Nucleic Acid Hybridization for Measurement of Effects of Antiviral Compounds on Human Cytomegalovirus DNA Replication

HÅKAN GADLER

Department of Virology, National Bacteriological Laboratory, and Department of Virology, Karolinska Institute, S-105 21 Stockholm, Sweden

A nucleic acid hybridization technique has been developed to study the effect of different antiviral compounds on the replication of human cytomegalovirus in vitro. One laboratory strain of human cytomegalovirus, Ad. 169, and six clinical isolates were studied. Doses needed for 50% inhibition of viral DNA replication were calculated for foscarnet, acyclovir, and arabinosyladenine. The mean 50% inhibition dose values obtained were 179 μM for foscarnet, 82 μM for acyclovir, and 44 μM for arabinosyladenine. This method yields values that agree with earlier reports, and it offers great advantages over usual methods to date for studying inhibition of viral DNA replication.

Human cytomegalovirus (HCMV) belongs to the herpesvirus family. Newly developed antiviral compounds, originally evolved against herpes simplex virus, have been investigated in vitro for possible use in clinical therapy of serious HCMV infections in immunocompromised patients and congenitally infected newborns. Substances shown to be active against HCMV in vitro include foscarnet (phosphonoformic acid [PFA]) (8, 19, 20), acyclovir (ACV) (10, 13, 17), and arabinosyladenine (ara-A) (6). The specificity of most antiviral compounds is at the level of the herpesvirus-induced DNA polymerases (9).

The effect of antiviral substances is usually studied by determining the reduction in production of infectious virus particles, e.g., the plaque reduction technique or titrations of produced virus particles. Other techniques include enzyme-linked immunosorbent assay for the effect on the synthesis of viral proteins (18) and labeling of viral and cellular DNA in cell culture with quantification of viral DNA synthesis after isopycnic banding of DNA in cesium chloride gradients (11). Several new antiviral compounds have been developed to the point at which they are used clinically. This makes it necessary to have rapid techniques for detecting the possible emergence of resistant virus strains (4, 12). Nucleic acid hybridization can be utilized to determine the amount of replication of virus DNA. In the present work, I show that this technique can also be used to study the effect of antiviral substances on HCMV replication in vitro. Fifty percent inhibition doses (ID_{50}s) for inhibition of viral DNA synthesis have been

determined for one laboratory strain of HCMV (Ad. 169), and for six clinical isolates.

MATERIALS AND METHODS

Cells. Human embryonic lung cells, grown in glass bottles, were used between passages 10 and 22. Eagle minimal essential medium with 7.5% calf serum was used for growth and with 2% serum for maintenance. The cells were regularly negative for mycoplasma contamination by the method of Chen (3).

Chemicals. PFA (Astra Läkemedel AB, Södertälje, Sweden) was evaluated at twofold dilutions, starting with 1,000 μM. ACV (gift from Astra Läkemedel AB, Södertälje, Sweden) was assessed beginning at 500 μM, and ara-A (Sigma Chemical Co., St. Louis, Mo.) was assessed beginning at 200 μM. [α-^{32}P]dCTP (2 to 3,000 Ci/mmol) was obtained from Amersham International (Amersham, England).

Virus assay. Microtiter culture plates with 24 or 96 wells and areas of 1.9 or 0.31 cm^2 per well were seeded with human embryonic lung cells. After reaching confluence (approximately 10^5 cells per cm^2), the plates were inoculated with HCMV, either laboratory strain Ad. 169 or fresh clinical isolates. The inoculum was chosen so that at least 50% of the cells showed cytopathic effect after 3 days of culture. Most often a 1:3 to 1:4 dilution of the trypsinized cell monolayer of the twice-passaged HCMV isolates was found to be suitable. Ad. 169 was tested at a dilution of 1:3 with a multiplicity of infection (MOI) of ~0.5 and at a dilution of 1:9 with an MOI of ~0.1. After an adsorption period of 60 to 90 min at 37°C, the inoculum was aspirated, and fresh medium containing different concentrations of antiviral compounds was added (Fig. 1A). Controls to monitor the replication of virus in the absence of antiviral substance were included, as was an uninfected cell control. The cell cultures were then maintained at 37°C in 5% CO_2 for 3 to 4 days. Thereafter the
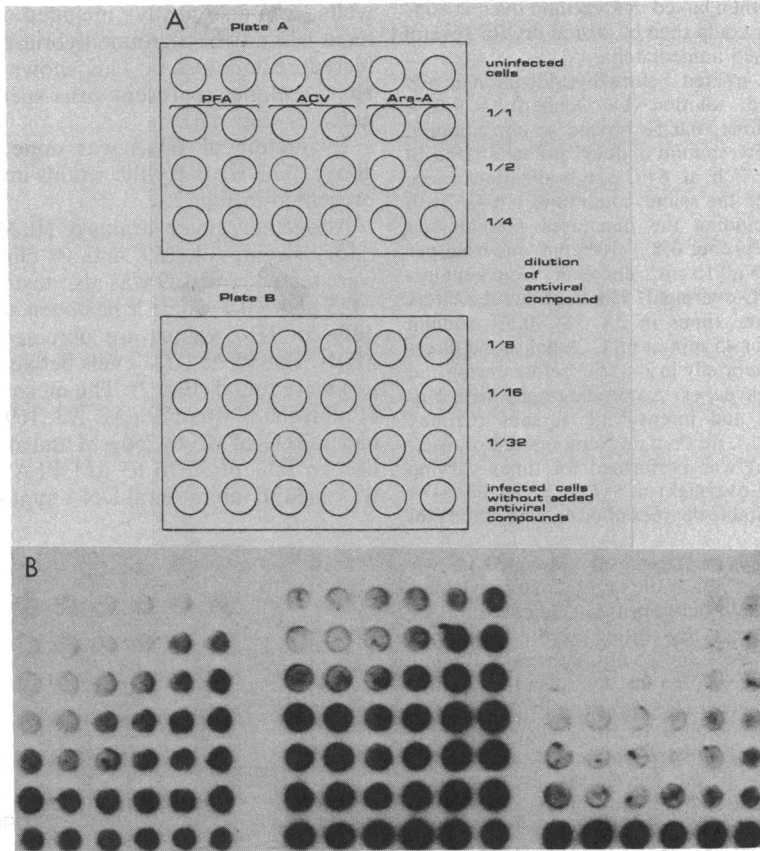
FIG. 1. (A) Layout of well disposition in 24-well plates. (B) Photograph of autoradiography film with three different strains of HCMV, Ad. 169, and two clinical isolates, V1023 and V1252. The initial concentration of PFA was 1,000 μM, of ACV 500 μM, and of ara-A 200 μM. For each isolate well disposition was as in (A).

medium was aspirated, and the plates were stored at −20°C or analyzed immediately.

Nucleic acid hybridization probes. A library of HindIII fragments from purified DNA of Ad. 169 (15) was constructed in the plasmid pBR322 (H. Gadler, P. Stålhandske, E. Sølver, and V. Petterson, manuscript in preparation). Briefly, HCMV DNA purified from virions as described earlier (5) was cleaved with restriction enzyme HindIII to completion. DNA from plasmid pBR322 was likewise cleaved with HindIII and dephosphorylated with calf alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, West Germany). The viral DNA fragments were ligated with plasmid DNA at a molar ratio of 1.5:1 with T4 DNA ligase (New England Biolabs, Inc., Beverly, Mass.). Escherichia coli HB101 cells were used for transformation and later spread onto agar plates containing ampicillin. Colonies were picked and checked for tetracycline resistance. Ampicillin-resistant, tetracycline-susceptible clones were checked for identity of inserted DNA.

Four recombinant clones with the following HCMV DNA inserts (16) were picked at random to be used as probes: pHCMV24 (X), pHCMV88 (S), pHCMV128 (b) and pHCMV151 (a). In total, these fragments represent 6.4% of the HCMV genome. The recombi-

nant DNA molecules were nick translated (14) with 25 μCi of [α-^{32}P]dCTP to a specific activity of approximately 3 × 10^7 cpm/μg. A mixture of these four clones, in equal proportions with regard to radioactivity, was used in all hybridizations.

Hybridization conditions. To analyze the viral DNA in cells in the plates, 300 μl of trypsin per well for a 24-well plate or 50 μl per well for a 96-well plate was added, and the cells were dislodged. The suspended cells were slowly filtered through a nitrocellulose filter (BA 85; Schleicher & Schuell, Dassel, West Germany) wetted with 6× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) by using a Minifold filtration apparatus (SRC-96; Schleicher & Schuell) attached to a water suction outlet. With this filtration apparatus, it was possible to filter up to 96 samples simultaneously through a nitrocellulose filter with a size of 88 cm². The filter was dried at room temperature, and the DNA was denatured in situ by placing the nitrocellulose filter on Whatman 3MM filter papers soaked with the following solutions: 0.5 M NaOH for 6 min, 0.1 M NaOH–1.5 M NaCl for 10 min, 1 M Tris (pH 7.5) twice for 2 min each, and 0.5 M Tris–1.5 M NaCl for 5 min. A similar technique for denaturation of DNA has been described by Brandsma and Miller (1). After this treatment, the nitrocellulose filter was dried at room

temperature and then baked in a vacuum oven at 80°C for 2 h. The filter could then be stored dry for several weeks or processed immediately.

The filter was treated before hybridization in 6× SSC–5× Denhardt solution (1× Denhardt = 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll)–0.5% sodium dodecyl sulfate at 1 ml/10 cm^2 for at least 2 h at 65°C. Hybridizations were carried out under the same conditions but in fresh solution and including the denatured recombinant DNA mixture as probe; 0.5×10^6 cpm was used per 10-cm^2 filter at 0.5 ml/10 cm^2. The filters were kept in a plastic bag at 65°C overnight. The next day the filters were washed three times in 2× SSC–0.5% sodium dodecyl sulfate for 45 min at 65°C. Finally, the filters were washed once briefly in 2× SSC before drying and mounting on thick paper. Autoradiography with Kodak XAR-5 film and intensifying screens (Cronex Lightning Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) was performed for times varying between 1 h and overnight at −70°C. The time was chosen so that suitable degrees of darkening of the film were obtained.

Calculation of ID$_{50}$s. ID$_{50}$s were evaluated in two ways. Visual inspection of the autoradiogram yielded ID$_{50}$s as intermediate between two concentrations of the antiviral substance. By cutting out the spots from the nitrocellulose filter and counting them in a liquid scintillation counter, an exact determination of the ID$_{50}$ could be obtained. This was done by plotting the amount of hybridized radioactivity for each viral strain and concentration of antiviral compound and linearly interpolating between the concentrations. The ID$_{50}$ was the concentration of antiviral substance at which hybridized radioactivity was diminished by 50% as compared to the infected cell control without added antiviral compound.

The two methods were compared for all strains, and agreement was found in seven of eight strains. Since the exact ID$_{50}$s would be expected to vary somewhat between different experiments, the ID$_{50}$s are presented as values intermediate between the concentrations of antiviral tested.

RESULTS

Nucleic acid hybridization probes. The four recombinant HCMV DNA clones which were used as probes were picked at random (Gadler et al., manuscript in preparation). They were assayed with the Southern hybridization technique (15) against three HindIII-cleaved preparations from HCMV isolates. The hybridized regions were found to be of identical size in all isolates (data not shown), indicating that the clones represent viral DNA sequences conserved among the tested viral strains. They should thus be suitable for use as probes in a hybridization technique.

Analysis of hybridization results. To obtain reliable results, it is important that a suitable level of virus DNA replication occurs in cells infected without added antiviral substances. For this purpose, one row of wells in the plates served as a positive control (Fig. 1A). A negative control, consisting of a row of uninfected

wells, was also always included. In no experiment was any background hybridization to uninfected cells detected. This shows that the four HCMV clones represent virus-specific DNA sequences (Fig. 1B).

Evaluation of ID$_{50}$s was done as described above. The ID$_{50}$s for the strains investigated are presented in Fig. 2.

Altogether, seven strains of HCMV, one laboratory strain, Ad. 169, and six clinical isolates, were tested. Ad. 169 was also tested at MOIs of ~0.5 and ~0.1, since it has been shown (7) that different ID$_{50}$ values are obtained at different MOIs. For PFA, ID$_{50}$ levels between 16 and 500 μM were found (Fig. 2). The mean ID$_{50}$ for PFA of all isolates was 179 μM. Ad. 169 at high MOI had an ID$_{50}$ of 125 to 250 μM and at a lower MOI had an ID$_{50}$ of 32 to 63 μM PFA. When ACV was used to inhibit viral DNA synthesis, a mean
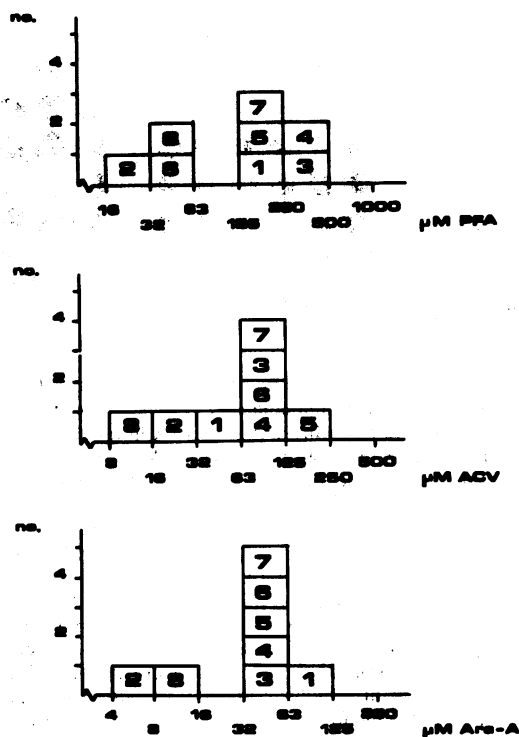

FIG. 2. Distribution of ID$_{50}$s for inhibition of viral DNA synthesis for HCMV isolates (numbers 1 to 6) and laboratory strain Ad. 169 (at an MOI of ~0.5 [no. 7] and at an MOI of ~0.1 [no. 8]). Spots were cut out from nitrocellulose filters, and ID$_{50}$s were calculated as the concentration of antiviral compound at which the amount of hybridization was diminished by 50% as compared to the positive control. The ID$_{50}$s are presented as intermediate between the concentrations of antiviral compound tested.

ID_{50} of 82 μM was obtained. Individual values varied between 8 and 250 μM ACV. The two different MOIs of Ad. 169 gave values of 63 to 125 and 8 to 16 μM ACV, respectively. Ara-A ID_{50} values varied between 4 and 125 μM, with a mean of 44 μM. The high MOI with Ad. 169 was inhibited to 50% by ara-A levels between 32 and 63 μM, whereas the low MOI was inhibited by 8 to 16 μM ara-A. Altogether, the laboratory strain Ad. 169 seemed to have ID_{50} values representative of fresh isolates of HCMV. Viral isolates having high ID_{50} values against one compound generally had high values against the other substances as well.

DISCUSSION

Earlier work on the effect of different antiviral compounds on viral DNA synthesis has used in vitro incorporation of [^{3}H]thymidine or $^{32}P_i$ into both viral and cellular DNA in cell culture in the presence of substance. DNA is then prepared, and viral and cellular DNAs are separated on cesium chloride gradients. The area of the radioactive peak corresponds to the amount of viral DNA synthesized in the presence of different concentrations of antiviral substances (11). This technique suffers from several drawbacks. Among them are the handling of large amounts of radioactivity, especially when working with $^{32}P_i$ and the limited number of samples that can be analyzed concomitantly. A more serious disadvantage is that some antiviral substances (ACV) compete with the radioactively labeled compounds ([^{3}H]thymidine), giving falsely high inhibition values for the antiviral compound in question. With the recombinant DNA technique, it has become possible to implement the nucleic acid hybridization for quantitation of the extent of viral DNA replication in infected cell cultures. Thus, it is possible to study the effect of different antiviral compounds directly on the level of viral DNA synthesis. The technique described in this paper is very well suited for this application, and preparation of DNA is very simple. A similar technique for quantification of specific DNA sequences in eucaryotic cells has been described by Brandsma and Miller (1).

The described technique is also suitable for studying the effect of substances with unknown antiviral activity and for examining possible resistance to different compounds in fresh clinical virus isolates. One prerequisite is that the tested antiviral compounds have an effect, direct or indirect, on the replication of virus DNA.

The ID_{50}s for inhibition of viral DNA synthesis were compared to those published by other authors using other techniques. It is mostly 50% plaque reduction values that have been published, but virus yield values have also been determined. The ID_{50}s of Ad. 169 agree with those found for PFA by Wahren and Öberg (20) and with those published for ACV by Lang and Cheung (10) (50% plaque reductions, 100 to 150 μM) and Plotkin et al. (13) (50% plaque reduction, 45 μM). As described by these authors, ACV is active against HCMV in cell cultures, although HCMV lacks a virus-specific thymidine kinase. The reason for this is at present unclear.

The ID_{50} values obtained for ara-A were compared with those found by Gephart and Lerner (6). Using a different technique, 50% inhibition of cytopathic effect, the values of ≥ 750 μM ara-A reported by Gephart and Lerner are substantially higher compared to the ID_{50} of 44 μM obtained by the hybridization technique. The difference is probably due to the different effect studied, inhibition of viral DNA synthesis, as measured by the hybridization method, being more sensitive.

In summary, the hybridization technique appears to be a suitable method for screening substances with an unknown activity on the replication of viral DNA and for studying the possible emergence of resistant virus variants in clinical isolates. The technique can equally well be applied to other DNA viruses or RNA viruses by using the appropriate probes.

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