

Persistent Infection of K562 Cells by Encephalomyocarditis Virus

INGRID U. PARDOE, KANWAL K. GREWAL, M. PAH BALDEH, JAWED HAMID,
AND ALFRED T. H. BURNES*

Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6

Received 29 May 1990/Accepted 13 September 1990

Infection of human erythroleukemic K562 cells by encephalomyocarditis virus readily resulted in establishment of persistently infected cultures. In contrast to the usual typical lytic infection by encephalomyocarditis virus, in which trypan blue staining of cells reaches close to 100% by about 15 h postinfection, K562 cell cultures required 3 to 4 days postinfection to reach a maximum of about 80 to 90% cell staining. The proportion of K562 cells taking up stain gradually decreased to about 10% of those present by about 13 days postinfection; during this time, virus yield per day measured by either plaque or hemagglutination titration fell about 10-fold. The decrease in percent staining was followed by waves of increased staining accompanied by increased virus production. Virus-producing cultures were maintained for over 3 months. Evolution of both virus and cells accompanied establishment of persistence in that plaque size changed from about 7 mm in diameter for the original virus to less than 1.5 mm by day 20 postinfection and most of the cells cloned from persistently infected cultures were resistant to superinfection with the original virus. Resistance was due, at least in part, to reduced virus attachment in that binding of ³H-labeled virus to cloned resistant cells was about 2% of that to uninfected cells.

The receptor for the picornavirus encephalomyocarditis (EMC) virus on human erythrocytes is glycophorin A, the major erythrocyte sialoglycoprotein (3). However, since human erythrocytes do not support EMC virus growth, it is debatable whether glycophorin A can be regarded as an authentic receptor.

Glycophorin A is found on the surface of human erythroleukemic K562 cells (13). Therefore, we decided to examine whether K562 cells can support EMC virus growth and, if so, whether glycophorin A is the receptor used by the virus to gain access to the cell interior. During these studies, we found that EMC virus could infect K562 cells but at a rate much slower than that found for infection of all types of cells previously examined. Further, since EMC virus has the reputation for being a highly cytolytic virus, we were surprised to find that the infected cultures were not completely destroyed but showed the ability to recover to some extent and that persistently infected cultures were readily established. We describe here some of the properties of the system.

MATERIALS AND METHODS

Virus. The K2 strain of EMC virus was grown in Krebs ascites tumor cells and purified as described previously (4). ³H-labeled virus was produced by addition of ³H-labeled leucine (Dupont, NEN Research Products) to the culture medium during virus growth.

K562 cell line. A seed of human erythroleukemic cell line K562, originally isolated by Lozzio and Lozzio (18), was kindly supplied by Alan Schechter, National Institutes of Health, Bethesda, Md. The cells were grown in suspension in RPMI 1640 medium (Flow Laboratories, Inc.) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Cell viability was measured by mixing equal volumes of a cell suspension with 0.1% trypan blue in phosphate-buffered saline (PBS) and counting total and unstained cells in a hemacytometer.

Plaque assays. Plaque assays were performed in 60-mm-diameter tissue culture dishes on confluent L-cell monolayers under a 5-ml overlay containing 0.85% agar in Dulbecco minimal essential medium-1% heat-inactivated horse serum-0.015% DEAE-dextran (Sigma Chemical Co.)-100 U of penicillin per ml-100 µg of streptomycin per ml. Plates were incubated for about 40 h at 37°C in 5% CO₂ before the cells were stained with 0.01% neutral red in PBS.

Cell cloning by limiting dilution. Cells were diluted to 10/ml in RPMI 1640 containing 10% fetal bovine serum, and 0.1 ml of this suspension was added to each well of a 96-well tissue culture plate. The plates, which were maintained at 37°C in 5% CO₂, were routinely examined under a microscope to identify wells containing single colonies. Individual clones were suspended in RPMI 1640-fetal bovine serum after 3 to 4 weeks and transferred first to 24-well and then to 6-well tissue culture plates to achieve suitable cell numbers for experiment.

Cloning in soft agar. To 5 ml of 0.3% agar in RPMI 1640 medium at 43°C was added 0.1 ml of a cell suspension containing 50 or 100 cells, and after rapid mixing, the suspension was immediately transferred to 60-mm-diameter plates. The plates were incubated in 5% CO₂ at 37°C, and after about 2 weeks, colonies were counted and individual clones were transferred in RPMI 1640 medium to 24-well tissue culture plates for amplification.

Protein A binding. To approximately 2 × 10⁶ packed K562 cells was added 50 µl of rabbit anti-glycophorin serum diluted 1/10 in PBS. After incubation for 1 h at room temperature, cells were washed twice in PBS to remove unadsorbed antibody and 100 µl of [³⁵S]protein A (Amersham Radiochemicals Ltd.) diluted 1/1,000 in PBS (approximately 14,000 cpm) was added. Incubation was continued for 1 h at room temperature, and then samples were made up to 1 ml with PBS, the cells were spun down, and the supernatant was collected. Cells were washed with 1 ml of PBS, which was saved, and the cells were suspended in 1 ml of PBS before measurement of the radioactivity in the cell sample, supernatant, and wash in 10 ml of Aquasol-2 (Dupont, NEN). Percent protein A binding was taken as the

* Corresponding author.

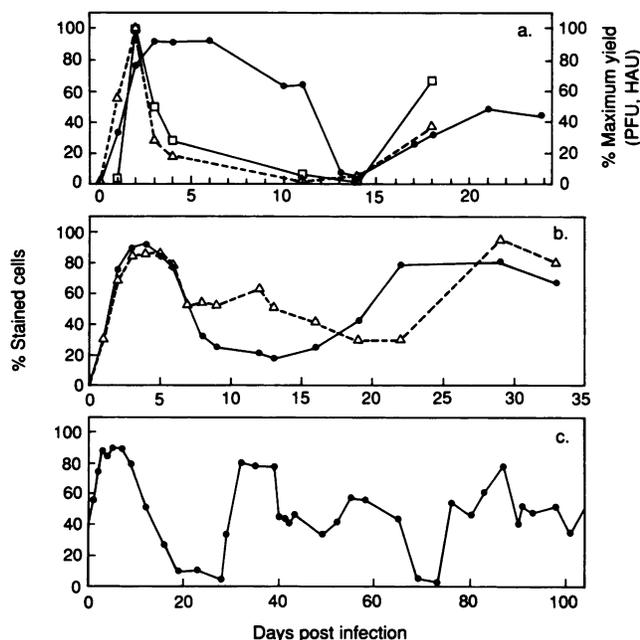


FIG. 1. (a) Establishment of persistent EMC virus infection of K562 cells as monitored by the percentage of cells becoming permeable to trypan blue (●) and the numbers of PFU (Δ) and hemagglutinating units (HAU) (□) released daily into the culture medium. (b) Comparison of the courses of EMC virus infection of K562 cells in washed cultures (●) whose medium was replaced with fresh medium daily and in nonwashed cultures (Δ) whose medium was supplemented with fresh medium about every 4 days to maintain a cell density of close to 2×10^6 /ml. (c) Extended persistent EMC virus infection of K562 cells maintained under nonwashed conditions and monitored by staining with trypan blue.

ratio of radioactivity in the cell sample to total radioactivity recovered.

Virus attachment. To measure virus binding, ^3H -labeled virus diluted to approximately 4,500 cpm in 0.1 ml of PBS was added to 2×10^6 packed cells. After incubation for 30 min at room temperature, the sample volume was made up to 1 ml with PBS before measurement of the radioactivity in the cell sample, wash, and supernatant as described for measurement of protein A binding.

RESULTS

Establishment of persistently infected K562 cells. The course of EMC virus infection can be monitored by plaque or hemagglutination assay or, more conveniently, by measuring cell viability by trypan blue staining. Usually, EMC virus causes cytolytic infection, resulting in close to 100% staining of cells in suspension culture in less than 15 h under one-step growth conditions (25).

It was surprising, therefore, to find that EMC virus infection of K562 cell suspension cultures proceeded more slowly, requiring 3 to 4 days to reach maximum cell staining, and that about 1 week later, cell viability apparently began to recover, reaching 80 to 90% at about 2 weeks postinfection (Fig. 1a). This time schedule refers to washed cultures in which the total supernatant fluid was collected daily and replaced with fresh, uninfected medium. Daily washing of cells was not required to establish persistence, unlike the situation reported for poliovirus (21). In nonwashed cul-

tures, to which fresh medium was added about every 4 days to maintain a cell density of approximately 2×10^6 /ml and released virus remained in contact with cells, viral persistence developed but recovery of maximum cell viability took closer to 3 weeks (Fig. 1b).

The phase in which cells recovered maximum viability was followed by alternating waves of decreased and then increased viability, the phases becoming successively less synchronized (Fig. 1c).

Plaque and hemagglutination assays of washed culture supernatants, each of which represented the total virus output in the preceding 24-h period, revealed that maximum virus release occurred on day 2 postinfection and before uptake of stain reached its maximum (Fig. 1a); typical titers on day 2 were 1×10^8 to 4×10^8 PFU/ml and 720 hemagglutinating units/ml. Virus yield then decreased on successive days until cell viability had recovered to 80 to 90%, by which time virus yield was less than 5% of maximum or about 2×10^6 PFU/ml and less than 40 hemagglutinating units/ml. However, virus yield again increased a few days later and before the next wave of increased permeability to stain by the cells, reaching 5×10^7 PFU/ml and 480 hemagglutinating units/ml (Fig. 1a). Virus output was not monitored routinely after this phase, because plaque size became minute, as explained below, and output was difficult to quantitate. However, a 3-month-old persistently infected culture still produced virus levels of about 5×10^6 PFU/ml per day.

Investigation of reasons for recovered cell viability. The number of K562 cells permeable to stain increased 3 to 5 days postinfection with EMC virus, in some cases to as high as 95%, which meant that about 1×10^5 to 2×10^5 cells per ml were viable, if trypan blue dye exclusion and viability are equated. After this phase there was apparent recovery, and about a week later, permeability to stain fell to 10 to 20%, or about 2×10^6 cells were viable (Fig. 1a). Possible reasons for recovery were that either cells became cured or a small percentage of resistant cells outgrew sick cells and took over the culture. To determine whether K562 cell growth rate was sufficient for 10^5 cells to give rise to 2×10^6 cells in less than 2 weeks, the rates of K562 cell growth in uninfected and virus-infected cultures were measured.

Uninfected cells seeded at 10^5 /ml grew with a mean generation time of about 1.2 days (Fig. 2), which was sufficient for 10^5 cells to give rise to 2×10^6 cells in less than 6 days. Since there were close to 10^5 viable cells per ml in persistently infected cultures at about 3 to 5 days postinfection, these could theoretically give rise to 2×10^6 cells per ml by 9 to 13 days postinfection growing at the rate of uninfected cells. In fact, infected cell cultures took slightly longer to reach this density of viable cells (Fig. 1). To determine why, the growth pattern of infected cultures was examined.

Within 24 h of infection, K562 cells showed a marked decrease in growth rate, the minimum coinciding with maximum permeability to stain at approximately 3 to 5 days postinfection (Fig. 2). This was followed by a lag period of 2 to 4 days, after which the rate of cell growth increased and a generation time of about 1.4 days was attained. Thus, the reason why it took infected cultures longer than uninfected ones to reach similar cell densities was the effect of a lag period and a slightly slower growth rate. Nevertheless, it seems reasonable to assume that the recovery of cell viability observed by about 2 weeks postinfection was due to self-renewal of cells rather than stained cells becoming cured. However, some stained K562 cell cultures did seem to recover viability, as the following studies suggested.

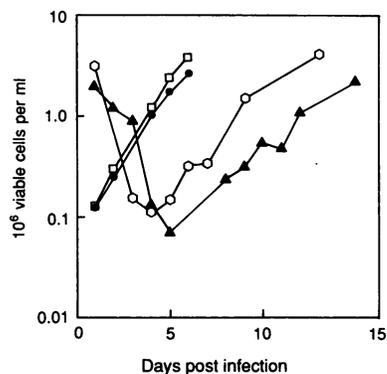


FIG. 2. Comparison of growth rates of infected and uninfected cell cultures. EMC virus-infected (● and ▲) and uninfected (□ and ○) K562 cell cultures containing 10^5 cells per ml were maintained under washed conditions (see the legend to Fig. 1b for a definition), and the number of viable cells per milliliter, as indicated by trypan blue dye exclusion, was recorded daily.

Origin of cells apparently resistant to virus infection. To determine whether the parental K562 cell line contained a small proportion of resistant cells which gave rise to the viable cells in the persistently infected culture or whether such resistant cells arose spontaneously and continuously, we cloned the parental cells in soft agar and tested each clone for susceptibility to infection.

On infection, the seven clones isolated followed a course already seen in the parental line, i.e., the percentage of cells taking up stain reached a maximum a few days postinfection, followed by recovery of viability which was so complete that several infected cultures of cloned cells attained levels of staining seen in uninfected cell controls (clones 4, 5, and 7) (Fig. 3a and b). Thus, it appeared that cells impermeable to stain in infected cultures were generated continuously and were not derived from a few resistant cells in the parental line.

The seven clones showed considerable heterogeneity in time course and extent of infection. Clones 1 to 3 reached high levels of cell staining (Fig. 3a) which remained high for a longer time than that usually found for the parental line (Fig. 1a and b). In contrast, uptake of stain by infected clone 5 was similar to that in uninfected cell controls, except for day 2, when staining reached 60% (Fig. 3a); this higher staining on day 2 was observed in a repeat experiment with clone 5 (data not shown). The time course and extent of

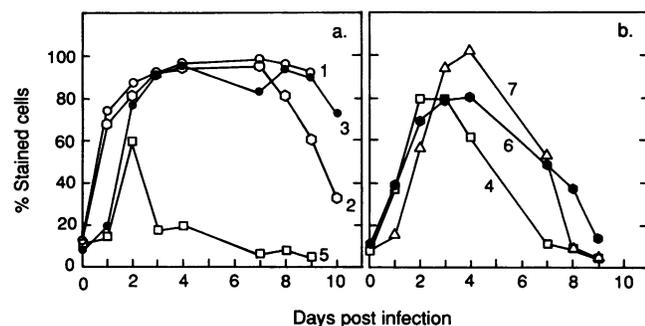


FIG. 3. Course of EMC virus infection as measured by cell staining in arbitrarily numbered cell clones isolated from the parental K562 cell line. a, Clones 1 to 3 and 5; b, clones 4, 6, and 7.

TABLE 1. Percent attachment of ^3H -labeled virus to K562 cell clones

Cell line or clone no.	% ^3H -labeled virus attachment	% Virus attachment ^a
K562	52.5	100.0
1	37.7	71.8
2	54.1	103.0
3	62.1	118.3
4	59.3	113.0
5	58.0	110.5
6	47.1	89.7
7	61.6	116.4

^a Normalized to 100% for parental K562 cell attachment.

staining for clones 4, 6, and 7 (Fig. 3b) were roughly intermediate between those for clones 1 to 3 and 5 (Fig. 3a). Despite this heterogeneity, all infected clones showed the propensity to recover viability, at least to some extent, during the 10 days when they were observed (Fig. 3a and b).

The number of cells permeable to stain fell from 59 to 17% in infected clone 5 between days 2 and 3 postinfection (Fig. 3a) and from 44 to 7% for infected clone 7 between days 7 and 8 (Fig. 3b). These two clones behaved similarly in a repeat experiment (data not shown). This change in staining pattern could not have been brought about by cell growth in the 24-h period and must have resulted from decreased permeability in previously permeable cells. This suggests that some apparently nonviable cells became cured.

The rate of progression and extent of infection varied between clones. For instance, after 24 h of infection, clones 3, 5, and 7 were less than 20% stained but clones 1 and 2 were over 70% stained. The possibility that this variability reflected differences in the numbers of virus receptors on the surface of the cells was considered. To test this possibility, ^3H -labeled EMC virus binding to the different cell clones was measured. However, little difference in virus binding was observed between the clones (Table 1), and the slight differences seen, if real, bore little relationship to differences in staining pattern exhibited by the infected cultures (Fig. 3a and b).

Nature of viable cells in persistently infected K562 cell cultures. The number of cells impermeable to stain in persistently EMC virus-infected K562 cultures increased to 80 to 90% by 2 to 3 weeks postinfection, despite the presence of infectious virus in the culture medium (Fig. 1a to c). To explore the nature of this apparent resistance to infection, cell clones were isolated from a culture (Fig. 1c) at 17 days postinfection by limiting dilution to examine their properties.

The 20 clones isolated were all resistant to EMC virus infection on the basis of their appearance under a light microscope and cell staining, except for clone 4A which, for example, at 5 days postinfection contained 64% stained cells compared with 90% in the uncloned, parental culture infected at the same time (Table 2).

The parental culture from which the resistant clones were obtained and the clones themselves were maintained for more than 70 days in culture and routinely examined by light microscopy and cell staining. During this time, the parental culture went through several crises and staining frequently reached 60 to 80% (Fig. 1c). In contrast, the cloned cells apparently remained healthy on the basis of their appearance under a microscope and impermeability to stain (data not shown), which was similar throughout to that of uninfected cultures (i.e., less than 5%). These observations suggest that

TABLE 2. Effect of EMC virus on clones isolated from persistently infected K562 cell cultures

Cells infected	% of cells stained ^a	% ³ H-labeled virus attachment	% Glycophorin ^b
K562	90.0	47.2	100
Clone 4A	64.0	28.9	132
Other clones ^c	1.9	2.1	131

^a At 5 days postinfection.

^b Compared with uninfected K562 cells.

^c Averages for 19 clones are shown.

unlike the parental culture, the cloned cells apparently did not carry virus.

To determine whether the resistance of most of the cloned cells to EMC virus infection was related to the lack of virus receptors on the cells, ³H-labeled EMC virus binding to all 20 clones was measured. Under standardized conditions, about 50% of the radioactivity in ³H-amino acid-labeled EMC virus preparations bound to K562 cells (Table 2). In contrast, binding of ³H-labeled virus to most of the cell clones from persistently infected K562 cell cultures was close to background levels. The exception was clone 4A, which bound about 30% of the virus added or about 60% of that bound to parental K562 cells (Table 2).

EMC virus attaches to glycophorin A on human erythrocytes (3). Since K562 cells also contain glycophorin A (13), it was of interest to determine whether the lack of EMC virus binding to K562 cell clones was related to their glycophorin A content. All of the clones apparently contained slightly more glycophorin A than did parental K562 cells (Table 2). Thus, glycophorin content on the cell surface was not related to susceptibility to EMC virus infection or the ability of cells to bind virus (Table 2).

Changes in virus in persistently infected cultures. Under standard conditions, EMC virus produces 7-mm-diameter plaques on mouse L-cell monolayers. During the first few days of infection, virus grown in K562 cells also produced 7-mm-diameter plaques but this gradually changed so that virus produced plaques of 3 to 4 mm in diameter by about 10 days postinfection and 1 to 2 mm or even smaller by about 20 days postinfection.

DISCUSSION

Picornaviruses have the reputation of causing cytolytic rather than persistent infections. However, there are numerous reports of persistent infections caused by picornaviruses, including foot-and-mouth disease virus (14, 27), Theiler's virus (6, 17), coxsackievirus (22), and poliovirus (24) in animals and foot-and-mouth disease virus (10, 11), Theiler's virus (23), coxsackievirus (5, 19), echovirus (15), and poliovirus (1, 16) in cell culture. It should not be surprising to find that EMC virus can also cause persistent infections under the right conditions. In fact, EMC virus has previously been shown to infect mice (8) and selected Chinese hamster ovary cells (26) persistently.

An unusual feature of the establishment of persistently EMC virus-infected K562 cells was the ease with which persistence was initiated. In contrast to EMC virus infection of CHO cells, which infrequently resulted in persistence (26), we found that infection of K562 cells invariably gave persistently infected cultures. K562 cells obviously have the tendency to become persistently infected readily, since this also happens with herpesvirus 1, measles virus, and mumps virus (7, 12).

The molecular basis of viral persistence is not well understood (20). It has been reported that coevolution of virus and cells occurs in cultures persistently infected with reovirus (2) or foot-and-mouth disease virus (10). The results presented in the present report are consistent with continuous evolution of virus and cells. We believe that most of the K562 cells in the original cultures were susceptible to EMC virus infection. However, a few cells resisted infection and continued to grow, taking over the culture, and therefore apparently largely recovered viability as measured by relative impermeability to stain. In the meantime, the virus itself was evolving and variants able to overcome the inhibition in the resistant cells were produced. As a result, the cultures went through another crisis until a few cells resistant to the variant virus grew and reestablished the culture. These cells remained the predominant form until yet again new virus variants arose when most of the cells succumbed to the new variants, and so the process was repeated again.

Certainly, there was evidence that EMC virus evolved rapidly in K562 cell cultures, giving rise to small-plaque variants, a feature also seen for persistent infections with foot-and-mouth disease virus (9), coxsackievirus (5), and poliovirus (16). In addition, it was apparent that the K562 cells also evolved, since cloned cells gave rise to mixtures containing some cells resistant and some susceptible to infection.

Our studies are continuing. The basis of the resistance to infection in this erythroleukemic K562 cell line, which might be related to a change at the receptor level (Table 2), remains to be determined, as does the nature of the change(s) in the virus leading to small-plaque morphology.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Diabetes Association and the Medical Research Council.

We thank Anna Dominic and Nicola Will for technical assistance.

LITERATURE CITED

- Ackermann, W. W., and H. Kurtz. 1955. Observations concerning a persistent infection of HeLa cells with poliomyelitis virus. *J. Exp. Med.* **102**:555-565.
- Ahmed, R., W. M. Canning, R. S. Kauffman, A. H. Sharpe, J. V. Hallum, and B. N. Fields. 1981. Role of the host cell in persistent viral infection: coevolution of L cells and reovirus during persistent infection. *Cell* **25**:325-332.
- Allaway, G. P., and A. T. H. Burness. 1986. Site of attachment of encephalomyocarditis virus on human erythrocytes. *J. Virol.* **59**:768-770.
- Burness, A. T. H., S. M. Fox, and I. U. Pardoe. 1974. The polypeptide composition of the encephalomyocarditis virus particle. *J. Gen. Virol.* **23**:225-236.
- Cao, Y., and D. P. Schnurr. 1988. Persistent infection of YAC-1 cells by coxsackievirus B3. *J. Gen. Virol.* **69**:59-65.
- Cash, E., M. Chamorro, and M. Brahic. 1985. Theiler's virus RNA and protein synthesis in the central nervous system of demyelinating mice. *Virology* **144**:290-294.
- Cernescu, C. 1982. Persistent virus infections in the human myeloid K-562 cell line: genetic implications and practical consequences. *Rev. Roum. Med. Virol.* **33**:257-265.
- Cronin, M. E., L. A. Love, F. W. Miller, P. R. McClintock, and P. H. Plotz. 1988. The natural history of encephalomyocarditis virus-induced myositis and myocarditis in mice. *J. Exp. Med.* **168**:1639-1648.
- De la Torre, J. C., M. Davila, F. Sobrino, J. Ortin, and E. Domingo. 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. *Virology* **145**:24-35.
- De la Torre, J. C., E. Martínez-Salas, J. Díez, and E. Domingo. 1989. Extensive cell heterogeneity during persistent infection with foot-and-mouth disease virus. *J. Virol.* **63**:59-63.

11. **Dinter, Z., L. Philipson, and T. Wesslen.** 1959. Persistent foot-and-mouth disease infections of cells in tissue culture. *Virology* **8**:542–544.
12. **Fujii, N., K. Oguma, K. Kimura, T. Yamashita, S. Ishida, K. Fujinaga, and T. Yashiki.** 1988. Oligo-2',5'-adenylate synthetase activity in K562 cell lines persistently infected with measles or mumps virus. *J. Gen. Virol.* **69**:2085–2091.
13. **Gahmberg, C. G., M. Jokinen, and L. C. Andersson.** 1979. Expression of the major red cell sialoglycoprotein, glycoporphin A, in the human leukemic cell line K562. *J. Biol. Chem.* **254**:7442–7448.
14. **Gebauer, F., J. C. de la Torre, I. Gomes, M. G. Mateu, H. Barahona, B. Tiraboschi, I. Bergmann, P. Auge de Mello, and E. Domingo.** 1988. Rapid selection of genetic and antigenic variants of foot-and-mouth disease virus during persistence in cattle. *J. Virol.* **62**:2041–2049.
15. **Gibson, J. P., and V. F. Righthand.** 1985. Persistence of echovirus 6 in cloned human cells. *J. Virol.* **54**:219–223.
16. **Kaplan, G., A. Levy, and V. R. Racaniello.** 1989. Isolation and characterization of HeLa cell lines blocked at different steps in the poliovirus life cycle. *J. Virol.* **63**:43–51.
17. **Lipton, H. L.** 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. *Infect. Immun.* **11**:1147–1155.
18. **Lozzio, C. B., and B. B. Lozzio.** 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **45**:321–334.
19. **Maverakis, N. H., N. J. Schmidt, J. L. Riggs, and E. H. Lennette.** 1973. Carrier culture of human fetal diploid cells infected with coxsackievirus type B2. *Arch. Gesamte Virusforsch.* **43**:289–297.
20. **Oldstone, M. B. A.** 1989. Viral persistence. *Cell* **56**:517–520.
21. **Pasca, P.** 1961. Poliovirus-carrying lines of HeLa cells: their establishment and sensitivity to viruses. *Acta Microbiol.* **8**:329–332.
22. **Roesing, T. G., B. G. Landau, and R. L. Crowell.** 1979. Limited persistence of viral antigen in coxsackievirus B3 induced heart disease in mice. *Proc. Soc. Exp. Biol. Med.* **160**:382–386.
23. **Roos, R. P., O. C. Richards, J. Green, and E. Ehrenfeld.** 1982. Characterization of a cell culture persistently infected with the DA strain of Theiler's murine encephalomyelitis virus. *J. Virol.* **43**:1118–1122.
24. **Rozhon, E. J., A. K. Wilson, and B. Jubelt.** 1984. Characterization of genetic changes occurring in attenuated poliovirus 2 during persistent infection in mouse central nervous systems. *J. Virol.* **50**:137–144.
25. **Sanders, F. K., J. Huppert, and J. M. Hoskins.** 1958. Replication of an animal virus. *Symp. Soc. Exp. Biol.* **12**:123–137.
26. **Taber, R., V. Alexander, and N. Wald.** 1976. The selection of virus-resistant Chinese hamster ovary cells. *Cell* **8**:529–533.
27. **Van Bakkum, J. G., H. S. Frenkel, H. H. J. Frederiks, and S. Frenkel.** 1959. Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschr. Diergeneesk.* **84**:1159–1164.