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ENRICHMENT OF EARLY HSV-INDUCED PROTEINS IN PHOSPHONOFORMATE-TREATED CELLS

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Summary. — The early HSV-specified proteins were selectively enriched in phosphonoformate (PFA)-treated Vero cells, i.e. in the absence of virus DNA synthesis. By pulse labelling, immunoprecipitation and enzyme immunoassay studies, the replication cascade of HSV-1 was shown to be blocked to a given stage allowing the production of early viral polypeptides only. With a prolonged treatment some of the early functions of the HSV genome seemed to cease as shown by the depletion of the respective polypeptides. Two suggestions rise from the presented experimental data: (i) the action of PFA on the production of early viral proteins modifies the recovery of HSV replication after the drug removal, (ii) the analysis of HSV polypeptide synthesis during a prolonged PFA treatment may be used for the evaluation of important viral gene functions responsible for the development of virus latency.

Key words: herpes simplex virus; phosphonoformate; early viral proteins; replication

Introduction

Replication of herpes simplex virus (HSV) is a cascade regulated by virus-induced polypeptides. These polypeptides comprise three different classes, the synthesis of immediate early (or alfa-) polypeptides being a prerequisite for the synthesis of early (or beta-) polypeptides, which in turn proceeds the synthesis of late (or gamma-) polypeptides (Roizman and Furlong, 1974; Marsden *et al.*, 1976). Inhibitors of herpesvirus DNA-polymerase, such as phosphonoacetate (PAA) and phosphonoformate (PFA), block this replication cascade so that only immediate early and early virus specified polypeptides are synthesized (Honess and Watson, 1977). Little is known about the synthesis of these polypeptides if the virus DNA synthesis is blocked for a longer time. Pulse labelling studies with PAA have revealed that the synthesis of certain early polypeptides is slightly pronounced during the action of the drug (Honess and Watson, 1977). PAA treatment also seems to result in a prolonged expression of early polypeptides and it has been used to increase the yield of HSV encoded DNA-binding proteins of

this class (Conley *et al.*, 1981; Knipe *et al.*, 1982). Extended production of early viral polypeptides was observed during adenine arabinoside (Ara-A) treatment (Pedersen *et al.*, 1981). These studies, however, were mainly concerned with the phenomena seen during the replicative cycle of the virus, i.e. from 0 to 18 hr post-infection (p.i.). Attempts to characterize the stages of HSV polypeptide synthesis after a prolonged block of virus DNA synthesis to our knowledge have not been made. We have studied the protein synthesis of HSV in the presence of PFA to see whether an accumulation of HSV-specified polypeptides could be detected. Applying radioimmunoprecipitation (RIP), enzymeimmunoassay (EIA) and polyacrylamide gel electrophoresis (PAGE) to monitore viral protein synthesis we noted an enriched and partially prolonged production of early virus-specified proteins in the presence of PFA. Implications of this modification of the early HSV-induced polypeptide synthesis on the expression of the resident HSV genome in the PFA-treated cells are discussed.

Materials and Methods

Cells and viruses. African green monkey kidney (Vero) cells were cultured in Eagle's minimal essential medium (MEM) supplemented with heat inactivated 10% newborn calf serum and pest (penicillin and streptomycin). HSV-1 (strain Turku) was used at its early passages in Vero cells. Preparation of virus stocks was as previously described by Cremer *et al.* (1977).

Chemicals. The trisodium salt of phosphonoformate (PFA) was obtained as a gift from Dr. Bo Öberg (Astra Läkemedel, Södertälje, Sweden). Radioisotopes, ^{14}C-protein hydrolysate, code CFB:25, ^{35}S-methionine, code SJ:204 and ^{14}C-methylated protein mixture, code CFA:626 were purchased from the Radiochemical Centre, Amersham (Bucks., England).

Virus titrations. Virus infectivity was assayed by using Vero cells grown on multiwell plates. Tissue culture infectious doses ($TCID_{50}$) were calculated using the Reed-Muench formula.

Infection and labelling of infected cells. Nearly confluent monolayers of Vero cells were infected with HSV-1 at a multiplicity of infection (m. o. i.) of 10 $TCID_{50}$/cell. After adsorbtion of virus for 1 hr at 37 °C, the cells were washed twice with MEM and covered with fresh medium containing 2% of newborn calf serum. Labelling with ^{35}S-methionine was done in suspensions after trypsinization as previously described (Michelsson *et. al.*, 1979). The medium contained methionine deficient MEM and 943 kBq of ^{35}S-methionine/ml. After washing and low speed centrifugation the trypsinized cells were suspended in medium (5×10^6/ml), and the labelling was continued for 2 hr at 37 °C. It was stopped by pelleting the cells by low speed centrifugation after which the pellet was extracted for RIP studies. The ^{14}C protein hydrolysate HSV-1 infected Vero cells were washed twice with Hank's buffered salt solution (HBSS) and covered with medium containing one part of MEM and 4 parts of HBSS supplemented with 2% dialyzed calf serum and 188 kBq of ^{14}C-protein hydrolysate/ml; 2×10^6 cells were labelled with 566 kBq for 2 hr at various times p. i. At the end of the labelling periods, the cells were detached with a rubber policeman and pelleted by low speed centrifugation. The pellet was prepared for PAGE as described by Marsden *et al.* (1976).

Immunoprecipitation and polyacrylamide gel electrophoresis. After labelling with ^{35}S-methionine cells were pelleted and resuspended in ice-cold phosphate buffered saline (PBS) in which they were additionally washed two times using a refrigerated centrifuge (Sorvall RC-5). The pellet was suspended in A-SDS buffer (1% Triton X-100, 0.1% SDS, 137 mmol/l NaCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10% glycerol, 20 mmol/l Tris-HCl pH 9) and sonicated two times for 1 min in an ice-bath. After this, the non-solubilized material was pelleted by 100,000 g for 30 min in a Sorvall OTD-2 ultracentrifuge. The supernatant (1 ml) was allowed to react with preadsorbed rabbit anti-HSV-1 serum (Dako, Denmark, 10 µl of rabbit antiserum was incubated with 100 µl extracts of mock-infected cells overnight at 4 °C in A-SDS buffer). The reaction time between labelled viral polypeptides and preadsorbed antiserum was 30 min at 37 °C after which the

immunocomplexes were precipitated with 200 µl of Staphylococcal protein A absorbent, prepared by the method of Kessler (1975). The precipitation reaction took place in 4 °C for 30 min after which the absorbent was washed with A-SDS buffer and centrifuged at 2000 g for 6 min in RC-5 centrifuge. Before PAGE analysis, the pellet was washed once with distilled water. 25 µl samples were run on 10 or 15 cm long homogeneous SDS-gels with 5% stacking gels at constant current (25 or 35 mA respectively) for 3 hr. The ^{14}C-methylated protein mixture or unlabelled standard proteins (purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, code 17-0446-01) were included as molecular weight (m. w.) marker. A discontinuous buffer system according to Neville (1971) was applied. We used either fluorographic (Bonner and Laskey, 1974) or silver staining techniques (Morrissey, 1981) to obtain a high sensitivity.

Enzyme-immunoassay (EIA) was used for the quantitative detection of viral antigens. Samples from extracellular fluid and from intracellular antigens were released by two cycles of freezing and thawing and diluted in PBS. Polystyrene balls (Precision Plastic Ball Co., U.S.A.) and rabbit anti-HSV-1 serum (Wellcome, England) diluted in 0.1 mol/l carbonate buffer (pH 9.5) were incubated at 4 °C overnight. After rinsing with PBS, they were incubated with different antigen dilutions (1/5, 1/10, 1/20) in FP-9 cuvettes (Labsystems, Helsinki, Finland) fo 1 hr at 37 °C. The balls were washed three times with the mixture of PBS + 0.5% Tween 20, rinsed three times in distilled water. Thereafter, human serum with high levels of IgG class HSV-1 antibodies was added (diluted 1:50 in following buffer: PBS + 1% BSA + 0.05% Tween 20) for 1.5 hr at 37 °C. After washing, alkaline phosphatase-conjugated anti-human IgG (Miles, Yeddah Israel) diluted 1/1000 with the dilution buffer supplemented with 2% rabbit serum and 0.5 mol/l NaCl (final concentrations) was added for 1 hr at 37 °C. After washing, P-dinitrophenylphosphate (Sigma, St. Louis, Miss., U.S.A.) diluted in diethanolamine buffer (pH 10) was added, as substrate and optical density of the reaction was measured in clean cuvettes after 30 min incubation at 37 °C by FP-9 spectrophotometer (finnppipette-Labsystems, Helsinki, Finland) as previously described (Leinikki and Pässilä, 1976).

Results

Effect of PFA on the production of infectious virus

To prevent totally the synthesis of virus-specific DNA and infectious virus, PFA concentration as high as 2 mmol/l was used. This concentration had been shown slightly but not irreversibly toxic for host cells (Stenberg and Larsson, 1978; Datta and Hood, 1981). The synthesis of infectious virus was totally inhibited in the presence of PFA, even when high input of infectious virus (10 $TCID_{50}$/cell) was used (Fig. 1-I). RIP technique allowed to monitor the effects of PFA on the replication of HSV-1. The replication cascade took place in untreated HSV-1 infected cells only. RIP also demonstrated that two HSV-specified polypeptides with m.w. of 130,000 and 37,000 synthesized early p.i. could be precipitated predominantly from PFA-treated cells (Fig. 2).

We analysed the synthesis of early HSV-specified polypeptides by labelling with ^{14}C-protein hydrolysate. While the replication cascade continued in the untreated cells (as demonstrated also by RIP experiments) it was effectively blocked in the PFA-treated cells (Fig. 3). Two early polypeptides (37K and 130K) seemed to be synthesized at increased rate in the presence of PFA between 4 to 6 and 10 to 12 hr p.i. and the 37K also between 22 to 24 hr p.i. The synthesis of the most of early virus-specified polypeptides continued even after 48 hr p.i. (Fig. 3), while the 37K polypeptide was not expressed at all, indicating changes in the expression of the HSV genome during PFA treatment. The synthesis of late virus-specific polypeptides proceeded in the untreated cells.

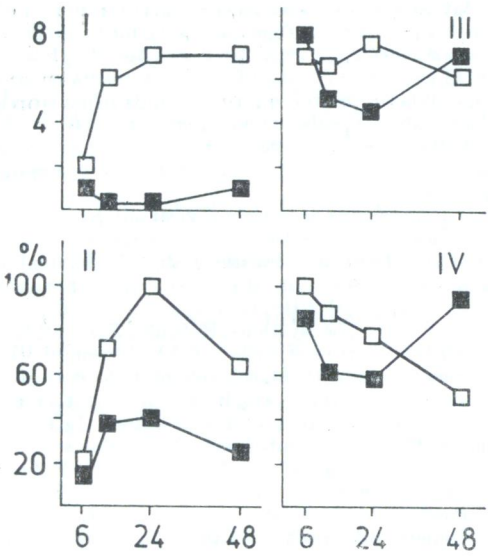

Fig. 1.
Effect of PFA on the production of intracellular virus and viral antigens

Amounts of viral antigens and infectious virus are expressed in relative units (%) calculated from the absorbency value for reference viral antigen and a negative control antigen from „mock" infected cells and in $TCID_{50}$ ($-\log_{10}$) values of infectious virus detected. (■ = = 2 mmol/l PFA, □ = 0 mmol/l PFA). Figures 1-I (upper left) and 1-II (lower left) represent the synthesis of infectious virus and viral antigens during PFA treatment, respectively. Figures 1-III (upper right) and 1-IV (lower right) represent the recovery of the synthesis of infectious virus and viral antigens after a given period of PFA treatment. The amounts of infectious virus and viral antigens were detected 18 hr after the removal of PFA.
Abscissae: hr p.i.
Ordinates: upper panels (I, III): $\log_{10}$ $TCID_{50}$; lower panels (II, IV): relative absorbency (EIA), per cent units.

Accumulation of HSV-induced early polypeptides in the absence of virus DNA synthesis

By comparing dilutions of the antigen samples in the EIA we obtained semiquantitative data on the accumulation of viral antigens. Our previous results had shown that rabbit antiserum bound also to early viral polypeptides and that only these polypeptides were synthesized in the PFA-treated cells (as also demonstrated by the absence of late polypeptide synthesis in Fig. 3 and by the absence of infectious virus in Fig. 1). Our assay demonstrated the rapid accumulation of early viral antigens within the PFA-treated cells. Their amounts reached a plateau and declined with prolonged treatment (Fig. 1-II). Silver staining and PAGE techniques also showed that the total amounts of early viral polypeptides were not markedly increased by the prolonged PFA treatment (Fig. 4). This suggests that the turnover of early viral polypeptides was rather rapid even when the virus DNA synthesis was blocked. The proportion of the 37K polypeptide was increased at 12 hr p.i., but it seemed to be absent from cells treated for 48 hr with PFA.

Recovery of infectious virus and viral antigens after prolonged PFA treatment

To evaluate the effects of PFA treatment on the recovery of HSV replication, we determined the amounts of infectious virus and viral antigene 18 hr after removal of the drug, i.e. after one replicative cycle should havs been over. Short PFA treatment had no effect on the recovery of infectious virus or viral antigens, whereas longer treatment seemed to affect both

synthesis of infectious virus and of viral antigens. The yield of the recovered infectious virus was by 3 logs lower after 24 hr treatment with PFA as compared to 6 hr treatment (Fig. 1-III). Also the amounts of viral antigen detected in cells were markedly lower (Fig. 1-IV). If PFA treatment was prolonged to 48 hr, the detrimental effect on the recovery of viral replication was abolished.

Discussion

PFA inhibits the replication of herpesviruses by binding to the virus--specified DNA polymerase (Reno *et al.*, 1978). Even concentrations as low as 0.1—0.5 mmol/l of PFA greatly reduced the amounts of infectious virus synthesized within the infected cells (Helgestrand *et al.*, 1978; Svennerholm *et al.*, 1979); the HSV DNA synthesis was also diminished at these low concentrations (Reno *et al.*, 1978). With higher concentrations such as 2 mmol/l, the cellular DNA-polymerases were inhibited and thymidine incorporation techniques revealed only traces of DNA-synthesis (Stenberg and Larsson, 1978).

When high virus input was used for labelling experiments and antigen assays, higher concentrations of PFA were needed for total inhibition of virus DNA synthesis. Under these conditions, pulse labelling studies demonstrated a clear-cut block in the shift from the synthesis of early viral polypeptides to the late ones. The synthesis of early viral polypeptides was very much prolonged during PFA treatment. Two polypeptides (37K and 130K) were synthesized at increased rate as revealed by RIP. The 130K may correspond to ICP 136,000 described by Matis and Rajčáni (1980), which may in fact represent two different polypeptides (ICP 0 and ICP 8, i.e. an immediate early and an early protein, respectively). PFA treatment seemed to increase the synthesis of the latter, which is also known to be the major DNA-binding HSV-1 coded protein (Knipe *et al.*, 1982). The PFA treatment also resulted in selective enrichment of the 37K polypeptide within infected cells. Prolonged or enhanced synthesis of early HSV-specific polypeptides have also been found during cytosine arabinoside (Ara-C), PAA and Ara-A treatments (Powell *et al.*, 1975; Honess and Watson, 1977; Pedersen *et al.*, 1981). The 37K polypeptide seems to be augmented in Raji cells persistently infected with the virus (Lehtinen *et al.*, unpublished data).

PAA and PFA have been used to induce latent stage of HSV in permissive cells (Colberg-Poley *et al.*, 1979; Lehtinen *et al.*, manuscript in preparation). Absence of an early function of the HSV genome seems to be responsible for the virus not entering the lytic cycle (Colberg-Poley *et al.*, 1981; Wigdahl *et al.*, 1982). This could be due to an increase of some early HSV-specified polypeptides, with abnormal regulatory functions (Jofre *et al.*, 1981; Knipe *et al.*, 1982). It was, therefore, of interest whether our experimental conditions produced changes in the protein synthesis semiprescent of latency. Results of the recovery of HSV replication after PFA treatment showed the transient inhibitory effect of the treatment even at high virus input. The inhibition

was not due to cell toxicity of PFA because there were no major differences between short and prolonged PFA treatments. The recovered viruses were also PFA-sensitive. Thus the PFA induced inhibition of HSV recovery in our assay conditions was transient, indirect but PFA-dependent.

It is tempting to assume that the early HSV specified proteins play a role in the PFA-induced effects. We showed that early viral antigens and especially the 37K polypeptide were enriched in the PFA-treated cells during the first 12 hr p.i. followed by a gradual depletion thereafter. The enrichment of the early polypeptides, especially the 37K protein, corresponded to the inhibitory effect of PFA treatment on the recovery of HSV replication, whereas their depletion paralleled "normal" start of the replication after the drug removal. A 37K protein coded by HSV-2 has been associated with its transforming gene sequence and possibly also with a protein kinase activity of the virion (Suh, 1982; Lemaster and Roizman, 1980, respectively). Thus it may have regulatory functions in the HSV replication. It will be interesting to find out whether the polypeptide of similar m.w. described by us has a role in the development of the PAA-, Ara-C- or PFA-induced HSV-1 latency.

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Explanation of Figures (Plates I–III):

Fig. 2. PAGE analysis of rabbit hyperimmune serum immunoprecipitates from HSV-1 infected Vero cells labelled with ^{35}S-methionine between 4–6 hr and 10–12 hr p. i., in the presence or absence of PFA. Same volumes of the precipitated material were applied to the gel, standard m. w. are given in the left. Early HSV-1 specified polypeptides are marked (●), HSV-1 specified late polypeptides are marked (○). (A = no PFA 4–6 hr, B = PFA 4–6 hr, C = no PFA 10–12 hr, D = PFA 10–12 hr p. i.).

Fig. 3. PAGE analysis of ^{14}C-protein hydrolysate-labelled infected cell specified polypeptides. HSV-1 infected Vero cells were labelled for 2 hr various times p. i., in the presence or absence of PFA. Equal amounts of infected cell specified proteins were applied to the gel. Standard m. w. are given in the left. Polypeptides specific for HSV-1 infected cells labelled early after infection are marked (●) and those labelled late after infection (○). (A = PFA 4–6 hr,

B = no PFA 4–6 hr, C = PFA 10–12 hr, D = no PFA 10–12 hr, E = PFA 22–24 hr, F = no PFA 22–24 hr, G = PFA 46–48 hr, H = no PFA 46–48 hr p. i.).

Fig. 4. PAGE analysis of HSV-1 infected Vero cells extracted after various times p. i. The gel was stained with the silver stain procedure as described by Morrissey (1981). Equal amounts of protein were applied to the gel, standard m. w. are given in the left. Early HSV-1 specified polypeptides are marked (●), late HSV-1 specified polypeptides (○). A = no PFA 6 hr, B = = PFA, 6 hr, C = no PFA 12 hr, D = PFA 12 hr, E = no PFA 24 hr, F = PFA 24 hr, G = no PFA 48 hr, H = PFA 48 hr p. i.).