Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease

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Adenovirus (AdV) infection in the course of allogeneic stem cell transplantation (SCT) is associated with high transplant-related morbidity and mortality. Disseminated AdV disease is lethal in most instances. Early detection of AdV infection and identification of patients carrying a high risk of disseminated disease therefore remain a major challenge. In view of the large number of existing AdV types, we have established real-time polymerase chain reaction (PCR) assays permitting sensitive detection and quantification of all 51 currently known human AdV serotypes. In a series of 132 consecutive pediatric patients undergoing SCT, more than 5000 samples derived from peripheral blood (PB), stool, urine, and throat were screened for adenovirus infection by PCR during the posttransplantation period. Thirty-six patients (27%) tested positive by PCR, revealing AdV types of the subgenera A, B, C, D, and F. Except for enteritis in some patients with AdV positivity in stool, detection of the virus at sites other than PB was not associated with clinical signs of virus disease, and transplant-related mortality was not significantly different from AdV-negative patients. By contrast, 82% of patients who had detectable AdV in PB died from infectious complications ($P < .001$). Monitoring of PB specimens by real-time PCR permitted early diagnosis of invasive AdV infection in all instances. In patients who developed disseminated AdV disease, detection of the virus in PB preceded onset of clinical symptoms by a median of more than 3 weeks. The observation of AdV in peripheral blood may therefore serve as a basis for early initiation of preemptive antiviral treatment. (Blood. 2003;102: 1114-1120)

Introduction

Adenoviruses are pathogens causing serious infections in patients undergoing allogeneic bone marrow transplantation (allo-BMT).[1-3] To date, 51 different human adenovirus (AdV) serotypes have been identified.[4,5] They are divided into 6 major subgroups (subgenera or species A-F) on the basis of their oncogenic, hemagglutinating, morphologic, and DNA sequence properties.[6-8] In immunosuppressed patients, any adenovirus species may cause life-threatening infections.[2,3,8,9] In most clinical situations involving adenovirus infection, species identification of an AdV isolate is as informative as a finer identification by serotype.[6]

In different studies, adenoviruses have been found to infect up to 20% of patients receiving allo-BM transplants, with a particularly high incidence of infection and virus disease in children.[8,10,11] Infections can be asymptomatic or cause localized disease such as enteritis, upper respiratory tract infection, or cystitis.[12] However, AdV infections in allo-BM transplant recipients tend to become invasive, and disseminated disease is associated with very high mortality.[8,10-17]

Earlier diagnostic approaches to AdV detection relied mainly on serologic tests and cell culture.[18-23] In immunocompromised patients, however, the use of serologic tests is limited because of the impaired immune response, and evaluation of positive cultures is a relatively slow method.[24] The introduction of polymerase chain reaction (PCR)–based assays has opened new ways to rapid, specific, and highly sensitive AdV detection.[4,12-15,17,25]

In view of the fact that many of the published diagnostic approaches do not effectively cover all AdV types, we have established species-specific real-time PCR assays permitting reliable detection and quantification of all 51 currently known human AdV serotypes. In the current study in pediatric patients after allogeneic stem cell transplantation, we have addressed the clinical significance of molecular AdV detection and investigated the potential of serial real-time PCR analysis to facilitate diagnosis of invasive infection early in its preclinical stage.

Patients and methods

Patients

All pediatric patients who underwent allogeneic stem cell transplantation (allo-SCT) at St Anna Children's Hospital, Vienna, Austria, between June 1996 and May 2002 were included in the study (n = 132). Written informed consent was obtained from each patient and/or the parents, and the transplantation protocol has been approved by the institutional review board of St Anna Children's Hospital. Patient characteristics and transplantation modalities are summarized in Table 1. Ex vivo T-cell depletion by $CD34^+$ positive selection was performed in case of antithymocyte globulin (ATG)–mismatched, unrelated, or haploidentical family donors. Patients with severe combined immunodeficiency, patients with severe aplastic anemia, and patients with mismatched or unrelated donors received ATG from day −3 to −1. Graft-versus-host disease (GvHD) prophylaxis consisted of cyclosporine A and, in recipients of non–T-cell–depleted grafts from unrelated donors, of an additional short methotrexate (MTX) course. All patients were nursed in laminar air flow units. Antiviral prophylaxis

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Table 1. Patient characteristics, transplantation modalities, and acute GvHD, N = 132

Observation time	Median, 23 mo (range, 2-71 mo)
Age at SCT	Median, 8 y (range, 0.1-20 y)
Diagnosis	Acute leukemia, n = 63
	Chronic leukemia/myelodysplasia/solid tumor, n = 23
	Severe combined immunodeficiency, n = 8
	Inborn errors/severe aplastic anemia, n = 38
Conditioning	Chemoconditioning, n = 80
	Irradiation (TBI/TLI)–containing conditioning, n = 52
	Additional antithymocyte globulin (ATG), n = 103
Donor	HLA-matched sibling donor, n = 35
	HLA-matched unrelated donor, n = 68
	HLA-mismatched family donor, n = 29
Graft	Bone marrow without $CD34^+$ positive selection, n = 62
	Peripheral blood stem cells without $CD34^+$ positive selection, n = 12
	Peripheral blood stem cells with $CD34^+$ positive selection, n = 58
Acute GvHD	All patients (pts) (n = 132)
	GvHD, any grade, n = 72
	GvHD, grade III-IV, n = 27
	AdV-negative pts (n = 96)
	GvHD, any grade, n = 51
	GvHD, grade III-IV, n = 18
	AdV-positive pts at any site, n = 36
	GvHD, any grade, n = 21
	GvHD, grade III-IV, n = 9
	AdV-positive pts in PB, n = 11
	GvHD, any grade, n = 5
	GvHD, grade III-IV, n = 3

consisted of acyclovir 30 mg/kg per day intravenously from day −7 until day +28 and immunoglobulin substitution every 3 weeks, until day +100.

Patients with a positive PCR test in peripheral blood for cytomegalovirus (CMV) or AdV were eligible for preemptive antiviral treatment, regardless of the viral load. Treatment was initiated on availability of PCR results, generally within 48 to 72 hours after sampling. Patients with CMV-DNAemia received primary preemptive therapy with ganciclovir and, in case of persistent DNAemia, secondary preemptive treatment with foscarnet, until 2 consecutive negative results were obtained. Patients with adenovirus-DNAemia undergoing transplantations between October 1998 and May 2002 (n = 7) received preemptive treatment with cidofovir (5 mg/kg per week according to a protocol requiring continuation of treatment until attainment of 2 negative PCR results).

Sample collection

Whenever possible, patients were tested prior to transplantation, and prospective virus screening in peripheral blood (PB), stool, urine, and throat for CMV, Epstein-Barr virus (EBV), AdV, human herpesvirus 6 (HHV6), and HHV7 was performed at 3- to 7-day intervals until day +28. Subsequently, the intervals in patients who tested negative were extended to 1 to 2 weeks, until day +100 after transplantation. In patients who tested positive for any virus until day + 28, the screening was continued at 3- to 7-day intervals, until 2 consecutive negative results were obtained. After day +100, tests were carried out only in case of clinically suspected viral infection. More than 5000 samples derived from the sites indicated, and occasionally from cerebrospinal fluid, organ biopsies, and bone marrow, were screened by PCR-based DNA analysis. The average number of samples investigated in each patient was 50 (range, 11-75).

Isolation of viral DNA

DNA extraction from largely cell-free liquids except urine and from peripheral blood leukocytes for detection of intracellular virus particles was performed using QIAamp DNA Mini Kit. Isolation of virus DNA from urine was done using the QIAamp Viral RNA Mini Kit, and from stool by the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations.

Quantitative PCR analysis of AdV

Six different primer/probe systems were designed on the basis of the available AdV DNA sequence information (National Center for Biotechnology Information [NCBI] database) to permit detection of the individual AdV species (subgenera) A to F. The AdV DNA sequences targeted by the primer/probe systems were derived from the hexon and VA RNA genes. The characteristics of the primers/probes used are indicated in Table 2. Reference strains of all 51 human adenovirus serotypes (kindly provided by H. Niesters, Department of Virology, University of Rotterdam, The Netherlands) were used as positive controls for the establishment of AdV-specific real-time PCR assays. Each of the 6 primer/probe systems were shown to permit sensitive and specific detection of all AdV serotypes belonging to the respective species. The only cross-reactivity observed involved the detection system for AdV species B that recognized also species E (including only serotype 4). By contrast, the detection system for species E detected exclusively this genotype under the conditions used.

PCR reactions were set up in a total volume of 25 μL, including 6 μL template DNA, and 12.5 μL TaqMan Universal Master Mix. The concentration of primers and carboxyfluorescein (FAM)–labeled probe for each detection system are displayed in Table 2. Amplifications were carried out using ABI Prism 7700 or 7900 Sequence Detectors (Applied Biosystems,

Table 2. Primers and probes for species-specific adenovirus detection by real-time PCR

Virus type	Target	Amplicon length, bp	Oligonucleotide sequence, 5′-3′, forward, probe, reverse	Concentration, nM	Nucleotide position	GenBank Accession no.
AdV A	Hexon gene	135	GGK CTG GTG CAA TTC GCC	300	17818-17835	X73487
			CCA CGG ACA CCT ACT TCA CCC TGG G	200	17840-17864	
			CAC GGG CAC AAA ACG CA	300	17936-17952	
AdV B (B + E)	Hexon gene	138	CGC CGG ACA GGA TGC TT	900	45-61	X76549
			AGT CCG GGT CTG GTG CAG TTC GCC	200	73-96	
			CTA CGG TCG GTG GTC AC	900	166-182	
AdV C	Hexon gene	138	ACC TGG GCC AAA ACC TTC TC	300	2884-2903	J01966
			AAC TCC GCC CAC GCG CTA GA	200	2910-2929	
			CGT CCA TGG GAT CCA CCT C	900	2940-2958	
AdV D	VA RNA gene	143	AAA AAC GAA AGC GGT TGA GC	300	2-21	U10675
			CCA ATA CCA CGT TAG TCG CGG CT	200	104-126	
			CGG GTC GAG ACG GGA GT	50	128-144	
AdV E	Hexon gene	75	CAA CAC CTA CTC GTA CAA AGT GCG	900	225-248	X84646
			CGC CCA CGG CCA GCG TGT	200	251-268	
			TAG GTG CTG GCC ATG TCC A	300	281-299	
AdV F	Hexon gene	128	GCA GGA CGC CTC GGA GTA	300	268-285	D13781
			TAC TTC AGC CTG GGG AAC AAG TTC AGA AA	200	329-357	
			TGT CTG TGG TTA CAT CGT GGG T	900	374-395	

Foster City, CA) for a total of 50 cycles. After an initial denaturation step for 10 minutes at 95°C, each cycle consisted of denaturation for 15 seconds at 95°C and annealing and primer extension for 60 seconds at 60°C.

Strict precautions were undertaken to prevent contamination of PCR reactions with exogenous products as described.[26] A deoxyuridine triphosphate (dUTP) glycosylase step was performed prior to each PCR reaction. Each DNA sample was analyzed in duplicate, and multiple negative controls were included in each assay.

For quantitative analysis of virus load, real-time TaqMan PCR was performed using the equipment indicated earlier. External standard curves for each AdV species were established by making serial dilutions of quantified virus preparations derived from reference strains. For assessment of virus copies per cell, a single copy gene (β2 microglobulin) was quantified in parallel by real-time PCR.[27] When investigating cell-free liquids, quantitative results were expressed as the number of virus copies per milliliter. In stool samples, the virus copies were calculated per gram material, and intracellular virus copies were indicated per 10^6 cells.

The sensitivity of the PCR assays permitted reliable detection of 2×10^2 virus particles/mL of the medium investigated. For reproducible quantification of virus load, however, the presence of 10^3 particles/mL or more was necessary. When analyzing cell material, sensitivity of the assays permitted detection and quantification of virus copies at a level of 10 particles/10^6 cells.

Statistical analysis

The risk factors for AdV infection analyzed in the univariate analysis included T-cell depletion in vivo and ex vivo, underlying disease, type of donor, graft-versus-host disease of all grades, and presence of other virus infections. Differences between patient groups, including (1) AdV-negative patients, (2) patients positive for AdV at 1 or 2 sites but negative in PB, and (3) patients positive for AdV in PB, were analyzed using the chi-square test. For analysis of small cohorts, Fisher exact test was used. The association between AdV infection and GvHD of all grades was analyzed by Mantel-Haenszel chi-square test.

The overall survival rate was estimated by the method of Kaplan-Meier. The cumulative incidence was estimated for the time to first AdV positivity in PB and time to onset of AdV disease. Depending on the subgroup analyzed, the intervals start at BMT, at first AdV positivity in PB, or at the time of AdV disease onset, respectively (Figure 1). For the comparison of patients who tested AdV positive in PB with patients who tested negative in PB, the interval for the latter group starts at the median time to AdV positivity (ie, day +16), to account for the time-dependent nature of AdV positivity in PB (Figure 1C). The effect of CMV positivity, AdV positivity, and AdV disease on survival was studied by including the respective variables as time-dependent covariates in a Cox regression model. The effect of CMV positivity on the time to AdV positivity in PB was analyzed in the same fashion. Relative hazard rates (relative risk) are indicated in addition to P values. All indicated P values are 2-sided. $P \leq .05$ was considered significant; P values of .01 or less were highly significant.

Definitions

Localized and disseminated adenovirus infection and definite and probable adenovirus disease were defined as described,[11,28] with minor modifications reflecting the use of PCR detection assays: patients with reproducibly positive adenovirus PCR screening assays in specimens derived from any site or positive tests from sources related to their clinical signs and symptoms were considered to have adenovirus infection. Disseminated (invasive) infection was defined as the presence of positive tests from 2 or more organ systems and/or positive tests from peripheral blood.

Definite disease was defined as the presence of typical adenovirus nuclear inclusions on routine histopathology, a positive culture from tissue (except from the gastrointestinal tract), or both. Probable adenovirus disease was defined as the presence of 2 or more positive PCR assays in samples from peripheral blood or from more than 2 different body sites, in association with compatible symptoms, without other identifiable causes. All patients with fever, unexplained symptoms, or laboratory test abnormalities were carefully tested for infections and for GvHD.

Results

Incidence and spectrum of adenovirus infections in children after allogeneic stem cell transplantation

Patients who tested AdV positive are summarized in Table 3. Thirty-six patients (27%) showed positive results in samples derived from at least one site. Among the AdV-positive patients, 25 (69%) had detectable virus at 1 or 2 sites, most commonly in stool or throat samples or both, but never tested positive in PB (Table 3, patients 1-25). In these patients, AdV positivity has appeared at a median of 18 days after transplantation (range, −7 to > +100 days). Patients who tested AdV positive at more than 2 different sites also showed presence of the virus in PB. Adenoviruses were detected in PB samples of 11 children, ie, 31% of the AdV positive cases, and 8% of all patients (Figure 1A; Table 3, patients 26-36). First appearance of adenoviral DNA in PB specimens has been observed at a median of 16 days after transplantation (range, −7 to > +100 days).

In our series, adenoviruses of all but one species were detected: groups A, B, C, D, and F were represented, but no sample was positive for species E, the smallest subgroup including only one serotype (no. 4). The most prevalent AdV species in the cohort investigated was group C (78% of all positive cases), whereas other types were observed less frequently (A, 8%; B, 8%; D, 8%, F, 8%). Four patients were positive for 2 different AdV species: In 2 instances, the species C+B were present, 2 other patients displayed

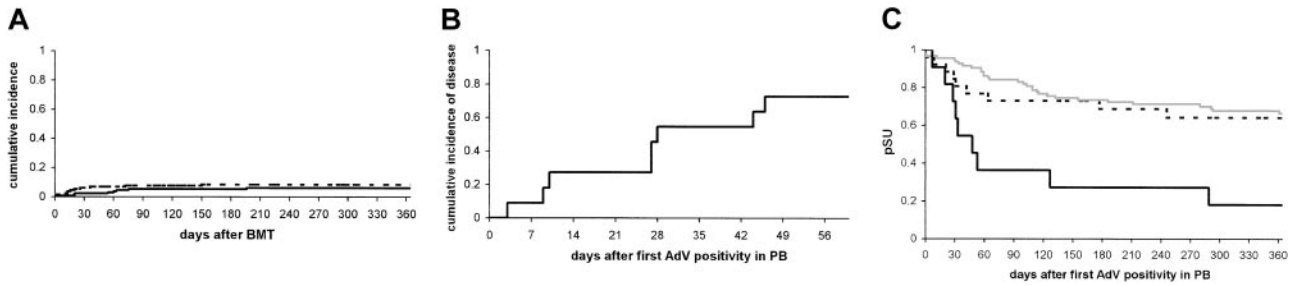

Figure 1. Incidence of invasive adenovirus infection, disseminated disease and overall survival. (A) Probability of invasive AdV infection and disease. In the cohort of patients investigated, the 1-year cumulative incidence of invasive AdV infection after SCT (dashed curve), documented by virus detection in peripheral blood, was 8% (± 8%), that of AdV-associated disease (solid curve) 6% (± 6%). (B) Probability of disease after AdV detection in PB. The cumulative incidence of disease was 73% (± 10%) within 60 days after the first AdV-positive PCR test in PB. (C) Probability of survival in patients with AdV-positive or -negative PCR tests. The black (bottom) curve indicates the overall survival in patients with AdV positivity in PB. The 1-year probability of survival (pSU) in this cohort was 18% (± 9%). The 2 upper, nearly identical curves illustrate the overall survival in patients who tested AdV negative in PB, but positive at 1 or 2 other sites (dashed gray curve; 1-year pSU of 64% [± 10%]), and in AdV-negative patients (solid gray curve; 1-year pSU of 67% [± 5%]). The difference between patients positive for AdV in PB and the other 2 cohorts illustrated is highly significant ($P < .001$). By contrast, there was no statistically significant difference in pSU between AdV-negative patients and patients who tested AdV positive at sites other than PB ($P = .706$).

Table 3. Characteristics of adenovirus-positive patients

Patient	Age, y	Diagnosis	Donor	Source	T-cell depletion	aGvHD during AdV infection/ grade*	AdV species	Site of detection	Day of CMV detection in PB before/after BMT†	Day of AdV detection before/after BMT‡	AdV disease	Outcome	Day of death after BMT	Cause of death
1	9	WG	MSD	BM	No	No	D	Stool	+56	+7	No	A/W		
2	3	AML	MUD	PBSC	1+2	No	C	Stool	−13	0	No	A/W		
3	20	ALL	MUD	BM	1	No	F	Stool, organ biopsy (postmortem)	ND	+59	No	Died	+101	CMV
4	1	HLH	MMFD	PBSC	1+2	NE	F	Stool, throat	−10	−7	Enteritis	Died	+2	HLH
5	8	ALL	MMFD	PBSC	1+2	No	C	Stool	+1	>+100	Enteritis	A/W		
6	3	ALL	MUD	PBSC	1+2	No	A	Stool	+17	>+100	Enteritis	Died	+131	Toxicity
7	8	ALL	MUD	BM	1	No§	C	Stool	+12	+90	No	A/W		
8	3	FHL	MUD	PBSC	1+2	No	C+B	Stool, throat (C)/stool (B)	−5	−7 (C)/>+100 (B)	No	Died	+153	Aspergillus
9	5	FA	MMFD	PBSC	1+2	No	C	Stool	ND	+49	Enteritis	Died	+70	Toxicity
10	2	FHL	MMFD	PBSC	1+2	No	C	Throat	ND	> +100	No	A/W		
11	10	ALL	MMFD	PBSC	1+2	No	C	Stool	ND	+91	No	Died	+120	Legionella
12	10	CML	MSD	BM	No	No	C	Skin	ND	> +100	Rash	A/W		
13	7	SAA	MFD	PBSC	1+2	No	C	Throat	ND	> +100	No	A/W		
14	3	AML	MUD	PBSC	1	No	C	Organ biopsy (postmortem)	ND	+39	No	Died	+39	Toxicity
15	16	SAA	MMFD	PBSC	1+2	NE	C	Throat	ND	+10	No	Died	+74	Aspergillus
16	4	XLP	MUD	PBSC	1+2	No	C	Stool	ND	+8	No	A/W		
17	11	AML	MSD	BM	No	No	D	Stool	+52	+10	No	A/W		
18‖	1	MPS I	MUD	PBSC	1+2	Yes/3	C	Stool	+6	+53	Enteritis	A/W		
19‖	1	MPS I	MUD	PBSC	1+2	Yes/3	C	Stool	ND	+23	Enteritis	A/W		
20	7	MDS	MSD	BM	No	No	C	Stool	−12	−7	No	A/W		
21	0	SCID	MMFD	PBSC	1+2	No	C	Stool	ND	+18	Enteritis	Died	+170	SID
22	9	ALL	MUD	PBSC	1	No	C	Stool	ND	−7	No	A/W		
23	1	HLH	MUD	PBSC	1+2	No	C	Stool	ND	−7	No	A/W		
24	8	AML	MUD	BM	1	No	D+F	Stool	ND	−7 (D)/+19 (F)	No	A/W		
25	1	CGD	MUD	BM	1	No	C	Stool	ND	−7	No	A/W		
26‖	5	ALL	MMFD	PBSC	1+2	No	C	PB	+4	+16	Disseminated	Died	+69	AdV
27	0	JMML	MUD	BM	1	No	C	PB, stool, throat	−11	+18 [+20]	No	Died	+424	CMV
28	5	ALL	MMFD	PBSC	1+2	No§	C	PB, stool, organ biopsy (postmortem)	> +100 [+139]	> +100 [+150]	Disseminated	Died	+198	AdV
29	3	AML	MMFD	PBSC	1+2	No	C	PB, stool, urine	+41	−7 [+13]	No	Died	+302	Relapse
30	9	FA	MUD	PBSC	1+2	No	C	PB, stool, urine, throat	+38	+72	Disseminated	Died	+79	AdV
31‖	9	CML	MUD	PBSC	1+2	No	C	PB, organ biopsy (postmortem)	−43	−7	Disseminated	Died	+8	AdV
32	2	JMML	MUD	BM	1	Yes/2	C	PB	ND	+13	No	A/W		
33	12	AML	MUD	PBSC	1	No	C	PB, stool, throat, urine, organ biopsy (postmortem)	+14	+36	Disseminated	Died	+64	AdV
34	3	AML	MUD	PBSC	1+2	No	A	PB, throat, liver	−10	−7	Disseminated	Died	+24	AdV
35	8	ALL	MMFD	PBSC	1+2	No	A+B	PB, stool, throat, urine, CSF (A)/stool (B)	ND	+11 (A)/−7 (B)	Disseminated	Died	+31	AdV
36‖	2	MPS I	MUD	PBSC	1+2	Yes/4	B+C	PB, stool, throat, urine, CSF, eye (C)/stool (B)	ND	−7 (C) [+26]/ > +100 (B)	Disseminated	Died	+151	AdV

Patients 1 to 25 are those who tested RQ-PCR positive at 1 or 2 sites other than peripheral blood. Patients 26 to 36 are those who tested RQ-PCR positive at sites including peripheral blood. AdV indicates adenovirus; CMV, cytomegalovirus; BMT, bone marrow transplantation; WG, Wegener granulomatosis; MSD, matched sibling donor; BM, bone marrow; A/W, alive and well; AML, acute myeloid leukemia; MUD, marrow unrelated donor; PBCS, peripheral stem cell transplantation; T-cell depletion 1, antithymocyte globulin (ATG); T-cell depletion 2, CD34+ positive selection; ALL, acute lymphoblastic leukemia; ND, not detected; HLH, hemophagocytic lymphohistiocytosis; MMFD, mismatched family donor; NE, not evaluable; FHL, familial hemophagocytic lymphohistiocytosis; FA, Fanconi anemia; CML, chronic myeloid leukemia; SAA, severe aplastic anemia; XLP, X-linked lymphoproliferative disease; MPS 1, mucopolysaccharidosis type 1; SCID, severe combined immunodeficiency; SID, sudden infant death; CGD, chronic granulomatous disease; JMML, juvenile myelomonocytic leukemia; CSF, cerebrospinal fluid.

*The highest grade of acute graft-versus-host disease (aGvHD) observed during the time of AdV infection is indicated. (For overall incidence of aGvHD during the posttransplantation period, see Table 1).

†Patient 31 is indicated as CMV positive on day −43. The availability of CMV data at this unusual time point is attributable to the fact that the patient had undergone his first SCT more than 3 months earlier. In patients who had both AdV and CMV detectable in PB (26-31, 33, 34), the median time point of first PCR positivity was day +9 (range, −43 to +139) for CMV and +14.5 (range, −7 to +150) for AdV. In one instance (patient 31), AdV detection in PB preceded that of CMV by 28 days, but in the remaining cases detection of CMV preceded first AdV positivity in PB by 3 to 36 days (median, 17). None of the patients who tested CMV positive prior to transplantation had evidence of active infection, as revealed by RQ-PCR monitoring.

‡The letters in parentheses indicate the AdV species. The appearance of AdV in PB is indicated in brackets if the virus was first detected at a site other than PB. Two patients (31 and 34) had AdV detectable in PB at the commencement of conditioning, but the transplantation could not be postponed because of the presence of leukemia in progression. In all other patients with AdV infection prior to transplantation, the virus was detectable in stool only.

§These patients had chronic GvHD during the course of AdV infection. In both instances, the GvHD was graded as extensive.

‖Patients who had undergone a prior SC transplantation.

the species A+B and D+F, respectively. Among patients who tested positive in PB, 9 (82%) displayed species C, and 2 (18%) species A.

Risk factors for AdV infection

T-cell depletion in vivo by ATG combined with T-cell depletion ex vivo by $CD34^+$ positive selection was associated with a significantly increased incidence of AdV infection ($P = .014$). T-cell depletion by ATG alone showed no significant association ($P = .493$). Children who received transplants from matched sibling donors (MSDs) had a significantly lower occurrence of AdV infection compared with the cohort of patients with matched unrelated donors (MUDs) and mismatched family donors (MMFD ($P = .014$).

No statistically significant correlation with AdV detection has been found for other parameters, including the presence of graft-versus-host disease (GvHD), regardless of the grade ($P = .725$), and for the underlying disease (nonmalignant versus malignant) ($P = .314$).

Association of AdV detection with presence of other viruses

Of the viruses tested in addition to AdV, including CMV, EBV, HHV6, and HHV7, only CMV was significantly correlated with detection of AdV in PB. Within the group of AdV PB-positive patients, 8 of 11 (73%) had detectable CMV in PB versus 33 of 96 (34%) in AdV-negative patients ($P = .020$). In contrast, the difference in the detection of CMV in PB between AdV-negative cases and patients who tested AdV positive at sites other than PB, 33 of 96 (34%) versus 10 of 25 (40%), was not significant ($P = .601$).

In the patients with positive PB tests for both AdV and CMV during the time of observation, all but one revealed CMV positivity before the first positive AdV test (Table 3). However, in contrast to the dynamics of AdV load observed in most of these patients ("Quantification of virus load in patients with invasive AdV infection"), there was no evidence of increasing CMV levels in PB. Cox regression analysis revealed that patients who tested CMV positive in PB had a 4.4-fold increased risk of invasive AdV infection. However, detection of CMV in PB per se, in the absence of expanding viral load, was not a significant factor for survival in the patients studied (relative risk, 1.3). Hence, despite a significant association with later occurrence invasive AdV infection, CMV was not an independent covariable with regard to transplant-related mortality (TRM).

Adenovirus infection and transplant-related mortality

In children with AdV detectable at sites other than PB, the only signs of adenoviral disease included enteritis in 7 patients with AdV positivity in stool specimens and generalized rash in one patient with AdV-positive skin biopsy (Table 3). By contrast, 8 of 11 patients who had detectable AdV in PB had clinical evidence of disseminated adenoviral disease (Figure 1B; Table 3).

In the group of AdV-negative patients, 28 children (29%) died during the observation period. In 8 instances (8%), the fatal outcome was attributable to disease relapse and in 20 cases (21%) to TRM. In the cohort of adenovirus-positive patients who had no detectable AdV in PB (Table 3, patients 1-25), 8 (32%) died. One patient (4%) died from the underlying disease, and 7 (28%) from TRM (ie, organ toxicity n = 3 or infectious complications, including CMV pneumonia n = 1, legionella n = 1, and invasive aspergillosis n = 2). The difference in TRM-related deaths between the 2 cohorts, 21% versus 28%, was not statistically significant (relative risk, 1.2; $P = .706$). In the group of patients who were AdV positive in PB (Table 3, patients 26-36), 10 (91%) died. One patient (9%) died from relapse, and 9 (82%) from TRM. The association of AdV positivity in PB with TRM was highly significant (relative risk, 5.8; $P < .001$) (Figure 1C). One of these patients died from CMV pneumonia more than 3 months after transient AdV positivity in PB, and 8 children developed disseminated AdV disease with multiorgan (hepatic, pulmonary, renal) failure. The correlation between onset of AdV-related disease and mortality was highly significant (relative risk, 31.4; $P < .001$). Adenoviral DNA was detected in PB at a median of 29 days before death (range, 7-112 days). The AdV types observed in PB of these patients belonged either to species C (n = 6) or A (n = 2).

Quantification of virus load in patients with invasive AdV infection

In patients who tested AdV positive at any site, virus load was monitored at 3- to 4-day intervals. The virus load was usually low in patients presenting with AdV positivity at sites other than PB ($< 2 \times 10^4$ virus copies per gram of tissue, eg, stool, or per milliliter of liquid, eg, urine), and there was mostly no evidence of rising virus copy numbers during the observation period. Only 3 patients (Table 3; patients 19, 35, and 36) showed dramatically increasing AdV levels in stool samples, reaching copy numbers of more than 10^8/g. During this period, one of the patients (19) had hemorrhagic diarrhea, which was treated with cidofovir, and resolved together with prompt reduction of AdV load in stool. Two other patients (35 and 36) later showed AdV positivity in PB with rapidly rising virus load and died shortly thereafter.

Among patients who tested AdV positive in PB (Table 3, patients 26-36), 3 showed only transient positivity with virus copy numbers ranging between 2×10^2/mL and 2×10^3/mL PB or per 10^6 leukocytes, without any dynamics indicative of virus proliferation. None of these patients had evidence of adenoviral disease during the observation period; one is alive and well 30 months after transplantation, one died from disease relapse, and one died from CMV pneumonia more than 3 months after AdV had become negative in PB (Table 3, patients 32, 29, and 27, respectively). All other patients who had detectable AdV in PB developed disseminated disease (Figure 1B). The maximum AdV load in PB detected in these patients prior to the onset of disease varied over more than 4 logs: 3 patients had very high maximum levels 10^7 or more (Table 3; patients 26, 28, and 34), 2 had intermediate levels of 10^5 and 5×10^6, respectively (Table 3; patients 35 and 36), but in 3 patients the maximum virus copy numbers detected were low, ranging from 1 to 9×10^3 (Table 3; patients 30, 31, and 33). With the exception of 2 children who died very shortly after first detection of the virus in PB (Table 3; patients 30 and 31), the virus load in PB could be monitored by quantitative PCR analysis during the course of infection. In these patients (Table 3; patients 26, 28, and 33-36), the kinetics of AdV proliferation revealed a 10-fold increase in virus load at a median of 21 days (range, 6-37 days) prior to the onset of clinical symptoms, and 26 days (range, 12-118 days) prior to fatal outcome.

Discussion

In the current study, pediatric patients undergoing allogeneic SCT were investigated for adenovirus infections by a panadenoviral PCR assay system. The observation of AdV infections in 27% of the children investigated is rather high compared with earlier

retrospective studies.[11,13,20,24] In the patients presented, detection of adenovirus at more than 2 sites was always associated with presence of the virus in peripheral blood (PB), indicating the presence of invasive virus infection.[11,15,21] In these patients, AdV was first detected at a median of 16 days after transplantation. In children with noninvasive AdV infections, with detectable virus at 1 or 2 sites not including PB, the virus was detected at a median of 18 days after SCT. However, the time range of first AdV detection was rather large in both groups (Table 3), as reported previously.[11,20,24]

In the clinical setting, species identification of an AdV isolate has been reported to be as informative as the analysis of individual serotypes.[6,25] Specific targeting of AdV DNA sequences by PCR is hampered by major genetic differences between the 6 species, with an overall sequence homology of less than 25%.[8,29,30] Most of the published PCR assays for AdV detection are based on amplification of fragments derived from the hexon gene, the most highly conserved gene in the AdV genome.[17,30,33] Comparative analysis of the currently available sequence information on the hexon gene from different types of adenovirus revealed a nucleotide homology of about 50% (NCBI database). This observation explains the apparent difficulty in establishing diagnostic assays that permit detection of the entire spectrum of human adenoviruses. Indeed, careful sequence analysis indicated that some of the published PCR tests targeting the hexon gene that were supposed to cover all AdV types are not likely to meet the expectations.[4,34-39]

For the purpose of this study, we have, therefore, established species-specific real-time quantitative PCR assays covering the entire spectrum of known human AdV types. Fatal infections were observed in patients infected with the AdV species A and C. Other studies in patients after allogeneic SCT, after chemotherapy of malignant disorders, or after organ transplantation have reported fatal outcome for infections with other AdV types, including also the species B, D, E, and F.[2,3,8,11,21,24,36,40] In our series, species C was by far the most prevalent type of adenovirus, and most of the fatal infections were associated with this AdV subgroup. However, the reported prevalence of individual AdV types associated with severe infections at various transplantation centers was quite heterogeneous.[2,3,8,11,21,24,36,40] The data available to date indicate that adenoviruses of any species may cause life-threatening infections in allografted patients. In view of the fact that there is no clear evidence against the association of individual AdV types with severe infections, the screening methods used in immunocompromised patients should be required to permit sensitive detection of all adenoviral serotypes.

Our experience, indicating that detection of adenovirus at multiple (ie, more than 2) sites reflects presence of invasive infection, is in concert with other reports.[15,20,21] In our series, however, 73% of the patients with AdV detectable in PB, but none of the patients with AdV detectable at sites other than PB, developed fatal disseminated AdV disease. First detection of the virus in PB preceded fatal outcome of the infection by a median of 29 days. In agreement with a recent retrospective pilot study,[13] our results demonstrate that detection of AdV in PB is highly predictive for TRM ($P < .001$).

Some of the patients who developed disseminated disease had very high levels of AdV copies in PB ($> 10^7$/mL or per 10^6 cells), whereas others had relatively low peak levels, between 10^3 and 10^4. Regardless of the maximum AdV levels reached, quantitative monitoring of virus load by real-time PCR usually revealed rising virus copy numbers before the onset of clinical symptoms. It should be noted that the documentation of virus proliferation virtually eliminates a problem inherent in PCR diagnosis, the occurrence of false-positive PCR tests because of inadvertent contamination with extraneous nucleic acids. Moreover, the assessment of virus proliferation kinetics permits clear distinction between latent and active adenovirus infection. We have shown previously for quantitative PCR assays that a 10-fold increase of the respective target copy number is well beyond the intrinsic variability of the method and provides reliable evidence of an expanding process.[41] Tenfold increase in virus load preceded clinical signs of adenovirus disease by a median of 3 weeks. These observations indicate that monitoring of adenovirus proliferation kinetics is more informative than the assessment of absolute virus load at a given time point. Our results, therefore, suggest that repeated detection of AdV in peripheral blood and documentation of rising virus copy numbers may provide the most reliable approach to diagnosis of invasive infection and prediction of adenovirus disease. It is necessary to keep in mind, however, that the options for effective therapy directed against adenoviral disease are still limited[12,15,23,42-46] and that delayed onset of treatment has been associated with a greater risk of treatment failure.[45] In view of the highly significant association of adenovirus detection in peripheral blood with the risk of fatal virus disease, a positive PCR test in PB should prompt the initiation of preemptive antiviral treatment. The availability of firm evidence for actively proliferating, invasive adenoviral infection, as revealed by rising virus numbers in PB, will gain clinical relevance when more effective antiadenoviral treatment becomes available. Currently, the main use of quantitative monitoring of adenoviral load is the assessment of the efficacy of treatment. The surveillance of patients after hematopoietic stem cell transplantation by molecular techniques that permit rapid and sensitive detection of the entire spectrum of human adenoviruses can be expected to contribute to an improvement of clinical outcome in patients with invasive adenoviral infections.

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