Rapid Epidemiologic Analysis of Cytomegalovirus by Using Polymerase Chain Reaction Amplification of the L-S Junction Region

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A technique based on polymerase chain reaction (PCR) amplification was developed to facilitate the study of the epidemiology of cytomegalovirus (CMV). Consensus oligonucleotide primers from repetitive DNA sequences were designed to amplify interspersed repetitive sequences in an area of heterogeneity within the L-S junction region of the CMV genome, and PCR products were detected by gel electrophoresis. Purified CMV DNAs from 25 CMV isolates, 13 from members of five families in which person-to-person transmission was documented, 9 random clinical isolates of CMV, and 3 laboratory reference strains of CMV (Towne, Davis, and AD169), were analyzed. The gel electrophoretic patterns of DNA bands, or PCR profiles, produced by amplification with the L-S primers were unique for epidemiologically unrelated strains and laboratory reference strains, yet similar patterns were observed for epidemiologically related strains isolated from members of the same family. This method of rapid fingerprinting of CMV DNA within the hypervariable L-S junction region by PCR to produce strain-specific, variably sized PCR products should simplify the molecular epidemiologic analysis of CMV.

Molecular epidemiologic analysis of cytomegalovirus (CMV) has enhanced the understanding of its transmission among immunocompromised hosts, neonates, and families, as well as in day-care and hospital settings (1, 3, 8, 14, 23, 29, 33-35). However, the standard molecular techniques used in these studies have several limitations that preclude their widespread application. For example, restriction enzyme analysis to detect polymorphisms arising from the absence or acquisition of restriction enzyme recognition sites requires relatively large quantities of purified viral DNA, which is produced by multiple in vitro passages of virus, and multiple viral passages, in turn, produce minor genetic changes that can alter the restriction enzyme profile (14, 15, 33). In addition, restriction enzyme analysis is limited because CMV strains have been shown to have approximately 80% homology in their nucleotide sequences, and this homology results in similarities in restriction enzyme profiles, even among unrelated strains (16). Epidemiologic analysis of CMV also has been accomplished by the use of Southern blot hybridization with cloned subgenomic CMV probes that define differences across the entire genome or alternatively at the heterogeneous L-S junction region (1, 3, 8, 9, 29). While relatively less viral DNA is needed for Southern blot methods, they have proven to be cumbersome and time-consuming procedures which require radiolabeled CMV DNA.

DNA amplification by the polymerase chain reaction (PCR) has been successfully applied to the detection of CMV in various specimens for diagnostic purposes (2, 5, 13, 26). More recently, PCR has been explored as a potentially useful tool for the epidemiologic analysis of CMV (4, 6, 36). Amplification has been accomplished at target sequences in regions encoding CMV DNA polymerase, envelope glycoproteins, and major immediate-early and structural proteins,

as well as short fragments in the L-S junction region of the CMV genome. However, all of these reported methods require multiple PCR amplifications and restriction enzyme analysis and are only able to define minimal differences between CMV strains.

In this study, PCR with consensus primers complementary to degenerate repetitive DNA sequences was applied to the rapid epidemiologic analysis of CMV isolates. This method required minute quantities of viral DNA and no restriction enzyme digestion. A region of the CMV genome known as the L-S junction was chosen for amplification by PCR because duplications and deletions frequently occur in this region during virus replication and produce interstrain nucleotide sequence variations (18, 27, 32). Degenerate repeat sequences in the L-S junction region selected from published sequence data for laboratory reference strains CMV Towne and AD169 were used to derive consensus oligonucleotide primers anticipated to provide amplification of strain-specific, variably sized PCR products (22, 31, 32). Amplification of a portion of the L-S junction region containing these degenerate direct repeats was successfully applied to distinguish both epidemiologically related and unrelated CMV isolates. Because of its relative simplicity, this method should facilitate further study of the molecular epidemiology of CMV.

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MATERIALS AND METHODS

CMV isolates. All CMV strains were isolated and propagated in human foreskin fibroblast cell cultures (Viomed, Minnetonka, Minn.) by standard techniques (30). The DNA from 16 isolates was extracted from low-passage CMV-infected fibroblasts contained in a 25-cm² flask and exhibiting 80 to 95% cytopathic effects, and this DNA was used for

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both Southern blot analysis and PCR amplification. The DNA from nine random clinical isolates was extracted from one or two cell culture tubes containing cells exhibiting low cytopathic effects. Extraction methods included either a manual modified Hirt procedure or the use of an automated nucleic acid extractor (Applied Biosystems, Foster City, Calif.) (11). Extracted DNA was resuspended in Tris-EDTA buffer (pH 8.0) and quantitated on a fluorescence spectrophotometer (Perkin-Elmer Cetus, Norwalk, Conn.). No further purification of viral DNA from cellular DNA was required prior to analysis.

Epidemiologically related CMV strains included 13 isolates from members of five families in which person-to-person transmission was documented by serial serology and viral culturing (7). Epidemiologically unrelated CMV strains were randomly selected from clinical specimens submitted to the Baylor College of Medicine CMV Virology Laboratory for viral isolation. These nine clinical isolates were from five congenitally infected infants, three transplant recipients, and a hospitalized patient with febrile illness. Laboratory reference strains AD169, Towne, and Davis were obtained from the American Type Culture Collection (Rockville, Md.) and were maintained in our laboratory by standard techniques (30).

Southern blot analysis. Isolates of CMV were analyzed by Southern blot hybridization with 32 P-labeled *Eco*RI junction fragments F and H (generously provided by D. Spector and S. Spector, University of California at San Diego, San Diego) to determine whether the molecular profiles of the CMV strains isolated from family members were similar. Southern blot analysis was performed with 6 μ g of purified viral DNA as previously described by Spector et al., with minor modifications (28).

Primers. Oligonucleotide primers were developed on the basis of published sequence data for the L-S junction regions of strains AD169 and Towne with the assistance of a computer program (OLIGO; National Biosciences, Hamil, Minn.) for selecting optimal oligonucleotide primers for in vitro amplification (25). Oligonucleotide primer LS-1 was derived from a consensus sequence of the degenerate direct repeat sequences present within the L-S junction region. Because of the variable numbers and locations of the degenerate direct repeats, primer LS-1 had the potential to bind to multiple sites within the L-S junction region (Fig. 1). A second consensus oligonucleotide primer, LS-2, was selected from a more constant region near the terminal sequence of the L-S junction region. The sequences of the consensus primers are as follows: CMV LS-1, 5'-GGGTGT GKYGGYIGGGTGTGTCRG-3', and CMV LS-2, 5'-ACGC CYGACATCCATTCCGGGCCG-3', where K = G+T, Y = C+T, R = A+G, and I = inosine.

Amplification. Each PCR was carried out with a microcentrifuge tube and a final total volume of 50 μ l. The reaction mixture contained 0.1 μ g of purified DNA, 50 pmol of each primer, 200 μ M each deoxynucleotide triphosphate (dATP, dTTP, dCTP, and dGTP), and 5 μ l of 10 $\times$ PCR buffer 50 mM KCl, 10 mM Tris-Cl, 1.5 mM MgCl₂, 0.01% gelatin, with sterile H₂O added to bring the volume to 49 μ l. The sample was overlaid with 50 μ l of mineral oil and heated to 100°C for 7 min in a heating block (VWR Scientific, Media, Pa.). Two units (1 μ l) of *Thermus aquaticus* (Taq) polymerase was added to the reaction mixture. Laboratory precautions were taken to minimize sample carryover as described by Kwok (19). CMV strain AD169 was used as a positive control, and uninfected human foreskin fibroblasts were used as a negative control. Each CMV isolate was run in duplicate.

FIG. 1. Structure of the CMV genome, which is composed of a unique long segment (U_L) and a unique short segment (U_S) with inverted repeats (ab and b'a'; a'c' and ca). The site at which U_L and U_S join is the heterogeneous L-S junction region. An expansion of the L-S junction region showing the nucleotide sequence of prototype strain AD169 is shown with potential sites of primer annealing (MBIR designation sequence, MBIR-HS5L5A). The locations of CMV LS-1 primer binding sites with $\geq 84\%$ bp homology with published nucleotide sequences are bracketed. The single target site of the CMV LS-2 primer is shown by shading. The arrows represent the various start sites of primer annealing and extension.

Conditions for PCR amplification were modified to enhance primer hybridization during the early cycles of amplification as previously described for human papillomavirus (10). In brief, a denaturing step at 94°C for 2 min was followed by a primer annealing step at 45°C for 2 min and a two-phase extension step beginning at 65°C, with a gradual rise to 72°C over a total of 5.5 min. These modifications allowed for amplification despite minor base-pair mismatches, strengthened low-energy base pairing with inosine in the target sequence, and maintained reproducible results. A total of 35 cycles were performed with a DNA thermal cycler (Perkin-Elmer Cetus). Samples were stored at 4°C at the completion of the reaction.

The amplified PCR products were detected by electrophoresis of 20 μ l of each sample in a 4% agarose (3% NuSieve GTG agarose-1% SeaKem ME agarose; FMC Bioproducts, Rockland, Maine) gel (15 by 20 cm; Bio-Rad, Hercules, Calif.). Electrophoresis was performed overnight at 50 V with Tris-borate-EDTA buffer. The DNA was stained with ethidium bromide and photographed under UV light. A ϕ X174 DNA *Hae*III digest (New England BioLabs, Beverly, Mass.) was used as the molecular weight marker.

FIG. 2. Representative Southern blot hybridization of ^{32}P -labeled CMV junctional fragment F to *Eco*RI-digested CMV DNA from members of four families (FS1 family members A, B, and C; FS15 family members C and D; FS21 family members A, B, C, and D; and FS30 family members A and C). Laboratory reference strain AD169 is included for comparison.

RESULTS

Southern blot analysis. Hybridization of the F and H probes to the L-S junction region confirmed that the strains transmitted within families were molecularly related (Fig. 2). These strains were therefore used to evaluate the application of this PCR method to determining strain relatedness.

PCR analysis. Target sequences within the L-S junction region for primer pair LS-1-LS-2 were successfully amplified in viral DNA extracted from all 25 CMV isolates. Electrophoresis of the amplified products revealed strain-specific PCR profiles consisting of three to eight bands ranging in size from 100 to 1,000 bp. No amplification occurred in DNA extracted from uninfected human foreskin fibroblasts, and reproducible PCR profiles were seen for positive control sample AD169 as well as for the other CMV isolates.

Epidemiologically related CMV strains isolated from members of the same family showed remarkably similar PCR profiles, with strains demonstrating between five and eight bands (Fig. 3A and B). Families FS1, FS15, and NN2-HS180 showed identical PCR profiles among their members. The PCR profiles were nearly identical for families FS21 and FS30, with only one minor band variation demonstrated among some members. Minor molecular variations within these two families also were noted when the strains were analyzed by Southern blot hybridization with the F and H fragments as probes for the L-S junction region (Fig. 4).

Laboratory reference strains Towne, Davis, and AD169, as well as the random clinical isolates of CMV, all produced unique PCR profiles (Fig. 5A and B).

FIG. 3. PCR products from epidemiologically related CMV strains amplified with primers CMV LS-1 and CMV LS-2, analyzed by gel electrophoresis, and visualized with ethidium bromide stain. (A) FS1 family members A, B, and C; FS15 family members C and D. (B) FS21 family members A, B, C, and D; FS30 family members A and C. NN2 and HS180 are a husband-wife pair. HFF, DNA extracted from uninfected human foreskin fibroblasts (HFF) and used as a negative control; AD169, laboratory reference strain of CMV used as a positive control; ϕ X174, molecular weight marker.

DISCUSSION

CMV oligonucleotide primers LS-1 and LS-2 successfully amplified the heterogeneous L-S junction region of the CMV genome and identified epidemiologically related isolates of CMV from five families (total number of isolates, 13) and 12 epidemiologically unrelated CMV isolates from infants and children. The similarities of the PCR profiles obtained for the epidemiologically related CMV strains demonstrated that the numbers and locations of the degenerate repeats in the hypervariable region of the genome were conserved between strains, even though the strains had undergone both in vitro passage in the laboratory and transmission between persons in the same family. Also, the unique patterns observed for the epidemiologically unrelated CMV strains, as well as the laboratory reference strains, confirmed that the locations and numbers of degenerate repeats in the L-S junction region

FIG. 4. Comparison of Southern blot hybridization with *Eco*RI junctional fragment F as a CMV probe (two left lanes) and L-S junction PCR analysis (two right lanes) of the FS30 family. Minor differences in band patterns (arrows) were detected by both techniques but did not preclude strain identification.

varied between strains, as was predicted by the sequence data available for the prototype CMV strains, Towne and AD169.

In this study, CMV strains were considered distinct when a difference of two or more bands was seen on the PCR profiles. For example, laboratory strains AD169, Davis, and Towne produced three common bands of PCR products, yet they also produced four unique bands, which allowed their distinction. Published *Eco*RI and *Hind*III restriction enzyme analysis profiles also showed comigrating DNA fragments when CMV strains Towne, Davis, and AD169 were compared, and reciprocal DNA-DNA reassociation kinetics indicated that strains Towne and AD169 share up to 90% of their nucleotide sequences. This observation suggested that significant genomic homology exists between these distinct strains and confirmed our observation that common bands of PCR products also may be produced by unique strains (11, 18, 24). In addition to observing that distinct strains may share bands, we noted that PCR profiles of related strains may differ by a single minor band, yet the strains are still considered related. For example, epidemiologically related strains from the members of family FS30, as well as family FS21, produced PCR profiles that differed by only one band, and this minor variation also was observed in Southern blot hybridization with the F and H fragments as probes for the L-S junction region (Fig. 4). Molecular analysis of CMV by use of restriction enzymes with or without cloned probes also has revealed similar minor variations in band patterns between epidemiologically related isolates (14, 23, 33). These relatively minor differences in band patterns observed in related isolates presumably indicate that some genetic alterations of the L-S junction region occur during in vitro passage or over a period of time as the virus is passed from person to person. Alternatively, minor changes in the virus may occur within the same person, or the person may be

FIG. 5. PCR products from epidemiologically unrelated CMV strains amplified with primers CMV LS-1 and CMV LS-2, analyzed by gel electrophoresis, and visualized with ethidium bromide stain. (A) PCR products from CMV laboratory reference strains AD169, Towne, and Davis. (B) PCR products from randomly selected clinical isolates of CMV. SC905, Y9304, SC566, Y8303, and SC849 were isolated from infants congenitally infected with CMV; AC406, AC1511, and AC1462 were isolated from hospitalized immunosuppressed transplant recipients; AC1477 was isolated from a hospitalized febrile patient. ϕ X174, molecular weight marker.

infected with more than one strain (15). Strains from families FS21 and FS30 were passed among members over 60 and 36 weeks, respectively, while the other families with identical PCR profiles transmitted CMV over a much shorter period, 12 to 16 weeks (7). Further support for the hypothesis that minor variations in the L-S junction region of the genome occurred over time in a distinct strain of CMV was provided by Tamashiro and Spector (32). Variability in the L-S junction region of laboratory strain AD169 was documented by examination of the virus over a period of 3 years, particularly with regard to the numbers and positions of the degenerate repeats in the α sequence. Mocarski et al.

reported less variability of the α sequence in CMV strain Towne on passage, but over an undefined period of time (22). Analysis of the Towne or AD169 strain by the PCR technique described here could further define the stability of this region over time.

While no previous reports have delineated differences between CMV strains by use of PCR with consensus primers complementary to degenerate repetitive DNA sequences, the analysis of CMV strain variability by use of several sites of PCR amplification followed by restriction enzyme digestion was recently evaluated (4, 6, 36). Chou amplified the CMV genome in conserved areas of the unique long segment and, using restriction enzymes in a stepwise manner, attempted to produce distinct profiles among CMV strains (4). The epidemiologic analysis of CMV by this method appeared less rapid and more complex than ours, as it required amplification with a minimum of three sets of PCR primers and digestion with six restriction enzymes to distinguish 20 strains. A recent analysis by Zaia et al. of differences among CMV strains in the L-S junction region and at two sites in the unique long sequence of the CMV genome involved PCR amplification followed by restriction enzyme digestion (36). Primers for the L-S junction region amplified a conserved region of the α sequence, with a PCR product of only about 138 bp. The use of seven restriction enzymes demonstrated slight fragment size differences in unrelated CMV isolates, while some unrelated isolates remained identical in the PCR analysis. In addition, several clinical isolates and reference strain Davis could not be amplified. In fact, our initial attempts at the epidemiologic analysis of CMV by PCR involved a similar segment of the α sequence and yielded an amplified product of approximately 100 bp (6). We found that restriction enzyme digestion with *Sma*I yielded identical patterns among related CMV strains. However, unrelated CMV strains were difficult to distinguish as unique because restriction enzyme digestion yielded PCR products that often differed in size by less than 10 bp. We therefore designed the primers described in this report to amplify a much larger segment of the variable L-S junction region; these primers incorporated direct repetitive sequences to optimize strain differences. A similar strategy of using PCR amplification with primers complementary to interspersed repetitive DNA sequences also has proven valuable in the analysis of yeast artificial chromosome clones and somatic cell hybrid lines for the presence of human genomic DNA, as well as fingerprinting of DNA extracted from biological evidence in the forensic identification of individuals (9, 12, 17, 20, 21).

In summary, the novel consensus PCR primers CMV LS-1 and LS-2 successfully amplified a portion of the L-S junction region containing interspersed degenerate repeat sequences and produced variably sized PCR products that permitted strain identification of CMV. In addition, this method provided an advantage over previous molecular techniques in that significantly less viral DNA was required for evaluation by PCR (0.1 μ g) than for Southern hybridization (6 μ g), and no radioisotope or restriction enzyme digestion was required. This approach allowed for more rapid, more convenient, and less hazardous processing of samples. Because of these advantages, L-S junction region PCR may provide an expedient means for analyzing the large number of specimens usually required for epidemiology studies.

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