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Prospective Comparison of the Diagnostic Potential of Real-Time PCR, Double-Sandwich Enzyme-Linked Immunosorbent Assay for Galactomannan, and a (1→3)-β-D-Glucan Test in Weekly Screening for Invasive Aspergillosis in Patients with Hematological Disorders

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The establishment of an optimal noninvasive method for diagnosing invasive aspergillosis (IA) is needed to improve the management of this life-threatening infection in patients with hematological disorders, and a number of noninvasive tests for IA that target different fungal components, including galactomannan, (1→3)-β-D-glucan (BDG), and *Aspergillus* DNA, have been developed. In this study, we prospectively evaluated the diagnostic potential of three noninvasive tests for IA that were used in a weekly screening strategy: the double-sandwich enzyme-linked immunosorbent assay (ELISA) for galactomannan (Platelia Aspergillus), a real-time PCR assay for *Aspergillus* DNA (GeniQ-Asper), and an assay for BDG (β-glucan Wako). We analyzed 149 consecutive treatment episodes in 96 patients with hematological disorders who were at high risk for IA and diagnosed 9 proven IA cases, 2 probable IA cases, and 13 possible invasive fugal infections. In a receiver-operating characteristic (ROC) analysis, the area under the ROC curve was greatest for ELISA, using two consecutive positive results (0.97; $P = 0.036$ for ELISA versus PCR, $P = 0.055$ for ELISA versus BDG). Based on the ROC curve, the cutoff for the ELISA could be reduced to an optical density index (O.D.I.) of 0.6. With the use of this cutoff for ELISA and cutoffs for PCR and BDG that give a comparable level of specificity, the sensitivity/specificity/positive predictive value/negative predictive value of the ELISA and the PCR and BDG tests were 1.00/0.93/0.55/1.00, 0.55/0.93/0.40/0.96, and 0.55/0.93/0.40/0.96, respectively. In conclusion, among these weekly screening tests for IA, the double-sandwich ELISA test was the most sensitive at predicting the diagnosis of IA in high-risk patients with hematological disorders, using a reduced cutoff of 0.6 O.D.I.

Invasive aspergillosis (IA) is one of the most serious complications in patients with hematological malignancies. It has an extremely high mortality rate (11) and affects not only terminally ill patients with refractory leukemia or lymphoma but also patients who could otherwise be expected to experience a potential cure of the underlying leukemia or lymphoma. Among several factors that contribute to the high mortality rate, difficulties in establishing a reliable diagnosis early enough for successful intervention have been repeatedly discussed (10). A definitive diagnosis usually requires invasive tissue sampling, which is often hampered by the critical condition of the patients, while a delay in initiating antifungal therapy, or, conversely, a hasty use of empiric or prophylactic amphotericin B before making a definitive diagnosis may result in treatment failure for full-blown infection or excess toxicity, respectively.

To overcome this problem and to improve the treatment outcome, advances have been made over the past decade in the fields of both diagnostics and therapeutics, including improvements in diagnostic imaging (7, 8, 18) and histopathology (1), and the development of broad-spectrum antifungal agents with low toxicities (4, 24, 29, 33). In the field of diagnostics, much attention has recently been given to the development of several types of noninvasive laboratory tests for IA. These tests are designed to sensitively detect circulating *Aspergillus* components and include a double-sandwich enzyme-linked immunosorbent assay (ELISA) for galactomannan (GM) antigen (Platelia Aspergillus) (30), tests for (1→3)-β-D-glucan (BDG) (β-glucan Wako or FungiTec G test) (23, 25), and a number of PCR-based assay systems for *Aspergillus* DNA (5, 6, 12, 34).

The ELISA for GM uses a rat monoclonal antibody directed against the 1→5-β-galactofuranoside side chains of the GM molecule as both the capture and detection antibodies for ELISA and can detect as little as 1.0 ng of circulating GM per ml (30). The excellent sensitivity and specificity of this assay have been repeatedly demonstrated and validated in tests of patients with hematological disorders (22, 27, 32). BDG is a ubiquitous component of diverse fungal species and a possible target for the diagnostic detection of IA. Two assay systems are currently available for the sensitive detection of circulating

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BDG, and both are based on the *Limulus* reaction, in which a trace amount of BDG can trigger a horseshoe crab coagulation cascade through factor G (23, 25). The BDG test is a useful method for screening for invasive fungal infection (IFI) and is widely used in Japan. The other test that has long been under intensive investigation for the sensitive detection of IA is PCR amplification of *Aspergillus* DNA, mainly of the 18S ribosomal gene (5, 6, 12, 34). Moreover, recently introduced real-time PCR designs have made it possible to quantitatively evaluate a fungal load with high sensitivity (9, 17, 21).

With regard to an antifungal strategy, it would be interesting to determine which of these tests is the best for diagnosing IA in patients with hematological disorders. Although high sensitivity and specificity are reported for PCR-based assays, the question whether PCR-based assays are superior to GM ELISA is still controversial (3, 5, 19, 34). Previously, we developed a sensitive real-time PCR system for detecting *Aspergillus* 18S ribosomal DNA, with which as few as 40 copies of aspergillus DNA per ml of plasma could be stably detected. We reported that the sensitivity of our real-time PCR for IA in 33 IA patients was higher than those of the double-sandwich ELISA for GM and the BDG test, with only a slightly lower specificity than that of GM ELISA (17). However, this previous study may have been biased by its partially retrospective design, limited sampling points in each case or infectious episode, and use of an inappropriately high cutoff value for ELISA. In the present purely prospective analysis, we consecutively enrolled 96 patients with hematological disorders who were at high risk for IA, monitored the levels of *Aspergillus* DNA, GM, and BDG in plasma, as well as the development of IA, at weekly intervals, and evaluated their diagnostic potentials by using receiver-operating characteristic (ROC) analyses.

MATERIALS AND METHODS

Study population and design. From March 2001 through April 2002, a consecutive series of adult patients with hematological disorders who had been admitted to our hospital and were thought to be at high risk for IA were enrolled in the study, and their levels of *Aspergillus* DNA in plasma and GM in serum, and BDG in plasma were monitored weekly. Patients were considered to be at high risk for IA if (i) they underwent chemotherapy and were expected to be neutropenic (less than 500 neutrophils per μl) for at least 10 days, (ii) they had refractory disease or were neutropenic and presented for more than 96 h with persistent fever that was refractory to appropriate broad-spectrum antibacterial treatments, (iii) they had presented with acute graft-versus-host disease (GVHD) of grade 2 or greater or had extensive chronic GVHD, or (iv) they had received corticosteroids for more than 3 weeks within the previous 60 days. Plasma *Aspergillus* DNA levels, serum GM levels, and plasma BDG levels were to be measured once weekly whenever the patients were thought to be at high risk. Each period during which measurement was performed was defined as one treatment episode. Omission of sampling was permitted unless two consecutive samples were lacking. Treatment episodes with only one or two samples for each test were excluded from the analysis.

The level of *Aspergillus* DNA in plasma was measured using real-time PCR, as described previously (17). The ELISA for GM (Platelia Aspergillus; Sanofi Diagnostics Pasteur, Marnes-La-Cosuette, France) and the β-glucan Wako test (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) were performed as specified by the manufacturers. Each sample was tested twice for GM and BDG, and the average of the two measurements was taken.

Antifungal prophylaxis consisted of daily administration of 200 mg of fluconazole or itraconazole capsules with or without 15 mg of aerosolized amphotericin B or 10 mg of intravenous amphotericin B for patients with a suspected history of IA. Neutropenic fever was treated with broad-spectrum antibiotics in accordance with the published guidelines (16). Blood samples were used for bacterial, mycobacterial, and fungal cultures prior to the initiation of antibiotics. When IFI was suspected, treatment with 1 mg intravenous amphotericin B per kg was initiated. During the febrile period, patients were intensively surveyed for possible sites of infection and causative microorganisms. Diagnostic procedures included routine cultures of urine and stools, repeated cultures of blood and sputum, weekly chest X rays, high-resolution computed tomography (CT) scan of the chest, and, when possible, bronchoscopic examinations and open biopsies.

Case definitions. For each treatment episode, a diagnosis was made following the published case definition criteria for invasive fungal infections from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC-IFICG and NIAID-MSG) (2), with the necessary modification that the plasma GM level was not included in the microbiological criteria.

Statistical analysis. As described by Maertens et al. (22), we made a set of different estimates (A/B, C, and D) for the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each test, where different definitions of disease status for an episode were used to calculate these statistical indexes, since there is an intrinsic uncertainty regarding the true disease status of IA so that the calculation of these values could be significantly affected by the definition of the disease status. Estimate A/B defines "proven IA" and "probable IA" as truly positive and only "no IA" as truly negative, whereas estimates C and D incorporate "possible IFI" into the truly positive and truly negative groups, respectively. In all of the estimates, "no-IA" episodes were considered truly negative. Since our objective was to validate and compare the potentials of different diagnostic tests in a setting where these tests are performed weekly to monitor the development of IA, the positivity or negativity of a test was defined for each episode, where an episode was considered positive if at least one sample (method I), or any two consecutive samples (method II) became positive. There is also a practical reason for this approach. The onset and resolution of an IA episode are not always clear and, indeed, are rather poorly defined in many cases. Even in proven cases, there might be several febrile episodes and the onset might be insidious. In this setting, the sample-based calculation of sensitivity and specificity might be severely biased. In addition, we determined a proper cutoff value for each test through a ROC analysis, in which sensitivity and specificity were calculated as a function of the cutoff value. (1 − specificity) was plotted against the sensitivity, and the areas under the ROC curves (AUCs) were calculated. The significance of the difference in the AUCs of any two diagnostic measures was statistically tested as described above, and *P* values were calculated by the paired method under the null hypothesis that the two ROC curves represent random samples from similar underlying data for sensitivities and specificities (13). Therefore, the *P* values can be used only to compare two ROC curves at a time. The calculated *P* values reflect the one-tailed significance of difference between two ROC curves.

RESULTS

Study episodes. There were 149 treatment episodes in 96 consecutive patients, including 9 proven IA, 2 probable IA, 13 possible IFI, and 125 no-IA episodes. Of these, 56 episodes (38%) were associated with stem cell transplantation. The patient characteristics and sample distributions are summarized in Table 1. Nineteen treatment episodes had no host factors. Overall, 1,251 samples were analyzed by the real-time PCR assay, 1,233 were analyzed by double-sandwich ELISA for GM, and 1,243 were analyzed by the BDG test. On average, approximately eight samples were examined for each treatment episode. The characteristics of the 24 episodes of proven IA, probable IA, and possible IFI are shown in Table 2. There were 24 fatal episodes, of which 8 were proven IA, 1 was probable IA, 4 were possible IFI, and 11 were no IA. Autopsies were performed in 14 episodes (58%), including 6 proven IA and 8 no-IA cases. In the remaining 10 fatal episodes, autopsy was not permitted by the patients' families. The 3 proven IA episodes were diagnosed based on histopathology of a pharyngeal biopsy specimen, a surgical specimen of the brain, and a skin biopsy specimen, respectively. Although postmortem examinations disclosed superinfections of disseminated *Trichosporon* infection and atypical mycobacteriosis in episode 1 and

TABLE 1. Patient characteristics

Characteristic	Patients with:				Total[b]
	Proven IA	Probable IA	Possible IFI	No IA	
No. of episodes	9	2	13	125	149 (96)
No. of deaths	8	1	4	11	24
No. of autopsies	6	0	0	8	14
Age (yr)					
Mean	46	47	43	45	45
Median	42	47	40	47	46
Range	19–69	40–53	18–68	17–74	17–74
Sex (no. male/no. female)	6/3	2/0	12/1	82/43	102/47 (67/29)
No. with disease[a]					
AML	3	1	5	48	57 (29)
ALL	1	0	4	26	31 (19)
CML	0	1	2	8	11 (9)
MDS	3	0	2	11	16 (14)
NHL	2	0	0	28	30 (21)
AA	0	0	0	2	2 (2)
Other	0	0	0	2	2 (2)
No. with allografts	4	2	6	44	56
Duration of episode (days)					
Mean	126	92	78	50	57
Median	135	92	57	37	43
Range	36–234	50–134	35–172	11–181	11–234
No. with host factor:					
Neutropenia	7	1	8	86	102
Fever	6	1	7	37	51
GVHD	2	2	5	17	26
Steroid	2	1	4	28	35
None	1	0	0	18	19
Duration of neutropenia (days)					
Mean	63	10	42	16	21
Median	37	10	18	14	15
Range	0–205	0–20	0–162	0–120	0–205
No. of samples tested					
PCR	154	25	146	926	1,251
Mean (per episode)	17.1	12.5	11.2	7.4	8.4
Median (per episode)	17	13	9	6	6
Range (per episode)	7–32	6–19	4–24	3–26	3–32
GM	155	24	140	914	1,233
Mean (per episode)	17.2	12.0	10.8	7.3	8.3
Median (per episode)	18	12	9	5	6
Range (per episode)	7–30	5–19	5–24	2–26	2–30
BDG	158	24	147	914	1,243
Mean (per episode)	17.6	12.0	11.3	7.3	8.3
Median (per episode)	19	12	9	6	6
Range (per episode)	7–31	5–19	6–24	3–23	3–31

[a] AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; CLL, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; AA, aplastic anemia.

[b] Values in parentheses are numbers of patients. Other values refer to numbers of episodes.

episode 9, respectively, no invasive candidiasis was documented during the study period.

Among the 125 no-IA episodes, 11 deaths occurred, and the diagnosis of no IA was confirmed by autopsy in 8. The other three fatal episodes were not confirmed by autopsy and included two respiratory failures following chemotherapy and one case of severe stomatitis following a second bone marrow transplantation. One respiratory failure was due to bacterial pneumonia, in which *Pseudomonas aeruginosa* was cultured from the sputum and the blood. In the other episode, respiratory failure developed in association with rapid tumor growth. Although no pathogen was identified despite repeated cultures, we could not completely exclude a possible infectious origin of this episode. The episode of severe stomatitis became suddenly fatal after the patient aspirated the clot and was asphyxiated.

ROC analysis. Figure 1 shows ROC curves for each test, using different definitions of the disease status. First, we examined the behaviors of the ROC curves for different diagnostic tests by using an "ideal" estimate (estimate A/B), in which episodes were expected to be most accurately defined. ELISA has a larger AUC in both method I (ELISA, 0.93; PCR, 0.81; BDG, 0.85) and method II (ELISA, 0.97; PCR, 0.76; BDG, 0.79). To increase the sensitivity for GM, we could more easily decrease its cutoff value with a small decrease in specificity. In contrast, a higher sensitivity could be obtained for the PCR and BDG tests by decreasing their cutoff values, but this would be at a significant cost in terms of specificity. When we shifted the diagnostic algorithm from method I (one positive sample) to method II (two consecutive positive samples), the AUC for the GM test was further increased while those for the PCR and BDG tests decreased, indicating that the GM test has higher

TABLE 2. Diagnosis of IA and its documentation

Episode no.	Patient characteristics[a]: Age (yr)	Sex	IA	Primary disease	Status of primary disease	Outcome	Host factors	Clinical evidence	Culture and its source	Histological evidence	Maximum value (method I/method II) PCR (copies/ml)	GM (O.D.L)	BDG (ng/ml)
1	41	F	P	AML M1	Post-allo, RD	Dead	NF	Erosion of sinus walls	*A. flavus* and *A. fumigatus* from pharyngeal mucosa	Biopsy	2,000/200	3.8/3.6	19.7/4.7
2	32	M	P	MDS (RAEB-t)	Post-allo, CR	Dead	GS	Dyspnea, pleural effusion		Autopsy	32/0	1.3/1.0	60.5/36.5
3	58	M	P	AML M1	RD	Dead	NF	Halo sign		Autopsy	90/42.5	7.7/6.4	25/1.5
4	38	F	P	AML M2	Post-allo, CR	Alive	NS	Cavity within area of consolidation	*A. fumigatus* from broncheal lavage fluid	Biopsy	33.5/0	1.9/1.7	2.8/0
5	51	M	P	Macroglobulinemia	Stable disease	Dead	None	Extensive skull base destruction	*A. fumigatus* from epidural abscess	Biopsy	0/0	1.2/0.8	37.4/7.1
6	19	M	P	MDS RA	RD	Dead	NF	Multiple nodular lesions in the lung field, pleural effusion		Autopsy	3,500/1,000	2.5/1.5	155.5/59.2
7	42	M	P	MDS/AML	Post-allo, RD	Dead	NFG	Dyspnea, pleural effusion		Autopsy	24/9	2.4/0.6	0/0
8	63	F	P	ATL acute type	RD	Dead	NF	Dyspnea, pleural effusion		Autopsy	50/12.5	1.9/0.7	2.4/0
9	69	M	P	ALL PreB	RD	Dead	NF	No specific clinical evidence		Autopsy	100,000/5,000	4.2/1.1	171.7/12.6
10	53	M	PP	AML M2	Post-allo, CR	Dead	FG	Dyspnea, pleural effusion	*A. spergillus* spp. from broncho-alveolar lavage fluid	NA[b]	5/0	5.3/0.7	4.5/2.2
11	40	M	PP	CML CP1	Post-allo, CR	Alive	NGS	Halo sign	*A. fumigatus* from sputum	NA	11.5/7.5	2.3/2.0	0/0
12	68	M	PPP	MDS/AML	RD	Dead	NF	Multiple nodular lesions in the lung field, intraparenchymal brain mass lesion, seizure, hemiparesis		NA	155/100	2.2/1.5	18.3/16.6
13	24	M	PPP	AML M4E	CR, HDAraC	Alive	NF	Nodular skin lesion without any other explanation, multiple nodular lesions in the lung field		NA	20.5/0	4.5/0.3	0/0
14	61	M	PPP	AML M4E	CR, HDAraC	Alive	N	Halo sign		NA	1,000/9	0.2/0.1	3.5/2.9
15	30	M	PPP	ALL precursor B	Post-allo, CR	Alive	NFGS	Nonspecific abnormal shadow in lung field, pleural effusion		NA	60/60	0.6/0.4	0/0
16	61	M	PPP	AML M2	RD	Dead	NF	Multiple nodular lesions in the lung field, halo sign, cavity within area of consolidation		NA	84.5/0	1.1/0.7	2/0
17	68	M	PPP	CML BC	RD	Dead	NS	Dyspnea, pleural effusion		NA	165/0	0.3/0.2	0/0
18	25	M	PPP	ALL precursor B	RD	Alive	NG	Cavity within area of consolidation		NA	400/0	0.7/0.6	3.2/0
19	32	M	PPP	ALL PreB	Post-allo, CR	Dead	FGS	Dyspnea, pleural effusion		NA	27/1	0.7/0.5	3.7/2.4
20	18	F	PPP	AML M2	CR, HDAraC	Alive	N	Halo sign		NA	0/0	0.6/0.1	0/0
21	55	M	PPP	MDS RA	Stable disease	Alive	F	Cough, dyspnea, pleural effusion		NA	19/4	0.8/0.3	0/0
22	28	M	PPP	CML CP1	Post-allo, CR	Alive	G	Cough, dyspnea, pleural effusion		NA	0/0	0.4/0.3	0/0
23	40	M	PPP	CML CP1	Post-allo, CR	Alive	GS	Cough, dyspnea, new infiltrate not fulfilling the major radiological criteria without an alternative diagnosis		NA	6/0	0.5/0.4	0/0
24	54	M	PPP	ALL precursor B	Post-allo, CR	Alive	F	Dyspnea, new infiltrate not fulfilling the major radiological criteria without an alternative diagnosis		NA	10.5/0	0.5/0.3	0/0

[a] F, female; M, male; P, proven; PP, probable; PPP, possible; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB-t, RA with excess of blasts in transformation; ALL, acute lymphoblastic leukemia/lymphoma; CML, chronic myelogenous leukemia; CP, chronic phase; BC, blastic crisis; allo, allogenic hematopoietic stem cell transplantation; CR, complete remission; RD, refractory disease; HDAraC, high-dose cytrabine; N, neutropenia; F, persistent fever; G, GVHD; S, prolonged use of corticosteroid.

[b] NA, not available.

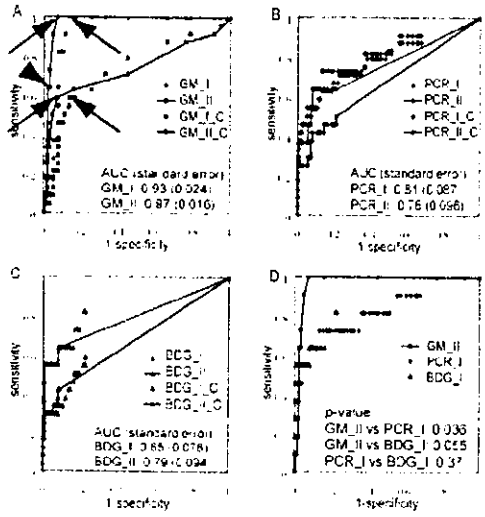

FIG. 1. (A to C) ROC curves of the GM (A), PCR (B), and BDG (C) tests for screening for IA. Both methods I and II were used. The ROC curves obtained by estimate A/B are shown in red, and those obtained by estimate C are shown in blue. The ROC curves obtained by method II are indicated by solid lines, and those obtained by method I are indicated by dotted lines. (D) Combination of ROC curves of the GM test (method II) and those of the PCR and BDG tests (method I).

reproducibility than the other two tests. The comparison of ROC curves of ELISA (method II), PCR (method I), and BDG (method I) is presented in Fig. 1D. When estimate C was applied for ROC analyses, these characteristics of the ROC curve for GM were partially obscured. In estimate C, a large decrease in sensitivity shifted the ROC curve downward and caused a significant reduction in AUC for the ELISA and BDG test, as expected. On the other hand, the ROC curve for the PCR test did not significantly change, since an expected decrease in sensitivity due to false-positive episodes in the possible IFI group is thought to be counterbalanced by a gain due to false-positive PCR results in these episodes. The ROC curves for the GM test in estimates A/B, C, and D, which is not presented but is similar to that for A/B, represent extreme cases, and the unknown "real" ROC curve might be mapped between these extremes.

Optimal cutoff value. Determination of an optimal cutoff value may be somewhat arbitrary depending on the purpose of the diagnostic test. A loss of specificity may be allowed to obtain a higher sensitivity. Based on the conventional or manufacturer-recommended cutoff values, an optical density index (O.D.I.) of 1.0, in two serial samples for GM (2, 22), i.e., 40 copies/ml for PCR and 11 pg/ml for the BDG test, all tests showed excellent specificity (0.98) in estimate A/B whereas their sensitivity was generally low (0.64, for GM, 0.45 for the PCR test, and 0.55 for the BDG test) even in estimate A/B, with further decreases as low as 0.33 for the GM test and 0.29 for the BDG test in estimate C. The current standard for ELISA (red arrowhead in Fig. 1A) seems to be inadequate. It could be reduced to 0.6 O.D.I. in method II (red arrows in Fig. 1A), or the criteria for positivity could be relaxed to those in method I while retaining the same cutoff (1.0 O.D.I.) (blue arrows), without great loss of specificity. With regard to specificity, the former may be recommended ($P = 0.0334$ by Fisher's direct test), which reflects a more leftward displacement of the ROC curve for method II. Both cutoff values represent the inflexion point of each ROC curve, around which the diagnostic efficacy is maximum for both cutoffs. The sensitivity/specificity and PPV/NPV of the GM test are 1.0/0.93 and 0.55/1.0 for a cutoff value of 0.6 O.D.I. in method II and 1.0/0.86 and 0.38/1.0 for a cutoff value of 1.0 O.D.I. in method I. Various diagnostic statistical parameters in different calculations are presented in Table 3. We may improve the diagnostic efficiency by using two or three tests in combination. In our analyses, however, we could not obtain better sensitivity by combination use of multiple tests employing much reduced cutoff values while maintaining high specificity (data not shown). This is also accompanied by significant delay of diagnosis.

Time interval between the first positive result and the antemortem diagnosis. Chronological relationships between the first positive results of different screening tests, histopathology, and diagnostic imaging are summarized in Fig. 2 and 3. For the PCR and BDG tests, the conventional cutoff was used, while the second of the first two consecutive results equal to or greater than 0.6 or 1.0 O.D.I. was plotted for ELISA. When the new reduced cutoff was used, the first positive date for GM was brought forward by a median of 10 (0 to 70, $n = 9$, mean = 24) days compared to the conventional cutoff value. Using the conventional cutoff, only one episode was identified to have a positive ELISA result before definitive treatment was started. In contrast, with the new reduced cutoff, the first positive ELISA result preceded the initiation of broad-spectrum antifungal treatment in seven IA-positive episodes (median, 31 days; range, 2 to 127 days; mean, 28 days). It became positive 51 days before a positive histopathology result (10 to 127 days; mean, 31 days).

Unfortunately, chronological comparisons between the three different assays were possible for only six episodes, in which patients had refractory leukemia and their IA tended to have a rapidly progressive course as a terminal infection (Fig. 3). In these episodes, ELISA gave positive findings earlier than (five episodes) or at the same time as (one episode) the BDG test (median, 16.5 days; range, 0 to 76 days). The PCR test was positive in 11 of 24 IA patients in estimate C. A comparison was possible in 5 of the 11 episodes, which were also positive for ELISA, but there was no significant difference in the date of the first positive result between ELISA and the PCR tests.

DISCUSSION

In this study, we compared the diagnostic potential of three different laboratory tests used to screen for IA in a prospective setting, where GM, DNA, and BDG levels in a cohort of patients at high risk for IA were measured weekly. The statistical parameters of a diagnostic test can be dramatically affected by the predetermined cutoff value, and when there is some uncertainty regarding the disease status, as in this case, they can also be influenced by the definition of the disease status. Therefore, to meaningfully compare the diagnostic potentials of these different tests, we performed an ROC analysis for each test by using the same cohort of patients with different positive result criteria (methods I and II) and various definitions of the disease status (estimates A/B, C, and D). As a

TABLE 3. Statistics for some selected thresholds

Method and threshold	Sensitivity A/B (C)	Specificity A/B (D)	PPV A/B (D)	NDV A/B (C)	Efficacy A/B (C)
Method I					
GM (O.D.I.)					
0.5	1.00 (0.88)	0.34 (0.33)	0.12 (0.11)	1.00 (0.93)	0.40 (0.43)
0.6	1.00 (0.79)	0.55 (0.54)	0.16 (0.15)	1.00 (0.93)	0.59 (0.59)
1.0	1.00 (0.58)	0.86 (0.85)	0.38 (0.34)	1.00 (0.91)	0.87 (0.81)
1.5	0.82 (0.46)	0.90 (0.89)	0.41 (0.38)	0.98 (0.90)	0.89 (0.83)
PCR (copies/ml)					
5	0.91 (0.88)	0.43 (0.41)	0.12 (0.11)	0.98 (0.95)	0.47 (0.30)
10	0.82 (0.79)	0.60 (0.55)	0.15 (0.13)	0.97 (0.94)	0.62 (0.63)
20	0.73 (0.67)	0.78 (0.75)	0.23 (0.19)	0.97 (0.92)	0.78 (0.77)
40	0.45 (0.46)	0.98 (0.93)	0.63 (0.36)	0.95 (0.90)	0.93 (0.89)
BDG (ng/ml)					
2	0.82 (0.58)	0.77 (0.76)	0.24 (0.21)	0.98 (0.91)	0.78 (0.74)
3	0.64 (0.46)	0.84 (0.82)	0.26 (0.23)	0.96 (0.89)	0.82 (0.78)
5	0.55 (0.29)	0.92 (0.92)	0.38 (0.35)	0.96 (0.87)	0.89 (0.82)
11	0.55 (0.29)	0.98 (0.97)	0.67 (0.60)	0.96 (0.88)	0.94 (0.87)
Method II					
GM (O.D.I.)					
0.5	1.00 (0.63)	0.84 (0.83)	0.35 (0.31)	1.00 (0.92)	0.85 (0.81)
0.6	1.00 (0.58)	0.93 (0.91)	0.55 (0.48)	1.00 (0.92)	0.93 (0.87)
1.0	0.64 (0.33)	0.98 (0.97)	0.70 (0.64)	0.97 (0.88)	0.95 (0.87)
1.5	0.45 (0.25)	0.98 (0.97)	0.63 (0.56)	0.95 (0.87)	0.93 (0.86)
PCR (copies/ml)					
5	0.64 (0.43)	0.87 (0.86)	0.30 (0.27)	0.96 (0.89)	0.85 (0.80)
10	0.45 (0.30)	0.94 (0.93)	0.38 (0.33)	0.95 (0.88)	0.90 (0.84)
20	0.36 (0.26)	0.98 (0.97)	0.67 (0.50)	0.95 (0.88)	0.93 (0.87)
40	0.36 (0.26)	1.00 (0.99)	1.00 (0.67)	0.95 (0.88)	0.95 (0.89)
BDG (ng/ml)					
2	0.64 (0.42)	0.91 (0.90)	0.39 (0.33)	0.97 (0.89)	0.89 (0.83)
3	0.55 (0.29)	0.95 (0.95)	0.50 (0.66)	0.96 (0.88)	0.92 (0.85)
5	0.55 (0.29)	0.98 (0.97)	0.67 (0.60)	0.96 (0.88)	0.94 (0.87)
11	0.45 (0.25)	0.99 (0.99)	0.83 (0.71)	0.95 (0.87)	0.95 (0.87)

result, the ROC curve for the GM test seemed to be better than those for the other two tests.

We previously reported that this real-time PCR for *Aspergillus* DNA was highly sensitive in vitro and with clinical samples (17): it could stably detect as few as 40 copies/ml in vitro and showed a higher sensitivity (79%) than those of the GM (58%) and BDG (67%) tests. In the present prospective analysis with consecutive patients, however, these results were not reproduced. This may be partly explained by the fact that our previous study included many retrospective samples. Furthermore, we intentionally selected IA patients and used a higher cutoff value for the GM test. Although several authors have also reported excellent sensitivity in PCR assays for IA (5, 6, 14, 34), we cannot directly compare those results with ours since there were differences in the target genes, methods of DNA extraction, starting materials, and designs of the PCR amplifications. Some form of standardization is required to make an international comparison possible. We used our real-time PCR system (GeniQ-Asper) (17) because it is most widely used in Japan. Several authors, including Loeffler et al. and Costa et al., also published excellent real-time PCR detection systems for *Aspergillus* DNA (9, 21, 26, 28), and their systems might produce superior results in the diagnosis of IA, which should be addressed in future studies.

As a diagnostic test, PCR requires more time and more complicated processing and thus costs more than the BDG and GM tests. It costs six times (15,700 yen/test) as much as the BDG and GM assays (2,700 yen/test) in Japan. A specialized laboratory as well as an expensive assay system and reagents are also required. These problems should be addressed before PCR is widely accepted as a standard screening test for IA, although it still seems to have value in making a diagnosis when a variety of clinical samples are used (20, 26, 28, 31).

The BDG test has also been widely used in Japan as a noninvasive diagnostic test for IFI. While it covers wide ranges of fungal species and may be potentially more useful as a screening test for IFI, it can cause frequent nonspecific reactions to various medical materials. Three kinds of assay systems for BDG have been developed in Japan: a chromogenic assay (FungiTec G test), β-glucan test Maruha) and a kinetic assay (β-glucan test Wako), but there is still some debate regarding their diagnostic potential. According to a sample-based analysis by Yoshida et al. (35), the chromogenic assay seems to be more sensitive (87.9 and 72.7%, respectively) than the kinetic assay but much less specific (43.3 and 75.2%, respectively) when the cutoff values recommended by the manufacturers are used. In the present study, where we used a kinetic assay, we could not obtain sufficient sensitivity even with the cutoff being maximally reduced. Furthermore, even if positive results were obtained, the positive results with the BDG test tended to occur later in the clinical course. The present result (55% sensitivity and 98% specificity) is consistent with our previous results (67% sensitivity and 84% specificity) using the chromogenic assay and also with other reports. This seems to be an inherent limitation of BDG assays

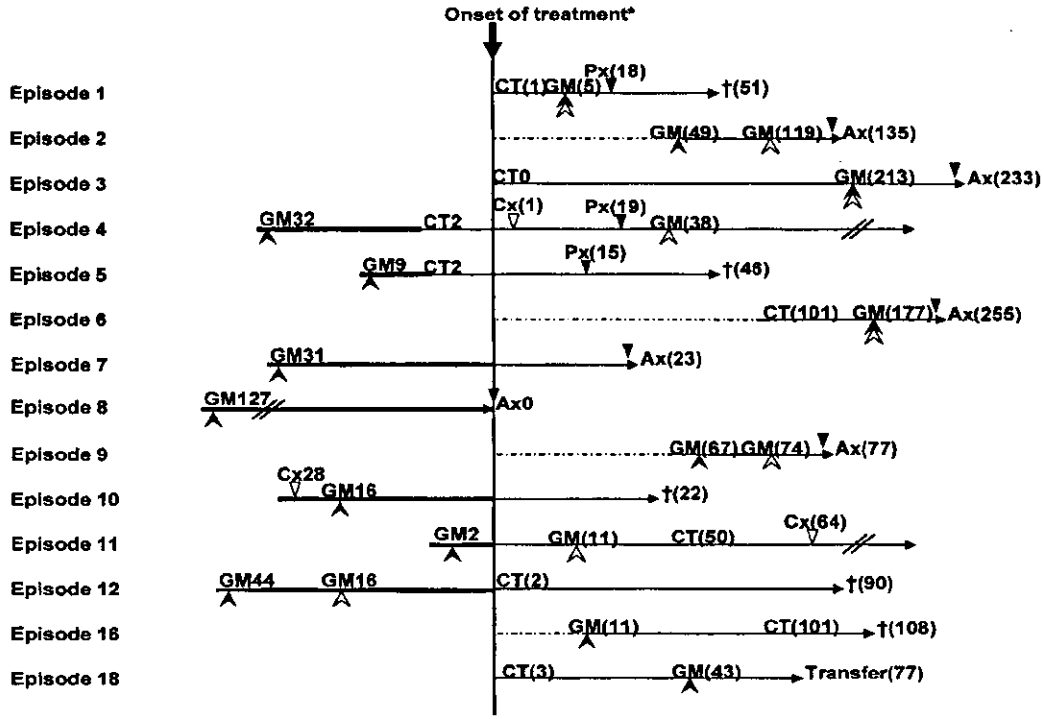

FIG. 2. Number of days from when GM assays become positive to the onset of treatment, using a threshold of 0.6 O.D.I. by method II (solid arrowheads) or 1.0 O.D.I. by method II (open arrowheads), or positive findings on CT. Open triangles indicate the date of positive culture, and solid triangles indicate when the histopathological diagnosis was made (Px, biopsy; Ax, autopsy). The values in parentheses indicate the number of days after the onset of treatment. For example, for episode 11, CT showed specific findings 50 days after the onset of treatment and the GM assay become positive 2 days before treatment. Episode numbers correspond to those in Table 2. Episodes whose GM assays did not reach the threshold are not shown. For episodes 2 and 9, a CT scan was not performed, and for episodes 7, 8, 10, 17, 19, 21, and 22, the CT findings were nonspecific and could not be used for decision-making. Each treatment was started at the discretion of the physician, taking into account various prices of clinical information, including CT findings and the results of GM assays. For Episode 8, IA was not suspected and no antifungal agent was administered. Therefore, the date of death was used instead of the date of treatment onset.

for the diagnosis of IA, although they show a very high sensitivity and specificity for candidiasis (25).

The diagnostic potential of double-sandwich ELISA for GM has been repeatedly validated in recent large-scale studies (15, 22). However, a direct comparison of the results of different studies, including ours, is not always easy and in fact can be quite difficult or impractical. Many factors can influence the apparent sensitivity and specificity and of course the PPV and NPV. Therefore, the important point is the way in which these results should be interpreted, and this depends on the objective and design of each study. From this perspective, our results are comparable to those of Maertens et al. (22) but in contrast to those of Herbrecht et al. (15). The latter addressed principally the diagnostic potential of the GM test in the presence of an unknown neutropenic fever or some respiratory signs and symptoms in cancer patients. On the other hand, in our study as well as in that of Maertens et al., the principal concern was the potential of the test in serial screenings with multiple measurements throughout the entire period of hematology care. For example, the mean numbers of measurements per episode in our study and that of Maertens et al. (8.3 and 11.2 per episode, respectively, with GM measured weekly) are significantly different from that in the study of Herbrecht et al. (5.5 per episode, with GM measured daily or weekly), consistent with the study designs. The difference becomes more prominent for proven IA episodes (17.3 and 19 versus 6.8). The differences in the mean number and timing of measurements clearly affect the apparent sensitivity and specificity of the studies. Hence, the apparent statistical values obtained by Herbrecht et al. are expected to be lower than ours and those of

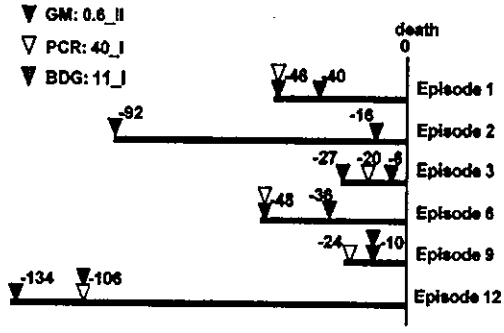

FIG. 3. Number of days before death that each test gave positive results. Solid triangles indicate the date when GM became positive, using a threshold of 0.6 O.D.I. by method II; open triangles indicate the date when PCR exceeded a cutoff value of 40 copies/ml; and shaded triangles indicate the date when the BDG test exceeded a cutoff value of 11 ng/ml, by method I. In episode 2, PCR never exceeded the cutoff value. Episode numbers correspond to those in Table 2.

Maertens et al., but they should provide a better approximation of the corresponding sample-based statistics, even though the patient population was more heterogeneous.

According to the ROC analysis of double-sandwich ELISA, the conventionally used cutoff seems to be too high: our recommendation is 0.6 O.D.I., and two consecutive positive results should be taken into consideration. With these new criteria, the GM test showed an excellent chronological profile. It gave the first positive diagnostic result in 9 of 14 GM-positive IA episodes and in 5 of 9 IA or possible IFI episodes where both CT and GM were positive. It preceded the initiation of empiric or definitive antifungal therapy in seven episodes. Using the novel criteria, positivity was ascertained a median of 10 days before conventional positivity was noted, and in six cases the GM test gave positive results only with the novel criteria. These chronological advantages were not observed with a threshold of 1.0 O.D.I. by method II: for episodes 5, 7, 8, and 10, the GM assay did not become positive; for episode 4, the GM assay exceeded the criteria 38 days after the onset of treatment; for episode 12, the GM assay gave positive results 16 days before the onset of treatment. According to the high PPV with the novel cutoff criteria (0.55 for proven or probable IA and 0.48 for proven, probable, or possible IFI) and the early timing of its positivity, we could have initiated antifungal therapy in a preemptive manner for episodes 4, 5, 7, 8, 10, 11, and 12.

Our result does not justify a discontinuation or moratorium of empiric antifungal treatment based only on a single negative result in the face of an impending threat of IA. It should be stressed that the extremely high NPVs provided here are episode-based calculations. Sample-based NPVs should be much lower, especially when patients are at high risk. We could not exclude a possibility of other IFI. Similarly, PPV does not always represent the probability of currently having IA but, rather, predicts the probability that the subject has or will have IA. In addition, while there was a sufficient number of no-IA episodes in this study to permit reliable estimations of specificity and NPV, there is much uncertainty regarding the estimations of the absolute values of sensitivity and PPV because of the small number of IA patients.

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A Prospective Trial to Evaluate the Safety and Efficacy of Pravastatin for the Treatment of Refractory Chronic Graft-Versus-Host Disease

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This prospective study evaluates the safety and efficacy of pravastatin for the treatment of chronic graft-versus-host disease (GVHD). We included 18 patients with refractory chronic GVHD. Oral pravastatin was started at 10 mg/day, and the dose was increased up to 40 mg/day in 4 weeks. This maximum dose was administered over 8 weeks. There were no severe adverse events caused by pravastatin. A clinical response was observed in the skin score in two patients, mouth score in five patients, eye score in two patients, liver score in three patients, platelet count score in one patient, and weight loss in two patients. The overall response rate was 28%. Immunophenotypic analyses showed that T-helper (Th)1 cells were dominant in all but one patient before treatment and that the Th1/Th2 ratio tended to be lower in the responders than in the nonresponders. A randomized controlled trial is warranted to evaluate the efficacy of pravastatin against chronic GVHD.

Keywords: Chronic graft-versus-host disease, pravastatin, treatment.

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Chronic graft-versus-host disease (GVHD) is one of the major complications after allogeneic hematopoietic stem-cell transplantation and develops in 25% to 80% of allogeneic transplant recipients (*1–3*). Corticosteroids and cyclosporine are most widely used to treat chronic GVHD, but they have demonstrated limited efficacy.

Pravastatin is a lipid-lowering agent that inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Recently, the immunosuppressive effect of statins has been highlighted in both clinical and laboratory studies. Pravastatin reduced the incidence of graft rejection after cardiac and kidney transplantation (*4, 5*). Statins also prevented islet allograft rejection in a mouse model (*6*). Two distinct molecular mechanisms of the immunosuppressive effect of statins have recently been proposed. First, statins suppress the induction of major histocompatibility complex-II expression by interferon-gamma on human endothelial cells and macrophages (*7*). Second, statins selectively inhibit the molecular association between leukocyte function antigen-1 and intercellular adhesion molecule-1 (*8*). With these data, we performed a prospective clinical trial to evaluate the safety and efficacy of pravastatin for the treatment of chronic GVHD.

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Patients aged between 20 and 70 years who had refractory pathologically proven chronic GVHD were eligible for the study. Refractory chronic GVHD was defined as chronic GVHD that was not improved by first-line treatment with corticosteroids at more than 0.5 mg/kg or cyclosporine at a therapeutic blood level for at least 2 weeks, or that showed progression during the tapering of first-line treatment. Patients had to demonstrate good hepatic and renal function as defined by serum bilirubin less than 85.5 μmol/L (5 mg/dL), alanine aminotransferase less than 500 IU/L, and serum creatinine less than 176.8 μmol/L (2.0 mg/dL). Patients with myopathy or who were receiving fibrates were excluded to avoid rhabdomyolysis. All of the patients provided their written informed consent. This study was approved by the institutional review board at each participating institution.

Pravastatin was started orally at 10 mg/day. The dose was increased to 20 mg/day after 2 weeks and finally to 40 mg/day after 2 additional weeks with close monitoring for adverse events. The maximum dose was continued over 8 weeks, unless grade 3 or 4 adverse events attributable to pravastatin were observed. Immunosuppressive agents that were being taken at study entry were continued at the same dose. However, once the dose of these immunosuppressive agents was increased or other immunosuppressive agents were added, the patient was withdrawn from the study and considered a nonresponder.

The incidences and severity of adverse events attributable to pravastatin were evaluated according to the National Cancer Institute Common Toxicity Criteria, Version 2.0. To evaluate the efficacy of pravastatin, chronic GVHD was graded at study entry according to Akpek's prognostic model (*9*). Response was evaluated every 2 weeks for 12 weeks after the initiation of treatment as an intent-to-treat basis. Response in individual organs was defined as follows: A marked response was a change from Akpek's code 2 or 3 to code 1, a

good response was a change from code 3 to code 2, and no response was no change in code or progression. An overall response to treatment was defined as a marked or good response in at least one organ, without progression in any other organs. We planned to include 18 patients with target and lower response rates of 40% and 10% and alpha and beta errors of 5% and 10%, respectively.

The trough blood concentrations of cyclosporine or tacrolimus and the peak plasma concentration of pravastatin were measured every 2 weeks to evaluate interaction between pravastatin and these immunosuppressants. Immunologic changes were evaluated at weeks 2, 4, 8, and 12 by quantification of the CD4/CD8 ratio, the T-helper (Th)1/Th2 ratio, and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes. Immunologic data were compared between responders and nonresponders using a repeated measures analysis of variance after logarithmic transformation.

Eighteen patients with a median age of 44 years (range 20–68 years) were included in the study. There were 14 men and 4 women. The underlying disease was acute myeloblastic leukemia in seven, chronic myeloid leukemia in four, non-Hodgkin's lymphoma in three, acute lymphoblastic leukemia in two, myelodysplastic syndrome in one, and aplastic anemia in one. Thirteen and five patients received grafts from a related or an unrelated donor, respectively. Ten of them demonstrated chronic GVHD of progressive onset. All patients but one demonstrated extensive chronic GVHD before starting pravastatin, and nine patients were receiving prednisolone. The grade of chronic GVHD at study entry according to Akpek's prognostic model is shown in Table 1. Seven patients, 10 patients, and 1 patient were grouped into the low-, intermediate-, and high-risk groups, respectively.

Pravastatin was well tolerated, and no patients developed grade 3 or 4 adverse events attributable to pravastatin. Treatment was discontinued in three patients between 14 and 41 days after starting pravastatin because of unrelated causes, including painful oral chronic GVHD, infection, and interstitial pneumonitis. According to each organ, a response was observed in the skin score in two patients, mouth score in five patients, eye score in two patients, liver score in three patients, and platelet count score in one patient (Table 1). An overall response was seen in five patients (28%). Pravastatin did not act through the interaction with cyclosporine or tacrolimus, because an increase in these blood levels was not observed after the administration of pravastatin (data not shown). The serum pravastatin concentration on day 42 was not different between responders and nonresponders (median 157.5 ng/mL vs. 253.1 ng/mL, $P=0.53$). The serum total cholesterol level significantly decreased from 6.37 mmol/L (standard deviation [SD] 1.79) before treatment to 5.67 mmol/L (SD 1.40, $P=0.0095$) and 4.77 mmol/L (SD 1.99, $P=0.0001$) on days 14 and 84 after starting pravastatin, respectively. The initial cholesterol response (ratio between cholesterol level on day 14 and before treatment) was significantly better in GVHD responders (0.78 vs. 0.95, $P=0.029$).

The Th1/Th2 ratio before the administration of pravastatin was greater than 1.0 in all but one patient. The Th1/Th2 ratio at study entry tended to be lower in responders than in nonresponders and became even lower after pravastatin treatment in responders, but not in nonresponders, although these differences were not statistically significant (Fig. 1, $P=0.22$). The CD4/CD8 ratio and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes did not change after treatment (data not shown).

This study demonstrated that pravastatin at 40 mg/day can be safely administered in patients with refractory chronic GVHD, including those taking cyclosporine. The overall response of 28% was similar to that with other alternative salvage treatments including tacrolimus, mycophenolate mofetil, thalidomide, and so on (*3*). However, considering the safety profile of pravastatin, it may be worthwhile for patients with chronic GVHD, especially in those with a coexisting infection that precludes severely immunosuppressive treatments. We chose pravastatin among many statins because it is hydrophilic and was considered to be less likely to cause rhabdomyolysis than other lipophilic statins (*10*, *11*). However, atorvastatin, lovastatin, and simvastatin have stronger in vitro immunosuppressive effects than pravastatin, and thus they may also have greater in vivo effects against chronic GVHD (*7*, *8*).

There is some controversy whether human chronic GVHD is a Th1 or Th2 disease. The immunophenotypic analyses in this study clearly showed that Th1 cells were dominant in patients with chronic GVHD. The efficacy of statin against rheumatoid arthritis, a Th1 disease, has been demonstrated clinically (*12*). In a mouse model of chronic and relapsing

TABLE 1. Severity of chronic graft-versus-host disease in each organ and the response to pravastatin

	Severity code before treatment				Response to treatment				
Each organ	1	2	3	NE	Marked	Good	NC	PD	NE
Performance status	16	2	0	0	0	0	18	0	0
Skin and fascia	6	6	4	2	1	1	13	2	1
Mouth	5	11	2	0	3	2	12	1	0
Eye	7	8	3	0	2	0	13	2	1
Liver enzyme	4	5	9	0	2	1	13	2	0
Thrombocytopenia	14	1	3	0	0	1	15	2	0

Overall response	Responder	5 (28%)
	Nonresponder	13

NE, not evaluable; NC, no change; PD, progressive disease.

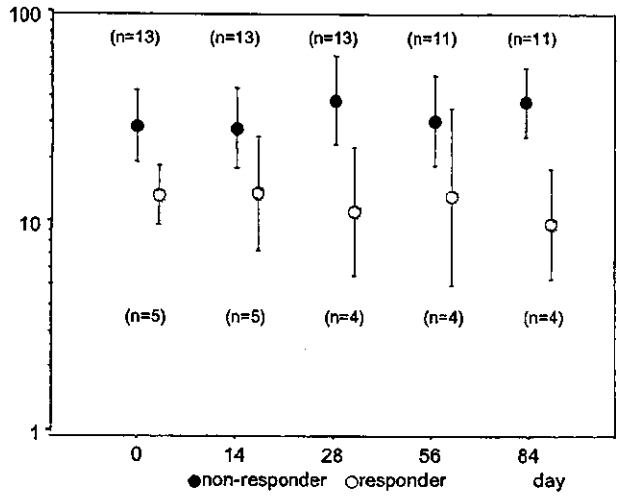

FIGURE 1. Serial changes in the T-helper (Th)1/Th2 ratio in responders and nonresponders. Data are shown as geometric mean and standard error.

experimental autoimmune encephalomyelitis, oral atorvastatin promoted a Th2 bias and reversed paralysis through the inhibition of STAT4 phosphorylation and the induction of STAT6 phosphorylation (*13*). Although we did not find a statistically significant association between the Th1/Th2 ratio and the response to pravastatin, pravastatin might have ameliorated chronic GVHD by inducing a Th2 shift.

In conclusion, our experience suggests that pravastatin may be safe and effective for the treatment of refractory chronic GVHD. However, a double-blind, randomized, controlled trial is needed to evaluate its true efficacy against refractory chronic GVHD.

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Value of chemotherapy before allogeneic hematopoietic stem cell transplantation from an HLA-identical sibling donor for myelodysplastic syndrome

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Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a curative treatment for myelodysplastic syndrome (MDS). The object of this study was to evaluate the impact of chemotherapy before allo-SCT. We analyzed the data of 283 patients who underwent allo-SCT from an HLA-identical sibling donor for MDS that were reported to the Japan Society for Hematopoietic Cell Transplantation. The cumulative incidence of grade II–IV acute GVHD was 33%. Overall survival (OS) at 5 and 10 years was 48.8 and 42.5%, respectively. Multivariate analyses identified karyotype, FAB classification, and the history of chemotherapy before allo-SCT as significant predictors for OS. OS at 5 years was 57% for patients who underwent allo-SCT as a primary treatment for refractory anemia with excess blasts in transformation (RAEB-t) or secondary acute myeloid leukemia (AML) and 54% for those who underwent allo-SCT in remission after induction chemotherapy ($P=0.81$). The proportion of patients with a poor karyotype was equivalent between the two groups ($P=0.44$). Although only a randomized controlled trial will be able to establish a definite conclusion, these results do not support the administration of induction chemotherapy for patients with RAEB-t or secondary AML before allo-SCT.
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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a curative treatment for patients with myelodysplastic syndrome (MDS).[1–4] While there is little chance of curing MDS by conventional measures, the timing and method of allo-SCT must still be refined, since allo-SCT is associated with a considerable transplant-related mortality. Recently, Cutler *et al* showed using a decision analysis that delayed BMT just before leukemic transformation is associated with maximal life-expectancy for patients with low- and intermediate-1-risk MDS grouped by the International Prognostic Scoring System (IPSS),[5] while immediate transplantation for intermediate-2- and high-risk disease is associated with maximal life-expectancy.[6] However, it is controversial whether remission induction chemotherapy should be performed before allo-SCT for MDS.[7]

We retrospectively analyzed the outcome of 283 Japanese adult patients who received allo-SCT from an HLA-identical sibling donor and were registered with the Japan Society for Hematopoietic Cell Transplantation (JSHCT). The primary object of this study was to evaluate the impact of chemotherapy before allo-SCT on the outcome of allo-SCT.

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Patients and methods

Patients

A total of 439 patients who underwent allo-SCT from an HLA-identical sibling donor for the first time between 1991 and 2001 for refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-t), chronic myelomonocytic leukemia (CMML), or transformation to acute myeloid leukemia (leukemic transformation (LT) were reported to the JSHCT. The diagnosis and classification of MDS was performed using the French–American–British (FAB) criteria.[8] Those younger than 16 years ($n=8$), those with a previous history of SCT ($n=1$), those who received a graft from a syngeneic donor ($n=9$), those who received a nonmyeloablative or reduced-intensity regimen ($n=25$), and those for whom we lacked information regarding the history of chemotherapy before transplantation ($n=113$) were excluded. Finally, 283 patients were analyzed. This study was approved by the Committee for Nationwide Survey Data Management of JSHCT. The blast cell count before allo-SCT was not included in the data set.

Cytogenetic evaluation

Cytogenetic data were available in 232 patients (82%). Patients were grouped according to the karyotype classification in IPSS.[5] Briefly, those who had a normal, del (5q), del (20q) or -Y karyotype, those who had complex abnormalities or chromosome 7 anomalies, and those who had other abnormalities were categorized into good, poor, and intermediate groups, respectively. In all, 51 patients (18%) without cytogenetic data were placed in the unknown group.

Transplantation procedure

The conditioning regimen consisted of either a total body irradiation (TBI)-based regimen ($n=173$) or a chemotherapy-based regimen ($n=110$). In all, 218 patients received bone marrow grafts, while 65 received peripheral blood stem cell

grafts. Prophylaxis against graft-versus-host disease (GVHD) mainly consisted of cyclosporine with or without methotrexate ($n=273$), whereas nine patients received tacrolimus-based prophylaxis. One patient received no prophylaxis. Acute and chronic GVHD were scored according to the classic Seattle criteria.[9,10]

Statistical considerations

The primary end point of this study was overall survival (OS) after allo-SCT. Disease-free survival (DFS) and cumulative incidences of relapse, nonrelapse mortality, and acute and chronic GVHD were evaluated as secondary end points. The probabilities of OS and DFS and the cumulative incidence of acute GVHD were calculated using the Kaplan–Meier method. Cumulative incidences of relapse and nonrelapse mortality were calculated using Gray's method, considering each other risk as a competing risk.[11] Univariate comparisons for dichotomous variables between groups were performed with Fisher's exact test or the χ^2-test and multivariate analyses were performed using logistic regression analysis. Proportional hazards modeling was used to assess the influence of confounding factors on time-to-event variables. Potential confounding factors considered in the analysis were recipient age, sex, FAB classification, karyotype, preparative regimen, stem cell source, and the history of chemotherapy before transplantation. The effect of the development of acute and chronic GVHD on the incidence of relapse was analyzed among patients who survived without relapse at 60 and 150 days after transplantation, respectively. This landmark analysis was used to exclude bias that may arise from including patients who died too early to develop GVHD in the group without GVHD.

Results

Patient characteristics

The patient characteristics are summarized in Table 1. There were 171 males and 112 females. The median age at allo-SCT was 41 years (range 16–65 years). The median duration from diagnosis to allo-SCT was 8 months (range 1–204 months). A total of 188 patients had received chemotherapy before allo-SCT (Chemo group), whereas 95 had not (NoChemo group). Among the Chemo group, 81 underwent allo-SCT in complete remission (CR), while 107 were not in remission (NR). The Chemo group included a significantly higher proportion of patients with advanced disease than the NoChemo group ($P<0.0001$). In addition, the proportion of patients with a poor karyotype was significantly higher in the Chemo group ($P=0.004$).

Survival

Of the 283 patients, 159 were alive with a median follow-up of 36.5 months (range 1–139 months). The Kaplan–Meier probability of OS was 67.9% (95% confidence interval (CI) 63.4–72.4%) at 1 year, 48.8% (95% CI 44.7–52.9%) at 5 years, and 42.5% (95% CI 38.8–46.3%) at 10 years (Figure 1). The OS and DFS curves are superimposed, since only 13 patients were alive after relapse at this analysis. In a univariate analysis, age younger than 40 years, disease duration of 1 year or longer, good karyotype, diagnosis of RA, and the NoChemo group were associated with longer OS (Table 2). Among these factors, FAB classification, karyotype, and the history of chemotherapy were identified as independent significant prognostic factors for OS (Table 2, Figure 2).

Table 1 Patient characteristics

		History of previous chemotherapy		
	Total	*Presence*	*Absence*	*P-value*
Age (years)				
≤40	136	94	42	0.38
>40	147	94	53	
Sex				
Male	171	116	55	0.61
Female	112	72	40	
FAB				
RA	61	29	32	<0.0001
RAEB	58	29	29	
RAEBt	70	55	15	
CMML	25	19	6	
LT	69	56	13	
Karyotype				
Good	131	84	47	0.004
Intermediate	60	31	29	
Poor	41	32	9	
Unknown	51	41	10	
TBI				
Presence	173	115	58	>0.99
Absence	110	73	37	
Stem cell				
BM	218	144	74	0.88
PB	65	44	21	

LT = leukemic transformation.

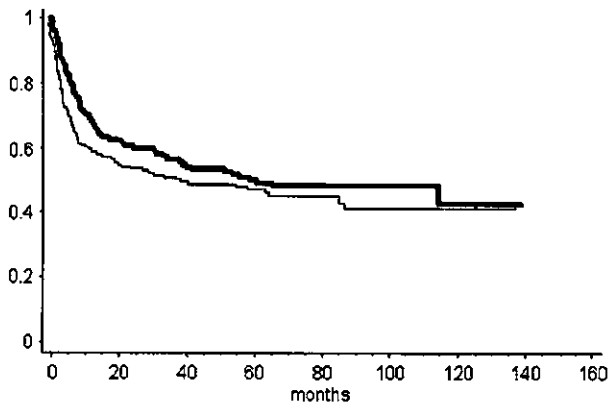

Figure 1 Overall survival (thick line) and disease-free survival (thin line) after transplantation.

Value of chemotherapy before transplantation

To determine the value of chemotherapy before transplantation in further detail, we only analyzed 139 patients with RAEB-t or LT to exclude biases, since the Chemo group included a significantly higher proportion of patients with RAEB-t and LT. Twenty-eight patients belonged to the NoChemo group and 111 patients belonged to the Chemo group. The proportion of

Table 2 Results of proportional hazard modeling for overall survival

Univariate	*Relative risk*	*P-value*
Sex		
Female	1.00	
Male	0.97 (0.68–1.39)	0.88
Age (years)		
<40	1.00	
⩾40	1.46 (1.02–2.09)	0.040
Duration from diagnosis to transplantation		
<1 year	1.00	
⩾1 year	0.64 (0.41–0.99)	0.045
Karyotype		
Good	1.00	
Intermediate	1.36 (0.83–2.21)	0.22
Poor	2.69 (1.65–4.37)	<0.0001
Unknown	1.47 (0.92–2.38)	0.11
FAB		
RA	1.00	
RAEB	1.84 (0.96–3.51)	0.065
RAEBt	2.42 (1.31–4.44)	0.0045
CMML	1.57 (0.71–3.50)	0.27
LT	3.16 (1.91–6.25)	<0.0001
Chemotherapy		
Absence	1.00	
Presence	1.92 (1.26–2.92)	0.0025
Unknown	1.70 (1.08–2.68)	0.023
Stem cell		
BMT	1.00	
PBSCT	1.33 (0.85–2.07)	0.21
Regimen		
TBI (–)	1.00	
TBI (+)	0.99 (0.69–1.42)	0.97
Multivariate		
FAB		
RA	1.00	
RAEB	1.82 (0.96–3.48)	0.069
RAEBt	2.00 (1.08–3.70)	0.029
CMML	1.31 (0.58–2.94)	0.51
LT	2.58 (1.40–4.76)	0.0023
Karyotype		
Good	1.00	
Intermediate	1.41 (0.86–2.30)	0.18
Poor	2.15 (1.30–3.55)	0.0028
Unknown	1.32 (0.82–2.14)	0.25
Chemotherapy		
Absence	1.00	
Presence	1.89 (1.19–2.99)	0.0065

patients with a poor karyotype was equivalent between the 2 groups (39 *vs* 45%, $P=0.44$). The duration from diagnosis to allo-SCT was shorter in the NoChemo group, with a marginal significance (161 *vs* 248 days in median, $P=0.07$). Among the Chemo group, 43 were in CR and 68 were in NR at allo-SCT. The survival curves of the NoChemo group and CR patients in the Chemo group were superimposed (57 *vs* 54%, $P=0.81$), whereas patients who underwent allo-SCT in NR after chemotherapy showed significantly shorter survival (20%, Figure 3a). The cumulative incidence of relapse was 26.2% in the NoChemo group and 27.6% in CR patients in the Chemo group ($P=0.83$, Figure 3b). Nonrelapse mortality was also similar between the two groups (18.8 *vs* 17.8%, $P=0.86$, Figure 3c). In contrast, the cumulative incidence of nonrelapse mortality was significantly higher in NR patients in the Chemo group than the NoChemo group (48.9 *vs* 18.8%, $P=0.014$), whereas difference in the incidence of relapse was not statistically significant (35.0 *vs* 26.2%, $P=0.44$).

The role of chemotherapy was also examined in patients with less-advanced MDS (RA or RAEB). Survival in the NoChemo group, CR patients in the Chemo group, and NR patients in the Chemo group was 73.4, 58.2, and 50.0% at 5 years, respectively. This difference was not statistically significant ($P=0.11$).

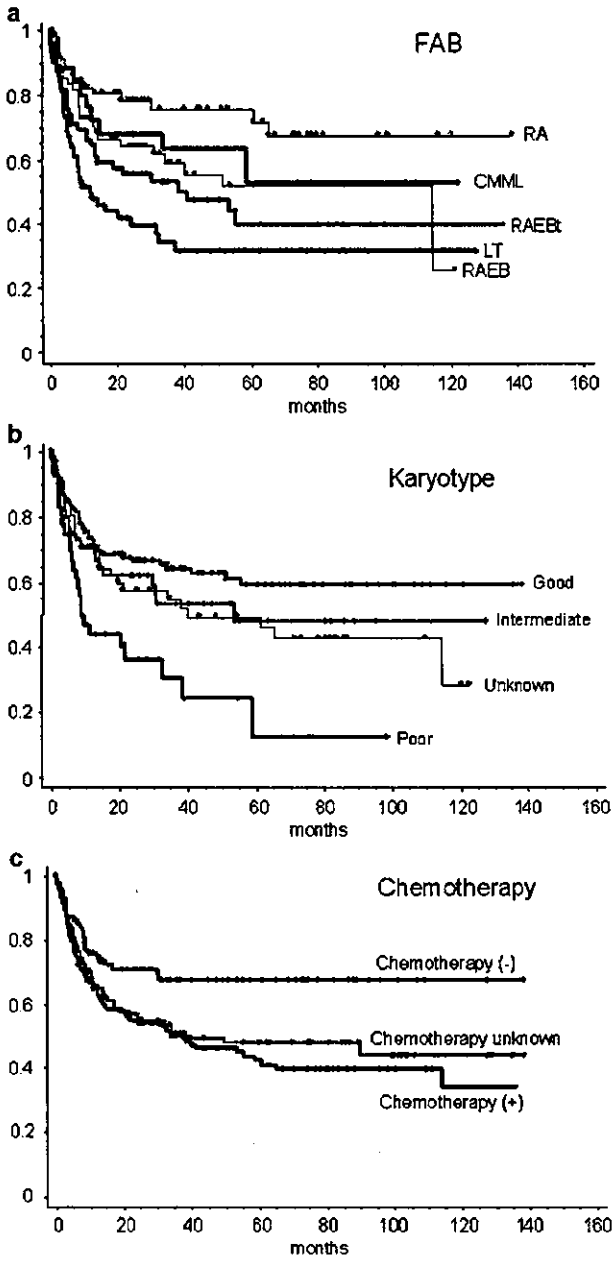

Figure 2 Overall survival grouped according to significant independent prognostic factors. Patients who lacked information regarding the history of chemotherapy before transplantation were included in (c).

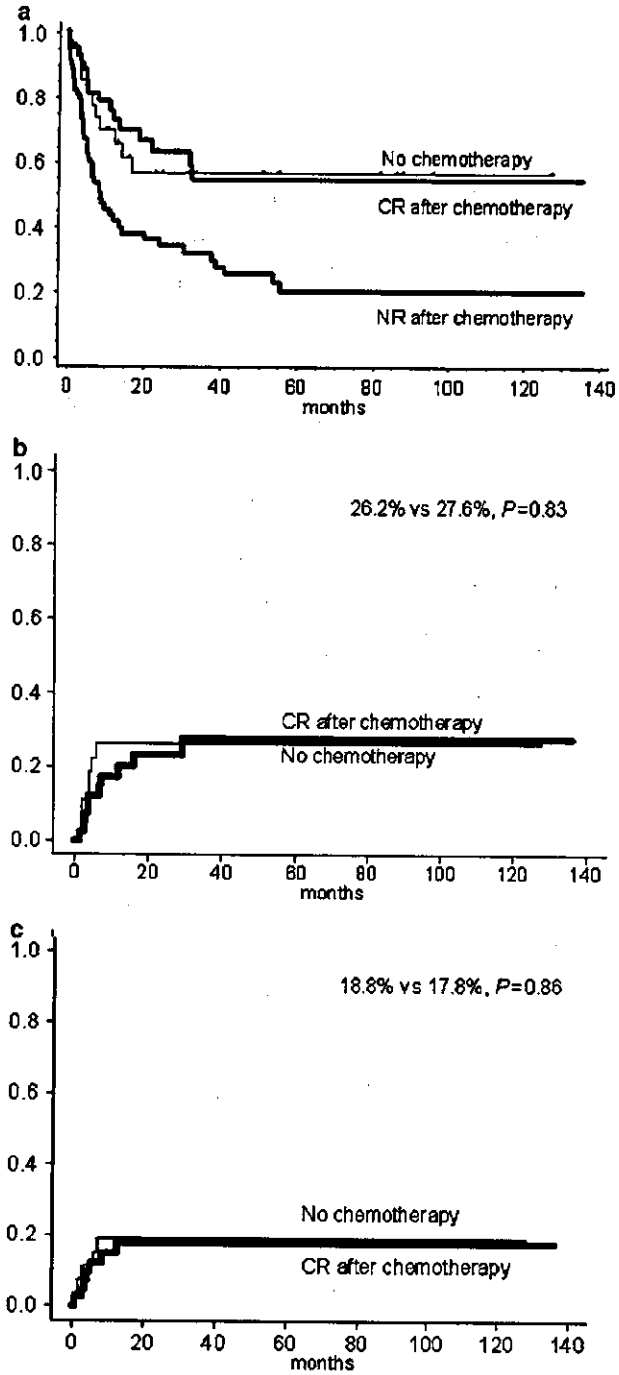

Figure 3 Overall survival (a) and cumulative incidences of relapse (b) and nonrelapse mortality (c) grouped according to the presence or absence of previous history of chemotherapy. These are compared between patients who achieved remission after chemotherapy and those who did not undergo chemotherapy. Only patients with RAEB-t or LT were included in the analyses.

Influence of acute and chronic GVHD on relapse

The cumulative incidence of grade II–IV and III–IV acute GVHD among those who achieved engraftment was 33 and 10%, respectively. Independent significant risk factors for grade II–IV acute GVHD were the karyotype (relative risk (RR) 1.98, 3.02, and 1.87, $P=0.016$, 0.0005, and 0.047 for intermediate, poor, and unknown groups, respectively) and the absence of a previous history of chemotherapy (RR 1.64, $P=0.033$). Chronic GVHD developed in 110 (53%) of 209 patients who survived more than 100 days. Logistic regression analysis identified higher age and the use of peripheral blood stem cell graft as independent significant risk factors for the development of chronic GVHD (RR 1.75 and 2.97, $P=0.049$ and 0.019, respectively). The cumulative incidence of relapse in those who developed grade II–IV acute GVHD and those who did not was 39.6 and 19.5%, respectively ($P=0.086$). The cumulative incidence of relapse in those who developed chronic GVHD and those who did not was 22.0 and 20.8%, respectively ($P=0.82$).

Discussion

Although intensive chemotherapy may cure rare young patients with advanced MDS,[10,12] allo-SCT is the only curative treatment for most MDS patients. The recent application of nonmyeloablative or reduced-intensity conditioning extended the indication of allo-SCT to older patients with MDS.[13,14] Factors known to be associated with the outcome of allo-SCT include patient age, disease duration, bone marrow blast counts, FAB classification, karyotype, and IPSS.[1–4,7,15,16] The fact that a lower bone marrow blast count before allo-SCT was associated with a better outcome after allo-SCT has encouraged physicians to administer intensive chemotherapy before allo-SCT. In fact, two registry reports from the European Group for Blood and Marrow Transplantation (EBMT) and National Marrow Donation Program showed that DFS for patients who underwent allo-SCT in first complete remission was better than that for patients who underwent allo-SCT as primary treatment for RAEB-t or LT.[1,2] However, this might only reflect the fact that patients with disease that originally responded well to chemotherapy were selected in the low blast count group. Also, these studies did not take into account patients who received induction chemotherapy but died or developed toxicity that precluded allo-SCT. Thus, these data do not justify the administration of induction chemotherapy before allo-SCT.

Runde *et al*[16] analyzed the outcome of patients who underwent BMT from HLA-identical siblings as first-line treatment for MDS and were reported to the EBMT. OS at 5 years was 42, 24, and 28% for patients with RAEB, RAEB-t, and LT, respectively. They concluded that allo-SCT should be performed as primary treatment for patients with a low blast cell count. Anderson *et al*[17] retrospectively compared the outcome of allo-SCT as an initial treatment for secondary AML and that of allo-SCT for chemotherapy-sensitive secondary AML. DFS at 5 years was not significantly different (24 *vs* 15%, $P=0.33$). The findings of the present analysis of JSHCT registry data were similar. OS at 5 years was 57% for patients who underwent allo-SCT for RAEB-t or LT without induction chemotherapy and 54% for those who underwent allo-SCT in remission ($P=0.81$). The difference was not significant even after adjusting for age and karyotype (RR 0.90, $P=0.80$). Although there might be a bias that patients with an indolent clinical outcome tended to undergo allo-SCT without induction chemotherapy, it was unlikely, because the duration from diagnosis to transplantation was rather shorter in the NoChemo group. Thus, the administration of remission induction chemotherapy before allo-SCT was not recommended from these data.

Information regarding the type of chemotherapy was not collected in this study and thus, it was supposed that low-dose

chemotherapy was also included, especially in patients with RA. However, most CR patients in the Chemo group must have received AML-type intensive chemotherapy, because CR can be rarely achieved with low-dose chemotherapy. A major shortcoming of this analysis was the lack of information regarding blast cell count at the time of transplantation. However, in the comparison between NoChemo group and CR patients in the Chemo group, blast count at transplantation was greater than 20% in the former group, whereas it was less than 5% in the latter group, because only patients with RAEB-t or LT were included in this analysis. Nevertheless, survival after transplantation was similar between the two groups.

We did not collect data of patients who underwent induction chemotherapy but did not proceed to allo-SCT. It was supposed that there were many patients who gave up transplantation due to lack of response to chemotherapy or toxicity of chemotherapy. Therefore, the CR patients in the Chemo group included only patients who had good response to chemotherapy and maintained good physical condition enough to undergo allo-SCT. Nevertheless, survival after transplantation was similar between the NoChemo group and the CR patients in the Chemo group.

OS at 5 years among patients with RAEB-t or LT in this study was better than those in previous reports. Considering the relapse rate of 26–27% and nonrelapse mortality rate of 17–18%, this good outcome resulted from the low nonrelapse mortality rate, probably due to the low incidence of acute GVHD. Not only the homogeneity of HLA and minor histocompatibility antigens but also the high frequency of the IL10-592A allele among the Japanese population may account for the low incidence of severe acute GVHD.[18–20]

The study from IBMTR did not demonstrate that the development of acute or chronic GVHD affected the incidence of relapse after allo-SCT from an HLA-identical sibling donor,[4] while the NMDP study showed that patients who developed grade II–IV acute GVHD had a lower incidence of relapse after allo-SCT from an unrelated donor than those who did not develop acute GVHD.[1] In this study, the development of GVHD did not appear to have a positive impact on the incidence of relapse. This discrepancy might be explained by a hypothesis that an anti-MDS effect associated with GVHD may be stronger in unrelated allo-SCT than in that from an HLA-identical sibling donor. Also, the anti-MDS effect associated with GVHD in this study might have been masked by the bias that the incidence of GVHD was significantly higher in patients with a poor karyotype.

In conclusion, this study confirmed that allo-SCT from an HLA-identical sibling donor offers the potential for long-term survival in patients with MDS. Induction chemotherapy before allo-SCT did not appear to offer any benefit. Although only a randomized controlled trial will be able to establish a definite conclusion, it seems that allo-SCT may be beneficial as primary treatment for patients with a low blast cell count or those with an advanced disease but with an indolent clinical course. Relapse is the major cause of failure after allo-SCT for advanced MDS, but we should not intend to induce GVHD, since GVHD did not appear to have a clear beneficial effect on the incidence of relapse.

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Unrelated donor transplants

Allogeneic peripheral blood stem cell transplantation from two- or three-loci-mismatched related donors in adult Japanese patients with high-risk hematologic malignancies

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Summary:

With the increasing frequency of haploidentical transplantation, it is becoming more important to establish the degree of HLA mismatch that can be accepted. We retrospectively analyzed clinical data of 50 adult Japanese patients with high-risk hematologic malignancies who underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from two- or three-loci-mismatched related donors with HLA class I and II gene disparities in the graft-versus-host direction. They were treated at 20 transplant centers between 1996 and 2002. In all, 18 patients received unmanipulated PBSC, while 32 received purified CD34+ blood cells. Conventional ($n=31$) or reduced-intensity ($n=19$) conditioning regimens were used. Of the 39 patients (78%) who survived for $\geqslant 28$ days after transplant, 37 (95%) achieved neutrophil engraftment, while graft failure and rejection occurred in two of 39 (5%) and three of 37 (8%) patients, respectively. Stepwise Cox regression analysis revealed a significantly lower incidence of grades II–IV acute GVHD in patients receiving purified CD34+ cells (hazard ratio 0.32; 95% CI 0.12–0.84; $P=0.022$). By 1 year post transplant, 28 patients (56%) had died of transplant-related problems, including infectious complications (30%). Although the number of patients is small, our data suggest that transplant-related problems, particularly infectious complications, are major obstacles to the success of this therapy.

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Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapeutic approach for a number of life-threatening hematologic malignancies.[1] Unfortunately, only 30–40% of patients have a matched related donor (MRD) or a one-locus-mismatched related donor (MMRD), mismatched for either HLA-A, -B or -DR antigens, availabe.[2] Therefore, most transplant centers perform allogeneic HSCT from a matched unrelated donor (MUD) as a second option for those who do not have an MRD. Even though a large number of volunteers are willing to donate bone marrow, there are candidate patients for whom an MUD is not available. Moreover, the aggressive nature of their diseases often precludes a lengthy search for an MUD. Recently, unrelated cord blood (CB) has been used as a source of allogeneic HSCT.[3] However, in some cases, it is difficult to collect a sufficient number of stem cells from CB to engraft adult patients, which has been reported as an important factor of improved event-free survival.[4] Therefore, two- or three-loci-MMRD, which are readily available, have been proposed as a potential stem cell source.

The degree of HLA disparity between patients and donors has been reported to have a major impact on the outcome of allogeneic HSCT, particularly on engraftment and acute GVHD.[5,6] Although the incidence of graft failure

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in BMT from a three-loci-MMRD or a haploidentical related donor has been reported to be higher than in BMT from a one-locus- or two-loci-MMRD,[7] allogeneic HSCT from a two- or three-loci-MMRD was demonstrated to be feasible for the achievement of stable engraftment.[8] Allogeneic HSCT from a two-loci-MMRD was reported to be associated with a similar incidence and severity of acute GVHD as conventional allogeneic HSCT from a one-locus-MMRD,[9] especially in patients receiving purified CD34+ blood cells.[10] It is becoming more important to establish the degree of HLA mismatch that can be accepted.

The Japanese population is believed to be relatively homogeneous both ethnically and genetically, with less HLA genetic diversity than Caucasians.[11,12] This could make unmanipulated allogeneic HSCT between two- or three-loci-mismatched pairs more feasible in this population because there may be lower incidence of graft failure and GVHD. Allogeneic peripheral blood stem cell transplantation (PBSCT) is increasingly undertaken in Japan since it became eligible for reimbursement from health insurance in the year 2000. We conducted a nationwide retrospective survey to investigate the time course of engraftment, incidence of acute and chronic GVHD, transplant-related problems and survival in patients undergoing allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the graft-versus-host (GVH) direction.

Patients and methods

Patient characteristics

This study included 50 Japanese patients over age 16 who underwent allogeneic PBSCT from two- or three-loci-MMRD with HLA class I (HLA-A and/or -B) and II (HLA-DRB1) gene disparities in the GVH direction for a variety of high-risk hematologic malignancies. They were transplanted between January 1996 and March 2002 at 20 different transplant centers in Japan. The patients consisted of 17 (34%) with AML, six (12%) with ALL, six (12%) with myelodysplastic syndrome (MDS), eight (16%) with CML, 11 (22%) with non-Hodgkin's lymphoma (NHL) and two (4%) with multiple myeloma (MM). All of them were considered to be at high risk for relapse or to be refractory to intensive chemotherapy: 18 patients (36%) were transplanted at primary refractory status, 17 (34%) at chemorefractory status, nine (18%) in relapse after prior autologous PBSCT, three (6%) in relapse after prior allogeneic BMT from an MUD (MUD-BMT), one (2%) in relapse after prior unrelated CB transplantation and MUD-BMT, and two (4%) after graft rejection following MUD-BMT for MDS. A total of 33 patients (66%) underwent primary allogeneic PBSCT and 17 (34%) underwent allogeneic PBSCT after prior autologous PBSCT ($n=10$, 20%) or prior allogeneic HSCT ($n=7$, 14%). The median time interval between prior autologous PBSCT or allogeneic HSCT and this therapy was 10.8 (range 3.1–27.3) or 5.3 months (1.7–29.2 months), respectively. Patient characteristics are summarized in Table 1. Treatment protocols were approved by local institutional review boards and all patients provided informed consent.

Stem cell collection and graft manipulation

G-CSF (Filgrastim, Kirin Brewery/Sankyo Co., Tokyo, Japan) was administered subcutaneously to donors at a dose of 400 $\mu g/m^2$ per day for 4 or 5 days as previously reported.[13] Leukaphereses were performed using a continuous-flow blood cell separator (Cobe Spectra, Cobe Laboratories, Lakewood, CO, USA) for 1–3 days beginning on day 4 of G-CSF administration until 3.0×10^6 CD34 cells/kg (patient body weight) had been collected. The percentages of CD34+ and CD3+ cells in the graft were determined by flow cytometry. Median doses of nucleated, CD34+ and CD3+ cells infused are shown in Table 1. Immunomagnetic selection was performed using a CliniMACS device ($n=17$; Kirin Brewery, Tokyo, Japan)[14] or an Isolex system ($n=15$; Baxter, Munich, Germany)[15] according to the manufacturer's recommendations. The graft was cryopreserved until infusion as previously reported.[16]

Conditioning regimen and GVHD prophylaxis

A conventional conditioning regimen was administered to 31 patients (62%). In all, 26 (52%) received 8–13.2 Gy TBI, and eight (16%) were given antithymocyte globulin (ATG) as an additional immunosuppressive. All patients treated with a conventional conditioning regimen received GVHD prophylaxis (CYA-based, $n=18$; FK506-based, $n=13$), as summarized in Table 2.

A total of 19 patients (38%) were treated with a reduced-intensity conditioning regimen: eight (16%) received 2–6 Gy TBI and 11 (22%) were given ATG. All but one of these patients treated with a reduced-intensity conditioning regimen received GVHD prophylaxis (CYA-based, $n=8$; FK506-based, $n=9$; prednisolone alone, $n=1$).

Definition of outcome

Neutrophil engraftment was defined as an absolute neutrophil count (ANC) exceeding $0.5 \times 10^9/l$ for 3 consecutive days after transplant. The day of neutrophil engraftment was determined to be the first of these 3 consecutive days. Platelet engraftment was defined as a platelet count exceeding $20 \times 10^9/l$ without platelet support. In all 42 patients (84%) received G-CSF until neutrophil engraftment after transplant. If the ANC never exceeded $0.5 \times 10^9/l$ or if it was not maintained above $0.5 \times 10^9/l$ for at least 3 consecutive days by day 28 post transplant, the patient was considered to have 'graft failure'. If ANC of greater than $0.5 \times 10^9/l$ could not be maintained after initial engraftment, the patient was considered to have 'graft rejection'.

The severity of acute GVHD was graded according to the consensus criteria[17] among 43 evaluated patients (86%) who developed acute GVHD within 28 days or who survived ≥ 28 days after transplant. Chronic GVHD was assessed and graded according to the standard criteria[18]