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Enhancement of Infectivity of Murine Cytomegalovirus *in Vitro* by Centrifugal Inoculation

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Centrifugation of murine cytomegalovirus inocula from a variety of sources onto secondary mouse embryo cell monolayers at 1,900 × *g* for 30 min regularly revealed 10- to 100-fold more infectious virus than could be found in the same materials using standard inoculation methods. Virus demonstrable only by centrifugation was present throughout the entire growth cycle in a constant proportion to virus measured without centrifugation. Extracellular growth curves of both populations revealed an 18- to 21-hr latent period, followed by a log-linear increase over the next 12 hr; final yield was 30 plaque-forming units (PFU) per cell. Centrifugation of cells prior to inoculation or after standard adsorption and removal of inoculum failed to result in any significant change in measured virus titer. However, even after 4-hr adsorption, the supernatant inoculum could be transferred and centrifuged onto a fresh monolayer resulting in the same increment of measurable virus. Neutralizing antibody and interferon were equally efficacious against 100 PFU of virus as defined by either method. Thus, this newly identified population of cytomegalovirus represents the vast majority of potentially infectious units and appears to differ solely in ease of adsorption onto cell monolayers.

Inefficiency of viral replication and transfer are virological phenomena which have frequently been invoked to explain histopathological features of malignant and degenerative diseases from which infectious agents cannot readily be isolated; ways to overcome or explain such inefficiency are of commensurate importance (10). Both human (16) and murine (11) cytomegaloviruses have been found particularly inefficient in reproduction of infectious particles, and the noninfectious nature of the vast majority of visualized particles has not been fully explained. In addition, the study of cytomegaloviruses has been hampered by the difficulty in achieving adequate multiplicities of infectious virus. The identification of several-fold more infectious units of cytomegalovirus than the number determined by standard assay is thus of both theoretical and practical interest.

Several investigators have utilized a technique of centrifugation of inoculum onto cell monolayers to improve the efficiency of infection with a variety of intracellular organisms (4, 6, 7, 13, 18). The magnitude of this increase in efficiency has ranged from threefold with myxoma (13) to more than 100-fold with relatively large microorganisms such as toxoplasma and trachoma (4, 7, 18). It has generally been supposed that this increase in efficiency resulted from an improved rate of adsorption and is related directly to size of the inoculated particle.

In the studies reported here, inocula of murine cytomegalovirus (MCMV) were found to contain 10- to 100-fold more plaque-forming units (PFU) when assayed by centrifugation of inoculum at 1,900 × *g* onto secondary mouse embryo cell culture (MECC) monolayers than when assayed by ordinary techniques; the increase in measurable infective units of virus appears to reflect improved efficiency of adsorption of MCMV.

MATERIALS AND METHODS

Cells and media. Primary mouse embryo cell cultures were prepared, as previously described (12), from 16- to 18-day Swiss mouse embryos. Confluent monolayers of primary cells were dispersed with 0.05% trypsin containing 0.05% ethylenediaminetetraacetate, and secondary cultures were seeded in 30-ml (1-oz) prescription bottles with no. 000 rubber stoppers. These were allowed to grow to confluency for 24 to 48 hr prior to use.

Cells were grown in medium 199 with 10% calf serum, bicarbonate buffer, penicillin, and streptomycin. After monolayers were inoculated, growth medium was replaced with either medium 199-5% calf serum (maintenance medium) or medium 199-10% starch gel overlay with 7.5% calf serum (5).

Viruses. MCMV of the Smith strain was kindly supplied for the initiation of these experiments by D. N. Medearis, Jr., and was passed serially by intraperitoneal injection in weanling mice. These were sacrificed 3 to 4 weeks postinoculation, and their submaxillary salivary glands were harvested aseptically and ground with mortar and pestle to a 10% (w/v) suspension in a 1:1 mixture of medium 199-5% calf serum and 50% sorbitol. Salivary gland extracts were used as inocula in all growth curve studies. Tissue culture fluid containing MCMV was harvested by decanting and was mixed 1:1 with 50% sorbitol. All specimens containing virus were stored in vials at −70 C until time of assay.

Throughout these experiments, MCMV was assayed by each of two methods. In the first, which will be referred to as the standard method, virus-containing inocula were added to cell monolayers and incubated in stationary position for 90 to 120 min, after which inocula were decanted. In the second, or centrifugation method, inocula were added in quantities of at least 1.0 ml to MECC monolayers in 30-ml (1-oz) bottles. These were then placed upright, cell side out, in a 28-cm (11-inch) basket head of an International model CM centrifuge and spun for 30 min at 3,500 rev/min (1,900 × *g*), after which the fluid was decanted. The drained monolayers were then overlaid with 4 to 5 ml of medium 199-10% starch, incubated for 72 hr, and again overlaid with starch containing 1:10,000 neutral red. Microplaques were scored under 10 times magnification 96 hr after inoculation.

Herpes simplex virus of the HF strain and pseudorabies virus were grown in MECC and then assayed by the two inoculation methods in secondary cultures of both mouse and chick embryo cells.

Plaque-reduction neutralization. Sera containing anti-MCMV neutralizing antibodies were prepared from rabbits, which had been repeatedly inoculated with suspensions of MCMV-infected mouse salivary gland at four weekly intervals. The sera were separated from clot by light centrifugation, inactivated at 56 C for 30 min, and stored at −20 C or 4 C until use. For assay of neutralizing antibody, duplicate serial twofold serum dilutions were prepared; each set was mixed with an equal volume of a dilution of stock MCMV calculated to contain 60 to 120 PFU of MCMV by either standard or centrifugal methods of assay. These mixtures, plus medium-virus controls, were incubated 60 to 90 min in a 37 C water bath with intermittent shaking and then assayed by the appropriate method. End points were defined as the reciprocal of the serum dilution at which 50% of control plaques were inhibited.

Interferon effect. Mouse serum interferon was prepared by exsanguinating adult mice 5 hr after intravenous injection of 10^8 PFU of Newcastle disease virus according to the method of Baron and Buckler (1) and assayed as previously described (12). Duplicate sets of monolayers were pretreated with serial twofold dilutions of interferon for 3 hr, and challenge doses of MCMV were added that were calculated to yield 60 to 120 PFU by the appropriate inoculation method. Plaque reduction end points (50%) were again used to designate titer.

RESULTS

Increased titer of virus with centrifugation inoculation. When replicate dilutions of a given inoculum were assayed by both standard adsorption and by centrifugal inoculation of monolayers, the measured titer was invariably 10- to 100-fold higher by centrifugal inoculation. Table 1 gives the results of comparative titrations of MCMV from various tissue culture fluids and from several infected mouse organ extracts. In each instance, several examples are cited to show that an increment of similar magnitude occurred over a wide range of concentrations of virus in the inoculum.

The centrifugation titer was at least as constant on repeated determination as was the standard assay titer, whether the source of virus was infected animal tissue suspensions or tissue culture fluid. Plaque morphology of the two populations appeared to be identical.

Effect of duration of centrifugation. The maximal effect of centrifugation was observed in the first 10 min, after which only slight additional increment in titer occurred (Fig. 1).

Growth curve of MCMV. Utilizing the method

TABLE 1. *Comparison of MCMV titers of various materials determined by standard and centrifugal methods of inoculation (PFU/ml)*

Source	Adsorption[a]		Fold increase (centrifugal/standard)
	Standard	Centrifugal	
Cell culture supernatant fluids	7.0×10^3	4.0×10^5	57.0
	1.0×10^4	7.5×10^5	75.0
	9.5×10^5	3.3×10^7	34.7
	3.2×10^6	1.0×10^8	30.0
Mouse spleen extracts	8.0×10^1	1.6×10^3	20.0
	8.0×10^3	1.4×10^5	17.5
	1.5×10^4	1.3×10^6	86.6
	8.0×10^4	8.0×10^5	10.0
	3.0×10^5	2.3×10^7	70.6
Mouse salivary gland extract	3.2×10^6	1.0×10^8	31.2
	1.0×10^6	5.4×10^7	54.0

[a] Data represent titers of an individual specimen assayed by two methods. The results of comparative titrations from several specimens are presented to illustrate relatively constant increment over a wide range of concentrations of infectious virus.

of centrifugal inoculation to achieve an adequate initial multiplicity, a growth cycle study was done. Inocula that contained $10^{7.8}$ PFU of MCMV by prior centrifugal assay were centrifuged onto replicate monolayers of MECC, composed of $10^{7.0-7.2}$ cells, giving a multiplicity of infection of about 5 PFU per cell. The inocula in these experiments were not decanted but to them was added maintenance medium to give a final volume of 5 ml per bottle. At each designated interval after inoculation, two or three bottles were removed from the 37 C incubator, and tissue culture fluids were decanted separately, mixed with sorbitol and stored in duplicate vials at −70 C. Replicate samples of fluid from each time interval post-inoculation were then assayed by both stationary and centrifugal inoculation methods (Fig. 2).

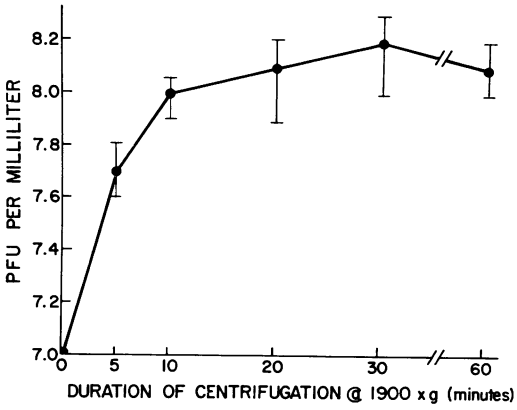

FIG. 1. *Effect of time of centrifugation on enhancement of MCMV adsorption onto secondary MECC monolayers. The mean and range of three or more determinations are indicated for each sampling interval.*

The extracellular growth curves determined by the two methods were parallel, suggesting that the phenomena accounting for increased infectivity with centrifugation pertained equally at all stages of the cycle of multiplication. After the initial adsorption, residual virus in the fluid remained at a constant concentration until 12 to 13 hr, at which time a secondary drop in titer was observed. At 18 to 21 hr, a log-linear increase in virus concentration began, persisting until about 30 hr. A second cycle apparently occurred with another 18-hr latent period, and further release of infectious virus occurred starting at 48 hr. When measured by standard assay, extracellular virus yield was barely equal to input; however, by centrifugation assay the infection was productive with 30 PFU per cell as an average final yield.

Mechanism of increased infectivity. To test the possibility that this newly identified component of inoculum represented a distinctly different population of MCMV, efficiency of neutralization and effect of interferon on the two virus

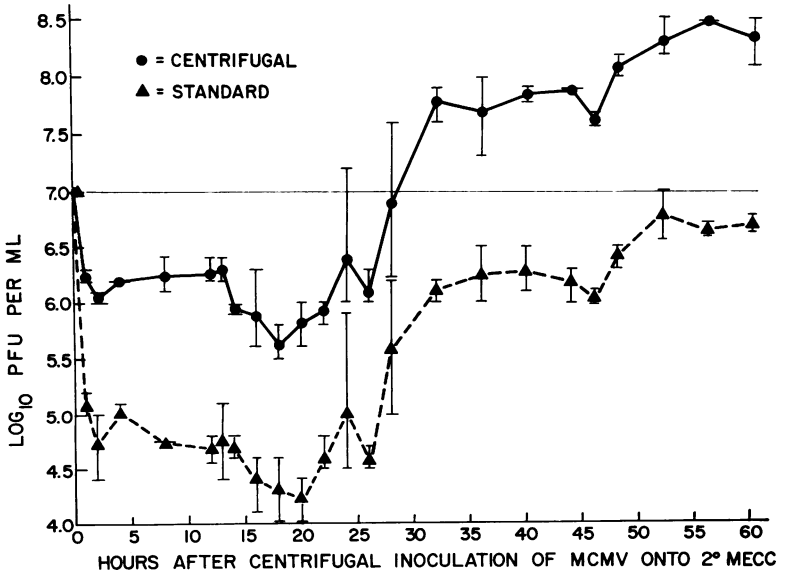

FIG. 2. *Extracellular virus yield from MCMV-infected mouse embryo cells; multiplicity of infection, 5. All monolayers were inoculated centrifugally at time-zero, following which identical samples were titrated by standard and centrifugal methods of assay. Data from three separate growth curve experiments are pooled. The means and ranges of two to five samples at each time interval are indicated as assayed by either method.*

populations were compared. It was found with several antisera and standard interferon pools that both the slopes and end points of plaque-reduction neutralization and interferon dose-response curves were essentially the same with 100 PFU of either "adsorption species" of MCMV.

The above experiments suggested that these were in fact two segments of the same virus population which were not obviously diverse in characteristics other than ease of infectivity. Studies were, therefore, undertaken to ascertain the mechanism of the centrifugation effect.

To test the hypothesis that centrifugal effects on the cells themselves allowed increased expression of virus infectivity at a stage after successful attachment, monolayers were centrifuged at 1,900 × *g* for 30 min prior to virus assay by standard adsorption. Final MCMV titer was the same on these monolayers as on control monolayers which had not been centrifuged previously.

The possibility was entertained that the additional virus, although adsorbed, required centrifugal force for further steps in the establishment of infection. However, after stationary adsorption, removal of inoculum, and washing of cells, centrifugation of monolayers failed to increase the titer over that measured by standard adsorption.

Attempts to improve the efficiency of the standard method of inoculation by increasing the adsorption period to 4 hr and rocking the monolayers throughout this period were unsuccessful. The increase in virus titer thus obtained was only 1.2-fold that of standard adsorbed controls.

If ease of adsorption were markedly different in the two MCMV populations, the newly identified virus might be recovered from the supernatant fluid after standard inoculation. Fluid decanted from bottles after 4-hr rocking incubation was inoculated onto fresh monolayers, and these were then spun at 1,900 × *g* for 30 min. The resultant titers on the fresh cells were essentially the same as when the original inocula were centrifuged directly onto the cell monolayers without intermediate stationary incubation of another cell sheet. Thus, the additional virus measured by centrifugation had remained intact in the supernatant fluid throughout the long stationary adsorption period and was readily retrieved by the centrifugal method of assay.

Specificity for cytomegalovirus. One hypothesis to explain centrifugal enhancement of infectivity would be that sedimentation per se allows maximal cell-virus contact and that efficacy would thus be directly related to particle size. If this were the sole or most important factor involved, it should follow that this property might be a feature of the entire herpesvirus group. Herpes simplex and pseudorabies viruses were, therefore, assayed by the two methods on mouse embryo and chick embryo cells, and the resultant increases in titer with centrifugation of these viruses are given in Table 2. The maximal increase of threefold corresponds to the magnitude of increment observed with myxoma (13) but is distinctly different from that observed with MCMV, indicating that virion size is not of cardinal importance in this enhancement phenomenon.

DISCUSSION

The studies reported here reveal that 10- to 100-fold more MCMV particles are capable of initiating infection than was recognized previously. The newly identified population of virus appears to differ from the standard measured infectious units largely in terms of ease of adsorption, and the deficiency in adsorption is reproducibly overcome by centrifugation onto susceptible monolayers at 1,900 × *g* for 10 or more minutes. The phenomenon cannot be explained simply on the basis of weight or size of particle, since only minimal increases in titer are measured when herpes simplex or pseudorabies inocula are similarly treated. The new population of virus thus identified appears to be otherwise identical to previously recognized MCMV in growth curve kinetics and in susceptibility to neutralizing antibody and to interferon effect.

TABLE 2. *Effect of centrifugation on titer of herpes simplex and pseudorabies viruses in cell culture fluids (PFU/ml)*

Virus	Cells for assay	Adsorption		Fold increase (centrifugal/standard)
		Standard	Centrifugal	
Herpes simplex	Mouse embryo	4.0×10^3	5.5×10^3	1.4
	Chick embryo	2.1×10^3	6.6×10^3	3.1
Pseudorabies	Mouse embryo	1.3×10^5	3.9×10^5	3.0
	Chick embryo	1.3×10^5	2.0×10^5	1.5

The growth curve constructed with the centrifugal method of inoculation and assay revealed several features worthy of comment. The final yield of infectious virus was less than 1 PFU per cell by standard measurements, but it was 30 PFU per cell by the new method of assay. It appeared that a single cycle of growth lasted about 30 hr; the pattern of rise in titer after that time suggested that synchrony of initial inoculation allowed a second discrete cycle to become apparent.

The latent period of 18 to 21 hr was considerably longer than that reported by Henson et al., who described a log-linear increase in MCMV beginning 10 hr after inoculation (9). Part of this difference may reflect the fact that only extracellular virus was measured in the present studies, whereas Henson et al. assayed virus after a single freeze-thaw cycle. However, unlike human cytomegalovirus, MCMV is rapidly released and relatively little cell-associated virus is found (9); thus, this difference in method probably does not fully explain the longer latent period. Another component of the discrepancy in results may be the fact that their study utilized tissue culture adapted MCMV, which may have altered its growth characteristics during passage.

The decline in titer observed in each of the two cycles just prior to appearance of new virus is unusual and difficult to explain. The stable titer prior to this might reflect a balance between thermal inactivation and elution which was temporarily altered in favor of the former effect after several hours. If such a balance does not occur, then it is noteworthy that MCMV is apparently surprisingly stable extracellularly in cell culture fluid for many hours at 37 C. Vonka and Benyesh-Melnick have described a similar stability of human cytomegalovirus at comparable temperatures (17).

In context of mouse infection with MCMV, it would be interesting to know if the newly identified virus population is capable of infecting in vivo. While it is difficult to answer this question definitively, it may be well to keep in mind that substantially more potentially infectious virus may be present in experimental animals than has been reported.

Other members of the herpesvirus group have been found to require special manipulation in order to achieve accurate assay (2, 3, 19). Smith (14) provided evidence for the occurrence of large aggregates of herpes simplex virus; dispersion by sonic treatment resulted in higher titers, although residual aggregates persisted even after the sonic treatment. It seems unlikely that disaggregation phenomena account for the increased MCMV titer found in these studies, since comparable increases were measured regardless of source or handling of infectious material.

Another aspect of inefficient production of infectious virions was studied by Smith (15), who found that nonenveloped particles of herpes simplex were essentially noninfectious. It may be that the inefficiency of MCMV adsorption reflects a similar deficit in envelope, which is overcome by application of centrifugal force.

Repeated electron microscopic revelations of viruslike particles in tissue obtained from individuals with various malignant or degenerative diseases add interest to the phenomena of inefficient transfer, since attempts to isolate and study such virions have thus far met with remarkably little success. An interesting case in point is the so-called EB virus associated with cultured Burkitt's lymphoma cells and serologically implicated in infectious mononucleosis (8), which appears to be a member of the herpesvirus group. Application of the centrifugal assay procedure to such material might distinctly facilitate recovery of infectious virus for identification and study.

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