The Molecular Epidemiology of Cytomegalovirus Transmission Among Children Attending a Day Care Center

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Cytomegalovirus (CMV) viruria was detected in 16 (25%) of 66 children attending a day care center. A significantly lower prevalence (6.9%) of viruria occurred among an age-matched control group of 1,457 hospitalized children. CMV DNA was compared by restriction endonuclease digestion of cell-associated CMV DNA, prepared by the Hirt procedure. CMV DNA fragments were detected directly on agarose gels by a 2-hr in situ hybridization with \(^{32}\)P-labeled DNA plasmids containing XbaI fragments of the Towne strain. EcoRI digestion of DNA isolated from 11 hospitalized children revealed 11 unique strains. A similar analysis, with EcoRI and several other endonucleases, of DNA isolated from the urine of 16 children attending the day care center revealed that one group of seven children and another group of four children were excreting identical strains of CMV. All seven children in the first group were <29 months old; six of these children shared the same classroom. All four children in the second group were >36 months old; three were assigned to the same room. These results prove that CMV was frequently transmitted among children attending the day care center.

Because of the high prevalence of cytomegalovirus (CMV) infections within many populations, because >99% of primary CMV infections are clinically inapparent, and because CMV produces both a chronic and latent infection with intermittent viral shedding, investigating the transmission of CMV from human to human with traditional epidemiological methods is imprecise. The antigenic differences that exist between different CMV isolates are too minor to allow for traditional serotype grouping. Hence, for epidemiological purposes, there is only one serotype of CMV. Epidemiologically unrelated isolates of CMV are genetically different from one another [1, 2]. This genetic diversity is detected by restriction endonuclease digestion of purified CMV DNA. Differences in restriction endonuclease patterns of the DNA of isolates have proven valuable for investigating possible CMV transmission within families [3, 4], hospital personnel [5, 6], and hospitalized infants [7], but the genetic diversity of epidemiologically unrelated strains of CMV has not been applied to a major epidemiological study of CMV transmission.

An obstacle to using CMV DNA analysis has been the difficulty of rapidly and simply analyzing large numbers of viral isolates. Previously reported methods of passing CMV in vitro and of purifying sufficient quantities of DNA for restriction endonuclease analysis and hybridization are slow and cumbersome. CMV grows slowly in tissue culture, and some methods require multiple virus passages (one to three weeks per passage) to produce sufficient extracellular virus [1-3, 5]. In addition, CsCl-equilibrium or sucrose-gradient centrifugation is frequently used to separate CMV DNA from cellular DNA [1-8]. Radiolabeling the CMV DNA with \(^{32}\)P in vivo is often required. These methods preclude the efficient and rapid purification and analysis of the DNA of multiple CMV isolates.

In my laboratory, a rapid, simple, and sensitive method was used for the analysis of the DNA of CMV isolates. This method required less than four weeks after initial virus isolation, was convenient for processing up to 100 isolates simultaneously, and did not require extracellular virus or the separation of CMV DNA from cellular DNA by either centrifugation in CsCl or sucrose. After restriction endonuclease digestion of small quantities of CMV DNA, restriction fragments were detected by hybridizing \(^{32}\)P-labeled plasmids containing cloned fragments of the Towne strain of CMV directly onto the agarose gel; this eliminated the conventional transfer of the CMV DNA to nitrocellulose [4, 9]. Using this tech-
nique, we demonstrated the frequent transmission of CMV among children attending a day care center.

Materials and Methods

Study populations. Two populations were studied. The first consisted of children hospitalized in the Children’s Medical Center of the Medical College of Virginia. The nursing units are arranged by age and include a 60-bed neonatal intensive care unit and units for children aged zero to two years, two to five years, five to 12 years, and 12 to 18 years. From these units had admission urine samples cultured for CMV. For children two to five years of age, urine samples continued to be cultured for CMV until November 1984. The hospitalized children were socially and economically diverse.

The second population studied was a group of 66 children attending a day care center on the hospital campus. The ages of these children ranged from three months to five years; one child was eight years old, and 13 children were less than one year old. The parents of the children attending the day care center were either hospital nurses or staff members. Children attending the day care center were surveyed three times (March, June, and September 1984) for CMV viruria. Over 75% of the children had two or more urine samples obtained for culture.

Statistical analysis. All comparisons were tested by using the χ² test with Yates’s correction and 1 df.

Tissue culture and virus isolation. Human embryonic lung fibroblasts (MRC-5) obtained from the ATCC (Rockville, Md) were propagated in minimal essential medium (MEM) supplemented with 20% fetal calf serum and containing 2 mM glutamine, 0.3% bicarbonate, and antibiotics (MEM-20). Cells were grown to confluency in 1 ml of MEM-20 in a 16-mm well (24 wells per plate; Costar, Cambridge, Mass). Cells were incubated at 37 C and maintained in 5% CO₂ and 100% humidity. We added 0.2 ml of urine or 0.2 ml of resuspended urine sediment (10 ml of urine centrifuged for 60 min at 31,000 g) directly to each well. After 24 hr, the cells were washed once with MEM, and fresh MEM supplemented with 5% fetal calf serum was added. The medium was changed weekly. At least weekly for six weeks, the cells were examined for CPE due to CMV. When CPE was noted, the cells were trypsinized and transferred to a 25-cm² flask (Costar) containing 5 ml of MEM-20 and freshly trypsinized confluent MRC-5 cells from a confluent 25-cm² flask and incubated. After 25%–50% of the monolayer showed CPE (approximately seven to 14 days), the cells were trypsinized and added to each of two 56-cm² petri dishes (Costar) containing 10 ml of MEM-20 and freshly trypsinized confluent MRC-5 cells.

Isolation of CMV DNA. When CPE involved at least 50% of the cell monolayer, CMV DNA was isolated by using the method originally described by Hirt [10]. After one wash with cold 0.01 M TRIS-HCl (pH 7.0), the cells were lysed in 1 ml of 0.6% SDS and 0.01 M EDTA (pH adjusted to 7.5). We added 0.66 ml of 5 M NaCl of lysate. After gentle mixing, the lysate was left at 4 C overnight but could be left refrigerated for at least two weeks. The lysate was centrifuged at 33,000 g for 40 min, the supernatant phenol extracted, and the ethanol precipitated. The DNA pellet was then redissolved in 100 µl of TE buffer (10 mM TRIS-HCl [pH 8.0] and 1 mM EDTA).

Restriction endonuclease digestion and gel electrophoresis. Restriction endonucleases were obtained from Bethesda Research Laboratories (Gaithersburg, Md). Conditions for complete digestion were established by using the DNA of the AD169 strain of CMV. Twenty microliters or less of each DNA was digested for at least 60 min at 37 C (final volume, 30 µl). The reactions were terminated with 20 µl of carrier tRNA (Escherichia coli, 10 mg/ml; Boehringer Mannheim, Federal Republic of Germany), 5 µl of 20% potassium acetate, and 100 µl of ethanol. After precipitation, the DNA was resuspended in 0.1% SDS, 0.025% bromophenol blue, and 6% glycerol, and the DNA fragments were separated by electrophoresis through a 0.8% agarose gel (15 × 30 cm) in TB buffer (0.089 M TRIS-borate, 0.089 M boric acid, and 0.01 M EDTA) at no greater than 5 V/cm, until the tracking dye had migrated 22 cm. After electrophoresis, the gel was soaked for 15 min in TB buffer with 0.5 µg of ethidium bromide/ml and the DNA fragments visualized under UV illumination (long wavelength).

Preparation of plasmid DNA. E. coli strain HB101 clones containing plasmid pACY184 with cloned CMV DNA fragments (Towne strain) were supplied by Dr. Mark Stinski [11]. Bacterial cells were grown to mid-log phase (OD₆₀₀ = 0.5) in LB broth (Difco, Detroit) with 25 µg of chloramphenicol/ml, and spectinomycin was added to a concentration of 300 µg/ml. After overnight aeration at 37 C, plasmid DNA was extracted at 4 C with lysozyme, EDTA,
and Triton and further purified by CsCl centrifugation [12].

Nick translation of plasmid DNA. We nick translated 1.2 μg of each plasmid DNA to a specific activity of >10⁶ cpm/μg, as described by Rigby et al. [13]. All reagents were obtained from Bethesda Research Laboratories. The nick-translated DNA was ethanol precipitated twice and redissolved in 100 μl of TE buffer.

In situ hybridization onto agarose gels. In situ hybridization directly onto the agarose gels was performed as described for the detection of human genomic sequences homologous to chemically synthesized oligonucleotides [14]. The gels were soaked at room temperature first in 0.5 M NaOH containing 0.15 M NaCl and then in 0.5 M TRIS-HCl (pH 8.0) and 0.5 M NaCl for 30 min. The gels were dried on 3-MM paper (Whatman, Clifton, NJ) under vacuum at 60°C and, after wetting and removal of the paper backing, incubated in a heat-sealed bag at 68°C for 2 hr in buffer (0.9 M NaCl, 90 mM TRIS-HCl [pH 7.5], and 6 mM EDTA). This buffer was replaced by fresh buffer containing 0.1% SDS and 6 × 10⁶ cpm of each of the 32P-labeled, heat-denatured DNA probes. One milliliter of buffer was used per 1 cm² of gel. After 2 hr at 68°C the gels were washed at 68°C in 2 × SSC (SSC: 0.15 M NaCl and 0.015 M sodium citrate) for 1 hr and then in 1 × SSC at 68°C for an additional hour. The gels were blotted dry, covered with plastic wrap, and exposed to x-ray film at room temperature with a Lightening-plus® intensifying screen (Dupont, Wilmington, Del). Exposure times varied from 1 to 48 hr, depending upon the quantity of DNA present.

Results

Two thousand seven hundred twenty-nine hospitalized children had admission urine samples cultured for CMV. The prevalence of viruria among these children was 5.1% (141 positive cultures), but significant age differences were observed (table 1). The prevalence of viruria in the Neonatal Intensive Care Unit (acquired plus congenital infections) was 3.3%. The highest prevalence of viruria (7%) occurred among children less than five years old. This was significantly higher than the prevalence of viruria among children five to 12 years old (4.2%) and children 12 to 18 years old (1.2%).

Of a total of 66 children attending the day care center, 16 (24.2%) had CMV viruria. Of 12 children less than one year old, two were viruric. The age differences between children with viruria (mean age, 3.4 years) and those without viruria (mean age, 2.4 years) were insignificant. From December 1983 through November 1984, CMV viruria was detected among 36 (6.8%) of 531 hospitalized children aged two to five years. This frequency of viruria was identical to the frequency of viruria in 382 children aged two to five years hospitalized in the previous 17-month period. The prevalence of CMV viruria showed no seasonal dependence. The prevalence of viruria among children attending the day care center was thus significantly higher than the prevalence of viruria in the 1,457 hospitalized children in the same age range ($\chi^2 = 24$, 1 df, $P < .0001$).

DNA was purified from each of the 16 CMV isolates obtained from children attending the day care center. Figure I shows an ethidium bromide-stained gel after EcoRI digestion of the DNA of 13 isolates. The size distribution and quantities of restriction endonuclease fragments for isolates A, B, C, D, F, and G were very similar to those for fragments H, I, J, K, L, and M. In order to enhance the sensitivity, to detect different nucleotide sequences present in fragments with the same electrophoretic mobilities, and to simplify interpretation of the gels, we used in situ hybridization to detect $\sim 0.1 \mu g$ of endonuclease-digested DNA from the isolates. Two sets of plasmids were used. One was made up of plasmids containing terminal repeats and inverted internal repeat sequences (AO, O, DG, D, I, and Q) and set two was made up of plasmids containing predomi-

<table>
<thead>
<tr>
<th>Age group</th>
<th>Hospitalized children</th>
<th>Children attending day care center</th>
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<tr>
<td>Newborns</td>
<td>18/551 (3.3)*</td>
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<tr>
<td>0–2 years</td>
<td>37/344 (6.8)</td>
<td>8/31 (25)</td>
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<tr>
<td>2–5 years</td>
<td>63/913 (6.9)</td>
<td>7/34 (20)</td>
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<tr>
<td>5–12 years</td>
<td>20/476 (4.2)†</td>
<td>1/1 (100)</td>
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<tr>
<td>12–18 years</td>
<td>3/243 (1.2) ‡</td>
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NOTES. Results are no. positive/no. cultured (% positive).

* $\chi^2 = 8.7$, 1 df, $P < .01$, when compared with hospitalized children in the 0–5 age groups.

† $\chi^2 = 3.9$, 1 df, $P < .05$, when compared with hospitalized children in the 0–5 age groups.

‡ $\chi^2 = 11$, 1 df, $P = .001$, when compared with hospitalized children in the 0–5 age groups.

Table 1. Comparison of the prevalence of CMV viruria among hospitalized children and children attending a day care center.

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The DNA of CMV isolates from urine samples of 13 children attending the day care center were purified, digested with EcoRI, the DNA fragments separated in agarose, and stained with ethidium bromide. Each lane is the digested DNA of a different isolate, as indicated by letter.

Fortunately unique sequences (S, L, K, E, B, J, R, N, T, C, and P). When either set was used for detecting the EcoRI fragments, one group of seven isolates (A, B, C, D, E, F, and G) had nearly identical digest patterns, as did another group of four isolates (H, I, J, and K; figures 2 and 3). The DNA of five isolates (L, M, N, O, and P) was unique. With the set-one plasmids used for detection of EcoRI fragments, each of 11 DNAs obtained from hospitalized children had a unique fragment pattern (figure 4), as did each of the DNAs of isolates obtained from 35 infants with congenital or acquired CMV infections (author's unpublished observations).

In order to confirm that the similarities in EcoRI digest patterns indicated identical strains, we digested the DNA of each isolate with these additional restriction endonucleases: HindIII, BamHI, and BglII (figures 5–7). When digested with BamHI (figure 5), an additional DNA fragment was present for isolates A, B, and C (arrow). This fragment was not present upon repeat digestion. The DNA of isolate E had a minor difference from the DNA of isolates A, B, C, D, F, and G after digestion with EcoRI, BamHI, and HindIII. The DNA of isolate K differed from the DNA of isolates H, I, and J when these were digested by EcoRI and BamHI. The DNA of isolate F had a single fragment variation when digested by HindIII, as did isolate J when digested by BglI. For isolates F, K, E, and J, these variations were considered minor (the presence of one or two additional fragments) when the overall similarities of their digest patterns by several enzymes were compared with the major differences (differing by more than two fragments) in patterns of the DNA of iso-
Figure 3. The purified DNA of CMV isolated from urine samples of 16 children attending the day care center were digested with EcoRI, and after electrophoresis, the gel was incubated with 32P-labeled plasmids containing the Towne strain XbaI fragments B, C, E, J, K, L, N, P, R, S, and T and autoradiographed. Each lane is the digested DNA of a different isolate, as indicated by letter.

Figure 4. The purified DNA of CMV isolated from urine samples of 11 hospitalized children were digested with EcoRI, and after electrophoresis, the gel was incubated with 32P-labeled plasmids containing the Towne strain XbaI fragments AO, DG, I, Q, O, and D and autoradiographed. Each lane is the digested DNA of a different isolate.

The DNA of isolates M and L had very similar fragment patterns when digested by EcoRI and when the DNA was detected by ethidium bromide staining (figure 1). Major differences in fragments, however, were present when the DNA was hybridized to plasmid DNA (figures 2 and 3). Major differences between the DNA of these isolates and of isolates H, I, J, and K were also detected after digestion with BamHI (figure 5) and HindIII (figure 6). Isolates L and M were thus different from each other and from isolates H, I, J, and K.

The children attending the day care center were grouped in five rooms by age (<13 months, 14–28 months, 29–35 months, 36–48 months, and >48 months). Contact between the children in the different age groups and rooms occurred daily during the outdoor play period and when children waited for tardy parents in a single room after the center closed each day. Each CMV isolate was coded and the restriction endonuclease patterns of the DNA analyzed and grouped without knowledge of the ages or room assignments of the children. Of the seven children shedding isolates A–G, six were between 14 and 28 months of age when viruric and were assigned to the same room (21 total children in this group). The seventh child of this group was five months old and shedding isolate F. Of the four children shedding isolates H, I, J, and K, three were aged 38, 36, and 36 months and shared a common room (17 total chil-
Figure 5. The purified DNA of CMV isolated from urine samples of 15 children attending the day care center were digested with BamHI, and after electrophoresis, the gel was incubated with the $^{32}$P-labeled plasmids listed in figure 2. Each lane is the digested DNA of a different isolate, as indicated by letter. The arrow indicates a DNA fragment for isolates A, B, and C that was not present upon repeat digestion.

Figure 6. The purified DNA of CMV isolated from urine samples of 15 children attending the day care center were digested with HindIII and after electrophoresis, the gel was incubated with the $^{32}$P-labeled plasmids listed in figure 2 and autoradiographed. Each lane is the digested DNA of a different isolate, as indicated by letter.
The purified DNA of CMV isolated from urine samples of 11 children attending the day care center were digested with BglI, and after electrophoresis, the gel was incubated with the $^{32}$P-labeled plasmids listed in figure 2 and autoradiographed. Each lane is the digested DNA of a different isolate, as indicated by letter.

of these children. The DNA of the isolates (P, M, and L) for three of these children was unique, a finding suggesting that these children were viruric upon entering the center. These three children, however, were in attendance at the center for > 30 days before their first urine cultures. A fourth child, in attendance for two months before his first culture, had viruria with isolate F, identical by DNA analysis to six other day care center isolates. A fifth child lacked viruria at enrollment but was viruric three months later with isolate G, an isolate similar to six others. Two other children, enrolled continuously at the day care center throughout the study period, initially lacked viruria but subsequently shed viruses A and K. These results prove that at least four children acquired CMV at the day care center.

Two of the 16 children with viruria were siblings, aged 19 and 36 months. Each was viruric with a different viral strain, D and I; however, each of these strains was identical to other strains in the day care center. Thus, transmission had occurred not between siblings but between contacts at the day care center.

The DNA of two or three consecutive isolates obtained at three-month intervals from four children at the day care center (I, E, H, and M) were digested with EcoRI and the DNA fragments detected by in situ hybridization. For each child, the digest pattern remained identical to the initial isolate, a result suggesting that reinfection with another viral strain had not occurred.

Discussion

The prevalence (24%) of viruria among the children attending the day care center was significantly higher — greater than threefold — than that of a large group of hospitalized children of similar ages. The higher prevalence of viruria was not attributable to the multiple culturing of the children at the day care center because only three of these children lacked viruria when initially cultured. The prevalence of inapparent CMV infections among children attending a day care center in a previous report was 57% (59 of 103 children) [15]. The lower prevalence of viruria among the children reported here was not attributable to the culturing of a single site. Virus excretion from saliva is not as prevalent as viruria in preschool children [15].

The analysis of viral DNA recovered from the children attending the day care center confirms that the majority of viruric children acquired CMV at the day care center. Sixteen children were viruric. A group of seven and a group of four were each shedding identical strains, a finding demonstrating that at least nine children had acquired CMV at the day care center. Seven of the 16 could have acquired the virus elsewhere. Of a total day care population of 66, these seven children represent 11%. This does not differ significantly from the 7% (100 of 1,457) prevalence in the control group of hospitalized children ($\chi^2 = 0.8, 1 df, P > .1$). The results of the DNA analysis are consistent with the epidemiological predictions and with the clustering, by age and room assignments, of the children shedding identical isolates.

The analysis of the CMV genome is important for strain differentiation in epidemiological studies. Although this technique has been used previously, its use has been limited to analyzing a few strains or involving single case reports [1, 3–7]. Previously used methods are not practical for large-scale epidemio-
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There are several advantages, however, to the methods used here. First, by using the 24-well plates, hundreds of specimens can be cultured simultaneously. Second, because cell-associated CMV of early-passage virus is used, purification of CMV DNA requires less than four weeks (usually two to three weeks). This avoids prolonged virus passage that, if done at too high a multiplicity of infection, results in genomic rearrangement and significantly alters the restriction endonuclease patterns [16]. Third, the Hirt procedure is simple and rapid. Within 48 hr, CMV DNA can be purified, digested, and electrophoresed. Less than 24 additional hours are required for in situ hybridization.

In situ hybridization directly onto agarose gels with plasmids containing the cloned XbaI fragments has several advantages over ethidium bromide staining. First, <0.1 μg of DNA is required for detection, compared with 1 μg with ethidium bromide. From two petri dishes of infected cells, ~2–5 μg of CMV DNA are obtained. Thus, by using in situ hybridization, many digests can be performed with the DNA prepared from only one or two 56-cm² petri dishes. As seen in figure 1, when ethidium bromide is used, all the DNA fragments are stained, and because many fragments have similar mobilities, the comparison of digest patterns for numerous isolates is tedious. By using a limited set of cloned fragments, this analysis is simplified. With use of only six plasmids containing the fragments with the terminal and internal inverted sequences, all the isolates studied can be differentiated.

In situ hybridization may also detect differences between viral DNA not readily apparent by ethidium bromide staining. The EcoRI digest patterns of the DNA of isolates L and M were similar to those of isolates H, I, J, and K when detected by ethidium bromide staining. When plasmid DNAs were used for detection, however, major differences were apparent. Even though the DNA of two isolates have identical digest patterns by using one or more endonucleases, the arrangement and frequency of nucleotide sequences (particularly inverted repeat sequences) within fragments of similar mobilities may not be identical. With uniformly labeled cloned fragments as hybridization probes, the sensitivity for detecting differences in nucleotide sequence arrangements within the CMV genomes may be enhanced over that obtained with only endonuclease digestion. When major differences between isolates are detected by this technique, the strains cannot be identical.

Minor differences occurred in the digest patterns for several isolates (F, K, E, and J). Minor differences in digest patterns have been detected by others [1, 2] who have not considered them significant because the isolates were epidemiologically related. Additional experience with many more isolates, using this and other techniques, is needed in order to establish the significance of minor variations in DNA sequence arrangements.

The data reported here prove that the children attending the day care center had an increased prevalence of CMV excretion because of viral transmission among the children. Restriction endonuclease analysis has been used twice to strongly suggest CMV transmission from a child to a parent and from a child to an aunt caring for that child [3, 4]. Current data suggest that mothers acquiring a primary CMV infection during pregnancy are at greatest risk of having infants with symptomatic congenital CMV disease [17]. In Richmond, Va, 50% of all mothers lack antibody against CMV; this is similar to other U. S. cities [15, 18].

Does the use of day care centers by seronegative mothers for their preschool children result in an increased frequency of congenital CMV infections among infants of these mothers? With what frequency is CMV transmitted to caretakers? These are the obviously important questions.

References