In Vitro Growth Characteristics of Monkeypox Virus[1] (36266)

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In another paper (1), we described the responses of monkeypox virus (MPV) infection in the chick embryo, specifically related to *in ovo* local pock formation and spread of MPV into tissues of embryos. In this study, we report *in vitro* growth characteristics of MPV to provide information with respect to virus interactions in tissue cultures.

Materials and Methods. Monkeypox virus. The MPV stock was the same one described in our earlier studies (1). This virus stock has an infectivity titer of $1.6 \times 10^{6.0}$ pfu/ml in CV-1 cells (1) cultured *in vitro*.

Tissue cultures. A continuous line of green African monkey kidney (CV-1) cells was used for characterizing growth properties of MPV. WI-38 and Kappa (2) cells were used for titration of the virus stock. Monolayers of CV-1 cells were either grown in Leighton tubes or in petri dishes. Tube cultures were seeded with $1.8 \times 10^{5.0}$ cells suspended in 1.5 ml of medium 199 containing 5% peptone and 5% fetal calf serum. Tissue culture plastic petri dishes (60 mm) were seeded with 6.0 ml of cell suspension containing approximately $8 \times 10^{4.0}$ cells/ml. Petri plates were incubated in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. The temperature of incubation was $37 \pm 0.5°$. Monolayer cell sheets formed in 3–4 days and were then used in experiments.

Methods for detection of virus. a. Cytopathic effect (CPE) method. The CPE method, identical to those of our earlier studies (2), was used to titrate the virus stock. Infectivity titers were calculated by the Reed–Meunch method (3).

b. Plaque assay. Full monolayers of CV-1 cells in petri dishes were washed twice with phosphate buffered saline (PBS) containing 0.05% bovine serum albumin (BAS). Aliquots (0.2 ml) of 10-fold dilutions of virus samples were delivered onto the cell sheet. After 1 hr of adsorption at 37°, infected cells were overlaid with 6 ml of a final concentration of 0.6% Ionagar prepared in maintenance fluid comprised of medium 199 containing 5% peptone and 2% fetal calf serum. Plaques were stained on day 6 by an overlay of an additional 6 ml of medium-Ionagar containing 1:2000 dilution of neutral red. Plaques were counted on the following day. The infectivity titer was expressed in plaque-forming units (pfu) per unit volume of the samples.

c. Chick embryos. The chorioallantoic membrane (CAM) of 10–11-day-old leghorn chick embryo was also used for titration of the virus stock. Graded dilutions of virus suspensions were inoculated onto the dropped membrane. Three days postinoculation pock lesions were counted; and the titer was expressed as pock-forming units (PFU).

d. Fluorescent antibody (FA) method. The direct FA staining method was used. Antisera were raised in rabbits as reported in an earlier study (4). The hemagglutination inhibition titer of this pooled sera was 1:160. The globulin fraction, obtained by $(NH_4)_2SO_4$ precipitation was conjugated with fluorescein isothiocyanate. Excess unconjugated fluorescein dye was removed by absorption with mouse liver powder. Methods for preparation of fluorescein-conjugated MPV antisera and for fluorescent staining

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have been described previously (4).

e. Hemagglutinin (HA) titration. Titration of HA of MPV-infected culture cells was done by the microtiter technique using chick red blood cells for agglutination. The HA titer was expressed as reciprocals of the final dilution of samples tested.

f. Cytochemical methods. Methods for hematoxylin and eosin (H–E) staining have been described by Preece (5), Feulgen reaction by Lillie (6), and acridine orange stain by Gurr (7).

Experimental approach. Monolayers of CV-1 cells grown in Leighton tubes were infected with MPV at multiplicity of infection of approximately 2 PFU/cell. After 1 hr of virus adsorption at 37°, infected cultures were rinsed 3 times with PBS to remove unadsorbed virions; after replacement of fresh maintenance medium, cultures were again incubated at 37°. At indicated intervals (see Results) of incubation, eight glass slips containing infected cells were removed from Leighton tubes for determination of plaque and HA titers, and for fluorescent and cytological study. For plaque assay and HA studies, each culture was divided into 2 fractions: the "cell-free fraction" representing the fluid overlay of infected cultures, and the "cell-associated fraction" comprised of cells scraped off the glass and resuspended in 1.5 ml of PBS/tube. These cell suspensions were then frozen and thawed 3 times. Techniques for the study of growth kinetics followed those described by Yohn *et al.* (8).

Results. Choice of cell cultures for assay purposes. Among 16 infected cultures (9), WI-38, Kappa, and CV-1 cells were found to be sensitive lines for studying MPV. Since CAM of chick embryos has been known to be a very sensitive system for assay of many pox viruses, this *in vivo* system was included in the tests to compare the relative infectivity titers of the virus stock. *In vitro* infectivity titer ($TCID_{50}$) based on CPE for the virus pool was $10^{5.6}$/ml in WI-38, $10^{5.6}$/ml in Kappa, and $10^{5.5}$/ml in CV-1 cells. A titer of $10^{5.8}$ PFU/ml was obtained on the CAM of chick embryos. The *in vivo* infectivity titer (ID_{50}) was $10^{5.8}$/ml intramuscularly in cynomolgus monkeys (2) and $10^{5.5}$/ml intradermally in rabbits (Cho, unpublished data).

TABLE I. Relationships Between Multiplicity of Infection of MPV and Appearance of Cytopathic Effect in CV-1 Cells.

MPV inoculum[a] (log_{10} $TCID_{50}$)	Cytopathic effect (day): Onset	Completed
5.5	0.3	1.0
3.5	2.0	5.0
2.5	2.0–3.0	5.0
1.5	4.0	6.0– 7.0
0.5	5.0–6.0	9.0–10.0

[a] In tube cultures on a rotating "drum."

From the knowledge that ID_{50} in the 3 tissue culture systems were essentially identical and of comparable sensitivity with CAM of chick embryos, we elected the CV-1 cell line for the following *in vitro* studies. Other reasons for using CV-1 cells relate to prolonged maintenance in culture, as well as background data on its sensitivity in measuring viremia in monkeys infected with MPV (2) and in rabbits infected with rabbit pox virus (10).

Cytopathic effects and plaque formation. The cytopathic effects of MPV in infected CV-1 cells were characterized by rounding up, granulation, and detachment from the side of the tube leaving microscopic visible "holes" in the cell sheet. The time required for onset and completion of CPE in MPV-infected CV-1 cells is shown in Table I. Time of onset of microscopically identifiable CPE was a function of multiplicity of infection, ranging from 8 hr to 6 days. Occasionally 10 days or more of incubation were required when only a very small amount of virus was present in the tested material. When MPV-infected monolayers of CV-1 cells grown in petri dishes were overlayed with agar and stained with neutral red, well-defined plaques of 2–3 mm in diameter were easily and regularly demonstrated. Thus, by this plaque method, quantitative studies of MPV could be performed with assurance and precision.

One-step growth curves. Infectivity titers of the cell-associated and of cell-free virus were measured independently by the plaque method. The time course of multiplication

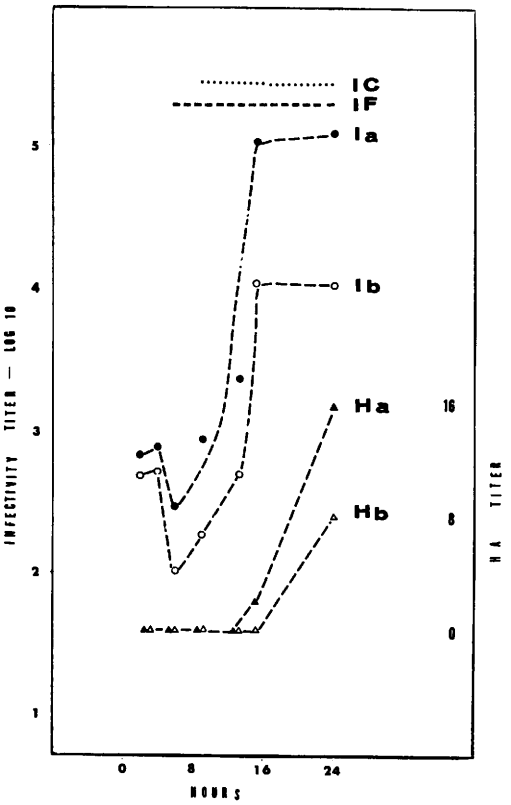

FIG. 1. One-step growth curves of MPV in CV-1 cells: The data were derived from multiplicity of infection of 2 pfu/cell. Infectivity titer is expressed in $\log_{10}$ pfu/0.1 ml (1a = cell-associated virus; 1b = cell-free virus). Hemagglutinin titer is expressed as reciprocals of dilution of infected culture (Ha = cell associated fraction, Hb = cell-free fraction); (top) the temporal course of development of cytoplasmic inclusions (IC) (. . . .); and of cellular immunofluorescence (IF) (- - -).

and release of virus in CV-1 cells is shown in Fig. 1. Following infection, an initial fall in virus input occurred during the first 6 hr. Between hours 6 and 9, virus titer regained level of input followed by an incremental surge of maximal levels by hour 14. The pattern of increase of cell-free virus followed closely that of the cell-associated virus, bearing a lag of approximately 3–4 hr. By the time the maximum growth of virus was reached, approximately 10% of the cell-associated virus was released into the fluid.

Development of HA. The sequential relationship between the development of HA activity and infectivity titer is depicted also in Fig. 1. Hemagglutinin developed at a late state of virus growth, presumably in relation to the maturation process of virions. Only minimal HA activity was measurable in the cell-associated fraction at 14 hr, at which time the infectivity titer had already reached its peak. However, from 14 to 24 hr, there was a progressive increase of HA titer approaching 1:16 at 24 hr. The lag in development of HA in the cell-free fraction was associated with the corresponding lag of virus release from the cell-associated fraction.

Development of inclusion bodies. The development of inclusion bodies in MPV-infected CV-1 cells also coincided with the kinetic aspects of viral replication. Cytoplasmic inclusions were first demonstrated 9 hr after infection at the time the infectivity titer began to rise, and shortly after onset of intracellular immunofluorescence. Inclusions increased numerically during the course of infection. At 14 hr, inclusions had developed in many infected cells (Fig. 2A). They varied in size and shape. During the early stage of virus growth, inclusions were small, spherical, and homogeneous in staining intensity; at later stages in cellular commitment, inclusions became larger, irregular in shape, and variable in staining intensity. Cells stained with hematoxylin and eosin contained eosinophilic inclusions; inclusions stained by Feulgen reaction were purplish and fluoresced yellowish with acridine orange indicating the presence of DNA. Nuclear changes characterized by coarsening and margination of chromatin were demonstrated best by Feulgen and acridine orange staining. Inclusions stained by FA were intensely fluorescent, indicating the presence of MPV antigen(s).

Development of immunofluorescence. Specific immunofluorescence was easily demonstrated as illustrated in Fig. 2B by direct staining of MPV-infected monolayers of CV-1 cells. Monkeypox virus antigen was revealed by hour 6, just before the detectable increase of infectious virus (Fig. 1). Specific fluorescence involved only a few cells in scattered foci. Immunofluorescence increased progressively and reached maximal cellular

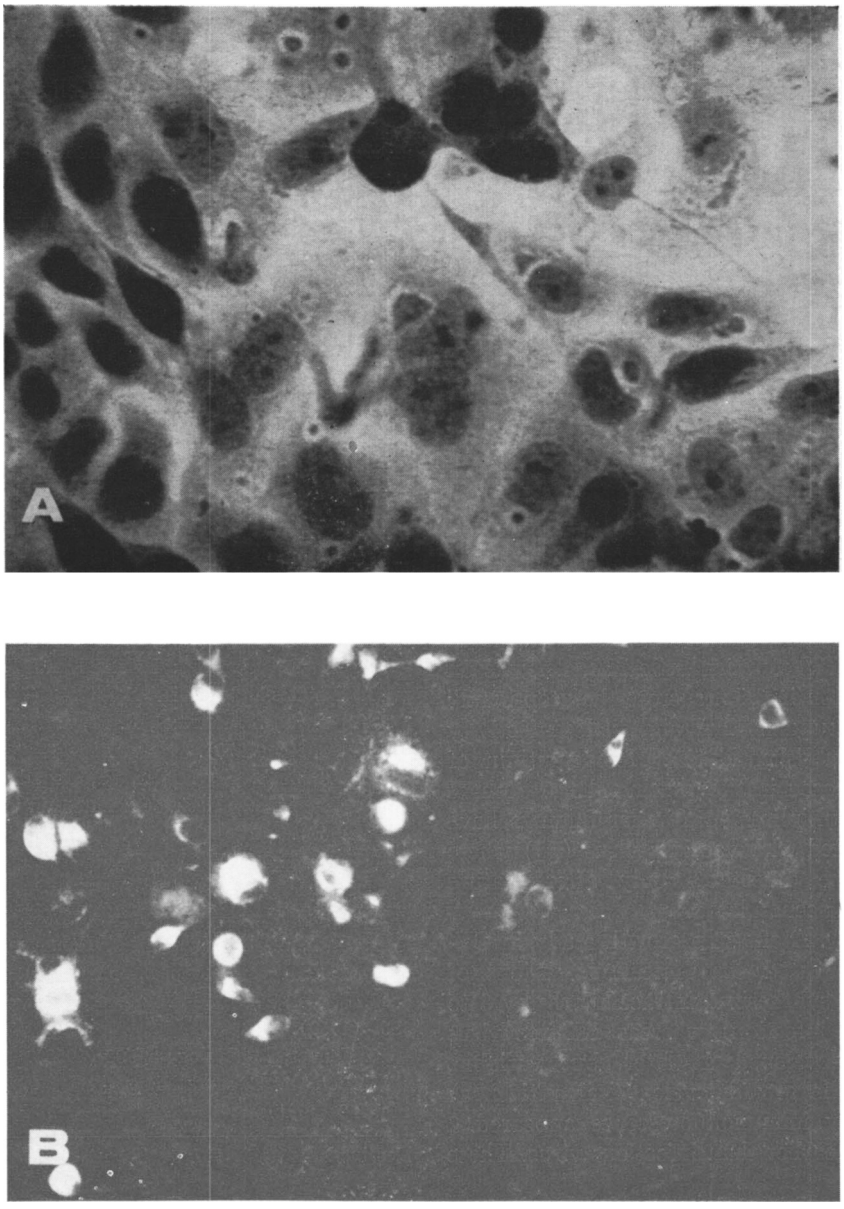

FIG. 2. Inclusion bodies and immunofluorescence in MPV-infected CV-1 cells: At hour 14 after infection (MOI = 2): cytoplasmic inclusion bodies (A) × 480; and viral antigens (B) × 250 are present in many of the infected cells.

spatiality and intensity by hour 14. Thereafter, appreciable differences were not found. Usually fluorescence was limited to cytoplasmic areas of cells, but not infrequently, FA staining involved the nuclear region or the long cellular bridges of infected cells (Fig. 2B).

Discussion. Monkeypox virus is a recently described member of the pox virus family. Related to vaccinia and variola viruses, it has a few properties linking it more closely with variola than with vaccinia, particularly with respect to pathogenesis in monkeys (2) and temperature dependence (1).

The kinetics of replication, and the development of viral antigen(s) in CV-1 cells infected with MPV parallel closely those of vaccinia and variola viruses in other tissue culture systems (11–13). The kinds of CPE produced by monkeypox and vaccinia viruses in CV-1 cells are indistinguishable. Indeed, the temporal events, including the latent period of utilization of available cell substrate, the kinetics of intracellular release of virions in relation to observed cytological changes and the development of inclusions and of viral antigens (HA) are very similar for all 3 members of this group of pox viruses.

MPV, like some other pox viruses, has associated hemagglutinins (14). Despite the time lag of HA detection, if not formation, release of hemagglutinins can be correlated with release of infective virions. However, HA is a nonessential part of the virion since purified MPV contains little HA, whereas on sedimentation in 36% sucrose the "top-band" contains abundant HA and little infectivity (Cho, unpublished data). Hemagglutinin of MPV increased in titer during the late phase of virus multiplication. Similar findings have been reported with vaccinia virus (15) where HA became detectable only after mature virions had accumulated.

Summary. A one-step growth study in CV-1 cells indicated that infectious virions of MPV were synthesized intracellularly by 8 to 9 hr after infection; whereas cell-free virus and HA developed at later, but variable, stages of the growth cycle. Viral antigen(s) detected by immunofluorescence appeared 2 to 3 hours earlier than infectious virus. These data indicate that MPV is similar to vaccinia and variola viruses in respect to the time course of *in vitro* intracellular multiplication and release of virus, in cytological alterations and in the intracellular development of viral antigens.

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