Table 2. *Clinical Characteristics of Patients with Invasive Aspergillosis*

Variable	CST (n = 21)	RIST (n = 14)	P value
Age, y, median (range)	38 (22-57)	54 (37-70)	<.01*
Antifungal prophylaxis, fluconazole/others	21/0	14/0	
Pulmonary complication before HSCT, yes/no	2/19	1/13	1.00
Risk of transplantation†, low/high	7/14	2/12	.26
Neutropenia at onset of IA, yes/no	3/18	3/11	.66
Graft-versus-host disease (GVHD), acute/chronic/none	11/7/3	5/7/2	
Use of corticosteroid‡, yes/no	17/4	13/1	.63
CMV infection (antigenemia positive), yes/no	4/17	5/9	.43
Onset day of invasive aspergillosis, median (range)	97 (11-885)	127 (35-364)	<.01
Treatment			
deoxycholate amphotericin B	14	10	.67
liposomal *amphotericin* B	2	1	
itraconazole	5	2	
no treatment	0	1	
Response to antifungal therapy, yes/no	6/15	5/9	.72
Mortality after IA diagnosis, within 30 days	7	8	.19

CST indicates conventional stem cell transplantation, RIST indicates reduced-intensity stem cell transplantation, CMV indicates cytomegalovirus.

*Statistically significant.

†We divided the risk of transplantation into 2 groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, and myelodysplastic syndrome. The other patients were defined as having high-risk diseases.

‡Use of corticosteroid during administration of antithymocyte globulin was excluded from this analysis.

acteristics and risk factors of IA. The cumulative incidence curves of IA were produced by using Gray's method [25], considering death without IA as a competing risk. The median follow-up period after transplantation was 518 days (range, 2-1874 days), and surviving patients were censored on the last day of follow-up.

Risk factors associated with IA were identified in univariate and multivariate Cox regression models. The variables analyzed included age, sex, primary disease and its status at transplantation, stem cell source, donor type, and type of transplantation (CST versus RIST). To evaluate the influence of the development of grade II to IV acute or chronic GVHD, proportional hazard modeling was used, with the onset of GVHD treated as a time-dependent covariate. Variables with a P value of <.10 were subjected to a multivariate analysis with backward stepwise proportional hazard modeling. P values <.05 were considered significant.

RESULTS

Incidence of IA

Thirty-five (5.6%) patients were diagnosed as having IA, which gave a 3-year cumulative incidence of 5.9%. Five of the 35 cases had proven IA, and the remaining 30 had probable IA according to the EORTC/NIH/MSG criteria [24]. The diagnosis of IA was established after death in 4 of the 5 patients with proven IA, and 1 of these cases was diagnosed with a transbronchial lung biopsy. Three of the remaining 4 proven cases had been diagnosed as probable IA while the patients were alive. The day of diagnosis of IA was defined as the day when the first diagnostic test was performed. Thirty patients were diagnosed as having probable IA on the basis of clinical, radiologic, and microbiological findings. All the patients showed some subjective or objective symptoms. The circulating galactomannan antigen assay tested positive in 26 patients. *Aspergillus* species were cultured from sputum in 3 patients, and bronchoalveolar lavage fluid was positive for galactomannan antigen in a patient. All of the 30 patients underwent chest computed tomography scan, and all of them showed some abnormal findings including halo and multiple nodules.

Clinical Characteristics of IA

The clinical characteristics of patients with IA are shown in Table 2. All cases developed after engraftment, whereas 3 patients each in the RIST and CST groups were still neutropenic at the presentation of IA. The median time to the onset after RIST (127 days) was later than that after CST (97 days; $P < .01$). All of the patients had been receiving fluconazole prophylaxis when the diagnosis of IA was established. Of the 35 patients with IA, 30 (86%) had GVHD (acute in 16 and chronic in 14). Thirty patients had been receiving corticosteroid treatment at the time of IA diagnosis, all for the treatment of GVHD. CMV antigenemia was positive in 9 of the 35 patients, and 3 of these progressed to CMV disease. Five patients with IA showed no signs of GVHD and were not receiving corticosteroid treatment when the diagnosis

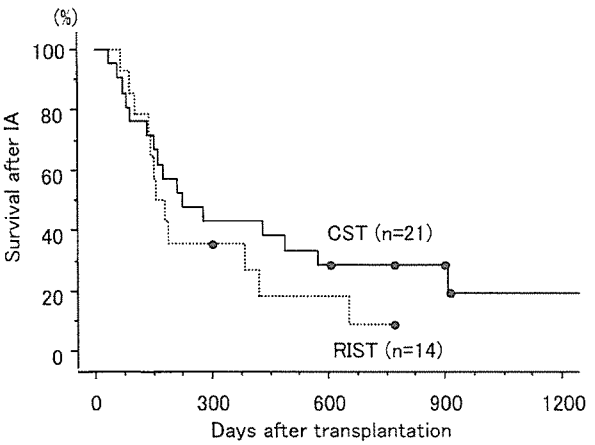

Figure 1. Overall survival after the diagnosis of IA. The survival rate at 1 year after the diagnosis of IA was similarly poor in both the RIST and CST groups (27% and 18%, respectively; P = .24).

of IA was established. The clinical characteristics of patients with IA after RIST (n = 14) were compared with those with IA after CST (n = 21), as summarized in Table 2. Except for age and onset of IA, no significant differences were observed between groups.

Treatment and Outcomes

Initial antifungal treatment consisted of deoxycholate amphotericin B (n = 24), liposomal amphotericin B (n = 3), and itraconazole (n = 7). One patient did not receive any antiaspergillus treatment because the diagnosis of IA was established after death. Sixteen (76%) of the 21 CST recipients and 12 (86%) of the 14 RIST recipients who developed IA died, and IA was the direct cause of death in 5 patients in each group. The 1-year survival rates after the diagnosis of IA were similarly poor in both groups (24% versus 19%; P = .19; Figure 1).

Risk Factors for IA

The actuarial frequency for the development of IA in the RIST group was 7.9% (14/178), which gave a 3-year cumulative incidence of 8.2%. In the CST group, 21 (4.3%) of 487 patients developed IA, to give a 3-year cumulative incidence of 4.5%. The probability of developing IA was significantly higher in the RIST group than in the CST group (P = .045; Figure 2). Old age (P = .0068), disease risk for transplantation (P = .0043), RIST (P = .045), and the development of GVHD (P = .00014) were significant risk factors for IA by univariate analysis (Table 3). Among these factors, only age >50 years and the development of GVHD were confirmed to be independently significant by proportional hazard models (Table 3). The difference in the types of preparative regimens was not significant after adjusting for age, donor sources, and the development of GVHD (RIST: relative risk, 1.52; 95% confidence interval, 0.64-3.6; P = .34).

DISCUSSION

This study was conducted to investigate the incidence and clinical features of IA after RIST compared with those after CST. IA is a significant complication in patients receiving CST, but little information is currently available on IA after RIST. Although the retrospective nature of this study is a limitation, this is one of the largest studies on IA after RIST and provides valuable information on this complication. We found that the incidence of IA was 4.3% and 7.9% in CST and RIST recipients, respectively. IA tended to be more common in older patients after RIST than after CST, suggesting that IA is still a significant complication in RIST, as well as in CST, in this setting. The incidence of IA in our study is lower than those in previous reports after CST [4] or RIST [18]. Although the reason for this difference is unclear, it might be associated with the lower incidence of GVHD and the consequent decrease in the use of steroids in our country [26]: both of these are known risk factors for IA [4].

The diagnostic criteria used in epidemiologic studies on IA are debatable. IA is difficult to diagnose while patients are alive, and it is frequently confirmed by autopsy. We strictly applied the diagnostic criteria recommended by EORTC/NIH/MSG [24]. However, in retrospective epidemiologic studies, the number of "IA" cases depends on whether both possible and probable IA cases are included. Because we included only probable and proven cases in this study and excluded possible cases, the rate of IA might have been underestimated. Nevertheless, we believe that the primary end point was achieved, because we com-

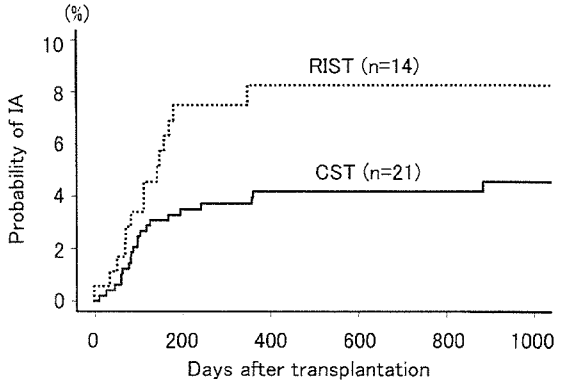

Figure 2. The incidences of invasive aspergillosis after RIST and CST. The cumulative incidences of IA at 3 years after RIST and CST were 8.2% and 4.5%, respectively, with a statistically significant difference (P = .045).

Table 3. *Risk Factors for Invasive Aspergillosis*

Factor	Variable	n	Incidences	P value
Univariate analysis				
Pretransplant factors				
Sex	Male	419	5.9%	.48
	Female	245	4.9%	
Age	<50	471	4.1%	.0068*
	≥50	193	9.2%	
Risk for transplantation	High-risk	486	7.2%	.0043*
	Low-risk	178	3.9%	
Donor	Matched related	343	5.6%	.39
	Mismatched related	39	11.7%	
	Matched unrelated	233	4.7%	
	Mismatched unrelated	49	4.1%	
Graft source	Bone marrow	413	4.1%	.091
	Peripheral Blood	251	7.8%	
Preparative regimen	Conventional	486	4.5%	.045*
	Reduced-intensity	178	8.2%	
Use of ATG	With	81	5.4%	.91
	Without	583	5.6%	
Posttransplant factor				
GVHD (acute and/or chronic)	Presence vs Absence		6.55 (2.49-17.2)	.00014*

Factor	Relative Risk	95% Confidence interval	P value
Multivariate analysis			
Age older than 50	2.12	1.08-4.17	.03*
GVHD (acute and/or chronic)	6.2	2.4-16.4	.0002*

ATG indicates Antithymocyte globin; GVHD indicates graft-versus-host disease.
*Statistically significant.

pared the 2 groups of patients by using the same criteria.

The onset of IA is bimodal, peaking 16 and 96 days after transplantation [3]. Recent studies, including ours, have shown that the development of IA has shifted to a late onset [4,18]. Several factors have contributed to the shift of IA development. First, most recipients undergo transplantation in a laminar air flow–equipped room with or without high-efficiency particulate air filters, which have been demonstrated to be protective against aspergillus infection [3]. Second, early-onset IA tends to occur among patients with incubating or occult aspergillus infection, and these high-risk patients usually receive some antiaspergillus agents before or immediately after transplantation. Third, the development of GVHD, which frequently necessitates corticosteroid therapy, is delayed in RIST compared with CST [27]. Both GVHD and the use of corticosteroids are independent risk factors for IA after HSCT [4]. Finally, the development of CMV infection, which is associated with IA [28], is also delayed in RIST [29]. We should therefore recognize that IA has been well characterized as a late-onset complication in RIST, and any preventive strategy for IA should be extended to cover the possibility of late aspergillosis. Prolonged prophylaxis with antiaspergillus agents such as voriconazole and itraconazole might be beneficial for reducing its incidence.

We found that IA is associated with a poor prognosis in RIST as well as in CST. In this study, only 2 of the 14 RIST recipients and 5 of the 21 CST recipients who developed IA survived. Although most patients with IA had been intensively treated with intravenous amphotericin B, the response rate to treatment was only 36% and 29% in RIST and CST recipients, respectively. Despite the recent availability of new antifungal agents such as voriconazole, itraconazole, and lipid-complex amphotericin B preparations, treatment of IA in recipients of HSCT is still a difficult task without the recovery of host immunity.

It is important to determine the risk factors of IA to identify high-risk patients and to enable the introduction of prophylaxis for more intense infection. The primary risk factor for IA in our study was the development of GVHD and the closely associated use of corticosteroid, because they further delay immune recovery to open the way for the development of IA. Because the incidence of GVHD after RIST is similar to that after CST in this study, it is quite reasonable that the clinical significance of IA is comparable to that after CST. Although use of antithymocyte globulin might be associated with an increase in the risk of IFI, this was not observed in our study. We speculate that the decrease in the incidence of GVHD with the introduction of antithymocyte globulin (10% in this study) contributed to the decrease in the use of steroid and, hence, decreased the risk of IA. Because the dose

of antithymocyte globulin we used was lower than those used in other institutes [12,30], the mild suppression of immune recovery might be another reason for the lower incidence of IA.

We believe that this study will provide some useful information on IA; however, there are several limitations. We compared the incidence of IA between the 2 treatment groups. They had different backgrounds, and many different preparative regimens were used for different underlying diseases, thus making it difficult to draw definite conclusion on IA from this study. The reliability of patient diagnosis is another concern. Although we made an IA diagnosis by using the EORTC/NIH/MSG criteria [24], only 1 of 7 IA patients had histopathologic evidence of IA. The EORTC/NIH/MSG criteria are the best currently available consensus on IFI in immunocompromised patients. However, it is well known that it is difficult to make an accurate diagnosis of IA in recipients of allogeneic HSCT, and most cases are diagnosed by postmortem examination. Additionally, the retrospective nature of this study provides a potential bias. Development of IA is associated with GVHD and corticosteroid use. Further study is required to investigate the association between IA development and other variables, including the severity of GVHD and the dose and duration of corticosteroid use. A prospective evaluation is warranted to further clarify the clinical features of IA after RIST.

In conclusion, we showed that IA is a major complication of RIST that affects older patients and is associated with significant mortality. Special attention should be paid to the effect of steroid treatment on complicated GVHD, especially in older patients. Currently, high-efficiency particulate air filters and correctly sealed rooms are accepted as proper prophylaxis for IA. However, these measures are not realistic in RIST, in which the onset of IA averaged beyond 100 days, when patients are generally followed up in the outpatient clinic [31]. The effectiveness of chemoprophylaxis with newly developed drugs against aspergillus [32,33] and of changing the home environment to eliminate the routes of infection for aspergillosis should be seriously investigated.

REFERENCES

1. Wingard JR, Merz WG, Rinaldi MG, Johnson TR, Karp JE, Saral R. Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N Engl J Med.* 1991;325:1274-1277.
2. Martino R, Subira M, Rovira M, et al. Invasive fungal infections after allogeneic peripheral blood stem cell transplantation: incidence and risk factors in 395 patients. *Br J Haematol.* 2002; 116:475-482.
3. Wald A, Leisenring W, van Burik JA, Bowden RA. Epidemiology of Aspergillus infections in a large cohort of patients undergoing bone marrow transplantation. *J Infect Dis.* 1997; 175:1459-1466.
4. Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood.* 2002;100:4358-4366.
5. Kami M, Tanaka Y, Kanda Y, et al. Computed tomographic scan of the chest, latex agglutination test and plasma (1-3)-beta-D-glucan assay in early diagnosis of invasive pulmonary aspergillosis: a prospective study of 215 patients. *Haematologica.* 2000;85:745-752.
6. Kami M, Fukui T, Ogawa S, et al. Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. *Clin Infect Dis.* 2001;33:1504-1512.
7. Stynen D, Goris A, Sarfati J, Latge JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. *J Clin Microbiol.* 1995;33: 497-500.
8. Herbrecht R, Denning DW, Patterson TF, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med.* 2002;347:408-415.
9. Arathoon EG. Clinical efficacy of echinocandin antifungals. *Curr Opin Infect Dis.* 2001;14:685-691.
10. Ribaud P, Chastang C, Latge JP, et al. Survival and prognostic factors of invasive aspergillosis after allogeneic bone marrow transplantation. *Clin Infect Dis.* 1999;28:322-330.
11. Giralt S, Estey E, Albitar M, et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood.* 1997;89:4531-4536.
12. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood.* 1998;91:756-763.
13. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood.* 2001;97:3390-3400.
14. Or R, Shapira MY, Resnick I, et al. Nonmyeloablative allogeneic stem cell transplantation for the treatment of chronic myeloid leukemia in first chronic phase. *Blood.* 2003;101:441-445.
15. Michallet M, Bilger K, Garban F, et al. Allogeneic hematopoietic stem-cell transplantation after nonmyeloablative preparative regimens: impact of pretransplantation and posttransplantation factors on outcome. *J Clin Oncol.* 2001;19:3340-3349.
16. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med.* 2000; 343:750-758.
17. Hagen EA, Stern H, Porter D, et al. High rate of invasive fungal infections following nonmyeloablative allogeneic transplantation. *Clin Infect Dis.* 2003;36:9-15.
18. Fukuda T, Boeckh M, Carter RA, et al. Risks and outcomes of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants after nonmyeloablative conditioning. *Blood.* 2003;102:827-833.
19. Mossad SB, Avery RK, Longworth DL, et al. Infectious complications within the first year after nonmyeloablative alloge-

neic peripheral blood stem cell transplantation. *Bone Marrow Transplant.* 2001;28:491-495.
20. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995;15:825-828.
21. Sullivan KM, Agura E, Anasetti C, et al. Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Semin Hematol.* 1991;28:250-259.
22. Kanda Y, Mineishi S, Saito T, et al. Long-term low-dose acyclovir against varicella-zoster virus reactivation after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2001;28:689-692.
23. Pizzo PA. Management of fever in patients with cancer and treatment-induced neutropenia. *N Engl J Med.* 1993;328:1323-1332.
24. Ascioglu S, Rex JH, de Pauw B, et al. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis.* 2002;34:7-14.
25. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med.* 1999;18:695-706.
26. Morishima Y, Morishita Y, Tanimoto M, et al. Low incidence of acute graft-versus-host disease by the administration of methotrexate and cyclosporine in Japanese leukemia patients after bone marrow transplantation from human leukocyte antigen compatible siblings; possible role of genetic homogeneity. The Nagoya Bone Marrow Transplantation Group. *Blood.* 1989;74:2252-2256.
27. Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood.* 2003;102:756-762.
28. Junghanss C, Marr KA, Carter RA, et al. Incidence and outcome of bacterial and fungal infections following nonmyeloablative compared with myeloablative allogeneic hematopoietic stem cell transplantation: a matched control study. *Biol Blood Marrow Transplant.* 2002;8:512-520.
29. Nakai K, Kanda Y, Mineishi S, et al. Suspected delayed immune recovery against cytomegalovirus after reduced-intensity stem cell transplantation using anti-thymocyte globulin. *Bone Marrow Transplant.* 2002;29:237-241.
30. Saito T, Kanda Y, Kami M, et al. Therapeutic potential of a reduced-intensity preparative regimen for allogeneic transplantation with cladribine, busulfan, and antithymocyte globulin against advanced/refractory acute leukemia/lymphoma. *Clin Cancer Res.* 2002;8:1014-1020.
31. Imataki O, Kami M, Kim SW, et al. A nationwide survey of deep fungal infections and fungal prophylaxis after hematopoietic stem cell transplantation in Japan. *Bone Marrow Transplant.* 2004;33;1173-1179.
32. Marr KA, Crippa F, Leisenring W, et al. Itraconazole versus fluconazole for prevention of fungal infections in patients receiving allogeneic stem cell transplants. *Blood.* 2004;103:1527-1533.
33. Winston DJ, Maziarz RT, Chandrasekar PH, et al. Intravenous and oral itraconazole versus intravenous and oral fluconazole for long-term antifungal prophylaxis in allogeneic hematopoietic stem-cell transplant recipients. A multicenter, randomized trial. *Ann Intern Med.* 2003;138:705-713.

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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 (Platellia 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 nyelogenous 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, allogencic 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.

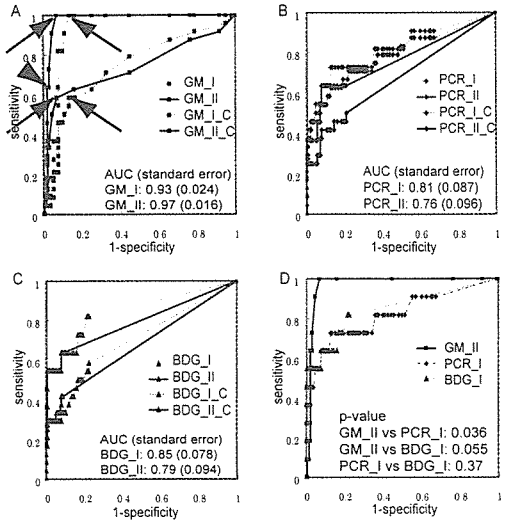

FIG. 1. (A to C) ROC curves of the GM (A), PCR (B), and BDG (C) tests for screening for IA. Both methods I and II were used. The ROC curves obtained by estimate A/B are shown in red, and those obtained by estimate C are shown in blue. The ROC curves obtained by method II are indicated by solid lines,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

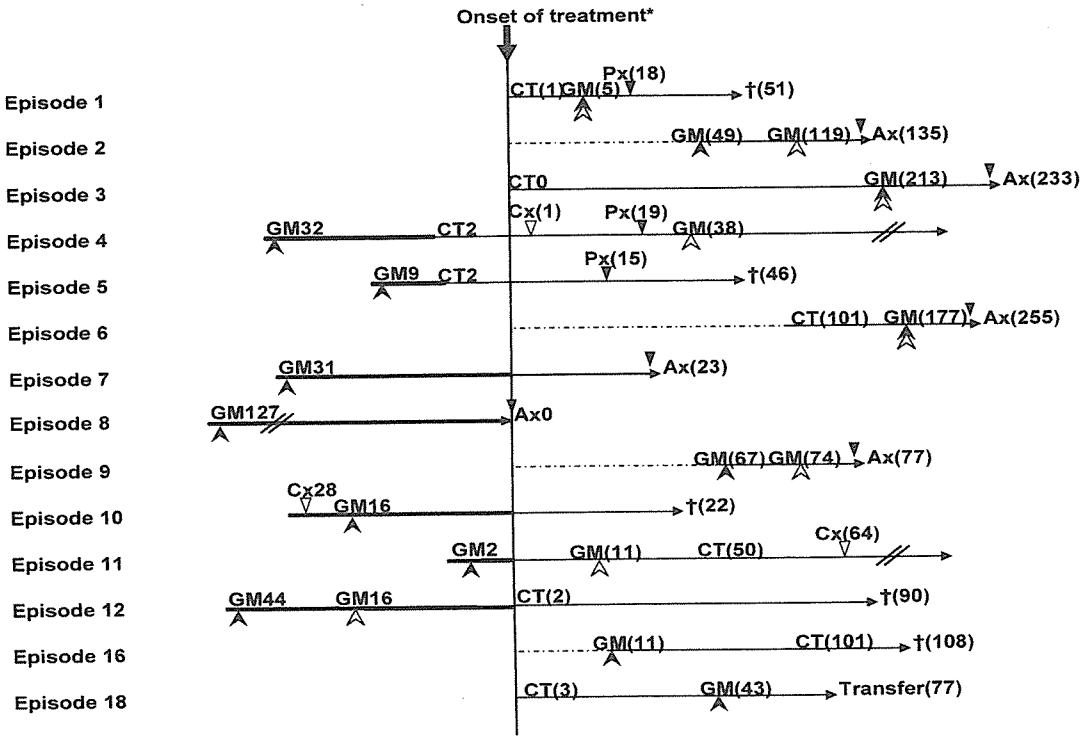

FIG. 2. Number of days from when GM assays become positive to the onset of treatment, using a threshold of 0.6 O.D.I. by method II (solid arrowheads) or 1.0 O.D.I. by method II (open arrowheads), or positive findings on CT. Open triangles indicate the date of positive culture, and solid triangles indicate 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

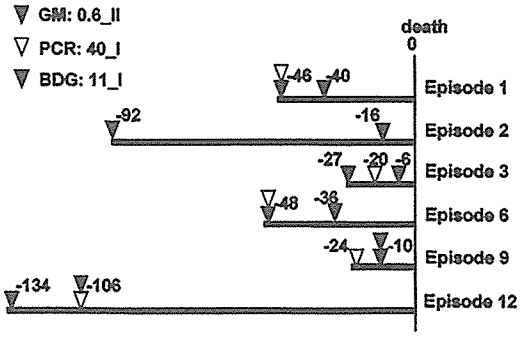

FIG. 3. Number of days before death that each test gave positive results. Solid triangles indicate the date when GM became positive, using a threshold of 0.6 O.D.I. by method II; open triangles indicate the date when PCR exceeded a cutoff value of 40 copies/ml; and shaded triangles indicate the date 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.

REFERENCES

1. **Andreas, S., S. Heindl, C. Wattky, K. Moller, and R. Ruchel.** 2000. Diagnosis of pulmonary aspergillosis using optical brighteners. Eur. Respir. J. **15**:407–411.
2. **Ascioglu, S., J. H. Rex, B. de Pauw, J. E. Bennett, J. Bille, F. Crokaert, D. W. Denning, J. P. Donnelly, J. E. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. Maertens, J. F. Meis, T. F. Patterson, J. Ritter, D. Selleslag, P. M. Shah, D. A. Stevens, and T. J. Walsh.** 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin. Infect. Dis. **34**:7–14.
3. **Becker, M. J., S. de Marie, D. Willemse, H. A. Verbrugh, and I. A. Bakker-Woudenberg.** 2000. Quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in an experimental rat model. J. Clin. Microbiol. **38**:1434–1438.
4. **Bowden, R., P. Chandrasekar, M. H. White, X. Li, L. Pietrelli, M. Gurwith, J. A. van Burik, M. Laverdiere, S. Safrin, and J. R. Wingard.** 2002. A double-blind, randomized, controlled trial of amphotericin B colloidal dispersion versus amphotericin B for treatment of invasive aspergillosis in immunocompromised patients. Clin. Infect. Dis. **35**:359–366.
5. **Bretagne, S., J. M. Costa, E. Bart-Delabesse, N. Dhedin, C. Rieux, and C. Cordonnier.** 1998. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. Clin. Infect. Dis. **26**:1407–1412.
6. **Buchheidt, D., C. Baust, H. Skladny, J. Ritter, T. Suedhoff, M. Baldus, W. Seifarth, C. Leib-Moesch, and R. Hehlmann.** 2001. Detection of *Aspergillus* species in blood and bronchoalveolar lavage samples from immunocompromised patients by means of 2-step polymerase chain reaction: clinical results. Clin. Infect. Dis. **33**:428–435.
7. **Caillot, D., O. Casasnovas, A. Bernard, J. F. Couaillier, C. Durand, B. Cuisenier, E. Solary, F. Piard, T. Petrella, A. Bonnin, G. Couillault, M. Dumas, and H. Guy.** 1997. Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computed tomographic scan and surgery. J. Clin. Oncol. **15**:139–147.
8. **Caillot, D., J. F. Couaillier, A. Bernard, O. Casasnovas, D. W. Denning, L. Mannone, J. Lopez, G. Couillault, F. Piard, O. Vagner, and H. Guy.** 2001. Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. J. Clin. Oncol. **19**:253–259.
9. **Costa, C., J. M. Costa, C. Desterke, F. Botterel, C. Cordonnier, and S. Bretagne.** 2002. Real-time PCR coupled with automated DNA extraction and detection of galactomannan antigen in serum by enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis. J. Clin. Microbiol. **40**:2224–2227.
10. **Denning, D. W.** 1998. Invasive aspergillosis. Clin. Infect. Dis. **26**:781–803.
11. **Denning, D. W.** 1996. Therapeutic outcome in invasive aspergillosis. Clin. Infect. Dis. **23**:608–615.
12. **Einsele, H., H. Hebart, G. Roller, J. Loffler, I. Rothenhofer, C. A. Muller, R. A. Bowden, J. van Burik, D. Engelhard, L. Kanz, and U. Schumacher.** 1997. Detection and identification of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. **35**:1353–1360.
13. **Hanley, J. A., and B. J. McNeil.** 1983. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology **148**:839–843.
14. **Hebart, H., J. Loffler, C. Meisner, F. Serey, D. Schmidt, A. Bohme, H. Martin, A. Engel, D. Bunje, W. V. Kern, U. Schumacher, L. Kanz, and H. Einsele.** 2000. Early detection of aspergillus infection after allogeneic stem cell transplantation by polymerase chain reaction screening. J. Infect. Dis. **181**:1713–1719.
15. **Herbrecht, R., V. Letscher-Bru, C. Oprea, B. Lioure, J. Waller, F. Campos, O. Villard, K. L. Liu, S. Natarajan-Ame, P. Lutz, P. Dufour, J. P. Bergerat, and E. Candolfi.** 2002. Aspergillus galactomannan detection in the diagnosis of invasive aspergillosis in cancer patients. J. Clin. Oncol. **20**:1898–1906.
16. **Hughes, W. T., D. Armstrong, G. P. Bodey, A. E. Brown, J. E. Edwards, R. Feld, P. Pizzo, K. V. Rolston, J. L. Shenep, and L. S. Young.** 1997. 1997 guidelines for the use of antimicrobial agents in neutropenic patients with unexplained fever. Infectious Diseases Society of America. Clin. Infect. Dis. **25**:551–573.
17. **Kami, M., T. Fukui, S. Ogawa, Y. Kazuyama, U. Machida, Y. Tanaka, Y. Kanda, T. Kashima, Y. Yamazaki, T. Hamaki, S. Mori, H. Akiyama, Y. Mutou, H. Sakamaki, K. Osumi, S. Kimura, and H. Hirai.** 2001. Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. Clin. Infect. Dis. **33**:1504–1512.
18. **Kami, M., Y. Tanaka, Y. Kanda, S. Ogawa, T. Masumoto, K. Ohtomo, T. Matsumura, T. Saito, U. Machida, T. Kashima, and H. Hirai.** 2000. Computed tomographic scan of the chest, latex agglutination test and plasma (1AE3)-beta-D-glucan assay in early diagnosis of invasive pulmonary aspergillosis: a prospective study of 215 patients. Haematologica **85**:745–752.
19. **Kawamura, S., S. Maesaki, T. Noda, Y. Hirakata, K. Tomono, T. Tashiro, and S. Kohno.** 1999. Comparison between PCR and detection of antigen in sera for diagnosis of pulmonary aspergillosis. J. Clin. Microbiol. **37**:218–220.
20. **Kawazu, M., Y. Kanda, S. Goyama, M. Takeshita, Y. Nannya, M. Niino, Y. Komeno, T. Nakamoto, M. Kurokawa, S. Tsujino, S. Ogawa, K. Aoki, S. Chiba, T. Motokura, N. Ohishi, and H. Hirai.** 2003. Rapid diagnosis of invasive pulmonary aspergillosis by quantitative polymerase chain reaction using bronchial lavage fluid. Am. J. Hematol. **72**:27–30.
21. **Loeffler, J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele.** 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J. Clin. Microbiol. **38**:586–590.
22. **Maertens, J., J. Verhaegen, K. Lagrou, J. Van Eldere, and M. Boogaerts.** 2001. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood **97**:1604–1610.
23. **Mori, T., H. Ikemoto, M. Matsumura, M. Yoshida, K. Inada, S. Endo, A. Ito, S. Watanabe, H. Yamaguchi, M. Mitsuya, M. Kodama, T. Tani, T. Yokota, T. Kobayashi, J. Kambayashi, T. Nakamura, T. Masaoka, H. Teshima, T. Yoshinaga, S. Kohno, K. Hara, and S. Miyazaki.** 1997. Evaluation of plasma (1→3)-beta-D-glucan measurement by the kinetic turbidimetric *Limulus* test,for the clinical diagnosis of mycotic infections. Eur. J. Clin. Chem. Clin. Biochem. **35**:553–560.
24. **Nakai, T., J. Uno, K. Otomo, F. Ikeda, S. Tawara, T. Goto, K. Nishimura, and M. Miyaji.** 2002. In vitro activity of FK463, a novel lipopeptide antifungal agent, against a variety of clinically important molds. Chemotherapy **48**:78–81.
25. **Obayashi, T., M. Yoshida, T. Mori, H. Goto, A. Yasuoka, H. Iwasaki, H. Teshima, S. Kohno, A. Horiuchi, A. Ito, et al.** 1995. Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet **345**:17–20.

26. **Rantakokko-Jalava, K., S. Laaksonen, J. Issakainen, J. Vauras, J. Nikoskelainen, M. K. Viljanen, and J. Salonen.** 2003. Semiquantitative detection by real-time PCR of *Aspergillus fumigatus* in bronchoalveolar lavage fluids and tissue biopsy specimens from patients with invasive aspergillosis. J. Clin. Microbiol. **41**:4304–4311.
27. **Salonen, J., O. P. Lehtonen, M. R. Terasjarvi, and J. Nikoskelainen.** 2000. Aspergillus antigen in serum, urine and bronchoalveolar lavage specimens of neutropenic patients in relation to clinical outcome. Scand. J. Infect. Dis. **32**:485–490.
28. **Sanguinetti, M., B. Posteraro, L. Pagano, G. Pagliari, L. Fianchi, L. Mele, M. La Sorda, A. Franco, and G. Fadda.** 2003. Comparison of real-time PCR, conventional PCR, and galactomannan antigen detection by enzyme-linked immunosorbent assay using bronchoalveolar lavage fluid samples from hematology patients for diagnosis of invasive pulmonary aspergillosis. J. Clin. Microbiol. **41**:3922–3925.
29. **Stone, E. A., H. B. Fung, and H. L. Kirschenbaum.** 2002. Caspofungin: an echinocandin antifungal agent. Clin. Ther. **24**:351–377; discussion, 329.
30. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latge.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. **33**:497–500.
31. **Tang, C. M., D. W. Holden, A. Aufauvre-Brown, and J. Cohen.** 1993. The detection of *Aspergillus* spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. Rev. Respir. Dis. **148**:1313–1317.
32. **Verweij, P. E., D. Stynen, A. J. Rijs, B. E. de Pauw, J. A. Hoogkamp-Korstanje, and J. F. Meis.** 1995. Sandwich enzyme-linked immunosorbent assay compared with Pastorex latex agglutination test for diagnosing invasive aspergillosis in immunocompromised patients. J. Clin. Microbiol. **33**:1912–1914.
33. **Walsh, T. J., P. Pappas, D. J. Winston, H. M. Lazarus, F. Petersen, J. Raffalli, S. Yanovich, P. Stiff, R. Greenberg, G. Donowitz, M. Schuster, A. Reboli, J. Wingard, C. Arndt, J. Reinhardt, S. Hadley, R. Finberg, M. Laverdiere, J. Perfect, G. Garber, G. Fioritoni, E. Anaissie, and J. Lee.** 2002. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N. Engl. J. Med. **346**:225–234.
34. **Yamakami, Y., A. Hashimoto, I. Tokimatsu, and M. Nasu.** 1996. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. J. Clin. Microbiol. **34**:2464–2468.
35. **Yoshida, K., Y. Niki, H. Mitekura, M. Nakajima, H. Kawane, and T. Matsushima.** 2001. A discrepancy in the values of serum (1–3)-beta-D-glucan measured by two kits using different methods. Nippon Ishinkin Gakkai Zasshi **42**:237–242. (In Japanese.)

nature medicine

AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis

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Embryonic development of multilineage hematopoiesis requires the precisely regulated expression of lineage-specific transcription factors, including AML-1 (encoded by *Runx1*; also known as CBFA-2 or PEBP-2αB)[1–5]. *In vitro* studies and findings in human diseases, including leukemias[6,7], myelodysplastic syndromes[8] and familial platelet disorder with predisposition to acute myeloid leukemia (AML)[9], suggest that AML-1 has a pivotal role in adult hematopoiesis. However, this role has not been fully uncovered *in vivo* because of the embryonic lethality of *Runx1* knockout in mice. Here we assess the requirement of AML-1/*Runx1* in adult hematopoiesis using an inducible gene-targeting method[10]. In the absence of AML-1, hematopoietic progenitors were fully maintained with normal myeloid cell development. However, AML-1-deficient bone marrow showed inhibition of megakaryocytic maturation, increased hematopoietic progenitor cells and defective T- and B-lymphocyte development. AML-1 is thus required for maturation of megakaryocytes and differentiation of T and B cells, but not for maintenance of hematopoietic stem cells (HSCs) in adult hematopoiesis.

Using the Cre-*lox*P sequence-specific recombination system, we generated mutant mice in which exon 5 of the *Runx1* gene could be selectively deleted by the expression of Cre recombinase (Fig. 1a). We mated mutant animals carrying deleted (*Runx1*$^{-}$) or *lox*P-flanked (*Runx1*^{fl}) alleles, and observed lethal bleeding of *Runx1*$^{-/-}$ embryos as anticipated[1,2]. In contrast, *Runx1*$^{fl/fl}$ and *Runx1*$^{fl/-}$ mice were normal (data not shown). We then bred the mutant mice with *Mx-cre*-transgenic mice to generate *Runx1*$^{fl/+}$*Mx-cre* or *Runx1*$^{fl/-}$*Mx-cre* mice. In these mice, the *Runx1*^{fl} allele could be effectively deleted in hematopoietic progenitors by using injected polyinosinic-polycytidylic acid (pIpC) to induce expression of Cre recombinase[10]. Two months after pIpC injection, genomic Southern blot analysis of *Runx1*$^{fl/-}$*Mx-cre* mice revealed that ≥90% of the bone marrow or peritoneal exudative cells, ~80% of which were morphologically normal neutrophils, had biallelic *Runx1* deletion. This indicates that *Runx1* deletion was induced in most bone marrow cells, and that most myeloid progenitors lacking AML-1 could still differentiate into mature neutrophils (Fig. 1b,c). Efficient excision of one *Runx1* allele was observed in the hematopoietic cells, including lymphocytes, of *Runx1*$^{fl/+}$*Mx-cre* mice. In contrast, only 9%, 32% and 57% of the thymocytes, splenic T cells and B cells *of Runx1*$^{fl/-}$*Mx-cre* mice, respectively, had a *Runx1*$^{-/-}$ genotype. Therefore, a large proportion of mature lymphocytes originated from lymphoid progenitor cells still expressing intact AML-1, and AML-1 deficiency should be disadvantageous to lymphocyte development.

Injection of pIpC did not cause significant differences in neutrophil counts or hemoglobin levels among the *Runx1*$^{fl/-}$*Mx-cre* (floxed) mice, *Runx1*$^{fl/+}$*Mx-cre* mice and *Runx1*$^{+/+}$*Mx-cre* (control) mice (Fig. 2a). Immediately after pIpC injection, however, platelet counts for the floxed mice declined to one-third to one-sixth of those for the control mice (Fig. 2a). Lymphocyte counts were slightly depressed after more than 4 weeks in the floxed and *Runx1*$^{fl/+}$*Mx-cre* mice. These results suggest that AML-1 is required for the maintenance of platelets and lymphocytes, but not for the sustained production of erythrocytes and neutrophils.

We next examined bone marrow cell morphology in the pIpC-treated floxed mice to determine whether thrombocytopenia results from abnormal megakaryopoiesis. The cellularity of the bone marrow and morphology of myeloid cells in the floxed mice were not remarkably altered from those of the control mice, except for a slightly elevated myeloid-erythroid ratio (2.35 ± 0.70 in floxed mice compared with 1.71 ± 0.39 in control mice; $n = 7$ (paired), $P = 0.046$ by Wilcoxon signed-rank test; Fig. 2b and data not shown). However, Wright-Giemsa staining of floxed bone marrow revealed the absence of normal megakaryocytes with abundant cytoplasm and lobulated nuclei (Fig. 2b). Instead, the floxed bone marrow contained a small number of immature megakaryocyte-like cells with occasionally separated round nuclei,

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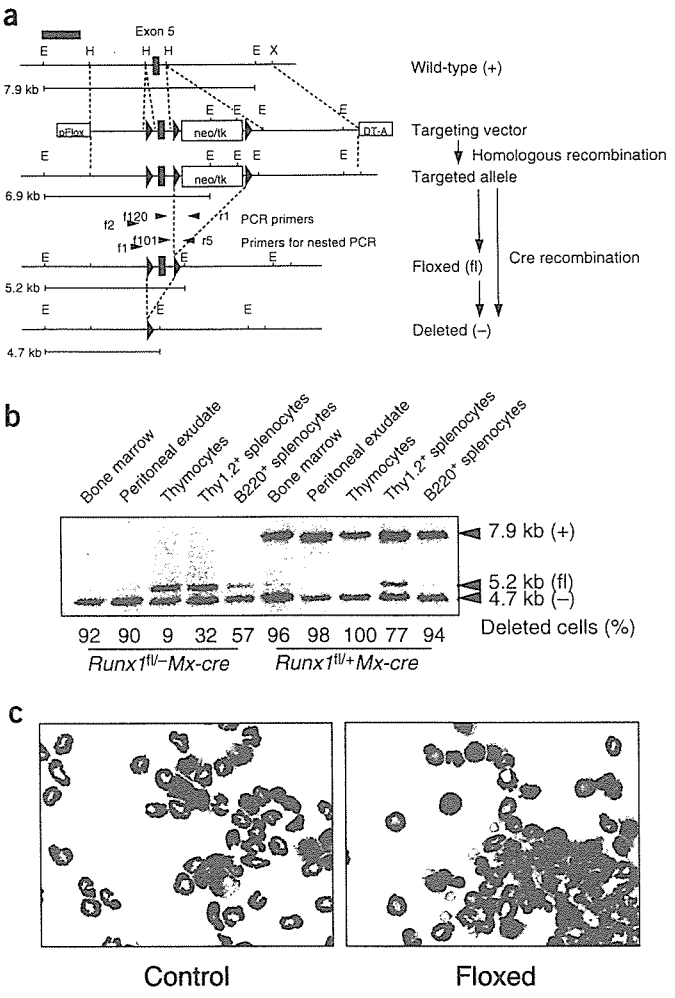

Figure 1 Inducible AML-1 knockout mice. (**a**) Schematic representation of conditional gene targeting of the *Runx1* gene. E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; gray box in upper left corner, 5′ genomic probe; *neo/tk*, PGK-*neo*/HSV-thymidine kinase positive selection cassette; *DT-A*, diphtheria toxin A chain negative selection cassette. (**b**) Southern blot genotyping of cells from hematopoietic organs of mice injected with pIpC. Numbers below lanes indicate proportion of *Runx1*-deleted cells. (**c**) Wright-Giemsa-stained, cytocentrifuged specimens of peritoneal inflammatory cells genotyped in **b**.

resembling the abnormal 'micromegakaryocytes' observed in human myelodysplastic syndromes (Fig. 2b). Consistent with this observation, a substantial increase in the number of small megakaryocytes was revealed in the floxed bone marrow by acetylcholinesterase staining, which specifically detects mature and immature megakaryocytes[11] (Fig. 2b).

Given the prominent immaturity of megakaryocytes in the floxed mice, we examined the ultrastructure of the megakaryocytes (Fig. 2c). In addition to their smaller size, floxed megakaryocytes showed poorly developed demarcation membranes compared with megakaryocytes from control (*Runx1*$^{+/+}$*Mx-cre*) mice. During the maturation process, megakaryocytes acquire high DNA ploidy (>4*n*) through a cell cycle process known as endomitosis[12]. As expected from the smaller size of their nuclei, CD41^{+} megakaryocytes from the floxed mice showed a markedly lower level of polyploidy (≤8*n*) than megakaryocytes from the control mice (modal ploidy, 16*n*), as revealed by flow cytometric measurement of DNA content. This demonstrates that AML-1 deletion also causes defective polyploidization in megakaryocytes (Fig. 2d). Although a recent report showed that AML-1 regulates the expression of megakaryocyte-specific genes in cooperation with GATA-1 (ref. 13), and although the small and immature megakaryocytes that we observed are reminiscent of those found in GATA-1 knockdown mice[14,15], the expression levels of megakaryocyte-specific transcription factors in floxed mice, including GATA-1, FOG-1 and NF-E2, remained unaffected according to RT-PCR analyses of lineage-negative (Lin^{-}) bone marrow cells (data not shown).

We subsequently conducted *in vitro* colony-forming assays to evaluate the frequency of hematopoietic progenitors. Floxed bone marrow cells formed more megakaryocytic colonies (CFU-Meg) than control bone marrow cells in semisolid media (Fig. 3a). The numbers of myeloid and mixed (myeloid and erythroid) colonies were also elevated in the floxed bone marrow. Using single-colony PCR genotyping, we detected the *Runx1*$^{-/-}$ genotype in 149 of 150 myeloid colonies, 48 of 50 erythroid colonies, 35 of 36 mixed colonies and 14 of 14 megakaryocytic colonies from pIpC-treated floxed bone marrow cells, thus excluding the possibility that those hematopoietic cells might be predominantly derived from progenitor cells still expressing intact AML-1 (data not shown). Using flow cytometry (Fig. 3b), we observed an increased number of cells in the Lin-c-Kit^{hi}CD41^{hi} fraction, which was presumed to contain CFU-Meg[16], in the floxed bone marrow. Similarly, the number of cells defined by CD34-Lin$^{-/lo}$c-Kit^{hi}Sca-1^{hi} (Fig. 3c), which represent the most immature hematopoietic progenitor cell population[17], was also elevated in the floxed bone marrow. Although development of both T and B cells from AML-1-deficient cells was impaired (Fig. 1b), the cells defined by Lin$^{-/lo}$IL-7Rα^{+}Sca-1^{lo}c-Kit^{lo}, previously reported to include common lymphocyte progenitors (CLPs)[18], were maintained in the floxed bone marrow (Fig. 3d).

Increased numbers of primitive hematopoietic progenitors, immortalized myeloid progenitor cells and arrested myeloid maturation have been observed in mice expressing the leukemic AML-1/ETO chimeric protein[19–21], which dominantly suppresses the normal function of AML-1. We observed an elevated colony-replating capacity of *Runx1*$^{-/-}$ hematopoietic cells, although replating could be repeated for only 2 months (Fig. 3e). Although AML-1-null neutrophils were morphologically normal, and freshly collected floxed bone marrow cells did not show altered apoptosis when assessed by annexin-V expression (data not shown), we detected increased apoptosis of myeloid colony-forming cells, suggesting that a certain degree of maturation arrest occurs in the myeloid lineage in the absence of AML-1 (Fig. 3f).

Because immature hematopoietic progenitor cell fractions were elevated in the floxed bone marrow, we used a competitive repopulation assay to assess their ability to reconstitute adult hematopoiesis[22]. We used isotypes of the pan-hematopoietic marker CD45 (Ly5) to distinguish the origins of the repopulating cells. Sublethally irradiated C57BL/6-Ly5.1 recipient mice (Ly5.1^{+}Ly5.2^{-}) were intravenously injected with a mixture of bone marrow cells from C57BL/6-Ly5.1/Ly5.2 F_1 competitor mice (Ly5.1^{+}Ly5.2^{+}) or floxed or control mice (Ly5.1^{-}Ly5.2^{+}) previously injected with pIpC. We then analyzed Ly5 isotypes on the repopulating cells by flow cytometry (Fig. 4a). Notably, the floxed bone marrow cells reconstituted neither peripheral T (Thy-1.2^{+}) nor B (B220^{+}) cells, whereas there were no significant differences in the mature neutrophil (Gr-1^{hi}Mac-1^{+}) and monocyte populations (Gr-1^{lo}Mac-1^{+})[23] (Fig. 4a). The contribution to the reconstituted bone marrow was not significantly different in neutrophils (test/competitor = 0.382 ± 0.083 for floxed mice (*n* =

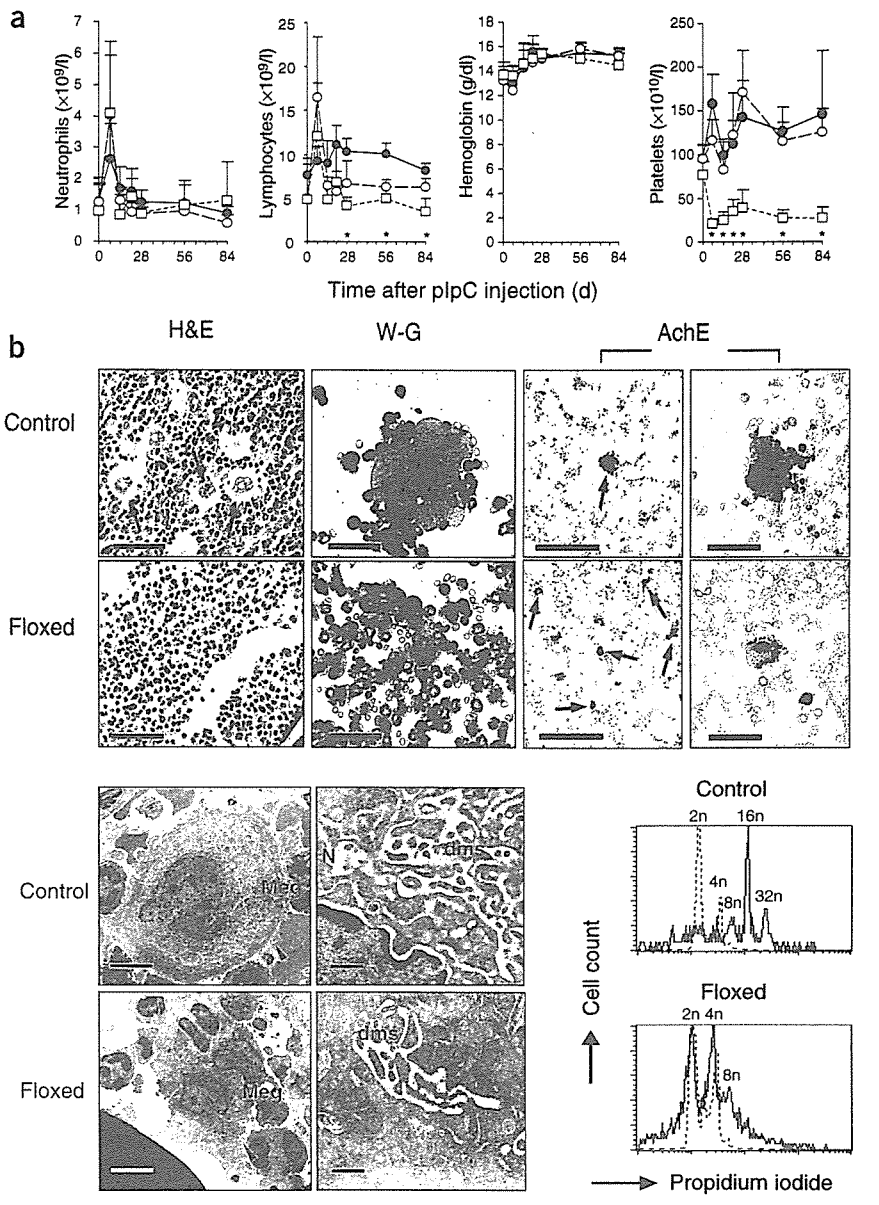

Figure 2 Thrombocytopenia and megakaryocytic maturation arrest of induced AML-1 knockout mice. (**a**) Peripheral blood cell counts of *Runx1*$^{+/+}$*Mx-cre* (control; ●), *Runx1*$^{fl/+}$*Mx-cre* (○) and *Runx1*$^{fl/-}$*Mx-cre* (floxed; □) mice injected with pIpC on days 0, 2 and 4. Results are shown as mean + s.d. (error bars) from four to seven mice. *, $P < 0.01$ for floxed compared with control mice (unequal-variance *t*-test). (**b**) Histochemical analyses of bone marrow. W-G, Wright-Giemsa; AchE, acetylcholinesterase. Arrows indicate megakaryocytes. Scale bars, 50-μm (H&E, W-G, and AchE right panel) or 250-μm (AchE left panel). (**c**) Electron micrographs of bone marrow megakaryocytes. Meg/arrowhead, megakaryocytes; dms, demarcation membranes; N, nucleus. Scale bars, 5-μm (left) or 500-nm (right). (**d**) DNA contents of CD41$^+$ bone marrow cells. – – –, CD41$^-$ fractions for 2*n* and 4*n* controls. Typical results from three experiments are shown (4–8 weeks after pIpC injection).

8), compared with 0.424 ± 0.118 for control mice ($n = 4$); $P = 0.93$ by Wilcoxon rank-sum test) or monocytes (test/competitor = 0.443 ± 0.082 for floxed mice ($n = 8$), compared with 0.940 ± 0.440 for control mice ($n = 4$); $P = 0.35$). The floxed cells also repopulated the megakaryocytic progenitors (Lin$^-$CD41$^+$; Fig. 4b). Given the inability of AML-1-deficient cells to reconstitute the T-cell lineage, we investigated thymocyte development in the absence of AML-1 by analyzing double-negative thymocytes of the recipient mice. We observed a significant block in the maturation of floxed T-cell progenitors at the transition from CD44$^+$CD25$^+$ (DN2) to CD44$^-$CD25$^+$ (DN3) (DN3/DN2 ratio of Ly5.1$^-$Ly5.2$^+$ cells = 0.025 ± 0.029 for floxed mice ($n = 5$), compared with 1.87 ± 1.51 for control mice ($n = 4$); $P < 0.05$; Fig. 4c), indicating that immature T-cell precursors had accumulated in the thymus. These findings, along with the observation that the CLP fraction was not affected in the floxed bone marrow, suggest that AML-1 is not necessary for the maintenance of CLPs, but is required in lymphoid precursors committed to T- or B-cell lineages. Consistent with this, a recent report and our experiments using *lck-Cre*-mediated, T-cell-specific AML-1-knockout mice show that the maturation of T-cell progenitors deficient in AML-1 is blocked at the DN3-DN4 transition (ref. 24 and T.A. *et al.*, unpublished data). Because *lck-Cre* expression becomes evident at the DN3 stage or later, the present study unveiled an important role of AML-1 in the DN2-DN3 transition. However, because of the insufficient contribution of the floxed cells to the B220$^+$ bone marrow fraction (test/competitor = 0.011 ± 0.012 ($n = 8$) compared with 0.306 ± 0.295 for control ($n = 4$)), we could not determine which step in B-cell development was affected by the absence of AML-1. The role of AML-1 in B-cell development might be revealed by other approaches, including the analysis of B-cell lineage-specific AML-1-knockout mice. Although lymphocyte development is substantially blocked at early stages in the absence of AML-1, the detection of AML-1-deleted T and B cells in the periphery of *Runx1*$^{fl/-}$*Mx-cre* mice (Fig. 1b) indicates that some lymphoid progenitors may still survive for a prolonged period and differentiate into mature lymphocytes.

Our data show that lack of AML-1 at the adult stage causes hematopoietic progenitor cell expansion, probably through a partial block in myeloid cell differentiation. This finding agrees with the phenotypes of previously reported mouse models of t(8;21)—carrying human leukemia, in which AML-1/ETO fusion protein is implicated in leukemogenesis through dominant-negative suppression of AML-1 function[19–21]. The loss or dominant-negative suppression of AML-1 function is also found in human myelodysplastic syndromes and familial platelet disorder with predisposition to AML[8,9], both of which are thought to be a preleukemic state. Taken as a whole, our observations suggest that the number of hematopoietic progenitor cells is negatively regulated by AML-1, and that the loss of AML-1 function triggers a preleukemic state. However, the myeloid immaturity and immortalization of bone marrow progenitors observed in AML-1/ETO mice could not be recapitulated in our *Runx1*$^{fl/-}$*Mx-cre* mice. Therefore, AML-1/ETO may not only inhibit AML-1 func-

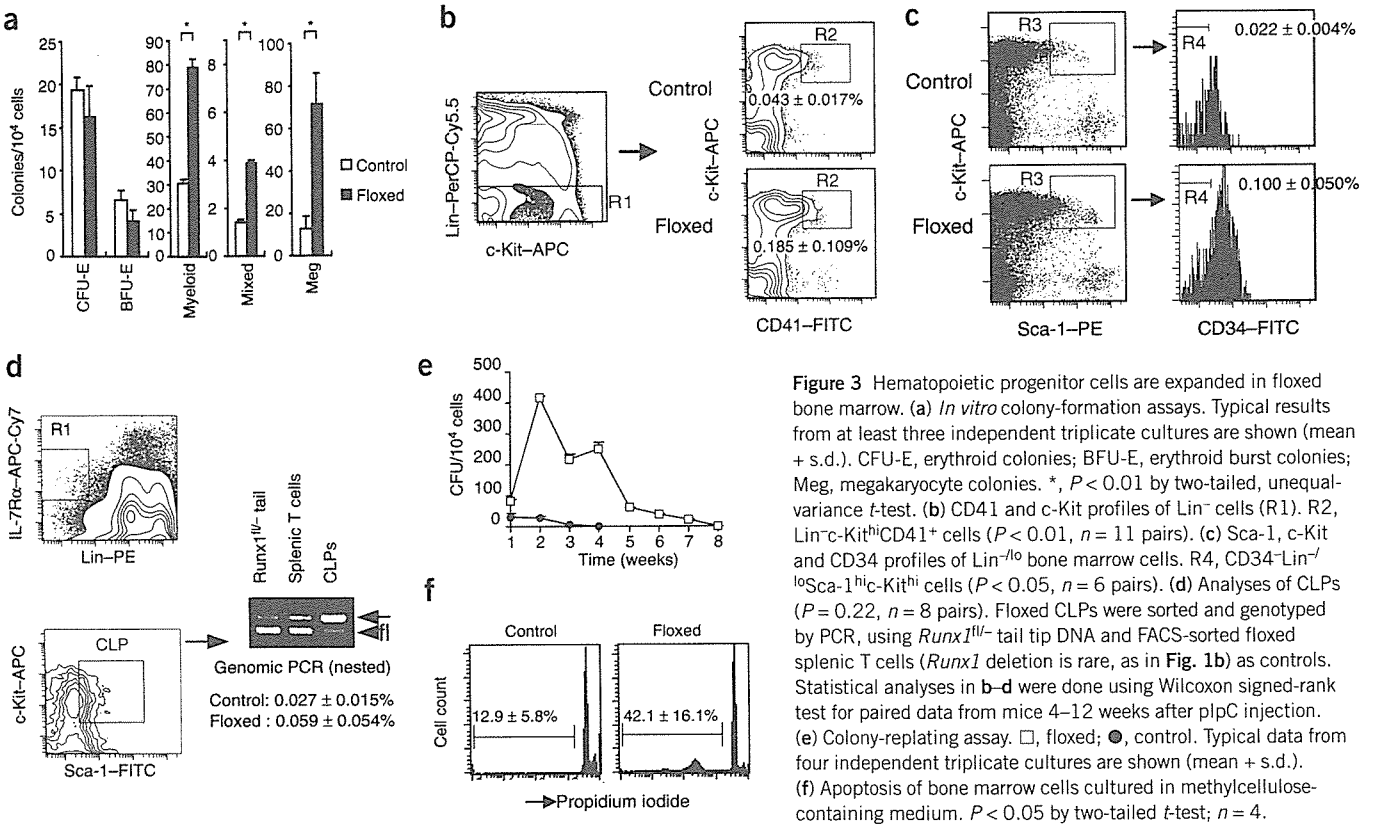

Figure 3 Hematopoietic progenitor cells are expanded in floxed bone marrow. (**a**) *In vitro* colony-formation assays. Typical results from at least three independent triplicate cultures are shown (mean + s.d.). CFU-E, erythroid colonies; BFU-E, erythroid burst colonies; Meg, megakaryocyte colonies. *, $P < 0.01$ by two-tailed, unequal-variance *t*-test. (**b**) CD41 and c-Kit profiles of Lin$^-$ cells (R1). R2, Lin$^-$c-Kit^{hi}CD41$^+$ cells ($P < 0.01$, $n = 11$ pairs). (**c**) Sca-1, c-Kit and CD34 profiles of Lin$^{-/lo}$ bone marrow cells. R4, CD34$^-$Lin$^{-/lo}$Sca-1^{hi}c-Kit^{hi} cells ($P < 0.05$, $n = 6$ pairs). (**d**) Analyses of CLPs ($P = 0.22$, $n = 8$ pairs). Floxed CLPs were sorted and genotyped by PCR, using *Runx1*$^{fl/-}$ tail tip DNA and FACS-sorted floxed splenic T cells (*Runx1* deletion is rare, as in **Fig. 1b**) as controls. Statistical analyses in **b**–**d** were done using Wilcoxon signed-rank test for paired data from mice 4–12 weeks after pIpC injection. (**e**) Colony-replating assay. □, floxed; ●, control. Typical data from four independent triplicate cultures are shown (mean + s.d.). (**f**) Apoptosis of bone marrow cells cultured in methylcellulose-containing medium. $P < 0.05$ by two-tailed *t*-test; $n = 4$.

tion, but may also have the ability to immortalize hematopoietic progenitors.

Although AML-1 is considered to be a master regulator of definitive hematopoiesis, our current study shows that AML-1 is dispensable for prolonged hematopoietic cell engraftment, as well as commitment to the myeloid lineage and at least to double-negative thymocytes in the lymphoid lineage. Our present data also indicate that AML-1 is essential for the terminal differentiation of hematopoietic progenitors of megakaryocytic and lymphocytic lineages, which establishes AML-1 as a regulator with multiple roles in the maintenance of lineage-committed cells in adult hematopoiesis. However, our results also suggest that the maintenance of HSCs and their commitment to more mature progenitors in adult hematopoiesis does not always require a transcription factor essential for hematopoietic ontogeny during embryogenesis, and can be induced by other hematopoietic genes. This is supported by a previous study

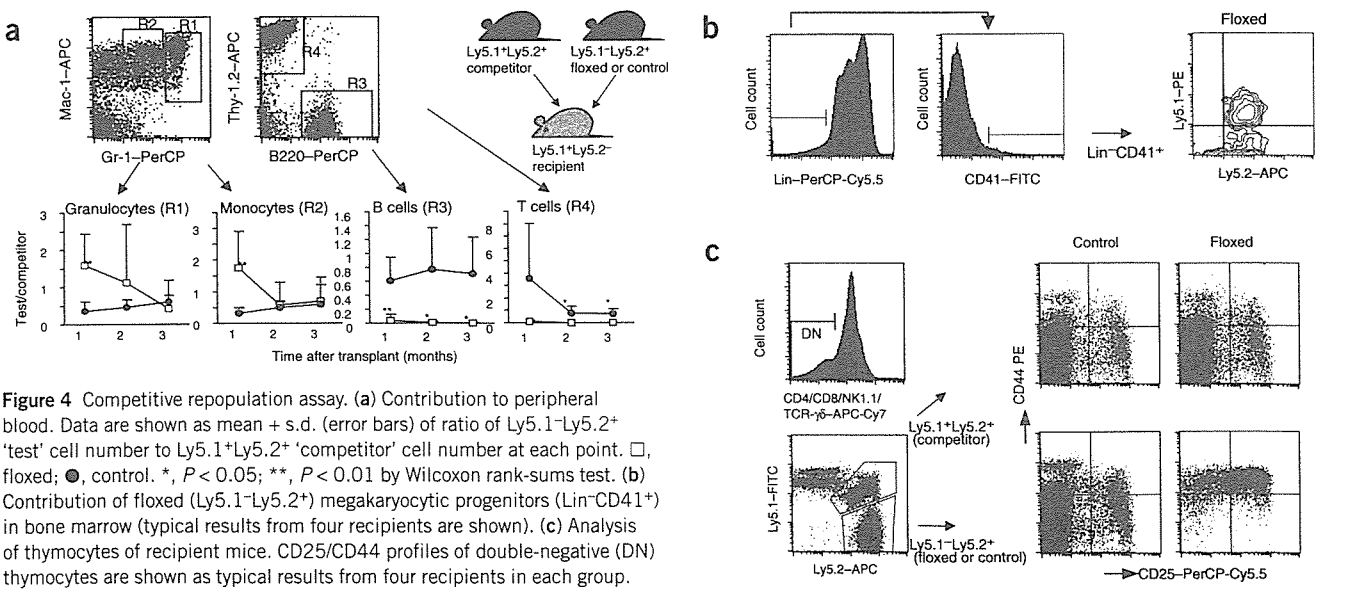

Figure 4 Competitive repopulation assay. (**a**) Contribution to peripheral blood. Data are shown as mean + s.d. (error bars) of ratio of Ly5.1$^-$Ly5.2$^+$ 'test' cell number to Ly5.1$^+$Ly5.2$^+$ 'competitor' cell number at each point. □, floxed; ●, control. *, $P < 0.05$; **, $P < 0.01$ by Wilcoxon rank-sums test. (**b**) Contribution of floxed (Ly5.1$^-$Ly5.2$^+$) megakaryocytic progenitors (Lin$^-$CD41$^+$) in bone marrow (typical results from four recipients are shown). (**c**) Analysis of thymocytes of recipient mice. CD25/CD44 profiles of double-negative (DN) thymocytes are shown as typical results from four recipients in each group.

that showed that SCL/*tal-1*, a transcription factor essential for the development of primitive hematopoiesis during the embryonic stage, is required for the proper differentiation of erythroid and megakaryocytic lineages, but not for the maintenance of HSCs in adult hematopoiesis[25]. To fully understand the intricate process of hematopoiesis, it will be necessary to determine the roles of those hematopoietic genes.

METHODS

Mice. We introduced the targeting vector (Fig. 1a) into TT2 embryonic stem cells[26], and transiently expressed Cre recombinase to generate embryonic stem cell lines carrying the *Runx1*^{fl} or *Runx1*$^{-}$ alleles. Chimeric mice raised by aggregation were crossed to the C57BL/6 background, and were mated to interferon-inducible *Mx-cre* transgenic mice[10]. *Mx-cre* expression was induced by intraperitoneally injecting 250 μg of pIpC, on three alternate days, into 4- to 8-week-old mice[10]. C57BL/6-Ly5.1 congenic and C57BL/6-Ly5.1/Ly5.2 F_1 mice were used for competitive repopulation assays. Mice were kept at the Animal Center for Biomedical Research, University of Tokyo, according to institutional guidelines.

Genotyping. For PCR genotyping, cells were lysed in a lysis buffer (0.3% Tween 20, 0.3% NP-40, and 120 μg/ml proteinase K in 1× TE buffer) at 55 °C for 1 h, followed by inactivation at 80 °C for 10 min. DNA was amplified using primers f2 (5′-ACAAAACCTAGGTGTACCAGGAGAACAAGT-3′), f120 (5′-CCCTGAAGACAGGAGAAGTTTCCA-3′) and r1 (5′-GTCTACTC-CTTGCCTCAGAAAACAAAAAC-3′) for the first PCR reaction, and nested primers f1 (5′-AAAACCTAGGTGTACCAGGAGAACAAGTC-3′), f101 (5′-TTCCAGGTCAACTCTCTCACCTCTC-3′) and r5 (5′-ATCTGAGTTGGCC TAATTTCCCTTTG-3′) for the second reaction, to detect the 280-base pair *Runx1*$^{-}$ and 220-base pair *Runx1*^{fl} products. Southern blot analyses of DNA samples digested with *Eco*RI were done according to standard protocols, using a 5′ *Eco*RI-*Bgl*II genomic probe (Fig. 1a).

Analyses of blood cells. Peripheral blood was counted using an automated hemacytometer, and leukocytes were morphologically classified to calculate neutrophil and lymphocyte counts. Mice were analyzed for bone marrow morphology 2 months after pIpC administration. For histological examination, sectioned femoral bone marrow specimens were stained with H&E, and cytocentrifuged specimens were stained with Wright-Giemsa or for acetylcholinesterase as previously described[11]. Peritoneal exudative cells were collected by washing the peritoneal cavities of mice 4 h after intraperitoneal injection of 2 ml of 2% casein in PBS. Splenocytes were labeled with antibodies to Thy-1.2 (for T cells) or B220 (for B cells), conjugated to magnetic microbeads to collect splenic lymphocytes using a MACS LS+ system (Miltenyi Biotec). The cells were checked for purity by flow cytometry (>97%; data not shown).

Ultrastructural studies. Femoral bone marrow samples prepared 4 weeks after pIpC injection as previously described[27] were examined with a JEOL 1200CX electron microscope.

Flow cytometry and cell sorting. All monoclonal antibodies and fluorochromes were purchased from BD PharMingen. To measure bone marrow progenitor cells other than CLPs, cells were stained with FITC-conjugated antibodies to CD41 or CD34, phycoerythrin (PE)-conjugated antibody to Sca-1, allophycocyanin (APC)-conjugated antibody to c-Kit, and biotin-conjugated antibodies to lineage (Lin) markers (CD3ε, CD4, CD8a, B220, Gr-1, Mac-1 and Ter119), and visualized with streptavidin-PerCP-Cy5.5. For CLP cell fractions, cells were stained with Lin-PE, Sca-1-FITC, c-Kit-APC and IL-7Rα–biotin/streptavidin-APC-Cy7. Stained samples were analyzed using either FACSCalibur or BD LSRII (BD Biosciences). For sorting CLP cells, Lin^{+} were predepleted from bone marrow cells using the MACS LD system (Miltenyi Biotec). The remaining fraction was stained for CLP as described above and sorted using a FACSVantage cell sorter. The cell sorter was calibrated to achieve >98% purity for Thy-1.2^{+} cells stained with Thy-1.2–APC. Approximately 1,000 CLP cells were genotyped by PCR. To analyze competitively repopulated cells, cells were stained with Ly5.2–FITC, Ly5.1–PE, Gr-1–biotin/streptavidin–PerCP and Mac-1–APC for myeloid cells, and with Ly5.2–FITC, Ly-5.1–PE, B220–PerCP and Thy-1.2–APC for T and B lymphocytes. Thymocytes were stained with Ly5.1–FITC, Ly5.2–APC, CD25– PerCP-Cy5.5, CD44–PE and biotin-conjugated antibodies to CD4, CD8, NK1.1 and TCR-γδ, and visualized by streptavidin–APC-Cy7 for analyzing double-negative thymocytes. Bone marrow megakaryocytic progenitors were stained with CD41–FITC, Ly-5.1–PE, Ly-5.2–APC and Lin–biotin/streptavidin-PerCP-Cy5.5. Two-color flow cytometric analysis of the DNA content of bone marrow megakaryocytes stained with CD41–FITC was performed as previously described[28].

***In vitro* hematopoietic colony-forming assays.** For CFU-Meg, 2.5×10^4 bone marrow cells from mice 4–8 weeks after pIpC injection were cultured in 1 ml of α-MEM containing 0.8% methylcellulose, 1% BSA, 30% FBS, 100 μM 2-mercaptoethanol and 10 units of mouse thrombopoietin. For other (myeloid and erythroid) colonies, 5×10^4 cells were cultured in 1-ml of IMDM containing 0.8% methylcellulose, 1% BSA, 30% FBS, 100-μM 2-mercaptoethanol, 100-ng/ml of mouse stem cell factor, 5-ng/ml of mouse interleukin-3 and 7.5 units/ml of human erythropoietin (all growth factors were generously provided by Kirin Brewery). Colonies were counted after 3 d (for erythroid colonies), 5 d (for erythroid bursts and myeloid colonies), 7 d (for mixed colonies) or 8 d (for CFU-Meg) of culture in 5% CO_2 at 37 °C. For replating experiments, the whole myeloid and erythroid culture was pooled on day 7 and washed twice, and 1×10^4 cells were subjected to subsequent culture in the same medium. Scoring for colonies and reculturing were repeated every 7 d.

Detection of apoptotic cells. Whole myeloid and erythroid methylcellulose cultures were pooled on day 7 of culture, washed, fixed and stained with propidium iodide for analysis by flow cytometry to detect apoptotic cells (DNA content < 2*n*) as previously described[29].

Competitive repopulation assay. X-ray-irradiated (9.5-Gy, 0.3-Gy/min, unfractionated) recipient mice were intravenously injected with a mixture of 1×10^5 each of unfractionated bone marrow cells from 'test' mice (floxed or control mice, 8–12 weeks after pIpC injection) and competitor mice. Peripheral blood was analyzed monthly, and bone marrow cells and thymocytes were analyzed 3 months after transplantation by flow cytometry.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G. & Downing, J.R. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321–330 (1996).
2. Wang, Q. *et al.* Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. USA* **93**, 3444–3449 (1996).
3. Mukouyama, Y. *et al.* The AML1 transcription factor functions to develop and maintain hematogenic precursor cells in the embryonic aorta-gonad-mesonephros region. *Dev. Biol.* **220**, 27–36 (2000).
4. Yokomizo, T. *et al.* Requirement of Runx1/AML1/PEBP2αB for the generation of haematopoietic cells from endothelial cells. *Genes Cells* **6**, 13–23 (2001).
5. North, T.E. *et al.* Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661–672 (2002).
6. Tenen, D.G., Hromas, R., Licht, J.D. & Zhang, D.E. Transcription factors, normal myeloid development, and leukemia. *Blood* **90**, 489–519 (1997).
7. Lutterbach, B. & Hiebert, S.W. Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. *Gene* **245**, 223–235 (2000).
8. Imai, Y. *et al.* Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis. *Blood* **96**, 3154–3160 (2000).
9. Song, W.J. *et al.* Haploinsufficiency of CBFA2 causes familial thrombocytopenia with pro-

pensity to develop acute myelogenous leukaemia. *Nat. Genet.* **23**, 166–175 (1999).
10. Kuhn, R., Schwenk, F., Aguet, M. & Rajewsky, K. Inducible gene targeting in mice. *Science* **269**, 1427–1429 (1995).
11. Jackson, C.W. Cholinesterase as a possible marker for early cells of the megakaryocytic series. *Blood* **42**, 413–421 (1973).
12. Kaushansky, K. The enigmatic megakaryocyte gradually reveals its secrets. *Bioessays* **21**, 353–360 (1999).
13. Elagib, K.E. *et al.* RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. *Blood* **101**, 4333–4341 (2003).
14. Shivdasani, R.A., Fujiwara, Y., McDevitt, M.A. & Orkin, S.H. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J.* **16**, 3965–3973 (1997).
15. Takahashi, S. *et al.* Role of GATA-1 in proliferation and differentiation of definitive erythroid and megakaryocytic cells *in vivo*. *Blood* **92**, 434–442 (1998).
16. Hodohara, K., Fujii, N., Yamamoto, N. & Kaushansky, K. Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). *Blood* **95**, 769–775 (2000).
17. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242–245 (1996).
18. Kondo, M., Weissman, I.L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661–672 (1997).
19. Rhoades, K.L. *et al.* Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood* **96**, 2108–2115 (2000).
20. Higuchi, M. *et al.* Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell* **1**, 63–74 (2002).
21. de Guzman, C.G. *et al.* Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. *Mol. Cell. Biol.* **22**, 5506–5517 (2002).
22. Szilvassy, S.J., Humphries, R.K., Lansdorp, P.M., Eaves, A.C. & Eaves, C.J. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc. Natl. Acad. Sci. USA* **87**, 8736–8740 (1990).
23. Lagasse, E. & Weissman, I.L. Flow cytometric identification of murine neutrophils and monocytes. *J. Immunol. Methods* **197**, 139–150 (1996).
24. Taniuchi, I. *et al.* Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111**, 621–633 (2002).
25. Mikkola, H.K. *et al.* Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* **421**, 547–551 (2003).
26. Yagi, T. *et al.* A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* **214**, 70–76 (1993).
27. Breton-Gorius, J., Reyes, F., Duhamel, G., Najman, A. & Gorin, N.C. Megakaryoblastic acute leukemia: identification by the ultrastructural demonstration of platelet peroxidase. *Blood* **51**, 45–60 (1978).
28. Jackson, C.W., Brown, L.K., Somerville, B.C., Lyles, S.A. & Look, A.T. Two-color flow cytometric measurement of DNA distributions of rat megakaryocytes in unfixed, unfractionated marrow cell suspensions. *Blood* **63**, 768–778 (1984).
29. Sherwood, S.W. & Schimke, R.T. Cell cycle analysis of apoptosis using flow cytometry. *Methods Cell Biol.* **46**, 77–97 (1995).