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# Inhibition of Herpes Simplex Virus Infection in Tissue Culture by Trisodium Phosphonoformate[1] (40502)

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Like phosphonoacetic acid (PAA) phosphonoformic acid (PFA) inhibits virus replication by interacting with virus specified polymerases (1–4). PAA and PFA both effectively inhibit herpes simplex virus (HSV) multiplication in cell culture by binding preferentially to HSV induced DNA polymerase in comparison to DNA polymerases of eucaryotic cells (5). The present study reports observations on the inhibitory effect of trisodium PFA on HSV replication, and on the selection of PFA resistant HSV strains.

*Material and methods. Cells.* Green monkey kidney cells (GMK AH-1) were cultured in plates and tubes using Eagles MEM supplemented with 10% fetal calf serum, 100 IU of penicillin and 100 μg of streptomycin/ml. The same medium supplemented with only 2% serum was used as maintenance medium.

*Virus.* Strains F and B 4327UR, of HSV-1 and HSV-2 respectively, and 80 recently isolated strains of HSV were studied. The strains were typed (6) including typing by immunoelectroosmophoresis against type-specific antisera (7). Titration of infective virus was carried out in monolayer cultures of GMK AH-1 cells by plaquing of virus and by 50% endpoint titrations. Titers were expressed as pfu and $ID_{50}$.

*PFA.* A stock solution containing 100 mM of PFA (gifted by Astra, Södertälje, Sweden) in Hanks' was prepared. Aliquots of this solution were diluted in cell culture medium. Adjustment of pH to neutrality was performed with NaOH.

*Statistics.* The Student *t* test was used for estimation of probability.

*Results. Inhibition of HSV-1 and HSV-2 in cell culture by PFA.* The inhibition of HSV replication was studied by following $ID_{50}$ titers assayed on sets of five tube cultures per 10-fold virus dilution. The effect of PFA added to the cell culture medium in concentrations ranging from 0.001 to 1.0 m*M* was observed and the cultures were read after 4, 7 and 12 days of incubation at 37°.

There were no morphological signs of PFA induced toxicity on outgrown cell cultures at any of the concentrations studied. Figure 1 demonstrates the inhibition in log $ID_{50}$ of both types of HSV as a function of the concentration of PFA. A delay in appearance of cpe was evident and final titers were not recorded before the twelfth day of incubation. At a concentration of 0.25 m*M* of PFA both types of HSV were inhibited by nearly 2 log units of $ID_{50}$. The kinetics of the inhibition differed between the F and the B 4327 UR strains, the latter seemingly being the more sensitive. Within limits indicated by semilog plots of inhibition of HSV against concentration of PFA, the reduction in titers was considered to express the sensitivity of the strain tested.

Removal of PFA 2 or 7 days after inoculation of virus, by replacement of medium with fresh cell culture medium devoid of PFA, influenced the final titers (Fig. 2). Replication of HSV-1 arrested by presence of 0.5 m*M* of PFA in the initial culture medium was resumed. Similar results were obtained with HSV-2, and with concentrations of 1.0 m*M* of PFA (not shown).

The inhibition of HSV at different multiplicities of infection (moi) is illustrated by Fig. 3. The reduction in 24-hr production of HSV in GMK AH-1 cells maintained in a medium containing 0.10 or 0.25 m*M* of PFA was observed. The moi studied ranged from 0.005 to 60 pfu/cell. With both concentrations of PFA, virus inocula corresponding to

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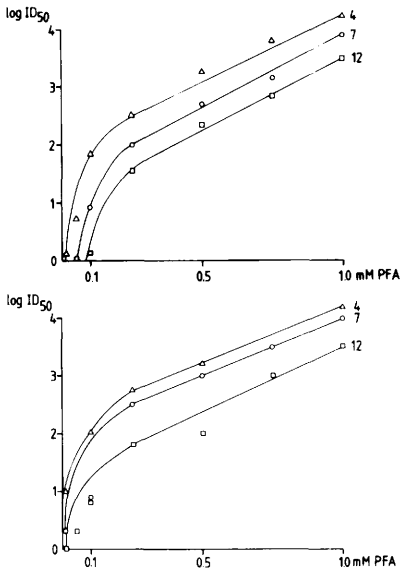


FIG. 1. Effect of varying PFA concentrations on replication of HSV-1 and HSV-2 in tissue culture. Log $ID_{50}$ titers were read after 4 (△——△), 7 (○——○) and 12 days (□——□). Upper set of curves represents PFA induced inhibition of HSV-1 (F) replication, while the lower figure indicates corresponding dose response curves of HSV-2 (B 4327 UR). Inhibition (log $ID_{50}$) is plotted against concentration of PFA.

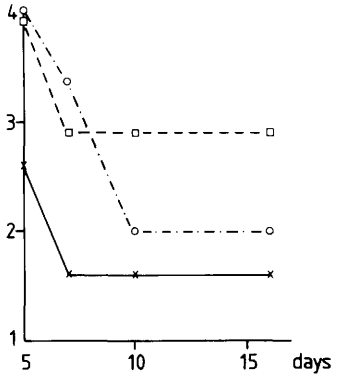


FIG. 2. Effect of removal of PFA from culture medium. At a concentration of 0.5 m*M* PFA, log reduction of $ID_{50}$ titers is plotted against the day of reading. Inhibition of HSV replication by PFA present during all of the 16-day observation period (□- - -□). Inhibition observed when PFA was removed 2 days after inoculation (×——×), and 7 days postinoculation (○-·-○).

moi ≥ 1 reduced the inhibitory PFA effect. At 0.1 m*M* PFA and a moi of 0.1 pfu/cell the yield of infective virus was about 20% of that demonstrable in cultures without PFA. The same concentration of PFA caused no inhibition of HSV production when the moi was increased to 60.

The experiments thus suggested that the inhibition of HSV replication by PFA was not an all or none phenomenon. The amounts of virus inoculated or produced in the culture and the time for PFA-HSV interaction, in addition to the concentration of PFA, were factors influencing the virus inhibitory effect of PFA.

*Sensitivity to PFA of newly isolated HSV strains.* HSV strains were isolated from patients with clinical herpetic infections. Eighty strains, 41 type 1 and 39 type 2, were selected for assay of their sensitivity to PFA. As a screening concentration we used 0.25 m*M* of PFA, inhibiting about 2 log units of $ID_{50}$ with the prototype virus strains. Following one passage each strain was titrated in tube cultures testing the same dilutions of virus in cultures with and without PFA. After 12 days incubation the final titers were read microscopically and the reduction in titers resulting from presence of PFA in the culture medium was calculated.

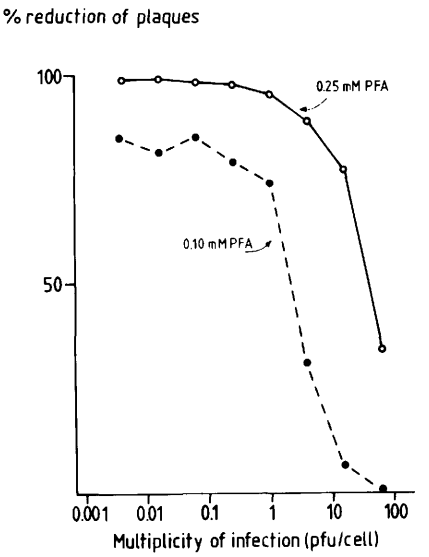


FIG. 3. Influence of 0.1 m*M* and 0.25 m*M* PFA on 24-hr production of HSV-1 (F) in GMK cells at different multiplicities of infection (moi). Moi from 0.005 to 60 pfu/cell were used. Yields of virus from cell cultures with and without PFA were assayed and subsequently % reduction of plaques was estimated and plotted against moi.

Figure 4 demonstrates the ranges of inhibition observed and the relative number of strains within each range. None of the strains was completely resistant to PFA and the reduction of HSV titers varied from 0.5 to more than 3 log units of $ID_{50}$. The differences in sensitivity between the strains were reproducible. The mean values for reduction of the 41 SV-1 strains was 1.71, while the corresponding mean for the 39 HSV-2 strains was 1.59 log $ID_{50}$. However, these mean values were not statistically different at the 0.05 level of probability.

*Development of PFA resistance.* Passage of HSV in the presence of 0.25 m*M* of PFA resulted in appearance of gradually more PFA resistant virus. The development of this resistance is illustrated in Fig. 5 by four strains of each type which to different extents became resistant to PFA after three–four passages in 0.25 m*M* PFA. The strains were selected on grounds of relatively high sensitivity to PFA in the screening procedure described in Fig. 4. The resistance acquired by passaging virus in presence of 0.25 m*M* PFA and by means of a limiting dilution procedure resulted for two strains of each type in an almost complete PFA insensitivity. Resistant strains did not revert to susceptibility after five consecutive passages in absence of PFA. To see if the parental strains contained sensitive as well as resistant mutants before being passaged in PFA containing cultures, one

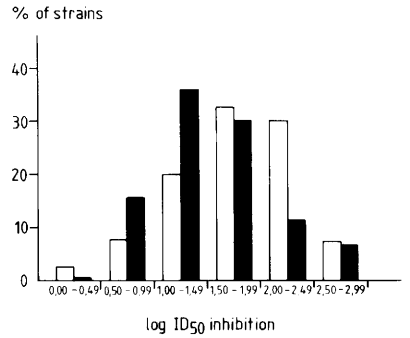


FIG. 4. Eighty newly isolated HSV strains, 41 type 1 and 39 type 2, were assayed for their sensitivity to 0.25 m*M* PFA. Following 12 days of incubation, titers were read and the log $ID_{50}$ inhibition of each strain was calculated. The log $ID_{50}$ inhibition is plotted against no. of strains (%) of HSV-1 (open bars) and HSV-2 (filled bars). PFA sensitivity observed is grouped according to ranges indicated.

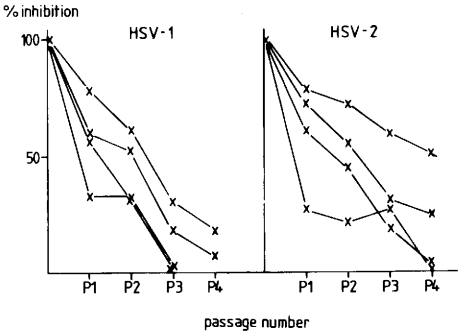


FIG. 5. Effect of consequentive passage of 4 HSV-1 strains and 4 HSV-2 strains in presence of 0.25 m*M* PFA. Increased development of resistance to PFA is indicated by changes in % plaque inhibition as a function of the passage number.

strain of each type was plaque purified and then exposed to PFA using the same limiting dilution procedure as mentioned above. No difference between patterns observed with plaque purified and original strains was demonstrable, suggesting that mutation(s) rather than selection of resistant variants occurred.

*Discussion.* Trisodium PFA inhibits HSV replication in cell-free, cellular and animal model systems (3). Cellular $\beta$ and $\gamma$ DNA polymerases are insensitive to PFA, and $\alpha$ DNA polymerase of eucaryotic cells is considerably less sensitive than the HSV induced DNA polymerase (5). These observations, resulting in low cell toxicity and high antiviral activity documented by therapeutic effects on experimental animal infections, makes PFA a candidate for clinical trials.

We found PFA inhibitory to HSV in concentrations comparable to those with the analogue PAA (8). Addition of PAA at any time before 10-hr postinfection is reported to depress the final 18 hr yield but the effect on the production of infective HSV is slowly and inefficiently reversed even when the drug is removed at early times (8). In addition to the concentration of the drug we found that the multiplicity of infection was important for the over all effect of PFA. In agreement the time for appearance of cpe in infected cultures was delayed by PFA and removal of the drug even one week after inoculation of cultures resulted in failure to achieve maximal inhibition of virus infection. These two latter findings we believe might depend upon mul-

tiplication of virus in the cultures leading to an increased multiplicity of infection. The relative amount of HSV polymerase per cell would influence the over all effect of the drug induced inhibition i.e. the binding of nucleoside triphosphatases to the polymerase.

Passage of HSV, 3 to 4 times in presence of PFA, resulted in increased resistance to the drug, as observed also with PAA (1, 9). PFA resistance is like resistance to PAA hereditable and persists through virus passage and cloning experiments. Cloning of HSV before exposure of virus to PFA demonstrated the same sensitivity and development of resistance to the drug of the clone as with the parental strain studied. Thus, mutations rather than selection of resistant variants seemed responsible for increased resistance to PFA, when virus was passaged in presence of the drug. Temperature sensitivity and sensitivity to PAA have been reported to be controlled by the same gene. In fact identification of the locus of PAA resistance has been employed to indicate the structural gene of the viral DNA polymerase on the HSV-1 genetic map. (10).

*Summary.* The present report describes the *in vitro* effect on herpes simplex virus (HSV) replication of trisodium phosphonoformate (PFA), a drug with low toxicity and which selectively inhibits the HSV induced DNA-polymerase and thus may have potential use in the treatment of HSV infection in man. The inhibitory effect of PFA on HSV replication was strictly dose-dependent and in the presence of 0.25 m*M* PFA the $TCID_{50}$ titers of HSV-1 and HSV-2 reference strains in green monkey kidney cells were decreased by 2 log units. The amount of virus inoculated or produced in the culture and the time for PFA-HSV interaction were factors influencing the virus inhibitory effect of PFA. None of 41 HSV-1 and 39 HSV-2 wild strains were resistant to the drug. However, passage of plaque purified HSV in the presence of 0.25 m*M* PFA resulted in appearance of mutants gradually more PFA resistant.

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