Interstrain Variations in the Cytomegalovirus (CMV) Glycoprotein B Gene Sequence among CMV-Infected Children Attending Six Day Care Centers

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Using the shell vial assay and sequence analysis of a variable region of the glycoprotein B (gB) gene, cytomegalovirus (CMV) excretion rates in urine and virus transmission were studied among 93 children from six day care centers (DCCs). During a 6-month period, excretion rates differed significantly between DCCs (P < .001). The 6 gB gene sequences, obtained from 24 CMV-infected children, were classified in four previously defined groups. In five DCCs, 2 or 3 strains cocirculated, and none was dominant. Infection could have been acquired outside the DCC for 2 children and inside it for 9. Two children from the same DCC had mixed infections. No differences in hygiene, child care practices, or experience and level of qualification of the staff could explain this wide variety of excretion rates between DCCs. The distribution of gB gene patterns observed does not suggest that 1 type was dominant or more efficiently transmitted.

Cytomegalovirus (CMV) infection is frequent in early childhood. Up to 15% of infants are infected by CMV at 6 months of age [1]. The earliest age at which CMV is acquired depends on socioeconomic factors, maternal antibody status, mode of feeding (breast vs. bottle), intrafamilial exposure to CMV, and environmental factors [2]. Congenital CMV infection is the most common intrauterine infection, affecting 0.4%–2.3% of live-born infants [2]. Perinatal infections refer to those acquired during delivery [1]. Very young children attending group day care centers (DCCs) are likely to acquire CMV and thus appear to be a major source of primary infection for pregnant women [1].

Molecular epidemiologic analysis has enhanced the understanding of CMV transmission among neonates [3], families [4], and immunocompromised hosts. Since the development of DNA amplification by the polymerase chain reaction (PCR), CMV strains can be compared by use of restriction enzyme or sequence analysis of the amplified products [5]. The envelope glycoprotein (gB) of CMV is essential for viral entry into host cells. It is an important target of the human immune response that induces the formation of neutralizing antibodies. Sequence data indicate that interstrain variations in gB are strongly clustered at two major distinct loci (codons 27–67 and 440–460). Analysis of gB variations led to the definition of four CMV groups [5].

To study the prevalence and the duration of CMV excretion in six DCCs, we looked for CMV excretion in 439 urine samples from 93 children < 1 year old. To specify the CMV transmission, DNA containing a partial gB gene was amplified from CMV-positive samples, and a variable region encompassing the cleavage site (codons 440–460) was sequenced. This approach allowed gB gene variability to be compared among CMV strains and the transmission pattern within the six DCCs to be analyzed.

Materials and Methods

Subjects and samples. CMV excretion was studied in urine samples collected from children attending six DCCs in the Val-de-Marne area near Paris. Urine samples from 93 children < 1 year old were examined. There were 11 children from DCC 1, 13 from DCC 2, 19 from DCC 3, 16 from DCC 4, 21 from DCC 5, and 13 from DCC 6. Each DCC handled 50–60 children. Urine samples (n = 439) were collected monthly from January to June 1990. At least 75% (70/93) of the children gave ≥4 urine samples for CMV detection.

CMV detection. Samples were inoculated onto human fibroblasts (MRC5; bioMérieux, Lyon, France). After 24 h of cell culture, immediate-early antigens were detected using a monoclonal
antibody (clone E13; Argene-Biosoft, Varilhes, France) and an immunofluorescence assay (IEAD).

**Viral DNA preparation and gB gene amplification.** Viral DNA was obtained by using a rapid preparation of virus particles as reported by Yamagushi et al. [6]. The urine pellet was suspended in 25 μL of deionized sterilized water. Oligonucleotide primers, derived from the gB gene sequence of Towne and AD169 strains, B2A (5'-GGGGATCAATCGGTGTTGGTGA-3') and B2B (5'-biotin-GTGTTCTGGCAGCGATCAAG-3') amplified a 320-bp fragment (gB bp 1441-1760) encompassing the variable region of gB (codons 440-460) [5]. "Hot-start" PCR was done with 1.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT) for 10 min at 95°C. Digested fragments were obtained by using a rapid preparation of virus particles as viral DNA with streptavidin-coated magnetic beads (Dynabeads M280; Dynal, Oslo) [7]. The bead pellet was suspended in 10 μL of deionized sterilized water. Oligonucleotide primers, B2A (5'-GCGGAATTCAATCGGTGTTGGTGA-3') and B2B (5'-GCGGTTA ATCCACACAC-3'), were biotinylated primer was used to purify single-strand DNA with streptavidin-coated magnetic beads (Dynabeads M280; Dynal, Oslo) [7]. The bead pellet was suspended in 10 μL of sequencing buffer, and the sequence analysis was performed according to the manufacturer's instructions (United States Biochemicals, Cleveland).

**DNA sequencing.** DNA sequencing. DNA sequencing. DNA sequencing. DNA sequencing. 

**Statistical analysis.** We used a χ² test and Yates's correction when the size of the calculated class was <5.

**Results**

**Detection of CMV excretion by IEAD.** Of 439 urine samples analyzed, 60 (13.7%) gave inconclusive results due to bacterial contamination or cytotoxic effect. Among the remaining 379 samples, 92 (24.3%) were CMV-positive. Of 93 children, 33 (35.5%) excreted CMV in their urine at least once during the study.

**Monthly CMV excretion rates differed significantly among the six DCCs (P < .001).** During the entire study period, no child from DCC I excreted CMV. In DCCs 2–5, 20%–26% of the children excreted CMV throughout the study. In DCC 6, the mean CMV excretion rate increased from the first 3 months (40%) to the last 3 months (81%; P = .01).

**CMV gB identification using PCR amplification.** Children's samples were selected for PCR assay at least one CMV IEAD result was positive, and all samples were analyzed when enough material was available. In total, 98 urine samples from 28 infected children were studied. CMV IEAD-negative samples from 2 children from DCC 1 were tested and remained negative for gB on PCR. After hybridization, 80 samples (65 of which had been IEAD-positive) from 24 children were positive on PCR.

**Sequence analysis.** CMV isolated from 24 infected children was subjected to sequence analysis. For 14 children, sequences of 2–5 samples were analyzed individually. Only 6 different viral genomic sequences were found in all the samples studied (table 1). gB gene sequences could be classified into the four CMV groups previously described [5]. Ten nucleotide sequences belonged to group I; 9 of those had a silent mutation (G→A) at position 1578 (strain T.1). Of 11 sequences belonging to group II, 3 (strain A.1) had a silent mutation (C→T) at position 1617. Four viruses were similar to strain C326 (group III), with a predicted glycine at codon 464 versus an aspartic acid in C326 gB protein (strain III.86). In our study, group IV sequences were detected in only 1 CMV strain (IV.1), which had 4 deduced amino acid (aa) substitutions in the gB variable region compared with the Towne strain. Furthermore, the 2 group IV-specific predicted aa at positions 474 (D) and 500 (T) were present. In analysis of 250 bp of the CMV gB gene, 1 base (0.4%) was changed within a group. Between 2 groups, sequences differed by 13–56 nt (5.2%-22.4%).

**Consecutive sample analysis and strain diversity among DCCs.** Five children from 3 DCCs were followed. For 1 child, CMV was detected in urine during 5 successive months. No mutation occurred during monitoring. For the other 4 CMV-infected children, analysis showed that no mutation arose between the first and last samples.

Two or three different viral sequences were found in each DCC. The CMV sequences obtained from 7 infected children from DCC 2 showed 3 concomitant circulating strains (AD169-like, A.1, III.86). DCCs 2–5 had cocirculation of different virus strains. Three strains were restricted to one DCC (table 1). On the other hand, strains T.1, AD169-like, and III.86 were found in three DCCs.

**Monitoring of a CMV outbreak in DCC 6.** Of 13 children monitored in this DCC, 2 did not excrete CMV during the study period. gB sequences were obtained from 8 of the 11 infected children. Three children were infected by strain T.1, 3 by AD169-like, and 2 (children A and B) became coinfected by group I and group II viruses.

For child A, direct sequencing of the PCR product initially showed a group I strain (T.1). Subsequent examination showed...
a mixed pattern (group I and II sequences) of the gB variable region (figure 1).

For child B, the first isolate belonged to group II (AD169-like), but a later sample analyzed by direct sequencing showed a mixed pattern (group I and II sequences). To further study these samples, PCR products were subjected to HinfI restriction enzyme analysis to differentiate group I from group II CMV strains and to confirm that strains from these 2 groups coexisted in samples from child B. Indeed, in consecutive samples from child B, the presence of the single AD169-like strain (group II) at the beginning of the study was confirmed and superinfection by the T.1 strain (group I) was demonstrated (figure 1).

Four children initially noninfected (i.e., gB PCR- and CMV IEAD-negative for at least the 2 initial samples) excreted CMV in subsequent samples. These children were thus considered to have acquired CMV infection within DCC 6. Furthermore, analysis of the double infections in children A and B (figure 1) suggested that coinfection occurred during the study. Thus, 6 (46.2%) of 13 children might have been infected in DCC 6 during the study.

Discussion

In children from six DCCs, throughout a 5-month study, the mean CMV excretion rate (24.3%) was similar to the prevalence previously observed [8, 9] but higher than the 9% reported by Pass and Hutto [10] for this age group. Mean CMV excretion varied from 0% in DCC 1 up to 60% in DCC 6 (P < .001). Such differences have been suspected of being due to child care or hygienic practices within the DCC [9]. We carefully questioned DCC staff to determine whether this hypothesis could explain the wide variations, but no differences in the workers’ skills or the DCC facilities were found.

On the other hand, prevailing virulent strains might explain such differences. Taken together, our results do not suggest that a specific gB sequence prevails. Indeed, in five of the six DCCs studied, 2 or 3 different sequences belonging to distinct CMV groups were found. Although these results differ from those previously described among children of the same age, which showed that CMV strains might be identical [8, 9], they are closer to those recently reported by Shen et al. [11], who used gB restriction enzyme analysis and found that most of the virus strains shed by children attending two DCCs were different.

Two gB sequences were observed only once (strains IV.1 and Towne, table 1). Those single infections suggest that the 2 children had been infected outside the DCC by a specific virus strain. Indeed, during the first year of life, the source of CMV infection might be the mother [2]. On the other hand, throughout the study, 9 children were probably infected while in the DCC. The results obtained during the outbreak in DCC 6 suggest that each of the cocirculating viruses could have infected the children and that none of them (group I or II viruses) was dominant or more efficiently transmitted. In contrast to our results, Grillner and Strangert [12] and Shen et al. [11] did not observe CMV transmission between children inside a DCC. However, in those studies, the children were 3–5 years old. Children’s behavior changes with age, and a lower frequency of CMV excretion in the older children might contribute to the slight horizontal spreading among their age group.

In 2 cases, we suspected double infections on the basis of sequence analysis. Superinfection was confirmed using HinfI restriction enzyme digestion and fragment analysis of consecutive samples. Indeed, a double infection was previously demonstrated in a healthy child by Shen et al. [13]. Double infection generally occurs in immunocompromised hosts [14]. Infants infected by CMV either congenitally or early in life might be reinfected during childhood due to immunologic tolerance of
the virus. Our analysis of 5 consecutive samples from 1 child confirmed that such children may shed CMV in their urine for a long period, perhaps even several years [1, 12].

In conclusion, we confirmed that CMV was shed at a high rate in five of the six DCCs studied. We could not prove a link between hygienic practices or DCC workers’ behavior and CMV transmission among children in the DCC. Furthermore, molecular epidemiologic results did not suggest a difference in transmission efficiency between most of the prevailing viruses (groups I and II). However, one DCC had a CMV outbreak and 2 children had CMV superinfection. Therefore, it will be useful to determine whether such differences between CMV excretion rates in a DCC might be linked to the children themselves.

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References