Porcine Parvovirus Infection *In Vitro*: A Study Model for the Replication of Parvoviruses
I. Replication at Different Temperatures[1] (36676)

P. A. BACHMANN[2]
(Introduced by James A. Baker)
Veterinary Virus Research Institute, Cornell University, Ithaca, New York 14850

Detailed *in vivo* studies of pathological changes in mouse embryos and newborn mice, particularly cerebellar changes, led Margolis and Kilham (1) to conclude that rat virus has an affinity for actively dividing cells. Subsequently these observations were extended to other parvoviruses, and the authors introduced the term "mitolytic" for this virus group.

Tennant and coworkers (2, 3) investigated the influence of the physiological state of the cell and the effect of X- and uv irradiation on the course of rat virus infection *in vitro,* and found that virus replication requires cell functions not needed by other DNA viruses which replicate in the nucleus.

It is well known that cell functions slow down at suboptimal temperatures but virus components are still synthesized (4–10). It might be expected that if parvoviruses would also grow at suboptimal or supraoptimal temperatures in cell cultures, this system could be used for further studies on the mode of replication of these viruses, and could provide important information for the understanding of the pathogenesis of these infections *in vivo*. Studies reported here are directed to this aim. The porcine parvovirus (PPV) grown in the SK cell line was chosen for these investigations.

Materials and methods. Virus. Porcine parvovirus, strain G10/1, which was originally isolated from a primary swine kidney cell culture (11, 12) and subsequently passaged in the SK cell line[3] was used. Passage numbers 14–32 were employed.

Cell cultures. The established pig kidney cell line SK between subcultures 149 and 165 was found to be most sensitive for PPV replication. The cell line was grown in Hanks MEM with 10% calf serum and 880 mg $NaHCO_3$/liter. For virus studies the serum content was reduced to 2% and $NaHCO_3$ increased to 1320 mg/liter. Falcon plastic bottles (21 cm^2) and 16 mm tubes were used for the cultures.

Preparation of virus growth curves. A 1-ml virus suspension was allowed to adsorb onto a confluent monolayer cell sheet for 60 min at room temperature in bottle cultures. The nonadsorbed virus was discarded before cultures were washed twice with 2-ml buffered saline (PBS), and 5 ml of virus medium were added. The multiplicity input was kept between 2 and 5 infectious units per cell. After infection the cultures were incubated at temperatures of 28, 33, 37 and 40°. Noninfected controls were treated in the same manner. Samples were taken at 0, 4, 6, 9, 12, 16, 24, 36, and 48 hr after infection and afterwards at 24 hr intervals up to 120 hr. At the respective times after infection two cultures were frozen and thawed twice and samples were taken for HA activity and infectivity titrations.

Infectivity titrations. Virus infectivity was assayed in tube cultures 24 hr after seeding and incubation at 37°. Virus samples were diluted tenfold, and 0.2 ml of each dilution was inoculated into four cultures. Titers were calculated as $TCID_{50}$/0.2 ml according to the method suggested by Kaerber (13).

Hemagglutination. HA activity was titrated at 4° using guinea pig red blood cells at 0.5% concentration in phosphate buffered

[1] Supported by Deutsche Forschungsgemeinschaft.

[2] Present address: Inst. Microbiology, Veterinarstr. 13, Munich 22, Germany.

[3] The SK cell line was established from pig kidneys by Dr. Karza at the Department of Pathology, Ohio State University in Columbus, OH. The properties have not been published so far.

saline at pH 7.6. All assays were carried out in the microtiter system. The end point was recorded as highest dilution of virus, giving complete hemagglutination after 4–6 hr.

Cell growth curve. 1 × 10^6 cells were seeded into plastic bottles and incubated at 28, 33, 37, and 40°. Samples were taken at 24 hr intervals until the cultures were confluent. From each sample the medium was decanted and the cells washed twice with 5 ml PBS, then 3 ml of a trypsin (0.05%)-versene (0.02%) saline solution was added. After coming off the plastic wall the cells were stained with 0.5% trypan blue and then counted in a Neubauer blood cell counting chamber. Three counts were made from each sample.

Mitotic index. Leighton tube cultures with cover slips were prepared as described and incubated at 37° for 24 hr before they were kept at the respective temperatures (28, 33, 37, and 40°). At 12–24 hr intervals one culture from each temperature was fixed in Bouin's solution for 20 min and stained with hematoxylin-eosin. A total of 4000 cells were counted and the number of mitoses per 1000 cells recorded.

Results. Growth of virus in SK cell cultures occurred at all temperatures employed. Consistent virus replication with occurrence of cytopathic effects was detected at the 28, 33, and 37° temperatures of incubation. The virus also multiplied at 40° to a certain extent. Surprisingly, no significant titer differences with respect to maximum HA activity and infectious virus were observed in the growth curves (Figs. 1 and 2). Multiplication at 40° stopped a few hr after virus multiplication began.

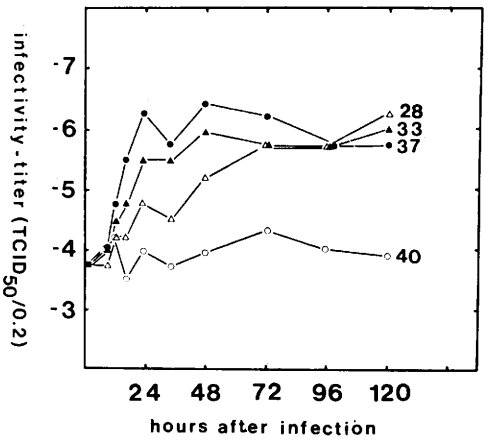

FIG. 1. Infectivity titers of growth curve of porcine parvovirus in SK cell cultures at 28, 33, 37, and 40°.

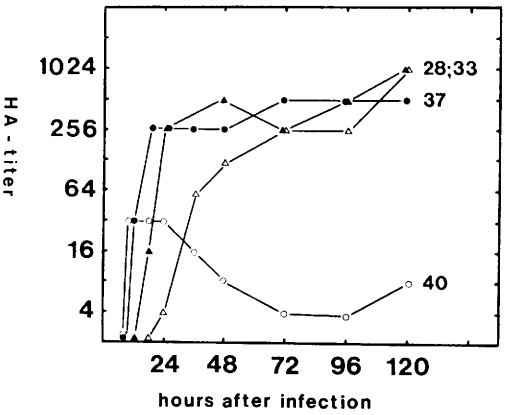

FIG. 2. Hemagglutinating titers of growth curve of porcine parvovirus in SK cell cultures at 28, 33, 37, and 40°.

Virus replication at 28, 33, and 37° led to the same virus and HA titers 72 hr after infection. Maximum titers reached were $10^{6.25}$ $TCID_{50}$/0.2 ml and 1:1024 at the 28°, $10^{6.25}$ $TCID_{50}$/0.2 ml and 1:1024 at 33° and $10^{6.5}$ $TCID_{50}$/0.2 ml and 1:512 HA titer at 37°. The respective maximum titers at 40° were $10^{4.25}$ $TCID_{50}$ and HA titer of 1:32. The greatest differences between the infectivity titers of the 72 hr samples were 0.5 log 10 and in HA activity one twofold dilution, as shown in the figures. Under the experimental conditions employed this difference is not significant.

However signficant titer differences in the replication of PPV-28, PPV-33, and PPV-37 were observed in the kinetics of growth. The growth curves showed a considerable delay in the formation of infectious virus and HA activity with decrease of incubation temperature. At 40° an initial fast replication was suppressed soon after its onset. Virus multiplication started approximately 2–3 hr earlier at 40° than at 37°. The first rise in HA and infectivity appeared in the infected cell fraction 9 hr after infection. At 37° new virus

TABLE I. Comparison of Infectivity and Hemagglutinating Activity Titers of Porcine Parvovirus (Strain G10/1) Grown at 28, 33, 37, and 40° at Different Levels.

Passage no.	28° HA/$TCID_{50}$/0.2	33° HA/$TCID_{50}$/0.2	37° HA/$TCID_{50}$/0.2	40° HA/$TCID_{50}$/0.2
PPV 20.SK	1:256/ $10^{5.50}$	1:128/$10^{5.75}$	1:256/$10^{5.5}$	1:64/ $10^{4.0}$
PPV 21.SK	1:32 / $10^{4.0}$	1:256/$10^{5.25}$	1:256/$10^{5.75}$	1:8 / $10^{3.0}$
PPV 22.SK	1:2 / $10^{2.5}$	1:512/$10^{5.50}$	1:256/$10^{5.50}$	0 /<$10^{1.0}$
PPV 23.SK	0 /<$10^{1.0}$	1:256/$10^{5.25}$	1:512/$10^{5.50}$	0 /<$10^{1.0}$
PPV 24.SK	0 /<$10^{1.0}$	1:512/$10^{5.75}$	1:512/$10^{5.25}$	0 /<$10^{1.0}$
PPV 30.SK	0 /<$10^{1.0}$	1:256/$10^{5.0}$	1:128/$10^{5.25}$	0 /<$10^{1.0}$
PPV 35.SK	n.d.[a]	1:512/$10^{5.50}$	1:1024/$10^{6.0}$	n.d.[a]

[a] n.d. = not done.

was detected at 12 hr post infection; maximum virus titers were obtained at 24 hr. There was good correspondence between HA and infectivity titers at 37° which was lacking at 40°.

At 33° the delay in virus replication as compared with 37° was between 4 and 6 hr. The first rise in HA and infectivity titers was seen at 16 hr post infection, maximum titers were obtained at 36–48 hr. At 28° there was a longer time interval compared to 33° in infectivity and HA, which was observed at around 36 hr post infection. Maximum titers occurred at 28° at 72 hr after infection. Continuous passages of the PPV material at different temperatures revealed that only at 33 and at 37° continuous passages were possible, at 40 and at 28° infectivity was lost after a few passages under comparable experimental conditions (Table I).

In addition to virus multiplication, a cytopathic effect (CPE) was observed in infected cultures at 28, 33, and 37°. CPE appeared first at 37° about 20 hr after infection, depending somewhat upon the infectivity input, and little later, at around 30 hr at 33°. At 28° CPE began 48 hr after infection. Although the cytopathic effect appeared later and progressed a little slower at 33°, the changes were more pronounced at 33° under identical conditions.

Experiments then were directed to find whether the replication of PPV at the four temperatures involved temperature-sensitive mutants. PPV-33 and PPV-37 were purified three times by the terminal dilution method after six passages at the respective temperatures, and then PPV-33 was grown in cells incubated at 37 and 28°, and PPV-37 was grown at 33 and 28°. There were no significant titer differences in both the HA activity and infectivity in the samples when titrated at 37° (Table II).

To find whether multiplication of PPV at the different temperatures was related to cell multiplication, cell growth curves and mitoses counts were carried out. Figure 3 shows that there was a temperature dependent multiplication of the cell line. The cells multiplied best at 37°, and the lowest rate of cell growth was recorded at 28° up to 96 hr after seeding; however, multiplication was observed at all four temperatures. The behavior of the cells at 40° was interesting: After an initial fast onset of multiplication up to 24 hr, the dividing rate declined and dropped to near zero at 48 and the following hr.

TABLE II. Comparison of Infectivity and Hemagglutinating Activity Titers of Porcine Parvovirus (Strain G10/1) Grown at 33° for Nine Passages and Then Incubated at 37 and 28° and Vice-Versa.

PPV-33 grown at:	37°	28°
Passage no.	HA/$TCID_{50}$/0.2	HA/$TCID_{50}$/0.2
PPV 16.SK	1:256/$10^{5.0}$	1:128/$10^{5.0}$
PPV 25.SK	1:128/$10^{5.5}$	1:128/$10^{5.0}$
PPV-37 grown at:	33°	28°
Passage no.	HA/$TCID_{50}$/0.2	HA/$TCID_{50}$/0.2
PPV 16.SK	1:512/$10^{5.5}$	1:256/$10^{5.25}$
PPV 25.SK	1:256/$10^{5.0}$	1:128/$10^{4.75}$

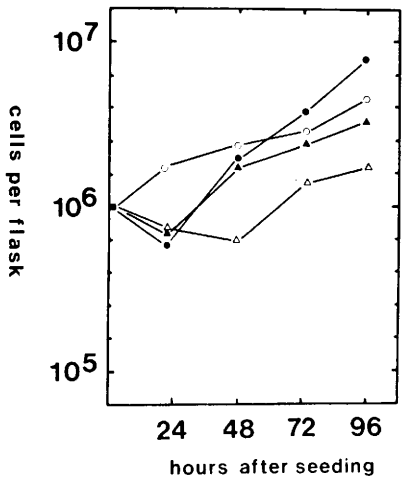

FIG. 3. Multiplication of SK cells at 28, 33, 37, and 40° under comparable conditions. △—△ 28°; ▲—▲ 33°; ●—● 37°; ○—○ 40°.

The mitotic index (Fig. 4) confirmed the results obtained in the cell growth curves (Fig. 3). Highest mitotic figures were observed at 40° with a sharp rise up to 12 hr, and then a sharp decline with an index close to zero at 48 hr. The maximum index rate at 37° was recorded at 24 hr. It was somewhat lower than at 40° and declined at slower rate to the stationary state. At 33° the peak was observed around 36 hr with a plateau of

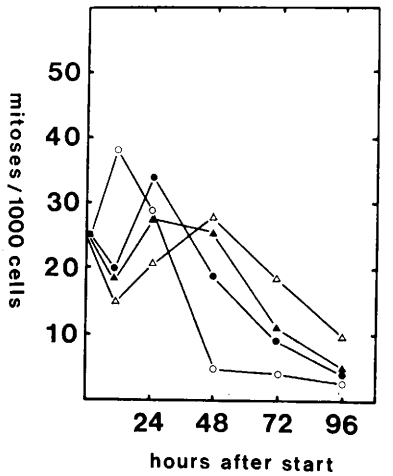

FIG. 4. Comparison of the mitotic index activity of the SK cell line at 28, 33, 37 and 40° over a period of 96 hr. △—△ 28°; ▲—▲ 33°; ●—● 37°; ○—○ 40°.

mitotic activity between 24 and 48 hr after tests began. The highest mitotic index was seen at 28° at 48 hr. The number of mitoses at a given time was always lower with decreasing temperatures. The curves presented in Fig. 4 show exactly the same pattern of rise and decline at each temperature; however, a clear shift to the right of the abscissa is recognizable.

Although there are clear differences in mitotic activity in the SK cell line at different temperatures, the numbers of mitoses was similar in all temperatures, when the figures obtained in the experiment were accumulated between 12 and 96 hr. There were 92 mitoses at 28°, 87 at 33°, 85 at 37° and 81 at 40°.

Discussion. The high temperature range between 28 and 40° at which the porcine parvovirus (PPV) replicates in a given cell system under experimental conditions employed is unexpected. The same virus and hemagglutinin yields are obtained at 28, 33, and 37°, although the kinetics of replication were different. This can partly be explained with a slow-down in cell functions at lower temperatures. Rott and Scholtissek (5, 6) showed a few years ago that viral RNA was synthesized at incubation temperatures of 23°. The virus used was not a *ts* mutant and the synthesis of DNA was slower than at 36°. That DNA viruses may grow at suboptimal temperatures is shown by the existence of temperature-sensitive mutants, which form infective virus particles.

Tennant and coworkers (2, 3) could show that parvovirus replication depends upon mechanisms of the cell functions not used by other DNA viruses. It was suggested that steps of cellular DNA synthesis are needed for parvovirus growth. This essential supporting factor reveals a large temperature optimum, but has a thermosensitive stage at 40°. At this temperature the HA activity was reduced 16–32 times and the infectivity yielded 100 infectious units less when compared with samples grown at 28–37°. Since at the elevated temperature the HA and infectivity titers did not correspond during the first 24 hr after infection, an abortive cycle of replication may well be possible. These results are

in agreement with reports on thermosensitive stages at elevated temperatures (7, 9, 10, 14, 15). The higher temperature usually restricts the formation of infectious virus, while virus-specific subunits are synthesized. These thermosensitive stages have an influence late in the replication cycle.

PPV replicates probably only in progressively dividing cells. A comparison of virus growth curves (Figs. 1 and 2) with the cell growth curve and the mitotic index shows a close relation betweeen the kinetics of virus replication and cell multiplication. Virus replication seems to start before the maximum of mitotic activity in the cell culture is observed. The delay of the onset of detectable virus replication corresponds well with the delay at which maximum mitosis rates are observed at the different temperatures. This supports the theory of Margolis and Kilham (1) directly, and the results show that cellular mechanisms needed for PPV replication involve early functions of the growth cycle. These results confirm suggestions of Tennant and Hand (3) that some of the steps of cellular DNA synthesis may serve as essential factors for parvovirus replication.

It is known that cells have a tendency to synchronize their growth cycle at nonoptimal temperatures, which includes a delay but not necessarily a reduction of the mitotic rate. Figure 4 supports this theory. There is a definite synchronization effect at the low temperatures as well as at 40°, and the accumulated numbers of mitoses between 12 and 96 hr after beginning of the experiment are roughly the same.

Apart from these obvious relations between virus replication and cell multiplication, it can only be speculated upon what mechanisms are involved in the growth of PPV at sub- and supraoptimal temperatures.

From the fact that PPV-28 and PPV-40 samples cannot be passaged continuously with the methods used and the results obtained in Table II after growing PPV-33 at 37° and PPV-37 at 33 and 28° it is believed that temperature-sensitive (*ts*) mutants are not involved in these experiments, since by definition *ts* mutants replicate at the permissive (low) temperature but are unable to complete a successful growth cycle at the higher temperature (16).

There have been no experiments with other pig cells either primary or in permanent cultures, but it cannot be excluded that the behavior of PPV at the sub- and supraoptimal temperatures may be closely connected to the growth properties of the SK cell line.

Since many of the functions playing a role in parvovirus replication have to be elucidated the PPV-SK cell line system may be an effective model for studying some of the aspects of parvovirus pathogenesis *in vitro* and *in vivo*.

Summary. The replication of a porcine parvovirus (PPV, strain G10/1) at different incubation temperatures was studied in the SK cell line. Temperatures of 28, 33, 37, and 40° were applied. No significant differences were observed between maximum virus and HA titers when grown at 28, 33, and 37°, however virus and HA production was delayed with falling temperatures. At 40° virus replication was suppressed shortly after its onset, resulting in probably an abortive infection. Temperature-sensitive (*ts*) mutants seem to be not involved.

The virus growth curves and the cell-multiplication and mitotic-index figures showed a close relation, indicating that certain functions of the cellular growth cycle are essential for PPV replication.

The results are discussed in connection with experiences with other viruses. The PPV-SK cell system is suggested as a model for further studies of parvovirus replication.

1. Margolis, G., and Kilham, L., "National Institute of Neurological Diseases and Blindness Monograph 2." (1965).

2. Tennant, R. W., Layman, K. R., and Hand, R. E., J. Virol. 4, 872 (1969).

3. Tennant, R. W., and Hand, R. E., Virology **42**, 1054 (1970).

4. Ghendon, Y. Z., and Ageeva, O., Arch. Gesamte Virusforsch. **30**, 7 (1970).

5. Rott, R., and Scholtissek, C., J. Gen. Virol. **3**, 239 (1968).

6. Scholtissek, C., Biochem. Biophys. Acta **155**, 14 (1968).

7. Lust, G., and Carmichael, L. E., J. Infec. Dis. **124**, 572 (1971).

8. Aurelain, L., J. Virol. **4**, 197 (1969).

9. Carmichael, L. E., and Barnes, F. D., J. Infec. Dis. **120**, 664 (1969).

10. Waroquir, R., Samaille, J., and Green, M., J. Virol. **4**, 423 (1969).

11. Mayr, A., Bachmann, P. A., Siegl, G., Mahnel, H., and Sheffy, B. E., Arch. Gesamte Virusforsch. **25**, 38 (1968).

12. Bachmann, P. A., Zentralbl. Veterinaermed. Reihe B **16**, 341 (1969).

13. Kaerber, G., Arch. Exp. Pathol. Pharmakol. **162**, 480 (1931).

14. Stephens, J. G., and Jackson, N. L., Virology **32**, 654 (1967).

15. Stephens, J. G., Virology **29**, 570 (1966).

16. DiMayorca, G., and Callendar, J., Progr. Med. Virol. **12**, 284 (1970).

Received April 24, 1972. P.S.E.B.M., 1972, Vol. 140.