Analysis of Acquired Human Cytomegalovirus Infections by Polymerase Chain Reaction

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We used the polymerase chain reaction and primers corresponding to three regions of the human cytomegalovirus (HCMV) genome to study HCMVs isolated from 16 children attending a single day-care center and the father of two children in the same center. When we analyzed isolates with primers for the pp65 and major immediate-early genes, we observed nearly uniform amplification yielding products of predicted sizes. By contrast, primers for the a sequence demonstrated variability among HCMV strains, supporting the use of these primers as an epidemiologic tool. Analysis of a-sequence products from two isolates demonstrated 50 to 70% nucleotide homology with the a sequence of HCMV Towne strain DNA. We observed 95% nucleotide homology for the two a-sequence products derived from the father-child pair. Analysis of day-care center isolates indicated that two children excreted two distinct HCMV strains during the study interval.

Children in group day-care centers in the United States, the parents or caretakers of such children, and adults with multiple sexual partners acquire human cytomegalovirus (HCMV) at rates that greatly exceed those of the general population (3). Rates of HCMV infection among day-care center workers, for example, range from 8 to 20% annually (4) versus 1 to 5% among persons lacking such exposures. Although the majority of these acquired infections do not produce disease, HCMV can cause an infectious mononucleosis syndrome in previously healthy individuals and may induce serious, life-threatening disorders in persons with impaired cell-mediated immunity (8).

Recent reports (6, 7, 12) indicate that the polymerase chain reaction (PCR) can be used to characterize the genetic variability of the virus, suggesting that PCR has potential as a method to investigate HCMV transmission. We used PCR with primers corresponding to three regions of the HCMV genome to study HCMV isolates derived from one large group day-care center. Our results indicate that PCR with an a-sequence primer can be used to characterize the epidemiology of HCMV transmission and provide additional evidence that children in group day-care centers can be reinfected with distinct HCMV strains.

MATERIALS AND METHODS

HCMV isolates. The virus strains analyzed in this study consisted of HCMVs isolated between May 1986 and March 1991 from (i) 16 children in a single large day-care center (19 total isolates distinct from those in prior reports (14)) and (ii) the father of two children in the above-mentioned center (one isolate). HCMV was isolated from this parent after the onset of hepatitis attributed to HCMV. The HCMV Towne strain was propagated separately as a positive control for PCR and restriction enzyme analysis.

Primary HCMV isolations were achieved by incubating urine or saliva on confluent monolayers of human foreskin fibroblasts (HFFs) grown in 24-well plates in 5% CO2 at 37°C. Monolayers were fed with Eagle's minimum essential medium containing 10% fetal calf serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 50 μg of gentamicin per ml and observed weekly. All isolates exhibited the characteristic HCMV-induced cytopathic effect, and 15 isolates were further confirmed by immunofluorescent staining with an HCMV-specific monoclonal antibody (MicroTrak; Syva Company, Palo Alto, Calif.).

PCR analysis. DNAs extracted from HCMV-infected HFF monolayers were amplified by PCR with five separate sets of HCMV primers (Table 1). Reaction mixtures for typical PCR amplifications consisted of 1.25 U of Taq polymerase, 200 μM each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl2, 100 μg of gelatin per ml, 20 pM each primer, and an approximately 1 μg of target DNA in a final volume of 50 μl. Typical PCR parameters consisted of an initial denaturation for 2 min at 94°C, which was followed by 25 to 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 1.5 min at 72°C, and then a final extension for 10 min at 72°C.

Controls for each run included amplification of (i) purified viral DNA extracted from HFF monolayers infected with the Towne strain of HCMV (courtesy of Mark Stinski, Department of Microbiology, University of Iowa), (ii) DNA from uninfected HFFs, and (iii) buffer with sterile, distilled H2O replacing target DNA. In several experiments, DNAs were coamplified with primers for beta-globin DNA (10) as an internal PCR control (primer 1, 5'-GGTTGCAACTACCTAC TCCCAGG-3'; primer 2, 5'-GCTCAGTGTGGGCAA AG-3'; product size, 536 bp). Appropriate precautions were taken to prevent contamination.

For initial size analysis, 8 μl of each PCR product was studied by electrophoresis in 1.8% agarose gels (SeaKem; FMC Corp., Rock Island, Ill.) run at 30 V for 4 h. Molecular size markers (123-bp ladder; Bethesda Research Laboratories, Gaithersburg, Md.) were incorporated in the initial and final lanes of each run. Gels were recorded by Polaroid photography, and bands were scored by two readers (J.F.B. and M.E.O.).

The specificities of PCR products were determined by Southern blot analysis with HCMV probes specific for the target region. Amplification products were separated on 0.9% agarose gels run at 60 V for 2 h and were transferred to Zetabind membranes (CUNO, Inc., Meriden, Conn.) by
using a vacuum transfer apparatus (Red-Evac; Hoefer Scientific Instruments, San Francisco, Calif.). Membranes were prehybridized according to the manufacturer’s instructions. Bound DNAs were hybridized with the appropriate probes end-labeled with [γ-32P]ATP and were visualized by autoradiography.

Restriction enzyme analysis. Several virus strains were studied by modifications of methods described previously (14). After three or four passages in HFFs, viral genomic and cellular DNA was harvested by proteinase K digestion, chloroform-phenol extraction, and ammonium acetate-ethanol precipitation. DNAs were resuspended in sterile H2O, and the concentration was adjusted to 1 μg/μl by the addition of H2O. DNAs were stored in H2O at 4°C until used.

For restriction enzyme analysis, 1 to 3 μg of DNA was incubated with EcoRI or HindIII overnight at 37°C in the appropriate buffer. Digested DNAs were separated by electrophoresis on 0.8% agarose gels for 2 h at 100 mA and transferred to Zetabind membranes (CUNO, Inc.), and the membranes were prehybridized for 3 h by procedures described by the manufacturer. Bound DNA was then hybridized overnight with an HCMV probe, the XbaI I D fragment of HCMV Towne (courtesy of Mark Stinski) labeled with [32P]dCTP by random priming, and visualized by autoradiography. This probe spans part of the long repeat, all of the short repeat, and part of the short unique regions of the HCMV genome (20). Our methods correspond to the junctional hybridization approach described by Spector et al. (18).

DNA sequencing. Selected PCR products were sequenced by the University of Iowa DNA Facility by using fluorescence automated DNA sequencing and an Applied Biosystems (Foster City, Calif.) model 373A automated DNA sequencer according to instructions provided by the manufacturer. The volumes of PCR reagents were doubled to provide 50 to 100 ng of PCR product in a final volume of 100 μl. Primers were provided at a concentration of 0.8 pm/μl. Hardcopy sequence data were reviewed and compared with respect to HCMV strains.

RESULTS

PCR analysis. Amplifications with one or both pairs of primers for the pp65 gene yielded predicted products for all but one HCMV isolate (344u). Amplifications with the IE-s primer, corresponding to exon 4 of the major immediate-early gene, resulted in uniform products for all but four isolates (329u, 344u, 356u, and 361u). However, a product was observed when 329u and 361u were amplified with the IE-m primer (corresponding to exons 3 and 4 of the major immediate-early gene), suggesting that these isolates contained nucleotide variations in the region of the IE-s primer. The nucleotide sequences of the primers used in these experiments are indicated in Table 1, and representative amplification products are shown in Fig. 1 and 2.

In contrast to the above-described results, considerable polymorphism was observed among HCMV isolates (Fig. 3) when analyzed with the a-sequence primer described by Zaia et al. (22). The isolates yielded a-sequence products of three sizes: approximately 220 bp (group A), approximately 180 bp (group B), and approximately 130 bp (group C). The product derived by amplification of purified HCMV Towne DNA was intermediate in size between group B and C products.

By using this schema, seven day-care isolates could be classified as group A, three could be classified as group B, and three could be classified as group C. Two additional isolates, shown in Fig. 3A, lane 11, and Fig. 3B, lane 5, had faint bands that appeared to categorize them as group A strains. Four isolates, shown in Fig. 3A, lanes 9 and 12, and Fig. 3B, lanes 9 and 13, had no a-sequence products. One of these isolates (364s) had a faint product on a duplicate run (Fig. 3B, lane 5), suggesting that it was a group A strain, whereas a group C product was observed in one run for 369s.

Beta-globin coamplification products were identified in the

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**TABLE 1. Primers for detection of HCMV genes**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size (bp) of predicted product</th>
</tr>
</thead>
<tbody>
<tr>
<td>a sequence&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A</td>
<td>130–220</td>
</tr>
<tr>
<td>Primer 1</td>
<td>TTC CCC GGG GAA TGA CAC AG</td>
<td></td>
</tr>
<tr>
<td>Primer 2</td>
<td>TTT TTA GCG GGG GGG TGA AA</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>GCG CCC GAC GTC GCT TTT AT</td>
<td></td>
</tr>
<tr>
<td>IE-s&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>CCA CCC GTG GTG CCA GCT CC</td>
<td>159</td>
</tr>
<tr>
<td>Primer 2</td>
<td>CCC GCT CCT GCT GAG CAC CC</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>CTG GTG TCA CCC CCA GAG TCC</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>CTT GTC CCC GCG ACT ATC C</td>
<td></td>
</tr>
<tr>
<td>IE-m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>TGT GGC GAA GAA TCC CTC AA</td>
<td>404</td>
</tr>
<tr>
<td>Primer 2</td>
<td>TCT GCA AAG ATC CTC CCA TC</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>GAT TAA GGT TCG AGT GGA C</td>
<td></td>
</tr>
<tr>
<td>pp65</td>
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<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>TAG GTG ACC AGT ACG TGA AG</td>
<td>223</td>
</tr>
<tr>
<td>Primer 2</td>
<td>TCC AGG ATG ATG TGC GAG ATC T</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>AAG GTC TTT TTT ATG AAC ATC A</td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>AAA GAG CCC GAC GTC TAC TAC AGC T</td>
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<tr>
<td>Primer 2</td>
<td>CCA GGT ACA GTC TGA GCT ACT GTC C</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>GTT GTC CAT GGA GCA AAC CAG GTC</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>GTG GGG</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Oligonucleotides 5'-3'.

<sup>b</sup> From Zaia et al. (22). The probe for a-sequence products was derived from the nucleotide sequence of isolate 311u.

<sup>c</sup> From Shibata (15).
runs for three of the negative isolates (361u, 364s [Fig. 3B, lane 9], and 369s), and elimination of the beta-globin primers gave similar results (data not shown). The three isolates had detectable products when amplified with an IE or pp65 primer. Thus, we believe that the results for these strains indicated nucleotide variations in the a sequence sufficient to inhibit PCR amplification.

When the groups of isolates were compared according to the dates of sample acquisition, it was found that group A isolates were recovered between November 1988 and March 1991, group B isolates were recovered between April 1988 and November 1988, and group C isolates were recovered between May 1986 and March 1988 (Table 2). The two isolates that had faint bands suggesting that they were group A strains were isolated in October and December 1987. Isolates without a-sequence products were recovered between December 1987 and June 1989. Thus, categorization by a-sequence product size generally correlated with temporal clustering of isolates, a feature compatible with horizontal transmission of HCMV strains in the day-care center.

Three sets of paired isolates obtained 12 or more months apart from each of three children were among the isolates analyzed in this study (Fig. 3 and 4). Two children (311 and 356) appeared to excrete two distinct strains during the study interval. Child 356 appeared to excrete a group A strain in saliva in 1987 (Fig. 3A, lane 11) and a group B strain in urine in 1988 (Fig. 3B, lane 11). Child 311 excreted a group C strain (Fig. 3A, lane 7, and Fig. 4, lane 2) in saliva in 1986 and a group A strain (Fig. 3A, lane 14, and Fig. 4, lane 1) in urine in 1989. A group A strain isolated in 1989 was also excreted in urine by the father of child 311.

Comparison of PCR and restriction enzyme analysis. We next compared the restriction endonuclease fragment patterns of several group A isolates. These results, shown in Fig. 5, suggested that at least two strains (isolates 368s and 378u) were closely related. The pattern shown by these isolates was similar to the pattern of the group A strains excreted by child 311 and his father. One isolate (356s) had a distinct restriction enzyme pattern differing from that of other group A strains by at least four bands in the combined digests.

Our results indicate that a-sequence primers have utility in investigations of HCMV transmission. We observed that

FIG. 2. Amplification of selected HCMV isolates with IE-m primers. Shown are an ethidium bromide-stained gel of PCR products (top) and an autoradiogram with 32P-labeled probe for the IE-m amplification product (bottom). Lanes: 1, no DNA (control); 2, HFF DNA; 3, HCMV Towne DNA; 4, 319u; 5, 318u; 6, 375u; 7, 312u; 8, 364s; 9, 364s; 10, 378u; 11, 361u; 12 and 13, 329u; 14, 368s. m, molecular size marker lanes.

FIG. 3. Amplification of HCMV isolates with a-sequence primers. (A) Lanes: 1, no DNA (control); 2, HFF DNA; 3, HCMV Towne DNA; 4, 329u; 5, 368s; 6, 310u; 7, 311s; 8, 365s; 9, 344u; 10, 380u; 11, 356s; 12, 369s; 13, isolate from a congenitally infected newborn; 14, 311u. m, molecular size marker lanes. (B) Lanes: 1, no DNA (control); 2, HFF DNA; 3, HCMV Towne DNA; 4, 319u; 5, 364s; 6, 318u; 7, 375u; 8, 312u; 9, 364s; 10, 378; 11, 356u; 12, 368s; 13, 361u. m, molecular size marker lanes. A, B, and C in the right margins correspond to the sizes of a-sequence products, as described in Results.

The a-sequence products from several group A isolates were further characterized by digestion with restriction enzymes BssHII and MnlI (data not shown). We observed identical digestion patterns for isolates 368s and 378u (cut by BssHII but not by MnlI), supporting the conclusion that these strains were closely related. The a-sequence amplification product of isolate 365s had a similar digestion pattern, whereas 369s did not.

Sequence analysis. We performed sequence analysis of two a-sequence products. This analysis demonstrated approximately 95% nucleotide sequence homology between isolates from child 311 and his father (Fig. 6A), group A strains known to have similar restriction enzyme profiles. Compared with the published nucleotide sequence for the HCMV Towne DNA a sequence (11), the clinical isolates exhibited 50 to 70% homology. The isolates demonstrated approximately 90% homology in the a-sequence pac-2 motif, conserving the six-nucleotide sequence (5'-TTTTAT-3') (Fig. 6B).

DISCUSSION

Our results indicate that a-sequence primers have utility in investigations of HCMV transmission. We observed that
HCMV isolates can be grouped according to the sizes of the a-sequence products and that strains with similar-size a-sequence products tended to cluster temporally in the day-care center, suggesting that these strains were horizontally transmitted. Transmission of certain group A strains was further supported by detection of similar restriction fragment patterns. Horizontal transmission of HCMVs among children in group day-care centers has been documented in prior United States studies (1).

However, a-sequence amplification alone may not completely distinguish HCMV strains. One isolate (356s), categorized as a group A strain, had a restriction fragment pattern substantially different from that of other group A strains, an observation that indicates that additional strategies, such as restriction enzyme digestion of a-sequence products (22) or amplification with other PCR primers (16), may be necessary to establish strain relatedness. We also observed that a-sequence amplification of some isolates did not consistently yield products, and we suspect that such isolates possess nucleotide variations within the a sequence. In vitro selection or alteration of the a sequence during passage could potentially affect strain characterization. Observations regarding the Towne strain indicate that the a sequence remains stable through repeated passage (13), whereas the AD169 strain may be unstable (21).
Our results indicate that certain gene regions are conserved among clinical HCMV isolates. Primers for pp65, a gene that encodes a tegument protein, and for the major immediate-early gene (IE-s primer) amplified most of these HCMV strains. This result is similar to that of Zaia and colleagues, who studied HCMV isolates from several clinical settings (22). However, recent data suggest that nucleotide variations occur within exon 4 of the major immediate-early gene, indicating that primers such as IE-s may not uniformly amplify all HCMV isolates (6).

Our data regarding the group A strains support the concept that certain HCMV strains may predominate in day-care environments. In a prospective study of 104 children in day-care centers, Adler detected 14 different strains among 55 HCMV-excreting children (1). However, three strains infected 44 of the children and were apparently transmitted within the day-care environment. Several factors could account for this observation. Excretion of similar HCMV strains could reflect person-to-person transmission during close contact (i.e., several children grouped together in one room) or transmission via fomites or caretakers (9). In the present study, group A strains were excreted by several different children in the day-care center over a period of at least 2 years.

The predominance of certain HCMV strains within a day-care center could also reflect the intrinsic characteristics of viral strains. The PCR data suggest that there are genomic differences in the a-sequence region among groups of HCMV strains. Although analysis with restriction enzymes has not identified biological differences among strains (19), variations in the HCMV genome may be important. Certain strains could differ in their replicative efficiencies, a feature that may influence viral titers in urine or saliva. Much of the pac-2 motif was conserved in the two isolates studied here, but the significance of the nucleotide variations within the remainder of the amplified a-sequence products is uncertain.

Our results indicate that children in group day-care centers can be reinfected with new HCMV strains. Two children in the present study excreted two strains with distinct a-sequence groupings. One child (311) first excreted HCMV in saliva in 1986. He subsequently had several negative saliva cultures and one positive urine culture in 1987. He was found to excrete HCMV in urine in 1989, coincident with excretion of HCMV by his younger brother and his father, who had viral hepatitis. We do not know whether child 311 acquired the new strain from his sibling, other children in the center, or, conceivably, his father, but we are intrigued by the observation that his second isolate was a group A strain, the predominant HCMV pattern.

Several studies of diverse human populations suggest that reinflection with HCMV occurs. Chandler and colleagues identified women who either shed multiple strains of HCMV simultaneously from different sites or had different HCMV strains detected during serial sampling (5). A similar phenomenon has been observed with homosexual men with AIDS (17). Our data and the results of Adler (2) regarding HCMV reinfection of children in day-care centers have
substantial relevance to the biology of HCMV infections in hosts without high-risk sexual behaviors or AIDS.

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REFERENCES