Abortive Replication of CV1 Strain of Vaccinia Virus in Human Embryo Kidney Cells (37681)

R. C. DUNLAP
(Introduced by H. M. Meyer, Jr.)
Bureau of Biologics, Food and Drug Administration, Rockville, Maryland 20852

The CV1 strain of vaccinia virus has been recommended by Kempe *et al.* (1) for use as a modified vaccine for protection against the complications of conventional smallpox vaccine. This strain has been administered to substantial numbers of eczematous children with generally good results. We have studied some of the properties of the CV1 strain in comparison to the U.S. Reference smallpox vaccine (CL) in an effort to characterize this strain more fully, and to attempt to establish laboratory markers for modification (2–4). A peculiarity of the CV1 strain is its inability to replicate well in human embryo kidney (HEK) cells, as first reported by Ellis and Fontes (5). The present study was undertaken to determine at what stage of replication normal CV1 virus production was blocked in HEK cells.

Materials and Methods. Cells and media. Primary human embryo kidney (HEK) cells were obtained in screw-capped tubes or 8 and 32 oz bottles from HEM Research, Inc., Rockville, MD. An embryonic monkey kidney cell line (MA 104) was obtained from the Cell Biology Branch of the Division of Virology, Bureau of Biologics, Bethesda, MD.

Eagle's basal medium No. 2 (6) with 0.03% (w/v) glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin was used to maintain the cell cultures. For the HEK cells, 10% (v/v) fetal bovine serum (FBS) was added and for the MA 104 cells, 2% FBS was used.

Antiserum and conjugate. Antiserum to the CV1 strain of vaccinia was produced in rabbits by a single course of 0.2 ml intradermal injections into 9 sites. The inocula consisted of serial 10-fold dilutions ranging from 4×10^8 virus particles (vp) to 4 vp. Each rabbit received all 9 dilutions. The animals were bled 8 wk postinoculation. Anti-rabbit serum prepared in goats and labeled with fluorescein isothiocyanate was obtained from Nordic Pharmaceuticals and Diagnostics Co.

Virus strains. The U.S. Reference Smallpox Vaccine, Lot No. 2 (CL), of calf lymph origin, is a lyophilized licensed vaccine purchased by the Bureau of Biologics and used as a reference vaccine in potency tests on other commercial smallpox vaccines. This reference lot was derived from the New York City Board of Health strain of vaccinia virus (7). The CV1 strain is a high passage chorioallantoic membrane (CAM) propagated virus originally derived by Rivers as a modified vaccinia strain (8). It too originated from the New York City Board of Health calf lymph strain but has been passed in rabbit testicles, chick embryo explants and the CAM as described by Kempe *et al.* (1). Our virus seed (CV1-78) was obtained from Wyeth Laboratories, Inc. and passed once in CAM in our laboratory.

Virus growth experiments. Virus yield was determined by inoculating tube monolayers of HEK cells with 0.1 ml of CL virus ($10^{6.3}$ vp/tube) or CV1 virus ($10^{7.3}$ vp/tube). After 1 hr for absorption, 1 ml of medium was added to each tube and to uninoculated control tubes, and the tubes were incubated at 37° for 5 days. The tubes then were frozen at −70°. When particle counts were done, the cells were thawed, scraped from the glass and sonic-treated by a Raytheon sonic oscillator to release the virus. Blind passages of the CV1 strain were made by inoculating tube monolayer cultures of HEK cells with 0.1 ml of a 10^{-2} dilution of the stock CV1 virus and incubating at 37° for 5 days. Cells

were scraped from the glass, sonicated and 0.1 ml of the suspension was used to inoculate new cells. Five such passages were made.

Absorption of virus by cells. HEK cells in tube culture were inoculated with 0.1 ml containing 2×10^6 vp/tube of either the CV1 or the CL virus strain. Immediately after inoculation, 2 tubes from each virus set were removed and 0.9 ml of medium was added to each tube. The monolayers were rinsed thoroughly and the medium was aspirated from each tube and frozen at −70°. The remaining tubes were incubated at 37° while virus absorption continued. At hourly intervals up to 5 hr, tubes were removed, cells were rinsed with medium and the fluid was removed and frozen. Subsequently the unabsorbed virus was titrated by plaque test on MA 104 cells by a standard procedure described elsewhere (3). Virus absorption was calculated by the formula:

$$\% \text{ virus absorbed} = 1 - \frac{\text{plaque titer at time } t}{\text{plaque titer at time } 0} \times 100.$$

Fluorescent antibody procedure. Monolayers of HEK cells were prepared in 60 mm petri dishes. Plates were inoculated with 4×10^4 vp of either the CL or CV1 strain. Uninoculated control plates were included in the test. Four hours of absorption time was allowed and medium was added. The plates were incubated at 36° in a 5% CO_2 atmosphere for 24 hr. The plates then were fixed and stained by the method of Thiel and Smith (9) with anti-vaccinial rabbit serum and goat anti-rabbit serum conjugate, both of which had been absorbed with calf liver powder. The number of cells showing cytoplasmic fluorescence per field was counted. Five randomly selected fields were examined in each of 12 plates for each virus strain (24 plates in all). The diameter of a field was measured using a hemacytometer grid, and equaled 1.225 mm. The area of a field was 1.18 mm^2 and the area of the plate was 804 mm^2, so that there were 681 fields/plate. The average number of infected cells per field was multiplied by 681 to obtain a titer of infected cells per plate.

Electron microscopy. Virus particle counts were made using the Sorvall SU counting rotor and electron microscopic counts of the precipitated particles according to the method of Sharp (10). Ultrathin sections of infected cells were prepared as follows: Infected cell monolayers and uninoculated control cells were fixed *in situ* with 2% paraformaldehyde–2% gluteraldehyde in a cacodylate buffer. The cells were treated with Dalton's chrome–osmium fixative and dehydrated with increasing concentrations of ethyl alcohol. Prior to final embedding, cells were carefully scraped from the glass and sedimented by low speed centrifugation. Final embedding of the cell pellets was done in Durcupan. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in an RCA EMU 3G electron microscope.

Results. Growth in HEK cells. Table I records the virus yields of the CL and CV1 strains after a 5 day period of growth in HEK cells. Also shown is the yield from the fifth passage of CV1 in HEK cells. The CL strain showed an increase of 2 logs in first

TABLE I. Growth of CL and CV1 Vaccinia Strains in HEK Cells.[a]

	Inoculum (vp/tube)	Yield (vp/ml)	Increase
CL	6.3[b]	8.3	2.0
CV1	7.3	7.8	0.5
CV1-5P HEK[c]	5.9	7.9	2.0

[a] Tube monolayer cultures of HEK cells were inoculated with 0.1 ml of the virus strains. After an absorption time of 4 hr, the cultures were rinsed with medium and 1 ml of fresh medium added. Cultures were incubated at 37° for 5 days, after which the cells were scraped from the tubes, the fluid and cells were sonic-treated and virus particle counts were made.

[b] Log_{10}.

[c] 5th passage of CV1 in HEK cells.

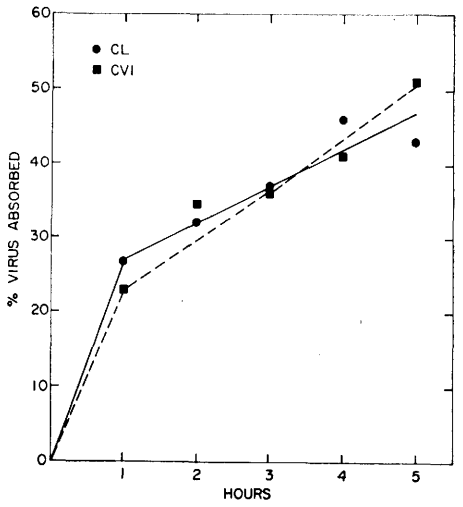

FIG. 1. Absorption of CL and CV1 vaccinia strains by HEK cells. Tube monolayers of HEK cells were inoculated with 0.1 ml of each virus strain (2×10^6 vp). At hourly intervals, sample tubes were removed and 0.9 ml of fresh medium was added. The monolayers were thoroughly rinsed and the fluid was removed. The unabsorbed virus was titrated in MA 104 cells. Percentage absorption was calculated as: $1 - (Pfu_T/Pfu_{T_0}) \times 100$.

passage, whereas the CV1 strain increased only 0.5 log over the input virus. After 5 passages in HEK cells, the CV1 strain then displayed a growth yield of 2 logs, comparable to the CL strain.

Absorption of CL and CV1 strains. Titration on MA 104 cells of virus not absorbed by HEK cells produced an almost identical absorption pattern for the CL and CV1 strains (Fig. 1). After 1 hr, 27% of the CL inoculum and 23% of the CV1 inoculum had been absorbed. After 5 hr, absorption had increased to 47% of the CL strain and 50% of the CV1 strain. Evidently both strains are taken into the cells at equivalent rates.

Fluorescent antibody staining of cytoplasmic viral antigen. The production of viral antigen, as shown by fluorescent antibody staining of viral antigen in infected cells, was approximately equal for the 2 virus strains (Table II). Calculations from the average count per field of infected cells and the areas of the field and the dish showed 2431 infected cells/plate for the CL strain and 2213 infected cells/plate for the CV1 strain. Production of viral protein apparently can proceed equally well with either strain.

Electron microscopy studies of progressive virus infection. HEK cells were infected at a multiplicity of 25–33 pfu (as measured in MA 104 cells)/cell with either the CL or the CV1 strain of virus. An absorption period of 1 hr was permitted after which the monolayers were rinsed and fresh medium was added. Incubation was at 36° for periods of 4, 8, 12, 16 and 24 hr and was terminated by fixation in paraformaldehyde–glutaraldehyde, and subsequent embedding in Durcupan. At 4 hr postinfection, the only evidence of infection by the CL strain was the presence of viral cores in the cytoplasm (Fig. 2A). At the same period, typical cores were not seen in the CV1-infected cells but several of the cells contained large swollen bodies with a dense central portion (Fig. 2B). By 8 hr, the CL strain was undergoing the normal early replicative pattern (Fig. 2C) with viroplasm being enclosed in the typical vaccinia membrane. The CV1 strain was in a similar stage of development as is seen in Fig. 2D.

At 12 hr the full panorama of vaccinial replicative stages were found in the CL cultures (Fig. 2E). Early forms with membranes in

TABLE II. HEK Cells Containing Viral Antigen as Shown by Indirect Fluorescent Antibody Staining.[a]

Virus	Inoculum (vp)	Infected cells/field (av)	Infected cells/plate
CL	4.2×10^4	3.57	2431
CV1	4.0×10^4	3.25	2213

[a] Monolayer cultures of HEK cells were inoculated with virus and incubated at 37° with medium for 24 hr. Cells were fixed and treated with specific antiserum and conjugate, and cells displaying bright cytoplasmic fluorescence were counted. Infected cells in 5 randomly chosen fields/plate were counted. Number of infected cells/plate was calculated from the area of the field and the area of the plate, and the average number of infected cells/field.

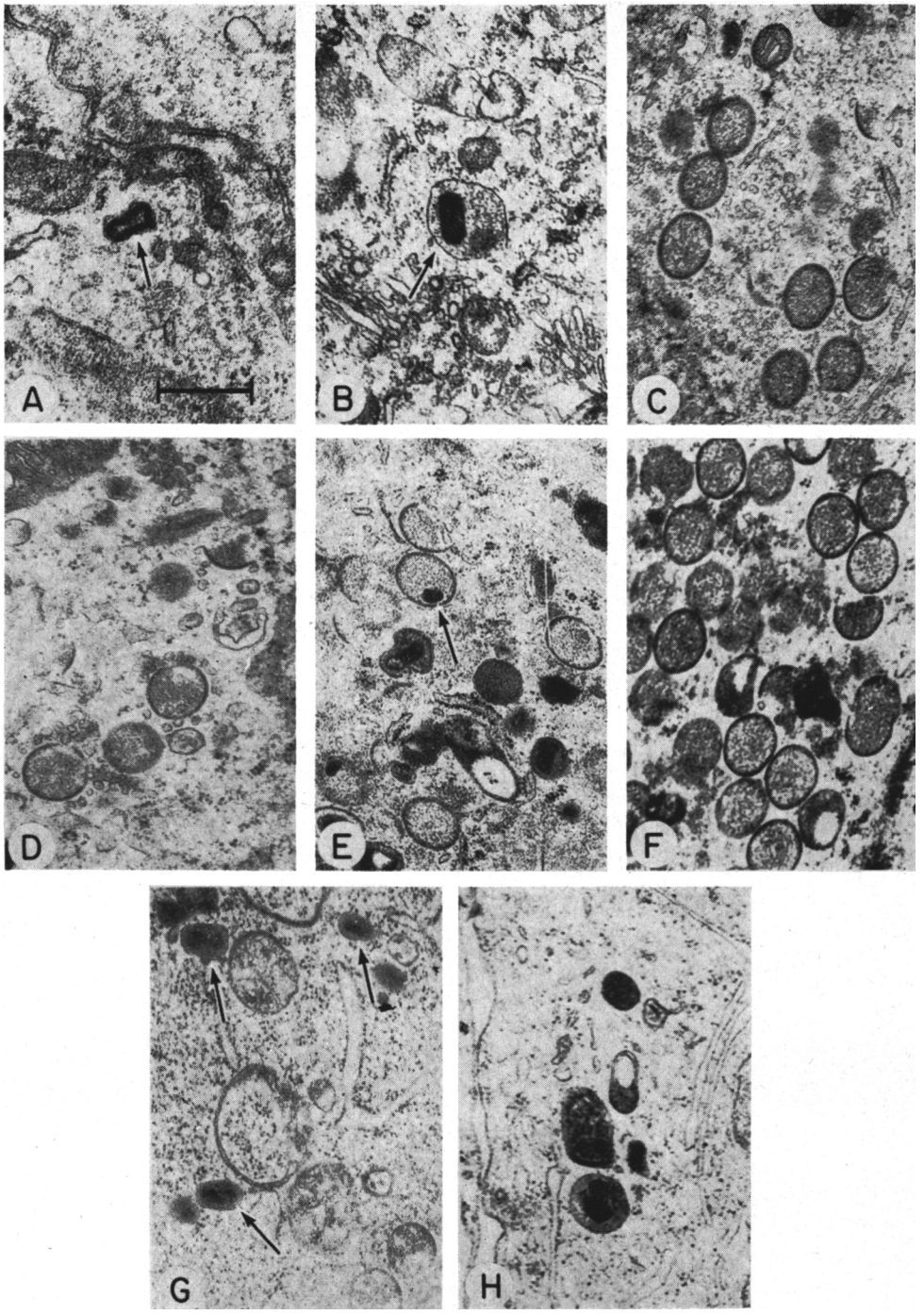

FIG. 2. Electron micrographs of developmental stages of CL and CV1 vaccinia in HEK cells. (A) CL; 4 hr culture. Arrow denotes viral core. (B) CV1; 4 hr. Arrow indicates core-like body. (C) CL; 8 hr. Early stage of viral replication. (D) CV1; 8 hr. Early stages of viral replication. (E) CL; 12 hr. Immature and mature forms of virus. Arrow indicates dense nucleoid inclusion. (F) CV1; 12 hr. Immature virus, none of which possesses a dense nucleoid. (G) CL; 16 hr. Mature and immature virus. Arrows indicate mature virus. (H) CV1; 16 hr. Bizarre bodies, possibly related to virus. Bar in A represents 500 nm and is the same for all micrographs.

varying stages of completion were present. Many early forms possessed a dense nucleoid. On the periphery of the "factory" area, fully mature particles possessing the dumbbell-shaped inner core could be seen. The CV1 cultures displayed many areas of infection but notable differences from the CL cultures were now evident (Fig. 2F). It was striking that the dense nucleoid body was missing from the immature forms. A few infected cells were found that possessed mature forms, normal in appearance. However, such mature particles were encountered much less frequently than in the CL cultures where most virus "factories" included mature forms.

At 16 hr the CL cultures continued to produce mature virus and all stages of virus development were still present (Fig. 2G). The CV1 cultures now showed numerous bizarre forms, totally unlike normal vaccinia virus (Fig. 2H). These forms were frequently grossly swollen or possessed a large vacuole. Again some normal mature particles were seen but in much lower numbers than in the CL cultures. By 24 hr, new cycles of replication were well underway, and again it was striking that the dense nucleoids common in the CL early replicative forms were absent from the forming particles of the CV1 virus.

An oddity encountered in two different CV1 cultures, one a 12 hr culture and the other a 24 hr culture, was the apparent presence of immature virus particles in the cell nucleus (Figs. 3A and B). Of particular interest is Fig. 3A, where apparent "viroplasm" lacking a viral membrane is present. The appearance of this group of particles is that of a typical cytoplasmic viral "factory." Although many nuclei were examined for virus particles, only 2 examples were seen. If this phenomenon is real and not an artifact of thin-sectioning, occurrence must be rare. No nuclear particles were seen in CL cultures.

Discussion. The CV1 strain of vaccinia seems to follow a normal course of replication in HEK cells through absorption into the cell, uncoating and production of viral antigen. Immature forms of virus are produced consisting of viroplasm enclosed in the typical vaccinia single membrane. However, very few of these particles possess the dense nucleoid that is thought to consist of viral DNA, and very few of the particles progress to full maturity. Instead, some bizarre, abnormal bodies appear in the cytoplasm which possibly are attempts at maturation that have gone awry. Rosenkranz *et al.* (11) studied the effect of hydroxyurea on vaccinia virus development

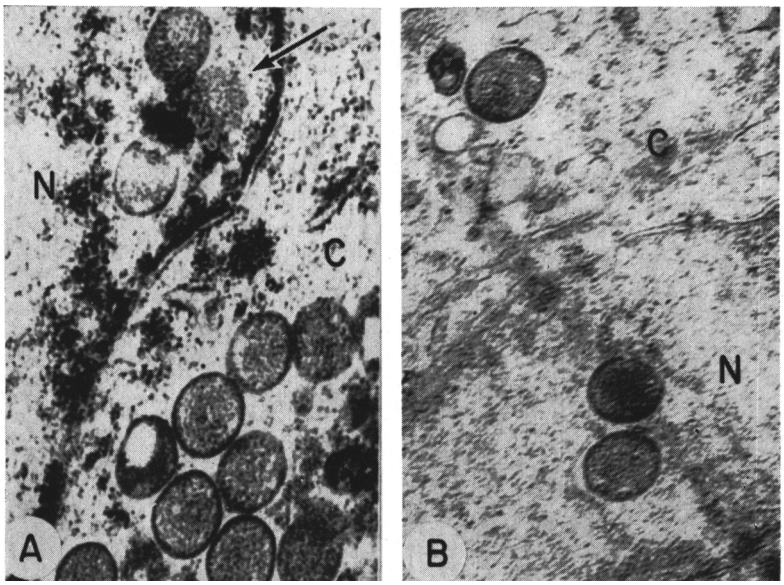

FIG. 3. CV1 vaccinia. Immature forms of virus in both nucleus (N) and cytoplasm (C). (A) 12 hr culture. Arrow indicates viroplasm without a limiting membrane. (B) 24 hr culture.

and found the same sequence of events culminating in immature forms without the dense nucleoid and little or no mature virus particle formation. Previous work by Rosenkranz and Levy (12) had determined that hydroxyurea blocked duplication of DNA. Since the nucleoid inclusion in immature forms is thought to consist of DNA (11), its absence in cells treated with hydroxyurea is understandable. Pogo and Dales (13) augmented the work of Rosenkranz *et al.* (11), with hydroxyurea and verified the finding that hydroxyurea interfered with viral DNA synthesis and the formation of nucleoids. Reversal of this inhibition then permitted formation of mature and infective virions.

The interruption of the normal replicative cycle displayed by the CV1 strain in HEK cells seems to indicate inability to carry on viral DNA synthesis, although the synthesis of early viral proteins does proceed. This failure can be overcome by repeated passages in HEK cells, since fifth passage CV1 virus showed a mature virus yield comparable to the control CL virus. However, attempts in our laboratory to clone the CV1 strain by single pock passage to select virus capable of replicating in HEK cells were a failure. Thus, it is still unclear whether the CV1 parent strain is a heterogeneous mixture of particles capable of replicating in HEK cells and particles which cannot carry on DNA synthesis in these cells or whether cellular factors are operating to change the characteristics of the virus.

It is interesting to speculate whether this abortive replicative cycle in a primary human cell system bears any relationship to the "modified" behavior of the CV1 strain when used as a vaccine in children with eczema. Neff *et al.* (14) reported that vaccination of healthy children with the CV1 strain of smallpox vaccine resulted in a considerably reduced percentage of "takes" and a drastic reduction in the production of post-vaccination neutralizing antibodies, compared with the response to a conventional calf lymph vaccine. Perhaps the CV1 strain replicates relatively poorly in humans compared to conventional vaccinia virus. This would be consistent with the performance of other modified viruses used as attenuated live virus vaccines (15).

Summary. The CV1 strain of vaccinia virus replicates poorly in human embryo kidney cells whereas most vaccinia strains such as the U.S. Reference smallpox vaccine replicate well in this host system. Absorption by the cell and production of viral antigen proceed at the same rate for both strains. Electron micrographs reveal that the CV1 strain produces immature forms of particles which do not possess a dense nucleoid of DNA material. Very few normal mature particles are formed and bizarre particles are found in the cytoplasm. CV1 DNA synthesis seems unable to proceed in the normal fashion in these cells.

The interest and counsel of Dr. L. F. Barker is deeply appreciated by the author. I am also grateful for the technical assistance of Mrs. Glennelle C. Washington.

1. Kempe, C. H., Fulginiti, V., Minamitani, M., and Shinefield, H., Pediatrics **42**, 980 (1968).
2. McGuire, P. M., Dunlap, R. C., and Sharp, D. G., J. Infec. Dis. **127**, 278 (1973).
3. Dunlap, R. C., and Barker, L. F., Arch. Gesamte Virusforsch. **42**, 107 (1973).
4. Dunlap, R. C., and Barker, L. F. Arch. Gesamte Virusforsch. **42**, 117 (1973).
5. Ellis, H. L., and Fontes, A. K., Bacteriol. Proc. Amer. Soc. Microbiol. p. 170 (1967).
6. Eagle, H., Science **130**, 432 (1959).
7. American Type Culture Collection. "Viral and Rickettsial Catalogue," 4th ed., p. 103 (1971).
8. Rivers, T., J. Exp. Med. **54**, 453 (1931).
9. Thiel, J. F., and Smith, K. O., Proc. Soc. Exp. Biol. Med. **125**, 892 (1967).
10. Sharp, D. G., "Proceedings of the 4th International Conference on Electron Microscopy," 1958, p. 542. Springer-Verlag, Berlin (1960).
11. Rosenkranz, H. S., Rose, H. M., Morgan, C., and Hsu, K. C., Virology **28**, 510 (1966).
12. Rosenkranz, H. S., and Levy, J. A., Biochim. Biophys. Acta **96**, 181 (1965).
13. Pogo, B. G. T., and Dales, S., Virology **43**, 144 (1971).
14. Neff, J. M., Speers, W. C., Wesley, R. B., Goldstein, J., Ruben, F. L., and Lourie, B., Pediat. Res. **6**, 386 (1972).
15. Meyer, H. M., Jr., J. Pediat. **73**, 653 (1968).

Received July 20, 1973. P.S.E.B.M., 1973, Vol. 144.