Use of Centrifugal Force to Promote Adsorption of Myxoma Virus to Cell Monolayers.* (27793)

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Myxoma virus adsorbs to cells in a monolayer so slowly that the process may not reach completion within a practical period of time(1). For some investigations, it is essential that a very short time be required for adsorption of virus to every cell in the system. Moreover, a long adsorption period is undesirable in a plaque assay system because the virus sample is subject to thermal inactivation during this time and sufficient virus may be denatured to affect the accuracy of the titration. Sharp and Smith(2) have employed centrifugal force to achieve a rapid adsorption of vaccinia virus to L cells. This report will show how centrifugal force can be used to improve the plaque assay of myxoma virus and to obtain virtually simultaneous attachment of virus to all cells in a monolayer.

Materials and methods. Detailed descriptions of the virus strain, tissue culture cells and procedures, and our standard plaque assay for myxoma virus have been reported(1). The Moses strain of myxoma virus was grown in rabbit kidney (RK) cells in medium 199 and 3% calf serum. RK cells were secondary cultures while the rabbit heart fibroblast (RFH-1) cells were serially cultivated. For plaque assays the cells were grown in 1- or 2-oz prescription bottles and 4-6 bottles were used for each experimental group. Virus dilutions were made in 199 and 3% calf serum. All plaque bottles received an inoculum of 0.2 ml of virus and additional diluent was added to bottles which were centrifuged to give the desired total volume.

Adsorption of virus. Stationary bottles were incubated at 37°C for 4 hours and the inoculum was redistributed over the monolayer once every hour. In our standard procedure adsorption occurred while the bottles were continuously rocked through a 60° arc on a tilting platform for 4 hours at 37°C.

Centrifugation of plaque bottles. One-oz bottles containing 1-2 ml total inoculum were placed upright at the periphery of a basket rotor 11″ in diameter with the side covered by cells against the outside wall of the rotor. A rotor of 11″ diameter holds 22 bottles so placed. During centrifugation the inoculum, which originally was at the bottom of the bottle, was forced up over the cell monolayer on the side of the bottle as a thin film. One ml was the minimum volume that would provide adequate coverage of the cells when centrifuged in this manner. Bottles with flat sides must be used to obtain even distribution of the inoculum. Prescription bottles (Moderne Ovals) from the Foster-Forbes Glass Co., Marion, Ind., were used in this study. Centrifugation was at 1900 *g* in an International, Model CM, centrifuge at room temperature. Breakage of bottles during centrifugation was negligible. After centrifugation the inoculum was poured off and 5 ml of agar overlay was added. After 5 days' incubation at 35°C, 3 ml of the appropriate indicator overlay(1) was added and plaques were counted on the next day.

Centrifugation of shell vials. Monolayers of RK cells grown on the bottom of shell vials (21 × 70 mm) were inoculated with 1 ml of virus. The vials were wrapped in cotton and placed in 50 ml centrifuge cups containing inverted rubber cushions. Centrifugation was at 1270 *g* at room temperature.

Results. Centrifugal force applied to plaque assay. Table I shows a comparison of the number of plaque-forming units (PFU) of myxoma virus detected using various methods of adsorption. It is evident that adsorption of virus was least efficient when the inoculum was distributed over the monolayers only occasionally. The efficiency of adsorption was substantially increased when the inoculum was continuously redistributed by use of a rocking platform as in the standard as-

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TABLE I. PFU of Myxoma Virus Detected Using Various Adsorption Methods.

Treatment of bottles during adsorption	Hr of adsorption	Avg No. of PFU	
		RK cells	RHF-1 cells*
Stationary	4	25	38
Tilted continuously	4	41	59
Centrifuged	½	58	97

* The experiment using RHF-1 cells was not done at the same time as that using RK cells and a different dilution of virus was used in each case. Therefore, a comparison should not be made between the number of PFU detected by the 2 cell lines.

say procedure. Centrifugation of the virus onto the monolayers for only 30 minutes, however, consistently resulted in detection of a significantly greater number of PFU. About 50% more PFU were detected after centrifugation than with the standard procedure and a 4-hour adsorption period.

In our standard assay procedure adsorption of myxoma virus to RK cells does not reach a plateau in 6 hours(1). An experiment was designed to determine at what point maximum adsorption would be accomplished using centrifugation to deposit the virus on the cells. It is evident from the curve in Fig. 1 that maximum adsorption was achieved in only 15 minutes. To determine whether all of the infectious virus had actually been deposited by 30 minutes of centrifugation at 1900 *g* a given dilution of myxoma virus was added to 6 plaque bottles of RK cells. The bottles were centrifuged for 30 minutes, then each supernatant fluid was transferred to a fresh plaque bottle. These 6 bottles were centrifuged for 60 minutes. The total number of PFU detected by the first 30 minutes of centrifugation was 350 (avg 58/bottle). Centrifugation of the supernatant fluids for 60 minutes yielded only 1 plaque. These results indicate that all of the virus in an inoculum can be deposited on a cell monolayer by 30 minutes of centrifugation.

In preliminary experiments centrifugation was carried out in a cold room. The results obtained were inconsistent. Centrifugation at 4°C followed immediately by addition of the agar overlay resulted in detection of fewer PFU than when centrifugation was carried out at room temperature. Prolonged use of the centrifuge at room temperature does result in some heating of the rotor, but 3 consecutive 30-minute runs were made using identical inocula without decreasing the number of PFU detected. Varying the total volume of the inoculum between 1 and 2 ml did not affect the number of PFU detected and even distribution of plaques was observed in all cases.

Centrifugal force applied to infection of all cells in a monolayer. Monolayers of RK cells in shell vials were inoculated with 1 ml of myxoma virus and centrifuged at 1270 *g* for 10 or 20 minutes. The supernatant fluids were saved. The cells were washed 5 times, 1 ml of diluent was added, and the cells were frozen and thawed once. Complete disruption of the cells was achieved by 5 minutes' treatment with an ultrasonic disintegrator (20 kc/sec). The amount of virus present in the inoculum, in the supernatant fluids, and in the disrupted cells was determined. The average number of cells/vial was also determined by trypsinizing cells in other vials and counting the cells in a hemocytometer. From the results shown in Table II it is evident that centrifugation for a few minutes is sufficient to deposit enough virus to infect every cell. In Experiment 2 there were 24 PFU of

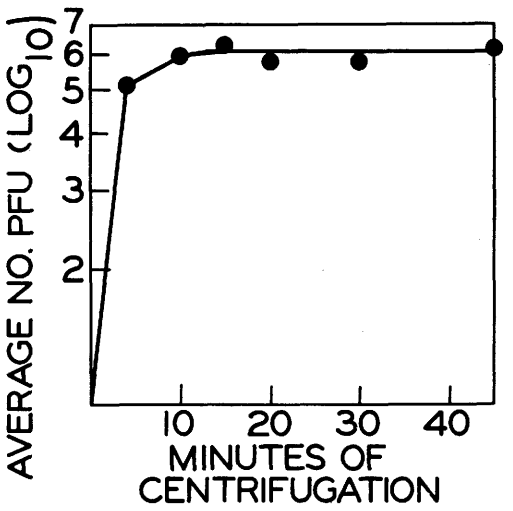

FIG. 1. Rate of adsorption of myxoma virus to RK cells when aided by centrifugal force. Two ml of diluted virus were centrifuged onto preformed monolayers at 1900 *g* for varying periods. Inoculum was removed and agar overlay added immediately.

TABLE II. Effectiveness of Centrifugation as an Aid in Infecting All Cells in a Monolayer.

Exp	Avg No. cells/vial	Total PFU of myxoma virus		
		Inoculum	Super. after cent.*	Disrupted cells
1	2.0×10^5	8.60×10^6	4.05×10^6	1.20×10^6
2	2.5×10^5	7.65×10^6	1.11×10^6	5.88×10^6

* The vials in Exp 1 were centrifuged 10 min while those in Exp 2 were centrifuged 20 min.

virus per cell attached to the cell monolayer even after thorough washing. The virus input was only 31 PFU/cell. As part of Experiment 2, some vials contained monolayers of RK cells growing on round cover-glasses on the bottoms of the vials. After centrifugation and washing, these vials received 1 ml of a maintenance medium and were incubated at 35°C for 8 hours. The cells on the cover-glasses were fixed in acetone, stained with fluorescein-conjugated anti-myxoma serum and examined by ultraviolet microscopy. At least 99% of the cells were found to contain viral antigen.

Discussion. Centrifugal force has been used to facilitate or enhance infection of tissue culture cells by psittacosis(3), trachoma (4), EEE(5), and vaccinia(2) viruses. Sharp and Smith(2) accomplished rapid adsorption of vaccinia virus to L cells by centrifuging a suspension of the two onto an agar block from which the cells could be removed for subsequent studies. Overman and Tamm (6) used centrifugation in a procedure to obtain counts of the absolute number of vaccinia virus particles present in a sample. This latter technic does not distinguish between infectious and non-infectious particles. Use of centrifugal force in a plaque assay system has not previously been reported. It might be used in two different ways. In the first method, suspended cells and diluted virus could be mixed and sedimented by centrifugation somewhat as described by Sharp and Smith(2). The cells could then be resuspended and planted in sufficient concentration to form a monolayer, or if only a few cells were used, they could be plated on preformed cell monolayers as in a titration of infective centers. In either case plaques would develop around infected cells. In the other method, as reported here, the virus particles could be centrifuged directly onto a preformed cell monolayer. We did not make a direct comparison of the two methods, but we chose the latter because it eliminated the possibility of loss of or damage to infected cells during the manipulations required for transferring and planting cells, and permitted the application of the agar overlay soon after the centrifugation.

Although virus adsorption in our standard plaque procedure with RK cells is terminated before a plateau is reached, the titration of myxoma virus by this method is as sensitive as the intradermal titration in rabbits(1). When adsorption is accomplished by means of centrifugation, however, the number of PFU detected is substantially increased. Similar results obtained with RHF-1 cells, which do show a plateau in virus adsorption at 4 hours in our standard procedure, demonstrate that centrifugation of the inoculum results in an absolute increase in the number of PFU that can be detected and not merely in a reduction in the time required to reach a plateau. The reduction in PFU detected by the standard procedure as compared with centrifugation may be due to heat inactivation occurring during the long adsorption period. By using centrifugation it appears possible, within the limits of the sensitivity of the RK cells, to detect all of the infectious particles in a sample. Other plaque assays depend for adsorption on chance collisions of virus and cell during varying periods of time. Under these conditions the rate of adsorption is slow and its efficiency is low(7). Also, thermal inactivation during this time may affect the accuracy of the titration. It is probable that such systems detect only a fraction of the infectious virus particles actually introduced. Since tissue culture cells will withstand much higher centrifugal forces than those used here, the centrifugation technic could be used with smaller viruses if a suit-

able container were found.

The rapid adsorption of myxoma virus to cell monolayers which is achieved by using centrifugal force will facilitate studies on the growth cycle of this virus and on virus-cell interactions during the early stages of viral replication. Also, it is now feasible to investigate such matters as rate of heat inactivation of myxoma virus at 37°C; a study which would have been meaningless with a plaque procedure requiring a 4-hour adsorption period.

Summary. Centrifugation of myxoma virus onto preformed monolayers of RK and RHF-1 cells substantially increased the number of PFU compared to other methods of virus adsorption. The maximum number of PFU was obtained after only 15 minutes of centrifugation at 1900 *g* at room temperature. Virtually simultaneous infection of all RK cells growing as a monolayer on the bottom of shell vials can be accomplished by centrifuging a low multiplicity of myxoma virus onto the cells at 1270 *g* for 10-20 minutes.

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Induced Latent Infection of Monkeys with Vacuolating SV-40 Papova Virus. Virus in Kidneys and Urine.* (27794)

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In nature, vacuolating or SV-40 virus(1), the simian member of the papova virus group (2), commonly infects rhesus (*Macaca mulatta*), less commonly cynomolgus (*M. irus* or *M. cynomolgus*), and apparently infects African primates (*Cercopithecus* spp., *Papio* spp.) only if these are placed in contact with infected Asian animals(1,3). When inadvertently given to human beings, low grade subclinical infection has occurred(4,5). Although no illness has been observed in primates (monkeys or man) infected with SV-40 virus, the agent is tumorigenic in newborn hamsters, producing fibrosarcomas(6,7).

The present report forms part of a study with the latent infection in African primates (green monkeys and baboons) infected experimentally with a purified line of the virus. Two features of the study reported here have been (a) to follow the animals for excretion of virus and subsequently of antibody in the urine, and (b) to follow the persistence of latent virus in the kidneys. The latter was done by carrying out repeated biopsies on the same monkey and testing the biopsy tissue for virus by direct and indirect means.

Materials and methods. The study included 23 primates: 7 baboons (*Papio doguera*) about 6 months old, and 16 green monkeys (*Cercopithecus aethiops*). The green monkeys included 8 young animals about 1 year old, 4 newborns about 7 days old and their respective mothers. All animals were flown from Africa directly to Houston or to New York, where they were kept separate and sent on within a day or two.

The routes and amounts of virus inocu-

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