PCR-RFLP Analysis of Cytomegalovirus Infections Associated with Bone Marrow Transplantation in Japanese Children

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Abstract: In order to investigate the longitudinal molecular epidemiology of cytomegalovirus (CMV) infections associated with bone marrow transplantation (BMT) in Japanese children, we analyzed 36 CMV strains from 11 cases. Three regions (DNA polymerase, glycoprotein H, and immediate-early regions) of CMV DNA were amplified by polymerase chain reaction (PCR), and amplified products were each digested with two restriction enzymes, followed by electrophoresis. These restriction fragment length polymorphism (RFLP) analyses allowed the differentiation of 36 strains into 13 genotypes. Each patient excreted his or her own CMV with distinct genotype over the study period of up to one year. CMVs of two different genotypes were recovered during a one-month study from one recipient, who received a peripheral blood stem cell transplantation. Although the majority of patients and donors were CMV-seropositive before BMT, multiple CMV infections might not be common and the reactivation of latently infected CMV might be prominent in Japanese children receiving transplants.

Key words: Cytomegalovirus, PCR-RFLP, BMT

Human cytomegalovirus (CMV) disease is a common infectious complication in allogeneic marrow transplant recipients, either due to reactivation of an endogenous strain or due to a newly introduced virus which is transmitted from the transplant bone marrow (1). Japan is a high endemic country regarding CMV infection, (8) and the majority of donors (D) and recipients (R) are CMV-seropositive (D+/R+) before bone marrow transplantation (BMT) (8, 14). However, whether the CMV disease after transplantation is due to reactivation or reinfection remains unanswered.

Chou (4) reported a useful method for molecular epidemiological studies of CMV, in which DNA polymerase (Pol), glycoprotein H (gH), and major immediate-early (MIE) regions were each digested with two restriction endonucleases after DNA amplification by polymerase chain reaction (PCR). Using this method, we analyzed here 36 CMV strains from BMT recipients to study the longitudinal molecular epidemiology of CMV infections associated with BMT in Japanese children, and obtained data suggesting that reactivation but not superinfection occurs frequently in patients receiving transplants.

Materials and Methods

Clinical specimens. The recipient subjects of this study underwent allogeneic BMT or peripheral blood stem cell transplantation (PBSCT) at the Department of Pediatrics of Tohoku University School of Medicine or the Department of Pediatric Oncology of the Institute of Development, Aging and Cancer, Tohoku University, between June 1988 and September 1996. A total of 79 recipients were monitored for CMV infection by culturing of urine and throat swabs obtained at one-week
intervals after transplantation during hospitalization from 6 months to 2 years.

**Virus isolation.** Both urine and throat swab samples were inoculated onto human embryonic fibroblasts (HEF) prepared in our laboratory (Virus Research Center). Characteristic cytopathic effect (CPE) and indirect immunofluorescent staining were used for the identification of CMV (13), after which the isolates were stored at −80°C until use. Two laboratory strains, AD169 and Davis, were employed as reference viruses.

**Serologic tests.** Serum specimens collected from all recipients and donors before transplantation were tested for anti-CMV antibodies by complement fixation and indirect immunofluorescent assay as previously described (8).

**Preparation of viral DNA.** DNA was extracted from CMV-infected HEF cells exhibiting CPE by phenol-chloroform extraction and ethanol precipitation (12). Briefly, cell pellets were resuspended in 300 µl of extraction buffer (10 mM Tris HCl, 1 mM EDTA, and 1.2% sodium dodecyl sulfate [SDS] [pH 8.0]) with proteinase K at a final concentration of 120 µg/ml. The mixtures were incubated at 55°C for 1 hr and then at 37°C for 2 hr. DNA was purified by two extractions each with equal volumes of phenol and phenol-chloroform-isoamyl alcohol (25:24:1), precipitated overnight in 0.3 M sodium acetate and a 2.5 volume of 100% ethanol, and then resuspended in 50 µl of Tris-EDTA buffer, pH 8.0.

**PCR protocol.** DNAs extracted from CMV-infected HEF were amplified by PCR with three separate sets of primers to obtain the following three fragments: the 3' half of the DNA polymerase gene (Pol, target length 2.0 kbp), the glycoprotein H gene (gH, target length 2.2 kbp), and the major immediate-early gene (MIE, target length 2.6 kbp) (4). The reaction mixture (50 µl) for PCR amplification contained 1.5 mM MgCl₂, 10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.08% Nonidet P40, 0.2 mM dNTP, 1 ng/µl of each primer set, 1 U of Taq DNA polymerase, and approximately 1 µg of target DNA template. Amplification was performed in a Thermal Cycler Model 480 (Perkin-Elmer, U.S.A.). A reaction cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and, extension at 72°C for 3 min was repeated 25 times, which was followed by additional 20 cycles of amplification (denaturarion at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min). The PCR products were separated by electrophoresis in 1.0% agarose gels and visualized by ethidium bromide staining. The AD169 and Davis strains were used as positive controls, and uninfected HEF as the negative control.

**Restriction fragment length polymorphism (RFLP) analysis.** The PCR products from three CMV genome regions were each digested with two enzymes each for 16 hr at 37°C. The restriction enzymes used were as follows: Mspl and HaeIII for the Pol region, HaeIII and Hhal for the gH region, and HaeIII and HinfI for the MIE region (all enzymes were purchased from New England Biolabs Inc., U.S.A.). Digested fragments were analyzed by electrophoresis in polyacrylamide gels and MetaPhor agarose gels (FMC, U.S.A.), and visualized by ethidium bromide or silver staining (New England Biolabs Inc.).

**RFLP patterns.** The system employed for the genome typing was as follows: the Mspl and HaeIII restriction patterns for the Pol region were named A, B, C, etc. and a, b, c (Fig. 1), etc., respectively. HaeIII restriction patterns for the gH region were named I, II, III, etc. and Hhal restriction patterns for the gH region were i, ii, iii, etc. HaeIII restriction patterns for the MIE region were named 1, 2, 3, etc. and HinfI patterns for the MIE region

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (Year)</th>
<th>Sex</th>
<th>Underlying disease</th>
<th>Day of transplantation</th>
<th>CMV serology prior to transplantation</th>
<th>Type of transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>M</td>
<td>AA</td>
<td>6/17/88</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>F</td>
<td>MPS (Type IV)</td>
<td>7/12/88</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>M</td>
<td>CML</td>
<td>9/19/88</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>F</td>
<td>CML</td>
<td>11/18/88</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>F</td>
<td>CN</td>
<td>5/19/89</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>M</td>
<td>AML</td>
<td>7/14/89</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>M</td>
<td>AA</td>
<td>1/12/90</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>F</td>
<td>ALL</td>
<td>6/18/93</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>M</td>
<td>Neuroblastoma</td>
<td>11/15/94</td>
<td>Positive</td>
<td>PBSCT</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>M</td>
<td>AA</td>
<td>1/13/95</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>M</td>
<td>WAS</td>
<td>5/22/96</td>
<td>Positive</td>
<td>AlloBMT</td>
</tr>
</tbody>
</table>

AA, aplastic anemia; MPS, mucopolysaccharidosis; CML, chronic myeloblastic leukemia; CN, congenital neutropenia; AML, acute myeloblastic leukemia; ALL, acute lymphocytic leukemia; WAS, Wiskot-Aldrich syndrome; AlloBMT, allogeneic BMT.
were α, β, γ, etc. According to the combinations of the RFLP for the three regions with the four restriction enzymes, CMV genome types were identified as Aαi1α, etc.

### Results

**Isolation of CMV from Children Who Underwent BMT**

A total of 79 recipients were monitored for CMV infection by viral culturing of urine and throat swabs obtained at one-week intervals after transplantation. Out of these 79 recipients, 28 (35.4%) proved CMV-positive at least once during the follow-up period, and 11 (13.9%) were positive twice or more. A total of 36 CMV strains from 11 recipients were used for RFLP analysis. Ten of the patients had received allogeneic BMT and one patient (case 9) had undergone PBSCT (Table 1). All recipients and donors were seropositive for CMV.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Day of sample collection</th>
<th>Sample</th>
<th>Pol</th>
<th>gH</th>
<th>MIE</th>
</tr>
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<tbody>
<tr>
<td>1/2/89</td>
<td>U</td>
<td>A A I i</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/12/88</td>
<td>T</td>
<td>A A I i</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/16/89</td>
<td>U</td>
<td>A A I i</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11/18/88</td>
<td>T</td>
<td>B A II i</td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/31/89</td>
<td>U</td>
<td>C A II i</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/14/89</td>
<td>U</td>
<td>D b I i</td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/12/90</td>
<td>T</td>
<td>E a III v</td>
<td>δ</td>
<td></td>
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</tr>
<tr>
<td>5/27/91</td>
<td>U</td>
<td>E a III v</td>
<td>δ</td>
<td></td>
<td></td>
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<tr>
<td>6/18/93</td>
<td>U</td>
<td>B a IV i</td>
<td>γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/15/94</td>
<td>T</td>
<td>B + D b + c I + II i + iii</td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/22/94</td>
<td>U</td>
<td>D b I i</td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/20/95</td>
<td>U</td>
<td>B a V iii</td>
<td>γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/25/95</td>
<td>U</td>
<td>B a VI iii</td>
<td>α</td>
<td></td>
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<td>5/10/96</td>
<td>U</td>
<td>B a VI iii</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/26/96</td>
<td>U</td>
<td>B a VI iii</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Davis</td>
<td>F</td>
<td>a I v i</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD169</td>
<td>F</td>
<td>a IV v i</td>
<td>e</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α: Day of transplantation.
β: CMV was derived from throat swab (T) or urine (U).
anti-CMV antibodies before transplantation, but none of
them were receiving anti-CMV drugs or presenting any
clinical manifestations of CMV disease. All transfused
blood for seropositive recipients was not screened for
anti-CMV antibodies, but was radiated and then filtered
to remove leukocytes.

**Restriction Analysis of Amplified Sequences**

DNA samples extracted from the 36 strains described
above and 2 control laboratory strains were amplified by
using each of the 3 primer sets (for the Pol, gH, and MIE
regions) to give products of the predicted sizes (data
not shown). The amplified sequences were digested
with restriction enzymes, and the resulting fragments
were analyzed by gel electrophoresis.

As can be seen in Table 2 and Fig. 1, there were 6 pat-
terns (A–F) for the Pol region digested with MspI, and 3
with HaeIII (a–c); 6 patterns (I–VI) for the gH region
digested with HaeIII, and 5 (i–v) with HhaI; and 4 pat-
terns (1–4) for the MIE region digested with HaeIII,
and 5 (α–ε) with HinfI. Combining the RFLP patterns
for these three regions with the four restriction enzymes
allowed us to classify 36 clinical strains into 13 CMV
genotypes and 2 laboratory strains into 2 genotypes.

All of the recipients except case 9 had their own dis-
tinct CMV RFLP patterns, demonstrating persistence of
the same CMV for up to one-year post-allogeneic BMT
(Table 2). In 7 of 8 recipients who were examined for 2
or more isolates from urine and throat swab samples,
identical CMV RFLP patterns were obtained for the
strains isolated from urine and throat swab samples.
However, in case 9, who underwent autograft trans-
plantation with PBSC (Fig. 2), the first isolate from a
throat swab showed a RFLP pattern mixing the patterns
of the second urine isolate and the third throat swab
isolate, suggesting that double-infection with two dif-
ferent CMVs occurred in this patient.

**Discussion**

CMV is a formidable barrier to successful marrow and
solid-organ transplantation (1, 10). The CMV serologi-
cal status of donor (D) and recipient (R) pairs con-
tributes to predict the risk of progression to CMV disease,
and the D+/R+ group had an intermediate risk of
CMV disease as compared with the D+/R− and D−/
R+ groups (5). The high seropositive rate in this study
is in accord with published seroepidemiological data
for Japanese pregnant women and infants (8). The
majority of donors and recipients were CMV-seroposi-
tive (D+/R+) before transplantation, in contrast to the
situation in Europe and North America (9) where D−/
R− is dominant.

CMV establishes a latent infection in the host and can
be reactivated with renewed shedding of infectious virus
years after primary infection. Thus, CMV infection
after transplantation could be either reactivation of latent-
ly infected virus, reinfection with CMV from a donor, or
both. However, it is difficult to differentiate the type of
infection by serological tests or virus isolation. Epi-
demiological studies of CMV using molecular tech-
niques afford a better understanding of transmission (4,
7, 11, 17–19). In our study using the PCR-RFLP
technique (4), a single genotype of CMV was detected in

![Fig. 1. RFLP variation of Pol PCR products digested with Hae-
III. The PCR products of the CMV Pol region show 3 different
patterns (a–c) when digested with HaeIII. M, molecular weight
marker of 100 bp DNA ladder (New England Biolabs Inc.).](image1)

![Fig. 2. PCR-RFLP analysis of CMVs isolated from Patient 9. The
patients obtained after HaeIII digestion of Pol PCR products
are shown. Lanes 1, 2, and 3 indicate the patterns of isolates
from throat swab at day 30, from urine at day 37, and from
throat swab at day 58, respectively. Note that the pattern of lane
1 contains all fragments found in the patterns of lanes 2 and 3.)](image2)
each of 10 cases who received allogeneic BMT (D+/R+) in spite of long-term and multiple site surveys, suggesting that superinfection may be quite rare, and that the majority of CMV infections arise by reactivation of latently infected CMV.

Horizontal transmission has been demonstrated in daycare centers (16) by the findings of excretion of CMVs closely similar to one another, reflecting transmission during close contact or via staff. Furthermore, nosocomial CMV infection has also been reported to occur in pediatric chronic care units (6). Seven (cases 1 to 7) of 11 pediatric cases studied here had been hospitalized at the same time. However, it appears that cross-infection did not occur since the genotypes of the CMVs isolated from these patients differed from one another.

In clinical specimens from one patient who underwent autograft transplantation with PBSC (case 9), two different CMVs were detected. This patient had no blood transfusion history, and no evidence for nosocomial CMV infection was obtained in the PCR-RFLP analysis. Thus, their origins remain unclear. Multiple strains of CMV were isolated from patients with sexually transmitted disease (2), human immunodeficiency virus infections (15), and from organ transplant recipients (3), but there were no reports presenting multiple CMV infections among patients that had undergone autograft transplantation with PBSC.

In summary, we demonstrated here that, although the majority of patients and donors were CMV-seropositive before BMT, multiple CMV infections are not common, and reactivation of latently infected CMV is prominent in Japanese children who receive transplants. Furthermore, molecular characterization of the CMVs isolated from 11 recipients in this study indicated the existence of many distinct genotypes. Recently, Meyer-Konig et al (11) reported intragenic variability of CMV glycoprotein B (gB) in clinical strains by employing RFLP after amplifying a fragment that corresponds to the glycoprotein B (gB) in clinical strains by employing PCR-RFLP. We need further studies to confirm our results with such a new technique.

References
