detection of viruses by immune staining versus CPE leads to a biased comparison that favors immune detection. The best comparison involves immune staining for viral antigens in both centrifuged and control cultures. For many centrifugation studies involving viruses, appropriate controls have not always been used. For example, it is not uncommon to find different cells, different culture conditions, different specimen volumes, etc., being used in the same study for both centrifuged and noncentrifuged cultures.

The treatment of virus-infected cells with certain chemicals such as DMSO can enhance viral transformation, increase plaque numbers and size, enhance antigen production, increase viral yields, and improve viral assembly and maturation. Chemicals such as DMSO, NaB, and DEX may be useful in certain diagnostic situations for enhancing viral infections. Heat shock also can enhance viral replication events. The effects of heat shock on viral replication are not well understood. Perhaps, heat shock or other cell treatments such as rolling, treatment with certain chemicals, or g forces activate cells to make them more permissible to viral infection and replication. Since the mechanism(s) of viral enhancement by rolling, centrifugation, heat shock, DMSO, or DEX is generally unknown, additional studies with these and other treatments are warranted.

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