JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1984, p. 1105–1108
0095-1137/84/121105-04$02.00/0

Vol. 20, No. 6

Use of Subgenomic Poliovirus DNA Hybridization Probes to Detect the Major Subgroups of Enteroviruses

HARLEY A. ROTBART,[1]* MYRON J. LEVIN,[1] AND LUIS P. VILLARREAL[2]

Infectious Disease Section, Department of Pediatrics,[1] and Department of Microbiology/Immunology,[2] University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 4 June 1984/Accepted 21 August 1984

Three nucleic acid hybridization probes were derived from DNA clones of the poliovirus type 1 genome. Used in dot hybridization experiments, the probes successfully detected members of each of the major enteroviral subgroups. The hybridization patterns obtained with the three probes suggested that a highly conserved nucleotide sequence existed among the enteroviruses tested, mapping between bases 220 and 1809 in the poliovirus genome. Two new antiviral agents capable of inhibiting enterovirus replication in tissue culture were used to demonstrate the specificity of the probes for viral RNA.

The enterovirus genus of viruses includes ca. 70 serotypes, many of which are important human pathogens responsible for a wide spectrum of human illness (9). Not all serotypes of enteroviruses can be readily detected in a conventional virology laboratory; even when isolation in tissue culture is successful, growth may take from several days to weeks (10). Ancillary methods, such as suckling mouse inoculation, are often cumbersome and time consuming. The application of rapid antigen detection techniques has met with only modest success in the identification of enteroviral infections because of the antigenic heterogeneity among and within the serotypes (4, 18, 19). To circumvent these problems, we prepared molecular probes from DNA clones of the poliovirus type 1 genome and used them to detect members of each of the major enteroviral subgroups.

(This study was presented in part at the meeting of the Society for Pediatric Research, San Francisco, Calif., 3 May 1984.)

MATERIALS AND METHODS

Cells. LLC-MK2 cells (American Type Culture Collection, Rockville, Md.; no. CCL 7-1), a continuous line of rhesus monkey cells, were maintained in Dulbecco modified Eagle medium plus 2% fetal calf serum. HEp-2 cells (no. 51519; Flow Laboratories, Inc., McLean, Va.) were grown in Connaught Medical Research Laboratory (1969) media (K. C. Biological Inc., Lenexa, Kans.) supplemented with 2% fetal calf serum.

Viruses. Poliovirus 1, poliovirus 2, and poliovirus 3 (Sabin strains) were generously provided by Lederle Laboratories, Pearl River, N.Y. Coxsackievirus A9 (Bozek strain), coxsackievirus B1 (Conn-5 strain), and echovirus 11 (Gregory strain) were obtained from the American Type Culture Collection. A clinical isolate of respiratory syncytial virus was obtained from our diagnostic virology laboratory stocks (P.G. strain).

Preparation of the cytoplasmic lysate. Cells were grown to confluence (ca. 10^5 cells) in 24-well cluster plates (16-mm-diameter wells; Costar, Cambridge, Mass.). Each well was inoculated with 10^2 to 10^3 50% tissue culture infective doses ($TCID_{50}$) of virus in 0.01 ml of Dulbecco modified Eagle medium. When 1+ to 2+ cytopathic effect was observed, the monolayers were drained and rinsed twice with cold Tris-saline (30 mM Tris and 150 mM NaCl [pH 7.0]), and the cells were lysed with 0.125 ml of 0.5% Nonidet P-40 (Bethesda Research Laboratories, Gaithersburg, Md.) in Tris-saline. The plates were then scraped, and the cell lysate was centrifuged at 15,000 × *g* at 4°C for 5 min. Samples of the supernatant fluid were immediately frozen at −70°C. For some experiments, 1 μg (final concentration) of candidate antiviral agents per ml was added to the wells just before virus infection. LLC-MK2 cells were used for all enterovirus infections. Cytoplasmic lysates of respiratory syncytial virus infections were prepared in HEp-2 cells. An uninfected culture of LLC-MK2 cells was grown and harvested under identical conditions.

Titration of virus in cell lysates. Flat-bottomed microtiter tissue culture plates (no. 3072; 96 wells; Becton Dickinson Labware, Oxnard, Calif.) were seeded with 0.2 ml of Dulbecco modified Eagle medium plus 10% fetal calf serum containing 2×10^4 LLC-MK2 cells. When confluent monolayers formed, the medium was removed, and 0.2 ml of 10-fold dilutions of cell lysate was added. Eight wells were used for each dilution. The plates were maintained in a CO_2 incubator for 7 days, and the wells were examined for cytopathic effect every other day. The $TCID_{50}$ per milliliter was calculated by the method of Kärber for the 50% end point (1).

Hybridization probes. Three different cDNA sequences of poliovirus 1 (Mahoney strain) were cloned by previously reported techniques in the pBR325 plasmid of *Escherichia coli* HB101 (16). Clone PDS 111 (generously provided by Bert Semler, State University of New York) contains the entire 7,524 nucleotides of the poliovirus 1 genome, except for the nucleotide segment 3235 to 3925. Clone PDS 14 (also from B. Semler) includes nucleotides 1 to 1809. Clone BAM 220 contains only the first 220 5′ nucleotides and was generated in our laboratory by restriction endonuclease digestion with *Bam*HI of the PDS 14 insert and religation of the plasmid with ligase. Each of the plasmid-insert complexes was then nick translated with ^{32}P-labeled dCTP (New England Nuclear Corp., Boston, Mass.) to a specific activity of 7.5×10^7 cpm/μg of DNA by standard techniques (14).

Dot hybridization. A 0.025-ml volume of each of the cytoplasmic lysates was thawed on ice, and 0.0025 ml of 200 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories) was added. Certain lysates were then further treated with proteinase K (0.1 mg/ml at 37°C for 30 min.;

* Corresponding author.

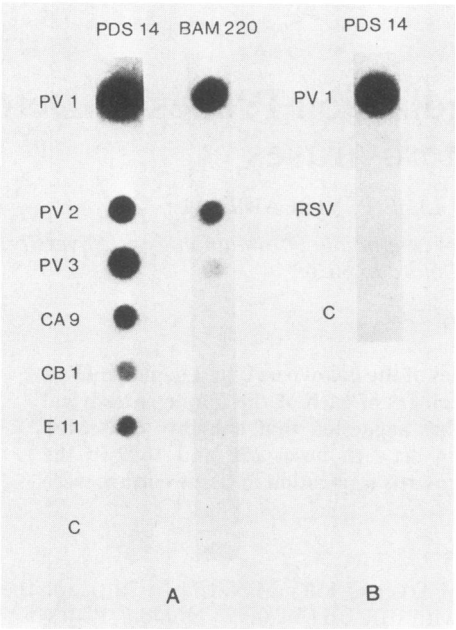

FIG. 1. Dot hybridization with poliovirus 1-derived probes of cell lysates from enteroviral infections. The probes used are shown at the top of each autoradiograph strip; the test viruses are listed vertically. Abbreviations: PV 1, poliovirus type 1; PV 2, poliovirus type 2; PV 3, poliovirus type 3; CA 9, coxsackievirus A9; CB 1, coxsackievirus B1; E 11, echovirus 11; C, uninfected cell lysate; and RSV, respiratory syncytial virus. Autoradiographs were exposed for 18 h.

Sigma Chemical Co., St. Louis, Mo.), boiling (100°C for 4 min), or EDTA (50 mM final concentration; J. T. Baker Chemical Co, Phillipsburg, N.J.). The lysate was then added to a vial containing 0.015 ml of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) and 0.01 ml of 37% formaldehyde (17), incubated at 60°C for 15 min, and applied to nylon membrane filters (Gene-Screen plus; New England Nuclear Corp.) under suction with a 96-well manifold apparatus (Schleicher & Schuell Inc., Keene, N.H.). The filter was dried at room temperature and prehybridized in a sealed plastic bag (Seal-A-Meal; Dazey Products Co., Industrial Airport, Kans.) containing 5× SSC and 5× Denhardt solution (2) for 4 h at 50°C with continuous agitation. The prehybridization solution was then removed and replaced by a hybridization mixture containing 10% dextran sulfate, 50% formamide, 5× Denhardt solution, and 5× SSC. The molecular probe (0.15 ml) containing 1.13×10^7 cpm was added to 1 mg of calf thymus DNA (in 0.1 ml of 10 mM Tris–1 mM EDTA), denatured at 100°C for 4 min, rapidly cooled, and added to the hybridization mixture. The plastic bag containing the filter and the hybridization solution was incubated at 50°C for 18 h with continuous agitation. After hybridization, the filter was removed and washed four times (10 min each) in 2× SSC containing 0.2% sodium dodecyl sulfate at room temperature and twice (40 min each) in 0.2× SSC plus 0.2% sodium dodecyl sulfate at 50°C. All washes were likewise continuously agitated. After drying at room temperature, the filters were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) in metal cassettes with a Du Pont Lightning-Plus intensifying screen for 18 h at −70°C.

Antiviral drugs. Arildone and WIN 51711 were generously provided by the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

RESULTS

Monolayers of LLC-MK2 cells were infected with representative members of each of the major enteroviral subgroups. Cytoplasmic lysates were then prepared and probed with cDNA to subgenomic fragments of poliovirus type 1 RNA. Cell lysates from cultures infected with each of six enteroviruses showed easily detectable hybridization with the 5′ 1,800-nucleotide PDS 14 probe after 18 h of autoradiograph exposure (Fig. 1A). Hybridization with the larger PDS 111 probe gave nearly identical results (Fig. 2, top row). However, when the smaller BAM 220 fragment was used as the probe, only lysates from cells infected with the polioviruses gave detectable hybridization signals. Strong signals were obtained with polioviruses 1 and 2, and a weaker signal was obtained with poliovirus 3 (Fig. 1A). Subsequent exposure for as long as 1 week failed to reveal hybridization of the nonpolio enteroviruses with the BAM 220 probe (data not shown). Negligible to undetectable signals were produced by lysates from the uninfected, control cytoplasmic preparations and from respiratory syncytial virus-infected cells (Fig. 1B). Treatment of infected lysates with proteinase K, boiling, or EDTA in addition to vanadyl ribonucleoside complex did not improve upon the hybridization signal obtained from lysates treated only with vanadyl ribonucleoside complex (data not shown).

Arildone and WIN 51711, two new antiviral drugs, were used to verify further the specificity of the PDS 111 and PDS 14 probes for viral RNA. Results were nearly identical with the two probes; only PDS 111 is shown (Fig. 2). No poliovirus RNA was detectable in cells which were treated with Arildone before infection; this drug, however, had no effect on the hybridization signals obtained with the nonpolio enteroviral RNAs. WIN 51711, on the other hand, eliminated all detectable enteroviral RNA hybridization signals. The drugs had no effect on cell viability at the concentration used (data not shown). The absence of detectable hybridization in cytoplasmic lysates which had been previously treated with these agents correlated with a reduction in viral replication in cell culture (Table 1). Arildone treatment resulted in an

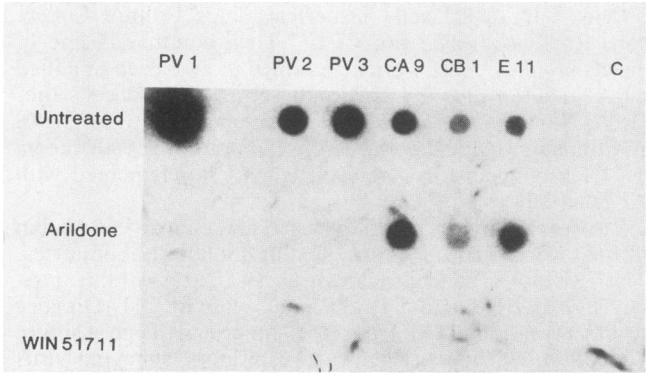

FIG. 2. Effect of antiviral drugs on hybridization of the PDS 111 probe with cell lysates from enteroviral infections. The viruses used to infect the cells are listed across the top of the autoradiograph. Abbreviations: PV 1, poliovirus type 1; PV 2, poliovirus type 2; PV 3, poliovirus type 3; CA 9, coxsackievirus A9; CB 1, coxsackievirus B1; E 11, echovirus 11; and C, uninfected cell lysate. The top row was dotted with lysates from cells which contained no antiviral agent. The middle row was dotted with lysates from cells which contained Arildone (1 μg/ml) during the virus infection. The bottom row was dotted with lysates from cells which contained WIN 51711 (1 μg/ml) during the virus infection. The autoradiograph was exposed for 18 h.

approximate 1,000-fold decrease in poliovirus titers, with no reduction in titer of the nonpolio enteroviruses. WIN 51711 treatment reduced all viral titers by 1.5 to 5 logs of $TCID_{50}$. The actual corresponding reductions in the amounts of viral RNA were not determined.

DISCUSSION

Numerous viruses causing human disease have been successfully detected, both in vitro and in situ, with specific nucleic acid probes (13). Antigenic variations within genera of these viruses are limited, and most have a surface antigen common to all members of the genus. It is not surprising, therefore, that significant genetic homology detectable by molecular probes exists within those genera. On the other hand, the marked antigenic heterogeneity in viral capsid proteins among enteroviral serotypes predicts little conservation in genetic sequences coding for those proteins. However, genetic relatedness might exist in areas of the enteroviral genome not expressed as surface antigens. Young (20) demonstrated a minimum of 3 to 5% genetic homology among the most distantly related enteroviruses with RNA-RNA hybridization experiments. Hewlett and Florkiewicz (5) showed identical base structure through the first 20 5′ nucleotides of poliovirus 1 and poliovirus 2 and that 17 of the first 20 5′ nucleotides are common to poliovirus 1 and coxsackievirus B1. Nomoto et al. (12) demonstrated absolute homology within the first 20 5′ nucleotides of all three poliovirus types and near identity of polioviruses 1 and 2 (one differing nucleotide) through their first 43 5′ nucleotides. Stanway et al. (15) have reported the complete nucleotide sequence of poliovirus 3 (Sabin strain), demonstrating absolute homology with poliovirus 1 (Mahoney strain) through the first 54 5′ nucleotides except at position 22. Larsen et al. (7) showed that the same sequence of poliovirus 1 RNA forms an internally complementary hairpin loop.

Our results have demonstrated the presence of some genetic homology among the enteroviruses tested. The superiority of the PDS 14 probe over the BAM 220 probe in detecting the RNAs of poliovirus 3 and the nonpolio enteroviruses suggests an area(s) of conserved sequence homology between nucleotides 220 and 1809 on the poliovirus 1 genome. It is known that the reading frame for poliovirus 1 begins at nucleotide 743, and it is suspected that the more proximal bases serve a regulatory role (3). The wide antigenic diversity among the enteroviruses would predict that the conserved sequences were largely located in nontranslated areas of the enteroviral genomes. These observations are substantiated by recently published data of Nomoto et al. (11), demonstrating a highly conserved region among the three poliovirus types between nucleotides 400 and 650. Stanway et al. (15) have shown absolute homology between bases 509 and 573 of polioviruses 1 and 3. These data also show that polioviruses 1 and 3 are ca. 84% homologous within the first 220 5′ nucleotides. The results of our BAM 220 probe studies would predict even greater homology between polioviruses 1 and 2 in this region.

Nomoto et al. (11) also showed areas of strong sequence homology among the three poliovirus types at their 3′ ends, especially in the terminal nontranslated segment. Additional evidence for nucleic acid homology among the enteroviruses at their 3′ ends has been reported recently by Hyypia et al. (6) with a probe derived from the 3′ end of coxsackievirus B3. Details of probe structure and quantitations of virus particles were not provided. The coxsackievirus B3 probe, like our poliovirus probes, appears specific for enteroviruses and sensitive enough to detect tissue culture infections. Our PDS 111 probe includes both the 5′ and 3′ noncoding end regions of the poliovirus 1 genome, as well as most of the translated sequences in between. This probe did not improve upon the hybridization signal obtained with the shorter PDS 14 probe. We are currently preparing specific 3′ probes for comparison with the probes we have described in this report.

TABLE 1. Inhibitory effects of Arildone and WIN 51711 on enteroviruses

Serotype	Viral titer (log_{10} $TCID_{50}$/ml)		
	Untreated	Arildone[a]	WIN 51711[a]
Poliovirus 1	8.42	5.64	3.26
Poliovirus 2	7.08	3.34	3.26
Poliovirus 3	8.47	5.39	5.11
Coxsackievirus A9	7.84	7.96	6.30
Coxsackievirus B1	8.21	8.45	6.55
Echovirus 11	8.70	8.95	6.24

[a] Concentration of 1 μg/ml.

Treatments of the cell lysates expected to disrupt intact virions (proteinase K, boiling, EDTA) did not improve upon the hybridization signals obtained with untreated lysates. This suggests that the techniques described here for dot hybridization result in liberation and detection of formerly encapsidated virion RNA as well as free viral RNA in the cytoplasmic lysates. The limit of sensitivity of our probes in cytoplasmic lysate preparations appears to be $10^{4.64}$ to $10^{4.95}$ $TCID_{50}$ per 0.025-ml dot ($10^{6.24}$ to $10^{6.55}$ $TCID_{50}$/ml of lysate) for the nonpolio enteroviruses (Table 1, Fig. 2). In reconstruction experiments with cerebrospinal fluid, we have found that the dot hybridization technique can detect $<10^3$ $TCID_{50}$ of purified polioviruses and $<10^4$ $TCID_{50}$ of purified nonpolio enteroviruses without special treatment to disrupt virion capsids (manuscript in preparation). The strength of the hybridization signals obtained in those experiments correlates directly with the titer of virions added. We have not yet determined the actual amount of viral RNA in the cytoplasmic lysates or cerebrospinal fluid reconstructions and therefore cannot estimate the limit of the sensitivities of the probes for detection of RNA. That work, now in progress, will allow us to predict and compare the relative homologies between the RNA of poliovirus 1 and the other enteroviral RNAs.

The inhibitory effect of two new antiviral agents was exploited to verify the specificity of our probes for viral RNA. These drugs appear to interfere with enteroviral replication by preventing uncoating (8). Arildone has been found to be particularly effective against the polioviruses and less so against many of the nonpolio enteroviruses, whereas the spectrum of activity for WIN 51711 appears to be broader with regard to the nonpolio enteroviruses (M. A. McKinlay, personal communication). The results obtained with our probes confirm that information. Arildone treatment of cells before infection reduced the titer and resulted in the absence of detectable hybridization signals with the three poliovirus types. Similarly, WIN 51711 treatment resulted in reduced viral titer and elimination of hybridization signals with all of the enteroviruses tested.

The potential utility of molecular probes which recognize many or all enteroviruses is great. The rapid diagnosis of these infections would result in significant reductions both in the hospitalization of ill neonates and children and in treatment with unnecessary antibacterial agents. Furthermore, a rapid diagnostic test for the enteroviruses might facilitate the clinical use of new and potentially effective antiviral agents.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Max Rotbart Memorial Fund of the University of Colorado Health Sciences Center, by a grant-in-aid from the Sterling-Winthrop Research Institute, and by University of Colorado School of Medicine Biomedical Research Support grant BRS 3779-11.

We thank Lewis Pizer, James Todd, Tom Dubensky, and Nancy Jorgensen for advice and encouragement; Janine Bellio, Neva Murphy, and Caroline Adams for expert technical assistance; and Rose Bradley for preparing the manuscript.

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