Replication Kinetics and Cytopathic Effect of Hepatitis A Virus

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SUMMARY

The replication kinetics and c.p.e. of hepatitis A virus (HAV) strain HM-175 were shown to depend upon the passage level of the cell line, and the passage level and method of selection of the virus population. Maximum virus production under single-step growth curve conditions occurred as early as 24 to 28 h or as late as 10 days post-infection. Although rapid replication of an isolate of HM-715 (pHM-175) occurred initially in BS-C-1 cells, its most pronounced c.p.e. was induced in FRhK-4 cells. The replication kinetics of pHM-175 in BS-C-1 cells were similar to those in FRhK-4 cells, although a higher yield of virus was obtained in the latter. The HAV that generated c.p.e. in FRhK-4 cells was obtained by two different selection processes: virus passage, or cloning of large focus-forming variants from the radioimmunofocus assay. The c.p.e. and yield of infectious pHM-175 in FRhK-4 cells could be reduced by 3 mM-guanidine. Another HAV isolate, strain MD-1, isolated directly from contaminated ground water in cell culture demonstrated c.p.e. in FRhK-4 cells after passage as persistently infected A-549 cells.

INTRODUCTION

The replication characteristics of hepatitis A virus (HAV), since its first propagation in cell culture (Provost & Hilleman, 1979), have been shown to be similar in numerous laboratories (Frosner et al., 1979; Dsemmer et al., 1981; Deinhardt et al., 1981; Flehmig et al., 1981; Flehmig, 1981). HAV replicates slowly when first isolated and requires 3 to 10 weeks for maximum virus production. More recently successive passage in cell culture has shortened significantly the replication cycle from weeks to days (Wheeler et al., 1986; Siegl et al., 1984; Anderson et al., 1986). In the early studies no lytic infection characteristic of picornaviruses was seen, and infected cells remained viable. Replication kinetics were not fully studied in the early systems where weeks were required for maximum virus or viral antigen production as determined by antigen detection assays such as radioimmunoassay, immunofluorescence and enzyme immunoassay (EIA). The development of the radioimmunofocus assay (RIFA) (Lemon et al., 1983) analogous to the plaque assay of lytic viruses, made feasible quantitative study of replication kinetics. Wheeler et al. (1986) employed this assay to demonstrate production of maximum infectious virus at 5 days post-infection (p.i.) under single-step growth curve conditions by a cell culture isolate selected for rapid growth.

We noted that an HM-175 isolate (pHM-175) generated by passage of persistently infected cells formed large foci, demonstrated by the RIFA, suggesting more rapid replication or cell-to-cell spread of this virus compared to parental stock that had not been passaged in persistently

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infected cells (Cromeans et al., 1986). More recently, we have shown that this rapidly replicating isolate of HAV can induce neutralizable c.p.e. These observations led to the development of a plaque assay (Cromeans et al., 1987). We have demonstrated rapid replication of this isolate with maximum production of infectious virus as early as 24 to 28 h p.i. (Cromeans et al., 1988). In the current report we compare the replication in BS-C-1 cells to that in FRhK-4 cells and also compare the replication kinetics of different HAV stocks in BS-C-1 cells.

METHODS

Cells and virus. FRhK-4 (foetal rhesus kidney) cells obtained from B. Flehmig, Tübingen, F.R.G., and BS-C-1 cells from S. Lemon, Chapel Hill, N.C., U.S.A. were propagated in Reinforced Eagle's MEM (REMEM) (Centers for Disease Control media unit) with 10% foetal calf serum (FCS) and 50 μg/ml gentamicin sulphate (Gibco). A-549 cells (human lung carcinoma) obtained from the American Type Culture Collection were propagated in Auto-POW MEM (Flow Laboratories) for the passage of persistently infected cells, and REMEM was used for acute infection of A-549 cells with HAV. Acute infection is defined as the infection of uninfected cells, whereas persistent infection or passage refers to the passage of previously infected cells plus virus.

Fig. 1 describes the origin and passage history of HM-175 in addition to sources of HAV stocks for kinetic studies, clonal isolation and c.p.e. HAV strain HM-175 was a human faecal isolate passaged six times in marmosets, 10 times in primary African green monkey kidney (AGMK) cells and six times in BS-C-1 cells (AGMK-derived cell line) (Binn et al., 1984). This HAV stock was inoculated into BS-C-1 cells which were then subcultured 20 to 22 times for persistent passage of the virus as previously described (Cromeans et al., 1987). Inoculum from persistently infected cells (designated pHM-175) was subsequently passaged by acute infection of FRhK-4 cells. Clone 24A and clone 24B were purified by cloning twice in BS-C-1 cells from foci of RIFA as described by Lemon & Jansen (1985).

MD-1, an HAV strain isolated from contaminated ground water (Sobsey et al., 1985) was propagated in primary AGMK cells. Cell lysate from two or three passages in AGMK was extracted with chloroform and then inoculated onto A-549 cells for an additional 15 to 24 passages as persistently infected cells. One rapid passage (3 day harvest) at a low m.o.i. of a pooled stock from persistently infected cells was made in A-549 cells before inoculation into FRhK-4 cells to obtain a c.p.e.

Kinetic studies. Confluent layers of 2 × 10⁵ to 5 × 10⁵ BS-C-1 cells in 35 mm dishes were inoculated with an m.o.i. sufficient to establish single-step growth curve conditions, i.e. an m.o.i. of 2 or more radioimmunofocus units (r.f.u.) per cell. The m.o.i. used for each experiment is stated in the figure legend. After a 90 to 120 min adsorption period cell layers were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and REMEM plus 2% FCS (MM) was added before incubation at 37 °C in 5% CO₂. At indicated times samples were harvested as previously described (Cromeans et al., 1987). Briefly, the supernatant was removed and pooled with two DPBS washes which constituted the released virus (RV) samples. For cell-associated virus (CAV), cell layers were frozen at −70 °C with another aliquot of MM, thawed, and the cell lysate plus two dish washes with MM were pooled. All samples were sonicated for 45 s at maximum power in a Sonic dismembranator Model 300 equipped with a cup probe (Fisher Company) prior to dilution for titration by RIFA or by a plaque assay.

Plaque assay and RIFA. Confluent layers of FRhK-4 cells in 60 mm tissue culture dishes (Falcon, Becton-Dickinson) were drained and inoculated with 0.25 ml of virus diluted in Earle's balanced salt solution plus 2% FCS. After 90 min of viral adsorption an overlay of 0.5% agarose (Seakem, FMC) in MM was added. After incubation for 8 days, the agarose overlay was removed and cell layers were stained with 0.065% crystal violet in 6% formalin-PBS. RIFA was performed as described previously (Lemon et al., 1983; Cromeans et al., 1987), except that the incubation periods of BS-C-1 were 6 to 7 days, 10 days or 14 days depending upon the rate of replication of the different stocks of HM-175.

ELA. This assay was performed as previously described (Cromeans et al., 1987). Briefly, rabbit anti-157S HAV IgG was employed as the capture antibody and biotinylated chimpanzee anti-HAV IgG was employed to detect HAV antigen. Streptavidin–horseradish peroxidase (Enzo Biochemicals) was reacted with bound biotinylated IgG and the indicator 3,3'-phenylenediamine dihydrochloride with H₂O₂ was added. Absorbance was determined at a wavelength of 493 nm (A₄⁹₃) using a microELISA MR580 autoreader (Dynatech Laboratories). A₄⁹₃ readings obtained for uninfected cell fractions, MM or mixtures of MM and DPBS were subtracted from readings obtained for CAV or RV fractions to obtain final values.

RESULTS

Replication kinetics in BS-C-1 cells

The isolate pHM-175 produced larger foci in passage 93 BS-C-1 cells than in passage 64 BS-C-1 cells using a 1 week incubation for RIFA (Fig. 2). This suggested potential differences in
Hepatitis A virus replication and c.p.e.

Fig. 1. Passage history of HM-175 in cell culture, derivation of c.p.e. and clonal isolates, and inoculum employed for kinetic studies (Gust et al., 1985).

pHM-175 replication kinetics for low passage level compared to high passage level BS-C-1 cells. Fig. 3(a, b) demonstrates single-step growth curves generated by pHM-175 in high (102) passage and low (63) passage BS-C-1 cells. Results obtained with passage 102 (Fig. 3a) are generally similar to those obtained with passage 93 BS-C-1 cells as previously described (Cromeans et al., 1987). CAV was present at its maximum by 32 to 40 h p.i., whereas cell-associated antigen production did not reach a plateau until 56 to 60 h p.i. Furthermore 50% of infectious virus was of the RV category. In the experiment with passage 93 cells (described previously) as much as 90% of infectious virus particles was released at later time points. However it is possible that the difference in quantity of RV was due to refeeding of cells in the experiment with passage 102.
Fig. 2. RIFA results. Dilutions of pHM-175 passage 21 inoculated onto BS-C-1 cells at passage 64 (a) or passage 93 (b). For (a), from top to bottom, are shown 1000-fold and 10000-fold dilutions; for (b), 10000-fold and 100000-fold dilutions.

![Cell images](image1)

Fig. 3. Production of infectious HAV and HAV antigen in passage 102 BS-C-1 cells infected with pHM-175 at an m.o.i. of 5 to 6 r.f.u./cell (a) or in passage 63 BS-C-1 cells infected at an m.o.i. of 3 r.f.u./cell (b). CAV (O) and RV (∆) as determined by RIFA; cell-associated antigen (●) and antigen in supernatant fluid (∆) as determined by EIA of duplicate samples at each time point.

When single-step growth kinetics were examined in lower passage BS-C-1 cells (passage 63 at an m.o.i. of 3 r.f.u./cell), two differences from the results obtained for higher passage BS-C-1 were found (Fig. 3b): firstly, fewer infectious virus particles were released (20 to 30% compared to 50%), and secondly, maximum cell-associated antigen production was not obtained until

cells at 50 h p.i., thus preventing cell degeneration or a partial c.p.e. The nutritive status of the cell has been shown previously to influence development of enterovirus c.p.e. (Koch & Koch, 1985).
Hepatitis A virus replication and c.p.e.

Fig. 4. Production of infectious HAV and HAV antigen in BS-C-1 cells infected with HM-175. (a) Passage 93 BS-C-1 cells infected at an m.o.i. of 2 to 3 r.f.u./cell. (b) Passage 60 BS-C-1 cells infected at an m.o.i. of 1 to 2 r.f.u./cell. Symbols as in Fig. 3.

140 h p.i. compared to 60 h p.i. with higher passage BS-C-1 cells. In another trial using passage 72 BS-C-1 cells, results were similar to those obtained for passage 63 (data not shown). Little or no viral antigen was detected in the supernatant fraction.

Replication under single-step growth curve conditions of parental stock HM-175 (passage 16, Fig. 1), never passaged persistently, is shown in Fig. 4(a, b). With an m.o.i. of 2 to 3 r.f.u./cell in high (93) passage BS-C-1 cells, CAV production first occurred between 40 and 80 h (p.i.). After a 12 to 16 h lag period relative to CAV production, RV production was concomitant with CAV production (Fig. 4a). After a plateau occurring at about 100 h p.i., a second logarithmic production of CAV and RV occurred. No cell-associated antigen as measured by EIA was detected until the second cycle of production of infectious virus. Viral antigen production continued until 500 h p.i., whereas infectious virus production plateaued at 260 h p.i. Viral antigen was detected in the supernatant fluid beginning at 200 h p.i., in contrast to pHM-175 replication where little or no viral antigen was detected in the supernatant. Fig. 4(b) shows results obtained with stock HM-175 one passage level later (seven passages in BS-C-1 cells) inoculated at an m.o.i. of 1 or 2 r.f.u./cell in low (60) passage BS-C-1 cells. These results are similar to those of virus one passage earlier, in that maximum infectious virus production was not obtained until approximately 200 h p.i., and 50% of infectious virus was released. Furthermore viral antigen production continued beyond the plateau of infectious virus production and viral antigen was detected in the supernatant fraction. A third single-step replication study in passage 72 cells with the same inoculum yielded similar results (data not shown). A common feature of these protracted (in comparison to pHM-175) replication cycles for HM-175 was the presence of two focus sizes, as shown by RIFA. Fig. 5(a) shows an example of the mixed population (virus passage 18) at 72 h p.i. in passage 72 BS-C-1 cells (data for this kinetic study are not shown). The smaller foci were more common in the first rise period, whereas the larger foci were more common in the second rise period and were the only foci present at later time points. In contrast homogeneous large foci were obtained by RIFA of clone 24A, virus passage 38 to 40 (Fig. 5b).

Cytopathic effect and effect of guanidine on c.p.e.

The c.p.e. of HAV has been previously demonstrated to be generated by pHM-175 after passage of persistently infected cells. By another method, clonal selection, we have also isolated cytopathic HAV using strain HM-175 that has never been persistently passaged. In the first
selection, virus from a large focus (15 mm by 14 days incubation on RIFA) of HM-175 passage 20 (Fig. 1) was cloned by the methods of Lemon & Jansen (1985) and designated clone 12A. When inoculated at a low m.o.i. in FRhK-4 cells this isolate generated c.p.e. which was evident by 2 to 3 days p.i. Control cells and cells inoculated with clone 12A at 5 days p.i. are shown in Fig. 6. Two characteristics of clone 12A match those of pHM-175: firstly, the c.p.e., and secondly, higher infectivity titres than HM-175. The second clonal selection of cytopathic HAV in the absence of persistent passage was by the isolation of large foci from a RIFA of CAV samples at 72 h p.i. in an HM-175 single-step replication kinetics study (Fig. 4b). Virus stocks from this procedure yielded an identical c.p.e. morphology and virus yields (data not shown). Thus, clonal selection of large foci on RIFA from virus stocks passaged at low or high m.o.i. produced variants of c.p.e.

The effect of 3 mM-guanidine on pHM-175 c.p.e. at 4 days p.i. in FRhK-4 cells is shown in Fig. 7. Control cells (Fig. 7a) appeared normal by light microscopy in the presence of MM containing 3 mM-guanidine. More advanced c.p.e. is evident in the absence of guanidine (Fig. 7c) than in the presence of guanidine (Fig. 7b) and differences in the c.p.e. were evident from day 2 p.i. Furthermore, in the presence of guanidine there was a 56% reduction in the infectivity titre of this pHM-175 stock at 5 days p.i. Similar results were obtained with clone 24B of pHM-175 at a high m.o.i. and the virus yield was reduced to $2.6 \times 10^6$ p.f.u./ml in the presence of guanidine from $3.8 \times 10^7$ p.f.u./ml in the absence of guanidine.

In FRhK-4 cells a c.p.e. was demonstrated with another isolate of HAV, strain MD-1. Fig. 8 shows FRhK-4 cells at 5 days p.i. with the MD-1 stock that had been passaged in A-549 cells as persistently infected cells as followed by rapid, low multiplicity infection of previously uninfected cells. The morphological appearance of the c.p.e. in these cells is similar to that seen with HM-175. After two rapid passages of MD-1 at a low m.o.i. in FRhK-4 cells, a c.p.e. together with complete destruction of an FRhK-4 monolayer was obtained by 9 days p.i. (data not shown). The titre of this lysate was $1.9 \times 10^7$ p.f.u./ml whereas virus stock from low m.o.i. passage in A-549 cells had a titre of only $5 \times 10^6$ p.f.u./ml. In a plaque neutralization assay of this cytopathic MD-1 stock, 87% neutralization was obtained by a 100-fold dilution of monoclonal antibody ascites fluid (K2-4F2, Commonwealth Serum Laboratories).

**DISCUSSION**

Kinetic analysis under single-step growth curve conditions of different populations of HAV strain HM-175, in different passage levels of BS-C-1 cells or in a different cell line, FRhK-4, yielded a spectrum of results ranging from a lengthy replication cycle with no c.p.e., to a rapid...
Fig. 6. C.p.c. in FRhK-4 cells inoculated with a low m.o.i. of clone 12A at 5 days p.i. (a) Control cells. (b) Infected cells. Bar markers represent 50 μm.
Fig. 7. Delay of c.p.e. in the presence of 3 mM-guanidine hydrochloride at 4 days p.i. (a) Uninfected cells with 3 mM-guanidine in the MM. (b) FRhK-4 cells inoculated with 5 or more p.f.u./cell of pHM-175 (passage 4 in FRhK-4 cells). (c) Same inoculation as for (b) but with 3 mM-guanidine included in the MM. Bar markers represent 50 µm.

replication cycle with a c.p.e. leading to complete cell destruction. Some findings, however, were common to all these kinetic studies. In all cases, infectious virus as detected by RIFA or plaque assay was released to various degrees, after a variable lag period, in parallel with CAV production. The lag period was much shorter (as little as 4 h) for the most rapidly replicating isolate. In FRhK-4 cells, the release of infectious virus occurred before a microscopically visible c.p.e. was apparent.

A second finding was that viral antigen production as detected by EIA did not necessarily correspond to infectious virus production. In particular, analysis of the culture supernatant by EIA was an unreliable indicator of the presence or absence of infectious virus. Interpretation of the data is dependent upon the sensitivity and specificity of the EIA. Data have been obtained with another strain of HAV (HAS-15) to indicate that a titre of $10^7$ r.f.u./ml is near the detection limit of the EIA. This observation may partially explain why viral antigen could be detected in the supernatant of FRhK-4 cells infected with pHM-175 when the titre was $10^9$ p.f.u./ml (Cromeans et al., 1988), whereas in all other infections with pHM-175 (or clone 24A) viral antigen could not be detected in RV when infectivity titres were only $10^7$ r.f.u./ml. However, in
infections with HM-175 (Fig. 3), RV antigen was detected only when titres remained at $10^7$
rf.u./ml. Perhaps in these infections more viral antigen is released from cells, whereas in the
infections with the more rapidly replicating virus, antigen or non-infectious particles are not
substantially released. Our antigen assay for HAV is quite specific because the rabbit antiserum
was raised against highly purified virions. It is therefore unlikely that there is an anti-cellular
component in the antiserum that would give false positive results. However if such a component
were present it could amplify detection in the cell-associated fraction as well.

Although initial production of cell-associated antigen occurs in synchrony with infectious
virus production, at later time points under single-step growth curve conditions there was no
indication of an increased quantity of infectious virus (Fig. 3b; Fig. 4a, b). This suggests that the
viral precursor protein (i.e. 5S or 14S units) and/or 80S particles are synthesized but not
packaged into infectious virions in those infections. In the most rapidly replicating systems, cell-
associated viral antigen production reaches a plateau at either 4 h (pHM-175 in FRhK-4 cells;
Cromeans et al., 1988) or 24 h (Fig. 3a) after infectious virus production reached a plateau. On
the other hand, in the protracted replication cycle of HM-175 (Fig. 4a, b) continued antigen
production was detected as late as 1 week after infectious virus production had reached a
plateau. Other investigators have found that antigen production continues after infectious virus
production reaches a plateau (de Chastonay & Siegl, 1987), which is consistent with the more
protracted HM-175 replication cycle observed here.
Replication kinetics for cytopathic pHM-175 in FRhK-4 cells were similar to those in BS-C-1 cells, although the latent period was shortened to 12 h from 16 h and peak production of infectious virus occurred a few hours earlier. In poliovirus infections an increased m.o.i. has been shown to shorten the latent period (Baltimore et al., 1966); however, in one HAV study using an m.o.i. of 96 p.f.u./cell, the latent period remained 12 h (Cromeans et al., 1988). The relative time (p.i.) of the appearance of c.p.e., as observed by light microscopy, is later for HAV than for poliovirus. In one experiment with an m.o.i. of 96 p.f.u./cell, c.p.e. reached its maximum (4+) earlier than in other experiments. This suggests that a higher m.o.i. could further decrease the time required for complete cell destruction, approaching perhaps the kinetic features of a poliovirus single-step growth curve. Compared to a poliovirus kinetic study, the lag and logarithmic phases of HAV replication are proportionally extended. This does not suggest a single block in the replication of HAV, but rather that each phase of the replication cycle is longer. On the other hand, a block or delay in replication could be at the level of uncoating only. If all infectious particles were not uncoated at the same time the logarithmic phase would be extended. Recently Anderson and coworkers have demonstrated that the pool of RNA available for replication is depleted by the encapsidation of viral RNA during HM-175 replication in BS-C-1 cells (Anderson et al., 1988). This finding could account for the lengthy logarithmic phase in HAV replication.

The yield of infectious virus per cell varied considerably in these systems. Yields as low as 10 r.f.u./cell were obtained with non-cytopathic pHM-175 in BS-C-1 cells and yields as high as 560 p.f.u./cell were obtained with cytopathic pHM-175 in FRhK-4 cells (Cromeans et al., 1988). Despite occupying a larger surface area per cell, FRhK-4 cells still yielded more infectious virus per surface area than BS-C-1 cells in both low and high multiplicity infections (data not shown). Yields of pHM-175 HAV from acute infection at low multiplicity of FRhK-4 cells from passage levels 72 to 100 did not vary significantly. However, plaque formation and c.p.e. were dramatically reduced in passage 33 FRhK-4 cells (data not shown). Other investigators have found a difference in the yield of virus from different passage levels at FRhK-4 cells (Robertson et al., 1988). In this instance high (258) passage cells were less permissive with persistent passage for HAV and low (78) passage cells were more permissive with acute infection as determined by antigen assay or yield of purified virions. Thus, the cell passage level can influence yield and c.p.e. in HAV infections.

Rapid replication of HAV appears to be a factor in the induction of a c.p.e., although one rapidly replicating cell–virus system, as determined by an antigen assay, was reported to be non-lytic (Kojima et al., 1981). As shown here, induction of a c.p.e. by HAV does not require virus from persistently infected cells or virus passaged in animals prior to cell culture. In this report we establish that persistent infection of cells is only one source or method of isolation of cytopathic HAV, but variants producing a c.p.e. can be obtained by clonal selection for rapid replication as indicated by focus size in RIFA.

The role of host cell factors in allowing for the different expression of viral genomes has long been recognized (Tamm, 1975). More recent studies have suggested that host shut-off mechanisms vary within the Picornaviridae family and depend upon the type of cell employed (Jen & Thach, 1982). Although host shut-off is probably not responsible for the late c.p.e. seen in picornavirus-infected cells (Schrom & Bablanian, 1981), their data suggest that virus effects on the host cell can be modulated by the cell type. In our study cell passage level and type of cell are shown to be determinants of HAV replication and c.p.e. Other investigators have reported HAV replication with no c.p.e. in RC-37 cells, but replication with a c.p.e. in FRhK-4 cells (Divizia et al., 1987).

The c.p.e. we have described represents morphological changes seen late in infection with picornaviruses. The physiological or biochemical changes that may be induced in this system are unknown. Guanidine has been shown to delay morphological development of c.p.e. in poliovirus-infected cells, but causes only a slight delay in the shut-off of RNA and protein synthesis (Bablanian et al., 1965). As guanidine reduced infectious poliovirus production by 1000-fold, it was suggested that viral production is necessary for a c.p.e. to occur. The partial inhibition of production of infectious HAV and delay of a c.p.e. by guanidine in our study
Hepatitis A virus replication and c.p.e. contrasts with results obtained by Siegl & Eggars (1982) for a non-lytic HAV, strain MBB, in PLC/PRF/5 cells. Whether this partial guanidine sensitivity is related to the cytopathic aspects of the virus should be determined.

The possible association of the c.p.e. with a specific mutation was not investigated in this study. Although rapid evolution of RNA genomes occurs and mutation rates may be high, repeated passage under the same conditions but at low m.o.i. can lead to stable populations (Holland et al., 1982). Furthermore, recent information indicates that certain mutants can replace wild-type virus in mixed infections (Whitaker-Dowling & Youngner, 1987). In this study, repeated passages of low m.o.i. were employed in the final isolation of the two cytopathic strains of HAV, and therefore the population should be relatively stable. Comparison of nucleotide sequences of these cytopathic isolates with non-cytopathic isolates could determine what possible mutations relate to the phenomenon of the induction of c.p.e. (S. M. Lemon et al., unpublished results).

The lytic properties of the virus in certain host cells in vitro may or may not be related to in vivo virulence. Hepatocellular damage in hepatitis A disease has been postulated by several investigators to be mediated by the immune system (Lemon & Binn, 1983; Kurane et al., 1985; Vallbrbracht et al., 1984). The non-lytic characteristic of HAV in cell culture has been considered supportive evidence for immune damage. It has been postulated that there could be early liver damage due to lytic virus infection and later damage that is mediated by the immune system (Feinstone, 1986). The HAV isolates, pHM-175 and MD-1, that generate a c.p.e. (later morphological changes in infected cell cultures) will be useful in determination of the mechanism(s) of liver damage, in addition to the determination of the molecular mechanism(s) of the induction of c.p.e. and the relationship of attenuation or virulence in vivo to c.p.e. in vitro.

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