High Levels of Adenovirus DNA in Serum Correlate with Fatal Outcome of Adenovirus Infection in Children after Allogeneic Stem-Cell Transplantation

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An increase in the incidence of adenovirus (AdV) infection leading to death among children who have undergone allogeneic stem-cell transplantation has made it necessary to find new ways to monitor AdV infection. In this retrospective study, levels of AdV DNA in serum samples obtained from 36 transplant recipients with stool cultures positive for AdV were measured by polymerase chain reaction (PCR) semiquantitatively by analyzing serial dilutions of the DNA template. Six (86%) of 7 children who died of AdV infection, compared with only 2 (7%) of 29 other patients, had high serum levels of AdV DNA (detectable by PCR at a 1/100-fold dilution of the DNA template; \( P < .0001 \)). High serum levels of AdV DNA were reached a mean of 18 days before death (range, 6–29 days). Quantification of adenoviral DNA in serum may prove to be a valuable tool to diagnose and monitor AdV infection and disease in immunocompromised children.

During the first months after undergoing transplantation, recipients of allogeneic stem-cell transplants are severely immunocompromised and, as a consequence, are susceptible to viral infections and reactivations. Since 1995, the incidence of adenovirus (AdV) infections among pediatric stem-cell transplant recipients has increased remarkably [1–12] (M. J. van Tol, A. C. Kroes, C. J. Schinkel, W. Dinkelaar, C. M. Jol–van der Zijde, and J. M. Vossen, unpublished data), and this infection has produced clinical manifestations such as hemorrhagic cystitis, enteritis, hepatitis, encephalitis, pneumonia, and multiple-organ failure [1–3, 5, 7, 11, 13, 14]. Disseminated infections frequently result in death.

In contrast, adenovirus infections in healthy children are usually not associated with serious clinical symptoms, indicating that effective immune responses to AdV in humans contain the infection. During the first years of life, children develop neutralizing antibodies and T cell responses against various strains of AdV [14, 15]. As a result, most adults have strong preexisting immunity to AdV, which is a complicating factor in the use of adenoviral vectors for gene therapy [16].

At present, 51 serotypes of AdV that are able to infect human cells have been identified, which are distinguished from each other by the antigenic determinants recognized by neutralizing antibodies. The different serotypes are grouped into 6 subgroups, A–F, according to their ability to agglutinate red blood cells and according to their DNA homology [17, 18]. At the DNA level, homology within a subgroup varies from 50% to almost 100%. Even strains belonging to the same serotype can differ in their DNA sequences [18]. Between subgroups, the homology can be as low as 4% [18]. Clinically, AdV
infections are detected and diagnosed by means of various techniques [14]. Isolation of the virus in culture and subsequent identification via immunofluorescence with AdV-specific antibodies is the most common approach, but it takes several days, depending on the virus load of the sample. Alternatives are direct immunofluorescence or detection of viral antigen by agglutination of antibody-coated latex beads, dot blot hybridization, and, more recently, detection of viral DNA by PCR.

Several PCR-based strategies have been developed. Because of the genetic variability within the group of human AdV, the choice of primers varies with the application. Some techniques detect AdV strains belonging to almost all serotypes by means of degenerate [19–22] or nondegenerate [20, 23–27] primer pairs. Sequences conserved in most of the 51 serotypes can be found in the hexon gene [19, 20, 22, 26, 27], EIA [20], and VA-RNA [21]. Analysis of the PCR products by restriction enzyme analysis or sequencing can supply sufficient information to assign an unknown isolate to a subgroup or serotype [19, 21, 22, 27–30]. Other approaches use subgroup-specific [28, 31] or serotype-specific [32] primer combinations.

In the present study, we performed a retrospective analysis of 36 children who had adenovirus infection after undergoing allogeneic stem-cell transplantation (SCT). A generic PCR that amplifies a conserved part of the AdV genome, allowing detection of all disease-causing AdV strains, was used [25, 33] in a qualitative as well as in a semiquantitative fashion. The presence of adenoviral DNA in serum and its level were determined at different time points after transplantation. Because high levels of AdV DNA correlated with fatal outcome, quantification of the AdV DNA load in serum may be a valuable tool to diagnose the dissemination of AdV infection in immunocompromised patients.

MATERIALS AND METHODS

Patients. Thirty-six patients from a cohort of 328 children who underwent SCT at the pediatric transplantation unit of the Leiden University Medical Center during 1985–1998 had ≥1 AdV-positive stool culture. Stool samples were tested by culture on Hep-2 cells, followed by immunofluorescence analysis (Imagen AdV; DAKO Diagnostics). The 36 patients were classified into 3 groups on the basis of their clinical symptoms (table 1). Group 1 consisted of 17 patients who did not show any clinical symptoms of AdV infection. Group 2 consisted of 12 patients who developed local clinical symptoms that were most likely associated with AdV infection. The localized clinical symptoms were enteritis (in 11 patients), hemorrhagic cystitis (in 2), and hepatitis (in 1). Group 3 consisted of 7 patients who died as a result of fatal disseminated disease caused by AdV. Clinical symptoms in group 3 were enteritis (in 5 patients), hemorrhagic cystitis (in 1), hepatitis (in 3), encephalitis (in 1), and pneumonia (in 1). AdV was recovered from cultures of samples of multiple sites, thus confirming dissemination. In 5 of the 7 patients, dissemination was also confirmed after autopsy.

AdV serotyping. Eight of the 36 AdV isolates were not serotyped (because of the lack of available antisera or the lack of an original isolate). The remaining 28 isolates were serotyped by use of standard techniques; 19 isolates (68%) belonged to subgroup C (AdV 1, 2, 5, and 6), 5 isolates (18%) belonged to subgroup A (AdV 12, 31), 2 isolates (7%) belonged to subgroup B (AdV 7), and 2 isolates (7%) belonged to subgroup F (AdV 41). No significant differences in serotypes were observed between patients with high virus loads compared with those with low virus loads.

SCT. Patients underwent SCT to treat immunodeficiency, hemopoietic defects, and leukemia (including familial hemophagocytic lymphohistiocytosis, severe combined immunodeficiency, Wiskott-Aldrich syndrome, metachromatic leukodystrophy, β-thalassemia, acute lymphoblastic leukemia, acute myeloid leukemia, myelodysplastic syndrome, Fanconi syndrome, and severe aplastic anemia). The sources of stem cells were human leukocyte antigen–identical related donors, other related donors, and matched unrelated donors. T cell depletion, if necessary, was performed by means of sheep erythrocyte rosetting and albumen gradient centrifugation and immunorosetting that used anti-CD2 and anti-CD3 (sometimes combined with anti-CD19 and anti-CD22) monoclonal antibodies [34] (<3-log T cell depletion) or administration of Campath-1G “in the bag” or enrichment of CD34+ precursor cells with use of a CliniMACS (>3-log T cell depletion; Miltenyi Biotec). Of the 36 patients who underwent SCT, only 2 patients developed graft-versus-host disease of grade 2 or higher. For comparison, serum samples from 17 healthy stem-cell donors (10 children and 7 adults) were screened as well.

Isolation of DNA. Serum samples had been stored at −20°C for 1–14 years. DNA was isolated from 200 μL of serum with use of QIAamp columns (QIAamp DNA blood mini kit; Qiagen), according to the manufacturer’s directions. Negative controls (water) were included in each run of extractions. DNA was eluted in 200 μL of water and stored at −20°C until further use.

PCR. Twenty microliters of template DNA was added to a final volume of 50 μL containing 1× Taq PCR Master Mix (Qiagen) and 0.2 μM of the primers Hex 3 (GACATGACTTTC-GAGGTCGATCCCATGGA) and Hex 4 (CCGGCTGAGAAG-CTCCGCCATATGTTT) [23, 25]. The primer pair amplified DNA from AdV strains belonging to serotypes 1–7, 7A, 8, 11, 12, 16, 19, 30, 34, 35, 37, 48, and 49 [25]; 31 and 41 (present study); and 6, 9, 40, 50, and 51 (authors’ unpublished data).

PCR amplification was performed with use of a Perkin-Elmer 2400 thermocycler. An initial denaturation at 94°C for 3 min
Table 1. Characteristics of adenovirus-infected recipients of allogeneic stem-cell transplants.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patient group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (n = 17)</td>
</tr>
<tr>
<td>Age, median years (range)</td>
<td>3.7 (0.6–15.8)</td>
</tr>
<tr>
<td>Donor type</td>
<td></td>
</tr>
<tr>
<td>HLA-identical related donor</td>
<td>4</td>
</tr>
<tr>
<td>Other related donor</td>
<td>6</td>
</tr>
<tr>
<td>Matched unrelated donor</td>
<td>7</td>
</tr>
<tr>
<td>T cell depletion</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>9</td>
</tr>
<tr>
<td>&lt;3 log&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>&gt;3 log&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>AGVHD&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>14</td>
</tr>
<tr>
<td>Grade 1</td>
<td>3</td>
</tr>
<tr>
<td>Grades 2–4</td>
<td>—</td>
</tr>
<tr>
<td>Adenovirus-related parameters</td>
<td></td>
</tr>
<tr>
<td>Stool culture result</td>
<td>Positive</td>
</tr>
<tr>
<td>Clinical symptoms present</td>
<td>No</td>
</tr>
<tr>
<td>Deaths</td>
<td>No</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of patients, unless otherwise indicated. AGVHD, acute graft-vs.-host disease.

<sup>a</sup> Depletion by means of erythrocyte rosetting plus albumin gradient centrifugation or immunorosetting with use of anti-CD2 and anti-CD3 (sometimes including anti-CD19 and anti-CD22) monoclonal antibodies [34].

<sup>b</sup> Depletion by means of Campath-1G “in the bag” or enrichment of CD34<sup>+</sup> precursor cells on a CliniMACS (Miltenyi Biotec).

<sup>c</sup> As defined in [35].

was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 15 s, and an elongation at 72°C for 30 s. Finally, a 7-min elongation completed the program. To obtain a semiquantitative measure of the load of AdV DNA in serum, the extracted template DNA was serially diluted (10-fold, 100-fold, and 1000-fold) in a 10 mM Tris solution before PCR analysis. The highest dilution that still yielded a PCR product was considered to indicate the load of DNA in the serum sample.

**Detection and identification of PCR products.** PCR products were detected by electrophoresis of 10 µL of the amplified product on a 1.5% agarose gel containing ethidium bromide. A molecular-size marker (SmartladderSF; Eurogentec) and an aliquot of the AdV-positive PCR control were mounted on the gel as well. Ultraviolet light visualized a positive PCR as a 139-bp amplified product. In addition, the results were confirmed by EIA hybridization with a digoxigenin-labeled probe (Hex-30; GACCCCACCCCTTCTTAGTTCTGT) [25] detected with antidigoxigenin antibody labeled with horseradish peroxidase.

**RESULTS**

**Qualitative analysis of serum samples for the presence of AdV DNA.** Serum samples from all 36 patients were analyzed for the presence of AdV DNA by PCR. We analyzed serum samples that had been obtained just before transplantation, approximately at the time of the first positive stool (or urine, throat, or sputum) culture, and just before the onset of AdV-related disease or the occurrence of death (to the extent possible; samples were not available from all time points for all patients, because of the retrospective character of this study).

A total of 156 samples was analyzed by PCR (group 1, 62 samples; group 2, 45 samples; group 3, 49 samples), of which 42 were positive for AdV. The 42 positive serum samples had been obtained from 17 patients: from 7 (41%) of 17 patients in group 1 (those who were asymptomatic), from 4 (33%) of 12 patients in group 2 (those with localized disease), and 6 (86%) of 7 patients in group 3 (those with fatal disseminated disease). This indicates that the presence of AdV DNA did not correlate with the presence of clinical symptoms. However, it
did correlate with fatal outcome when the group of patients who did die was compared with the 29 patients who did not die, but the statistical significance was low ($P = .04$; Fisher’s exact test) because DNA was present in the serum of 11 of 29 patients with a nonfatal course of disease.

**Semi-quantitative determination of virus DNA load.** To investigate whether a quantitative measure of the DNA load in serum would be more informative regarding the course of an AdV infection in these patients than the qualitative detection of DNA, semi-quantitative PCR was performed by analyzing serial dilutions of serum template DNA.

AdV DNA was detected in serum samples obtained from 7 of 17 asymptomatic patients (group 1) and 4 of 12 patients with localized disease (group 2) (figure 1). In 9 of these 11 patients, the level of AdV DNA was low. The 2 remaining patients (patients 168 and 286) demonstrated transiently high levels of AdV DNA—that is, a PCR product was still detectable after 1000-fold dilution of template DNA. In contrast, the AdV DNA load was high (detectable at 100-fold or 1000-fold dilution) in serum samples obtained from 6 of the 7 patients with fatal disseminated infection (group 3). These high loads were first detected a mean of 18 days (range, 6–29 days) before death, remained high until death, and were always accompanied and sometimes preceded by stool cultures positive for AdV. The only patient who died and whose PCR results remained negative had serum samples obtained until 3 weeks before death, after which no further serum samples were available.

In general, there was a poor correlation between the presence of AdV DNA in serum and AdV-positive stool cultures. For example, no AdV DNA was detected in serum samples obtained from some patients from groups 1 and 2, despite prolonged periods during which cultures of stool samples were positive for AdV.

Although a transient presence of AdV DNA occurred in patients without clinical symptoms of AdV infection, a persistently high level of AdV DNA correlated with fatal outcome. A high level of AdV DNA in serum (detectable at 100-fold or 1000-fold dilution) was observed for 6 of 7 patients who died. The sample that tested positive belonged to a 13-year-old child, but the sample was positive only when undiluted. Thus, AdV DNA may be detected occasionally in the serum of healthy subjects, but only at very low levels.

**DISCUSSION**

The data presented indicate that a high level of AdV DNA (i.e., detection of a PCR product after 100-fold or greater dilution of template DNA) in serum samples obtained from pediatric patients infected with AdV after undergoing SCT correlates with fatal disseminated AdV disease. A high AdV DNA load was detected in 86% of the patients who developed fatal disease, whereas only 7% of the patients who survived had similar high loads of AdV DNA ($P < .0001$). Recently, it has been reported that the detection of AdV DNA in serum by PCR was associated with fatal AdV disease [33]. However, in the present extended retrospective analysis, AdV DNA was also detected in a significant proportion of patients who survived, albeit at low levels. Thus, quantitative determination of AdV DNA in serum contributes significantly to the identification of patients with disseminated fatal AdV infection.

Only 2 patients without AdV-related clinical symptoms (patients 168 and 286) had high serum levels of AdV DNA, although only transiently. Patient 168 had severe combined immunodeficiency disease and was infected with AdV serotype 31. Before the patient underwent transplantation, stool and urine cultures were already positive for AdV, and a high load of AdV DNA was detected in serum. After transplantation, the DNA load in serum remained high until day 20 after transplantation, then declined until it reached undetectable levels at day 34. Stool culture results also became negative 2 months after transplantation. Patient 286, the other patient with a high level of AdV DNA in serum, underwent transplantation to treat acute lymphoblastic leukemia. Stool cultures were negative for AdV before SCT but became positive at day 6. The AdV isolate was typed as serotype 2. A serum sample obtained at day 21 after transplantation was strongly positive by PCR (at a 1000-fold dilution). Serum samples obtained on days 35 and 42 after transplantation were positive for AdV but at a lower load (at 10-fold and 100-fold dilutions, respectively), indicating a decline in the virus load. At day 70, AdV-DNA was no longer detected in serum, despite the continued presence of AdV in stool. Patient 286 died at day 107 after SCT as a result of a relapse of the leukemia. The reason that both patients had transiently high loads of AdV DNA in serum without any accompanying clinical symptoms is unclear. Interestingly, it appears that, in some patients, high loads of AdV DNA in serum resolved in the absence of any anti-AdV drug treatment.

The group of 36 patients whose serum was analyzed by PCR was originally identified by the detection of AdV in stool cultures. In the period 1985–1999, another 4 patients had
Figure 1. Results of the semiquantitative adenovirus (AdV) PCR for children with AdV infection following stem-cell transplantation (SCT). Children are grouped according to the absence or presence of clinical symptoms of AdV infection (group 1, AdV-infected patients without clinical symptoms; group 2, patients with AdV-related clinical symptoms; group 3, patients with AdV-related fatal outcome). Culture results for stool, throat, and urine samples (if performed), AdV PCR results for serum samples, and mortality (related to AdV infection or not related) are indicated in relation to the time of (first) SCT (dotted vertical line). Data to the far left (under the heading “/H11002 30/ /H11002 7”) were obtained 7–30 days before SCT. All stool culture results (negative and positive) are shown, but only positive urine and throat culture results are indicated. The symbols for PCR results represent the highest dilution of template DNA after which a positive result was still obtained. UPN, unique patient number.
Table 2. Level of adenoviral DNA in serum specimens obtained from adenovirus-infected stem-cell transplant recipients, as determined by semi-quantitative PCR.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n</th>
<th>Negative</th>
<th>Positive, according to dilutiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Relative virus load | Negative | Low | Low | High | High |

NOTE. *P < .0001, for groups 1 and 2 vs. group 3, threshold between 10-fold and 100-fold dilution.

a Highest dilution of template DNA after which a specific PCR product was obtained.

AdV-positive results of cultures of throat swabs in the absence of positive stool culture results, but these patients were all asymptomatic (not shown). Although it is difficult to be certain in this retrospective study, an AdV-positive stool culture appears to indicate all patients who may develop AdV-related clinical symptoms. However, not all patients with an AdV-positive stool culture result developed symptoms or disease, and, therefore, there is a need for an additional tool to differentiate between transient asymptomatic AdV infection and disease caused by AdV.

The results of this study demonstrate that semiquantitative data on AdV DNA load in serum provide clinically relevant information about the outcome of AdV infection, especially with respect to fatal outcome. The less severe clinical symptoms in group 2 were not associated with increased DNA loads in serum. However, this finding could be due to the lack of appropriate serum samples in this retrospective analysis. More-precise quantitative data based on the analysis of serum samples obtained more frequently and in a prospective study will demonstrate whether serum levels of AdV DNA can provide information that allows the course of infection to be predicted more accurately.

Because quantitative data on the AdV DNA load in serum appear to be important, we have started to develop a real-time PCR to detect AdV DNA in an automated and more quantitative fashion [36]. Detection of viral infections by PCR also has several other advantages. Results are obtained faster than with conventional culture techniques. Culture may require 3–4 days for results to be available, whereas PCR detection can be performed in 1 day. Eventually, multiplex PCR reactions may allow simultaneous detection of multiple viruses in one reaction tube.

In this study, a generic PCR that amplifies a conserved region of the hexon gene was used [23, 25, 33]. The advantage of this strategy was that all clinically relevant AdV strains were detected. An additional possibility might be that the availability of a segment of AdV DNA amplified from a clinical sample would provide the opportunity to type the infecting virus by sequencing the PCR product without the need for time-consuming virus neutralization tests. To investigate whether virus strains could be typed on the basis of this limited sequence information, PCR products of a number of serotyped clinical isolates were sequenced. Comparison of the sequences from the PCR fragments with published sequences revealed that sequence analysis, in this limited number of cases, could provide sufficient information to assign an unknown strain to a certain subgroup of human AdV but not to a specific serotype (data not shown). This single primer pair therefore seems suitable for detection and, potentially, for partial typing of all clinically relevant AdV strains.

Although the diagnosis of AdV infections may be improved by use of quantitative PCR, treatment options to reduce the number of fatalities are still very limited. Ribavirin and cidofovir therapy have been used in recent years with variable success [9, 10, 37–39]. No systematic study of the effectiveness of these drugs has been published. Preemptive treatment based on an increase in the AdV DNA load in serum followed by careful quantitative monitoring of DNA loads in patients with AdV infection could provide objective information on the antiviral efficacy of these drugs in immunocompromised patients. The time period between detection of a high level AdV DNA and death should be long enough to initiate treatment at a stage when the disease has not yet progressed to a terminal course. In this retrospective study, this time period varied from 6 to 29 days. It is possible that more-frequent sampling may have the additional benefit of detecting AdV DNA in blood earlier, allowing for the rapid initiation of treatment. Because quantification of the AdV DNA load during infection in this patient group appears to be a valuable tool for both diagnosis and clinical management of AdV infection, we have initiated a prospective study to address these issues more systematically.
References


