(Cell Signaling, Beverly, MA) and mouse anti-rabbit IgG-HRP (Cell Signaling, Beverly, MA). Thereafter, the sample was extensively washed with phosphate-buffered saline. The immunopositive band was detected by means of a light-emitting nonradioactive detection system (Amersham International plc, Little Chalfont, Buckinghamshire, UK) with Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY).

Data analysis

The pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka *et al.* [20]. The data were analysed using Student's *t*-test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $p<0.05$ or 0.01 was taken to indicate a significant difference between sets of data. The electrophoresis results were analysed by using NIH Image software.

Case Report

The patient was a 23-year-old man (56 kg) who had received bone marrow transplantation for acute myelogenous leukaemia 3 months before. He had been receiving immunotherapy with oral administration of Neoral® capsule (Novartis Pharma Co. Ltd.; CyA, 120 mg/day, 2 times a day). The trough blood concentrations of CyA were well maintained at about 100 ng/ml. However, he developed *Aspergillus* pneumonia, which was treated with intravenous infusion with Fungizone® injection (AMB, 1–30 mg/day, over 6 h). Figure 1 shows the relationship between the blood concentration of CyA and the dose of AMB or CyA. The patient was also receiving Saxizon® injection (Kowa Pharmaceutical Co. Ltd; hydrocortisone sodium succinate, 50–100 mg/day) and had been given a preparation of mixed amino acids and multiple vitamins since the transplantation.

The blood concentration of CyA in the patient gradually decreased to about one-third over the 12 days following the start of coadministration of AMB. Although the CyA dose was increased to 160 mg at day 17 after the start of AMB treatment, the blood concentration of CyA did not increase immediately. Subsequently the blood concentration of CyA slowly recovered, reaching about 210 ng/ml with 240 mg/day of CyA after 34 days.

The levels of γ-glutamyltranspeptidase (γ-GTP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), parameters of hepatic function, were initially 52, 45 and 65 IU/l, respectively, and subsequently remained stable within normal ranges. The value of serum creatinine as a parameter of renal function was initially 0.8 mg/dl, but increased slightly to 1.35 mg/dl at day 9 after the start of AMB treatment. Therefore, the AMB administration, which was initially 30 mg/day daily for the first 9 days, was reduced to the same dose every second day until day 40. Following the decrease of AMB dosage frequency, the serum creatinine level rapidly decreased to 0.8 mg/dl. These results suggest that the initial AMB treatment caused slight renal impairment.

Experimental Results

Pharmacokinetics of CyA in rats with AMB treatment

Figure 2 shows the blood concentration–time courses of CyA after i.v. administration of CyA (10 mg/kg) in control rats and in rats pretreated with AMB (1.5 or 3.0 mg/kg/day, i.v.) for 4 days. The blood concentration of CyA in the low-AMB group showed a significant, time-dependent decrease compared with the control, and that in the high-AMB group showed a similar decrease.

Figure 3 show the blood concentration–time courses of CyA after p.o. administration of CyA (10 mg/kg) in control rats and in rats treated with AMB. After p.o. administration, the concentration of CyA reached a maximum within 2–4 h in all three groups. The maximum blood concentrations (C_{max}) of CyA in the control, low-AMB and high-AMB groups were 1.16, 0.37 and 0.26 µg/ml, respectively. The blood concentration of CyA was significantly and dose-dependently decreased by the AMB treatment.

The pharmacokinetic parameters of CyA in the three groups are listed in Table 1. After the i.v.

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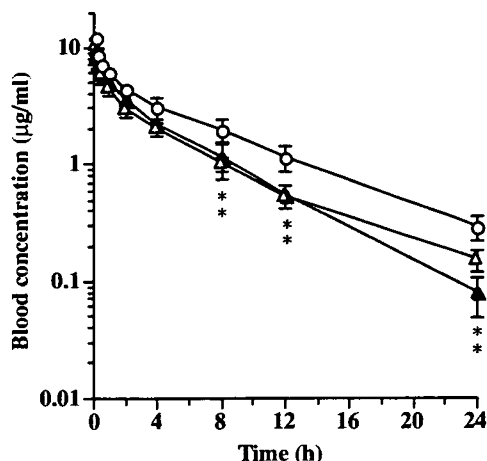

Figure 2. Blood concentration–time courses of CyA after an i.v. administration of CyA (10 mg/kg) in untreated rats (○) and rats treated with AMB at 1.5 mg/kg (△) or 3.0 mg/kg (▲) for 4 days. Rats were given CyA at 24 h after the last AMB treatment. Each point and bar represents the mean ± SD of four rats. **Significant difference between the control group and both AMB groups at $p<0.01$

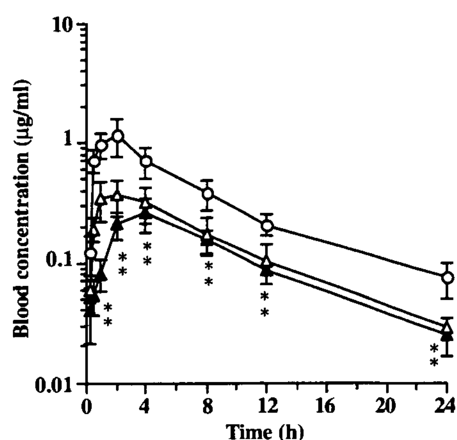

Figure 3. Blood concentration–time courses of CyA after a p.o. administration of CyA (10 mg/kg) in untreated rats (○) and rats treated with AMB at 1.5 mg/kg (△) or 3.0 mg/kg (▲) for 4 days. Rats were given CyA at 24 h after the last AMB treatment. Each point and bar represents the mean ± SD of four rats. **Significant difference between the control group and both AMB groups at $p<0.01$

administration, the values of the area under the blood concentration–time curve from 0 to 24 h ($AUC_{0\text{-}24\,h}$) in the two AMB groups were significantly decreased compared with the control,

Table 1. Pharmacokinetic parameters of CyA with or without AMB (1.5 or 3.0 mg/kg) in rats

Parameters	Non treatment	AMB treatment	
		1.5 mg/kg	3.0 mg/kg
i.v. administration			
$AUC_{0\text{-}24\,h}$ (μg h/ml)[a]	45.0 ± 2.7	28.4 ± 1.2**	30.5 ± 1.8**
MRT (h)[b]	5.96 ± 0.82	5.01 ± 0.51	4.67 ± 0.53*
CL_{tot} (ml/min)[c]	3.70 ± 0.22	5.87 ± 0.19**	5.47 ± 0.31**
Vd_{ss} (l/kg)[d]	1.32 ± 0.31	1.76 ± 0.35	1.53 ± 0.23
$t_{1/2}$ (h)[e]	5.87 ± 0.78	6.03 ± 0.68	4.18 ± 0.83*
p.o. administration			
$AUC_{0\text{-}24\,h}$ (μg h/ml)	8.53 ± 0.78	3.61 ± 0.41**	2.66 ± 0.23**
$t_{1/2}$ (h)	6.32 ± 0.95	5.86 ± 0.82	5.97 ± 0.93
Bioavailability (%)	19.0	12.7	8.7

Rats were intravenously or orally administered with CyA (10 mg/kg) at 24 h after the last AMB treatment in untreated rats and rats treated with AMB at 1.5 or 3.0 mg/kg for 4 days. Pharmacokinetic parameters were estimated according to model-independent moment analysis. Each value represents the mean ± SD of four rats.
*,**Significant difference between the control group and both AMB groups at $p<0.05$ and 0.01, respectively.
[a] Area under blood concentration–time curve from 0 to 24 h.
[b] Mean residence time from 0 to 24 h.
[c] Blood total clearance.
[d] Distribution volume at the steady-state.
[e] Elimination half-life.

and the values of total clearance (CL_{tot}) were significantly increased. The elimination half-life ($t_{1/2}$) in the high-AMB group was significantly faster than that in the control group. After the p.o. administration, the $AUC_{0\text{-}24\,h}$ values of the low- and high-AMB groups were significantly decreased to about 42% and 31% of the control, respectively. However, the $t_{1/2}$ values in the three groups showed no significant difference. The values of oral bioavailability of CyA of the low- and high-AMB groups were decreased to about 67% and 46% of the control, respectively.

Laboratory data in rats after AMB treatment

Table 2 shows the laboratory data for rats treated or not treated with AMB (1.5 or 3.0 mg/kg). There was no clear difference in the body weight among the three groups. The laboratory data reflecting hepatic function were unaffected by the AMB treatment, except for the A/G ratio. The values of blood urea nitrogen (BUN) and serum creatinine were significantly increased by the high-AMB treatment.

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RT-PCR analysis of mdr1a, mdr1b and CYP3A2 mRNAs in intestine and liver

Figure 4 shows the effect of AMB treatment (1.5 or 3.0 mg/kg/day, i.v., for 4 days) on the expression of mdr1a, mdr1b and CYP3A2 mRNAs in the duodenum, ileum and liver at 24 h after the last treatment. The expression levels of mdr1a and mdr1b mRNAs were significantly increased in the duodenum, but little changed in the ileum and liver by the AMB treatment. On the other hand, the expression of CYP3A2 was significantly induced, in the liver only, by both AMB treatments.

Table 2. Physical and biochemical data in rats treated with AMB

	Non treatment	AMB treatment	
		1.5 mg/kg	3.0 mg/kg
Body weight (g)	220 ± 7	227 ± 12	216 ± 11
AST (IU/l)	84 ± 5	82 ± 8	88 ± 14
ALT (IU/l)	40 ± 7	35 ± 2	38 ± 13
Albumin (g/dl)	4.07 ± 0.11	3.93 ± 0.15	3.67 ± 0.31
A/G ratio	2.23 ± 0.15	1.97 ± 0.25	1.70 ± 0.31*
Total bilirubin (mg/dl)	0.14 ± 0.04	0.15 ± 0.08	0.14 ± 0.06
BUN (mg/dl)	15.1 ± 1.8	26.1 ± 5.9*	46.8 ± 9.3**
serum creatinine (mg/dl)	0.26 ± 0.03	0.28 ± 0.03	0.37 ± 0.05**

Data were measured at 24 h after the last administration of AMB (1.5 or 3.0 mg/kg i.v.) for 4 days in rats. Each value represents the mean ± SD of four rats.

*,**Significantly different from the control at $p<0.05$ and 0.01, respectively.

Expression of P-gp and CYP3A2 in intestine and liver

Figure 5 shows the effect of the AMB treatment (3.0 mg/kg/day, i.v., for 4 days) on the expression levels of P-gp and CYP3A2 proteins detected by western blot analysis. The protein levels of P-gp in the duodenum and liver were significantly elevated compared with the untreated control level, but the level in the ileum was hardly changed by AMB. On the other hand, the protein level of CYP3A2 in the liver was significantly elevated compared with the control level, while the levels in the duodenum and ileum showed no significant change.

Discussion

In order to understand why the blood concentration of CyA in a patient receiving immunotherapy decreased following coadministration of AMB, the effect of repeated i.v. administration of AMB (1.5 or 3.0 mg/kg) for 4 days on the disposition kinetics of CyA was examined in rats after i.v. or p.o. administration (Figures 2, 3). A dose of 1.5 or 3.0 mg/kg was used in rats because the total clearance of AMB in rats is about five times higher than that in humans (0.5 mg/kg) [21]. As it was previously established that dexamethasone [9] and cyclophosphamide [10] induced P-gp and CYP3A2 within 4 days of coadministration, in this study AMB was coad-

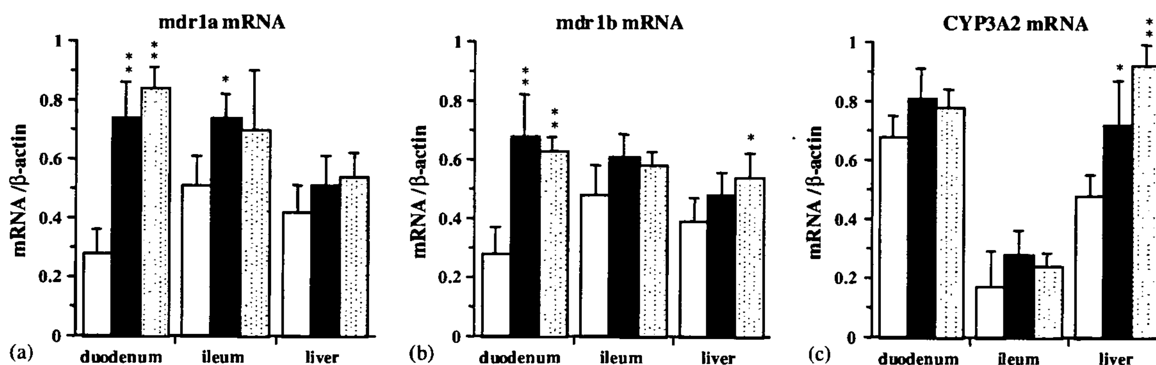

Figure 4. Effect of AMB on the expression of mdr1a, mdr1b and CYP3A2 mRNAs in duodenum, ileum and liver. The data represent the relative expression of the mRNAs obtained as mRNA/β-actin mRNA ratios in the control group (□), the low-AMB (1.5 mg/kg, ■) and high-AMB (3.0 mg/kg, ⬚) groups at 24 h after the last AMB treatment for 4 days. *,**Significant difference from the control group at $p<0.05$ and $p<0.01$, respectively

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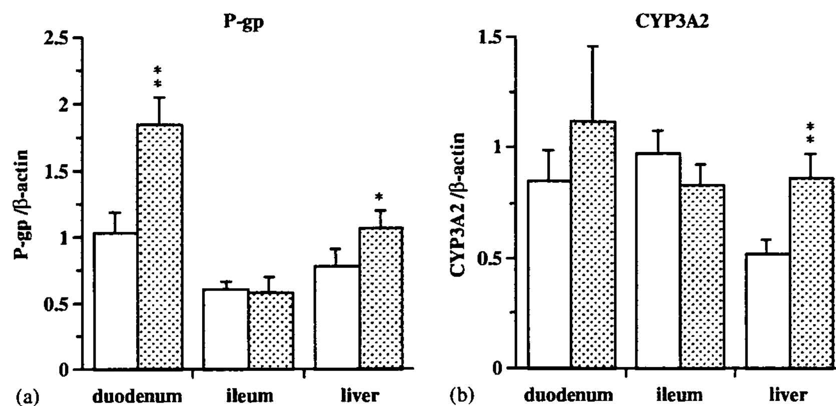

Figure 5. Western blot analysis of P-gp (a) and CYP3A (b) proteins in the duodenum, ileum and liver of rats with (□) or without (▒) the high-dose AMB (3.0 mg/kg) for 4 days, at 24 h after the last AMB treatment. *,**Significant difference from the control group at $p<0.05$ and $p<0.01$, respectively

ministered intravenously to rats for 4 days. The blood concentration of CyA after i.v. administration was significantly, though not dose-dependently, decreased in both AMB groups compared with the control. After p.o. administration, there was a significant, dose-dependent decrease of the blood concentration of CyA in both AMB groups. The oral bioavailability of CyA was clearly decreased by AMB treatment (Table 1).

It is well known that renal failure is the main side effect of AMB in clinical practice. There are some reports indicating that the disposition kinetics of CyA is influenced by renal failure in rats. Shibata *et al.* [22] reported that CYP3A and P-gp in liver and intestine are not likely to be involved in lowering the oral bioavailability of CyA in gentamicin-induced acute renal failure, and that a change in bile function is responsible for the marked decrease. Huang *et al.* [23] reported that the expression level of P-gp in rats with glycerol-induced acute renal failure was increased 2.5-fold in the kidney, but was not increased in the liver or brain. Leblond *et al.* [24] reported that chronic renal failure is associated with a decrease in total liver CYP450 (mainly CYP2C11, CYP3A1 and CYP3A2) activity in rats, and this leads to a significant decrease in drug metabolism. In the rats, hepatic function was not impaired, as judged from the laboratory data, whereas increases in the values of serum creatinine and BUN indicated an appreciable impairment of renal function by the high-AMB treatment (Table 2). However, the increases of serum creatinine and BUN were not large compared with the increases of about 3- to 5-fold and 2- to 5-fold in model rats with gentamicin- or glycerol-induced renal failure, respectively [22–24]. Therefore, it was considered that the AMB-induced renal impairment was not great even in the high-AMB group, and may have had only a slight influence on the excretion of CyA, which is metabolized mainly by CYP3A.

It was found that in our model the expression of mdr1a and mdr1b mRNAs was characteristically induced about 3-fold in the duodenum compared with the control, but was only slightly increased in the ileum and liver by both AMB treatments (Figure 4). Also, the expression of CYP3A2 mRNA was increased about 2-fold over the control in the liver. These results are broadly consistent with the previous findings in mice treated with dexamethasone (DEX) [25,26]. Unlike DEX, AMB did not induce a substantial increase of CYP3A2 mRNA expression in intestine, possibly because the dose levels of AMB were relatively low. This seems consistent with the relatively minor renal impairment. The studies with DEX [9–11,26] indicated that increased mRNA levels of mdr1a, mdr1b and CYP3A2 mRNAs are well reflected in increased levels of P-gp and CYP3A2 proteins. In this study also, the induction of the mRNAs by AMB treatment resulted in correspondingly increased levels of P-gp and CYP3A2 proteins (Figure 5). Therefore, it was considered that the oral bioavailability of CyA was reduced in the present

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animal model as a result of increased efflux transport via P-glycoprotein in the duodenum and an increased first-pass effect of hepatic CYP3A2 induced by AMB. Interestingly, there was not a great difference in the $t_{1/2}$ values of CyA among the three groups. Therefore, it appears that the induction of CYP3A2 in the liver only slightly influences the disposition kinetics of CyA. Previously it was suggested that, under physiological conditions, the oral bioavailability of CyA is mainly controlled by CYP3A in the upper intestine, rather than in the liver, but when P-gp is induced by steroid, the intestinal absorption of CyA may be inhibited [26]. Therefore, it is considered that the decrease of bioavailability of CyA caused by AMB treatment is mainly due to a decrease of intestinal absorption resulting from the induction of P-gp.

In the case of our patient, the stable trough values of blood CyA concentration during repeated p.o. administration of CyA were decreased after repeated intravenous infusion administration of AMB was started, and the decrease continued for 3 weeks. During this period, the laboratory data indicated relatively minor impairment of hepatic and renal functions by AMB. The patient had also been receiving hydrocortisone since the transplantation, but the low dose of hydrocortisone (50–100 mg/day) appeared to have had little influence on the plasma level of CyA prior to the start of AMB treatment. Therefore, we think that the clinical observations can also be explained mainly in terms of the induction of P-gp in the intestine by AMB.

It was previously clarified that the induction of P-gp and CYP3A2 continues for 2 weeks after the final DEX treatment [27]. Here, the patient received repeated administrations of AMB for 40 days, so it was difficult to predict when the levels of P-gp and CYP3A2 expression would recover to the control values; therefore, we chose to increase the dose of CyA gradually. The level of blood CyA concentration began to increase 7 days after the dose of CyA was increased from 120 mg/day to 160 mg/day, and after the dose of CyA was further increased to 240 mg/day, the blood CyA concentration reached 210 ng/ml. Therefore, we speculate that the blood CyA concentration might have recovered to about 100 ng/ml within 40 days after the start of AMB treatment, if the dose of CyA had been kept at 120 mg/day throughout. However, there are species differences in hepatic metabolism and susceptibility to metabolic changes. Therefore, it would be desirable to examine further the influence of AMB treatment on the metabolic activity, uptake and efflux of CyA in the intestine by means of *in vitro* studies using human hepatocytes, human hepatic microsomes, intestinal membrane, Caco-2 cells, etc.

In conclusion, the results indicate that the oral bioavailability of CyA is decreased by coadministration of AMB because of an increase of expression of P-gp and CYP3A induced by AMB treatment. The results in this rat model were consistent with the clinical observations. Therefore, the blood concentration of drugs that are substrates of P-gp and CYP3A, such as CyA, should be carefully monitored in patients when AMB is coadministered in combination with such drugs.

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DOI: 10.1002/bdd

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Bone Marrow Transplantation (2007) 39, 501–507
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ORIGINAL ARTICLE

Immature platelet fraction for prediction of platelet engraftment after allogeneic stem cell transplantation

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Platelet regeneration represents an important and separate element in the engraftment process for allogeneic stem cell transplantation. Fully automated flow cytometry using blood cell counters now allows reliable quantification of reticulated platelets, expressed as the immature platelet fraction (IPF). We studied the kinetics of IPF in six patients grafted with allogeneic peripheral blood stem cell transplantation (PBSCT), 12 patients with bone marrow transplantation (BMT) and seven patients with cord blood transplantation (CBT). Preconditioning therapy caused an immediate and rapid fall in tri-lineage hematopoiesis. IPF rose transiently above 3% after a mean duration of 11 days post-PBSCT, 18 days post-BMT and 19 days post-CBT. This was 1, 4 and 13 days earlier than platelet engraftment, respectively. A linear correlation model showed a close association between the rise of IPF and tri-lineage engraftment after transplantation. IPF counting may thus provide an accessible measure of thrombopoietic activity, leading to early evaluation of marrow function and allowing monitoring of platelet regeneration.
Bone Marrow Transplantation (2007) **39,** 501–507. doi:10.1038/sj.bmt.1705623; published online 5 March 2007
Keywords: reticulated platelet; immature platelet fraction; Sysmex XE-2100; IPF rise; allogeneic stem cell transplantation; cord blood transplantation

Introduction

Allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) have been used successfully to treat patients with high-risk or recurrent hematologic malignancies, bone marrow failure syndromes and solid tumors.[1] In addition, the recent establishment of unrelated cord blood transplantation (CBT) offers the possibility of cure even in adult patients lacking an HLA-matched related or unrelated donor.[2,3]

At present, the observation of rising peripheral blood neutrophil count offers the earliest practical measure of engraftment onset. Failure of neutrophil recovery may, however, reflect intercurrent events such as infection or graft-versus-host disease and can be difficult to interpret. Although reticulocyte counts performed by automated flow cytometric methods provide a good indicator of erythropoietic activity that may predict bone marrow regeneration, the duration, until increases in reticulocytes are seen, is usually later than or equal to that of neutrophils after transplantation.[4–6] Analysis of chimerism using fluorescence *in situ* hybridization (FISH) or polymerase chain reaction (PCR)-based methods can allow earlier demonstration of engraftment after transplantation,[7] but is costly and therefore unsuitable for routine monitoring.

Evaluation of thrombopoiesis by quantifying reticulated platelets, expressed as the immature platelet fraction (IPF), has recently been described.[8–12] Measurement of IPF is beneficial in determining whether thrombocytopenia is due to decreased production of platelets or to increased peripheral destruction, leading to avoiding the need for bone marrow examination as well as allowing optimal platelet transfusions.[9,13–17] We describe herein a study of thrombopoiesis following allogeneic stem cell transplantation using an automated blood cell counter for flow cytometric techniques with a nucleic acid-specific dye in the reticulocyte/optical platelet channel. Besides, we compared the IPF to the immature reticulocyte count (IRF)[18,19] to predict bone marrow regeneration.

Patients and methods

Patients

Between January 2005 and December 2005, 26 consecutive patients (15 men, 11 women) underwent unmanipulated allogeneic stem cell transplantation at our institute. The type of allografts used was determined at the reasonable discretion of the patients' physicians based on various factors such as the presence or absence of related donors, disease status, complications and immediacy of

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Received 24 April 2006; revised 2 January 2007; accepted 1 February 2007; published online 5 March 2007

Table 1 Patient characteristics

	PBSCT	BMT	CBT
n	6	12	7
Median age, years (range)	40 (17–60)	46 (26–64)	57 (48–67)
Sex (male/female)	4/2	8/4	3/4
Disease			
ALL	1	2	0
AML	4	3	1
CML	0	1	0
NHL	0	2	3
MM	0	0	1
MDS	0	1	2
SAA	0	2	0
ST	1	1	0
Donor type			
HLA-identical sibling	4	6	0
Mismatched related donor	2	0	0
Matched unrelated donor	0	6	0
Mismatched unrelated donor	0	0	7
Myeloablative regimen			
TBI + CY + CA	3	9	0
Reduced-intensity regimen			
TBI + Flu + Mel	2	1	7
TBI + Flu	1	0	0
TLI + Flu + CY	0	2	0
Flu + Bu	0	0	0
Median follow-up, days (range)	260 (31–381)	197 (28–428)	225 (71–380)
OS at 1 year	67%	83%	86%

Abbreviations: PBSCT = peripheral blood stem cell transplantation; BMT = bone marrow transplantation; CBT = cord blood transplantation; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CML = chronic myeloid leukemia; NHL = non-Hodgkin's lymphoma; MM = multiple myeloma; MDS = myelodysplastic syndrome; SAA = severe aplastic anemia; ST = solid tumor; TBI = total body irradiation; CY = cyclophosphamide; CA = cytarabine; Flu = fludarabine; Mel = melphalan; TLI = total lymphnode irradiation; Bu = busulfan; OS = overall survival.

transplantation. A total of 25 engrafted patients were analyzed (Table 1), excluding a 62-year-old male patient with secondary myelodysplastic syndrome receiving cord blood transplant who died of regimen-related toxicity at day 10 before engraftment. These 25 patients achieved complete donor chimerism as confirmed using FISH to detect X and Y chromosomes for recipients of sex-mismatched transplants and PCR-based analysis of polymorphic microsatellite regions for recipients of sex-matched transplants.[7] All patients were administered granulocyte-colony stimulating factor at a dose of 5 μg/kg/day starting 1 day after graft infusion until absolute neutrophil count (ANC) was $\geqslant 1 \times 10^9$/l for 3 consecutive days. Red blood cell (RBC) and platelet transfusions were given to maintain hemoglobin levels at >8 g/dl and platelet counts at $>20 \times 10^9$/l. All patients provided written informed consent to participate before entry in the study.

Measurements of peripheral blood counts

Peripheral whole blood samples (3 ml) collected into ethylene diamine tetraacetic acid (EDTA)-2K anticoagulant were analyzed at our institute. A normal reference range for IPF was established using 82 samples from healthy volunteers with a median age of 31 years (range, 21–63 years) and all routine full blood count parameters including platelets within the healthy reference range. All samples were kept at room temperature until analysis and were analyzed within 2 h after collection. Samples with platelet counts $<50 \times 10^9$/l were analyzed in duplicate and mean IPF was calculated and used for analysis.

IPF was measured using an XE-2100 fully automated hematology analyzer (Sysmex, Kobe, Japan) with upgraded software (XE IPF Master; Sysmex), as described previously.[8,9] Briefly, two fluorescent dyes, polymethine and oxazine, penetrate the cell membrane, staining the DNA and RNA in white blood cells (WBCs), and the RNA in the RBCs and platelets. Stained cells pass through a semiconductor diode laser beam, and the resulting forward light scatter (cell volume) and fluorescence intensity (RNA and DNA content) are measured. A computer algorithm discriminates between mature and immature platelets according to intensity of forward-scattered light and fluorescence. The XE-2100 instrument used in the study was equipped with upgraded software for data analysis of IPF, to apply a preset gate for separating the two platelet populations. Mature platelets appear as blue dots, whereas immature platelets characterized as showing increased cell volume and higher fluorescence intensity compared to mature platelets are displayed as green dots. IPF is calculated as the ratio of immature platelets to the total number of platelets. An absolute count of immature platelet can also be obtained as absolute immature platelet count (AIPC).

The IRF was simultaneously measured in the same channel on the XE-2100 analyzer.[19]

Hematologic parameters were measured as part of routine blood count analyses and results were available at the same time.

Statistical analysis

Engraftment of neutrophils, RBCs and platelets was defined as an absolute neutrophil count (ANC) $>0.5 \times 10^9$/l for three consecutive measurements on different days, a platelet count $>30 \times 10^9$/l for three consecutive measurements on different days in the absence of platelet transfusions for $\geqslant 7$ days, and a reticulocyte count $>30 \times 10^9$/l in the absence of RBC transfusions, respectively. Time to engraftment of neutrophils, RBCs and platelets was defined as the time from transplantation to the first day of engraftment of each lineage.

The relationship between recovery of IPF and engraftment was estimated by simple linear regression and correlation analysis. Unpaired or paired *t*-tests were used to determine the level of statistical difference. Values of $P<0.05$ were considered statistically significant.

Results

IPF values in healthy controls

IPF was measured in 82 healthy adults. Mean ($\pm$s.d.) IPF was 2.0 $\pm$ 1.1% (range, 0.5–5.7%) and mean AIPC was

4.5±1.9 × 10^9/l (range, 1.4–10.4 × 10^9/l). No significant differences were noted in IPF or AIPC between men and women.

Reproducibility and precision of IPF

The reproducibility of the IPF counting was measured by 20 repeated analyses using six different samples. In the three samples from healthy individuals with platelet counts within the normal reference range, the average mean for the IPF was 1.9% (range, 1.7-2.0%), the average s.d. for the IPF was 0.23% (range, 0.21–0.25%) and the average coefficient of variation (CV) for the IPF was 12.6% (range, 11.0–13.9%). For the remaining three samples from individuals with low platelet counts (mean, 29 × 10^9/l; range, 10–50 × 10^9/l), these parameters corresponded to 20.9% (16.6–25.4%), 2.4% (1.6–3.6%) and 11.1% (7.9–14.2%), respectively.

Hematologic recovery after stem cell transplantation

In all patients, the ANC, reticulocyte count, platelet count, IPF and AIPC fell progressively after onset of conditioning to a nadir that persisted for a variable period, irrespective of the intensity of each preparative regimen. Figure 1 shows the mean hematologic parameters in each transplant group.

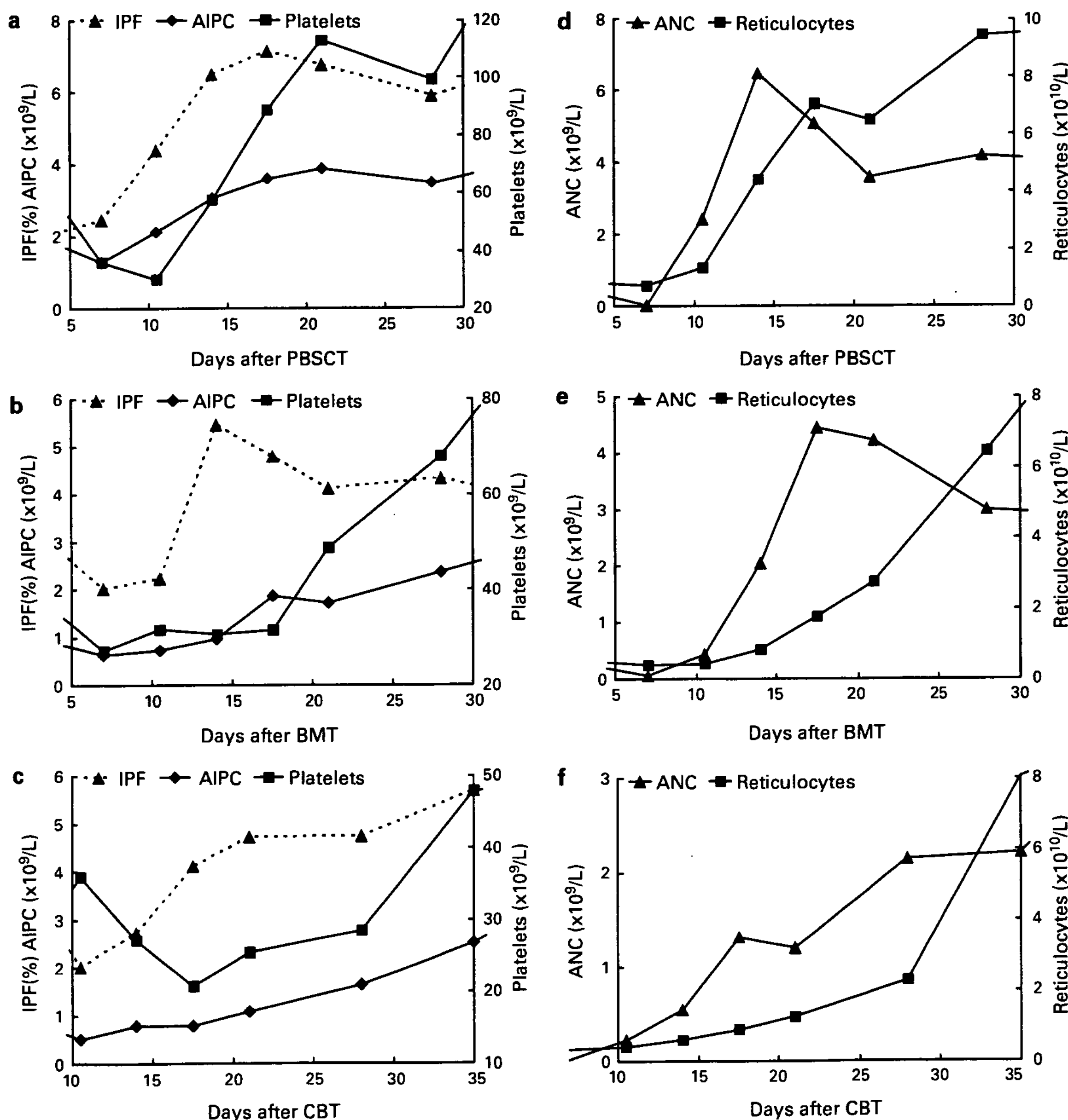

Figure 1 Mean values for hematologic parameters during the post-transplant period after peripheral blood stem cell transplantation (**a**, **d**), bone marrow transplantation (**b**, **e**) and cord blood transplantation (**c**, **f**). ANC represents absolute neutrophil count, IPF immature platelet fraction and AIPC absolute immature platelet count.

Table 2 Hematologic recovery data

	PBSCT	P	BMT	P	CBT	P
			Median % (range)			
IPF, minimum	1.8 (0.4–2.5)		1.0 (0.4–2.1)		0.8 (0.4–1.2)	
IPF, maximum	8.2 (5.1–34.0)		6.3 (4.2–12.9)		11.7 (4.2–19.4)	
			Mean days post transplantation (range)			
IPF≥3.0%	11 (7–13)	[a]	18 (6–22)	[a]	19 (15–29)	[a]
IPF≥3.5%	11 (7–13)		21 (15–22)		23 (15–29)	
IPF≥4.0%	12 (7–14)		21 (15–28)		24 (15–29)	
Platelet engraftment	12 (8–15)	0.3	22 (12–40)	0.01	32 (25–41)	0.01
RBC engraftment	14 (11–15)	0.2	25 (18–40)	0.02	36 (25–56)	0.01
Neutrophil engraftment	12 (10–18)	0.5	15 (11–20)	0.7	20 (12–28)	0.9
IRF≥10%	14 (10–15)	0.7	16 (13–20)	0.9	21 (15–29)	0.4
			Median number of sepsis[b] within 30 days post transplant (range)			
	1 (0–2)		1 (1–3)		2 (1–3)	
			Median unit of platelet transfusions within 30 days post transplant (range)			
	50 (20–100)		75 (40–140)		145 (110–200)	

[a]Represents reference.
[b]Sepsis was judged by persistent fever ≥38°C.

Table 3 Correlation of time of IPF rise (x) with time of engraftment (y)

	Platelet engraftment	Neutrophil engraftment	RBC engraftment
IPF≥3.0%	$r=0.95$, $y=1.37x$, $P<0.0001$	$r=0.92$, $y=0.94x$, $P<0.0001$	$r=0.92$, $y=1.55x$, $P<0.0001$
IPF≥3.5%	$r=0.96$, $y=1.22x$, $P<0.0001$	$r=0.95$, $y=0.86x$, $P<0.0001$	$r=0.94$, $y=1.38x$, $P<0.0001$
IPF≥4.0%	$r=0.95$, $y=1.16x$, $P<0.0001$	$r=0.95$, $y=0.81x$, $P<0.0001$	$r=0.93$, $y=1.30x$, $P<0.0001$

PBSCT restored tri-lineage hematopoiesis the fastest, followed by BMT (Table 2). Following the period of thrombocytopenia and <3% in IPF, a rise in IPF to ≥3% post transplantation occurred in all engrafted patients. The rise in IPF to ≥3% preceded platelet and RBC engraftment after a mean of 18 days versus 22 days ($P=0.007$) and 25 days ($P=0.02$) post-BMT, and 19 days versus 32 days ($P=0.007$) and 36 days ($P=0.009$) post-CBT (Table 2), respectively. However, this was not evident in the PBSCT group, with mean times of 11 days, 12 days ($P=0.3$) and 14 days ($P=0.2$) to IPF rise ≥3%, platelet and RBC engraftment. Recovery of AIPC appeared similar to that of platelet count. No significant differences were noted between time to IPF rise ≥3% and time to neutrophil engraftment in each transplant group.

Relationship with the IPF rise and engraftment
The minimum of IPF ranged 0.4–2.5% following conditioning regimen, and the maximum of IPF in the early post-transplant period ranged 4.2–10.4% (Table 2). Based on these findings, when time to IPF rise ≥3.0, ≥3.5, or ≥4.0% versus time to platelet engraftment was plotted for all patients, a marked association was evident in each case (Table 3). The correlation was maximal when the cutoff count for IPF was set at 3.5% (Table 3; Figure 2a). Of note, the IPF rise ≥3.0% preceded platelet engraftment in four PBSCT patients (67%), 10 BMT patients (83%) and all seven CBT patients, suggesting that detection of the IPF rise ≥3.0% can allow estimation of platelet engraftment after transplantation. IPF rise also predicted RBC engraftment (Figure 2c), but appeared coincident with neutrophil engraftment (Figure 2b).

Comparison of the IPF to the IRF
The recovery of the IRF, which was defined as an IRF value ≥10% according to previous reports,[14,17] took a mean of 14 days (range, 10–15 days) in patients undergoing PBSCT; a mean of 16 days (range, 13–20 days) in patients undergoing BMT; and a mean of 21 days (range, 15–29 days) in patients undergoing CBT (Table 2), which was similar to the recovery of IPF ≥3% ($P=0.7$, 0.9 and 0.4, respectively).

Discussion

Newly released platelets contain a coarse and punctuated reticulum, and have been termed 'reticulated platelets', as the platelet analog of red cell reticulocytes.[20] The number of reticulated platelets reflects the rate of thrombopoiesis,[13,15,21,22] increasing when platelet production rises and decreasing when production falls. Reticulated platelets contain amounts of cytoplasmic RNA, and can thus be distinguished from mature platelets poor in RNA by flow cytometric quantification using any fluorescent dye binding RNA.[21] Several studies have clearly shown that platelet RNA content correlates directly with megakaryocyte activity under conditions of thrombocytopenia.[13,15,16,22]

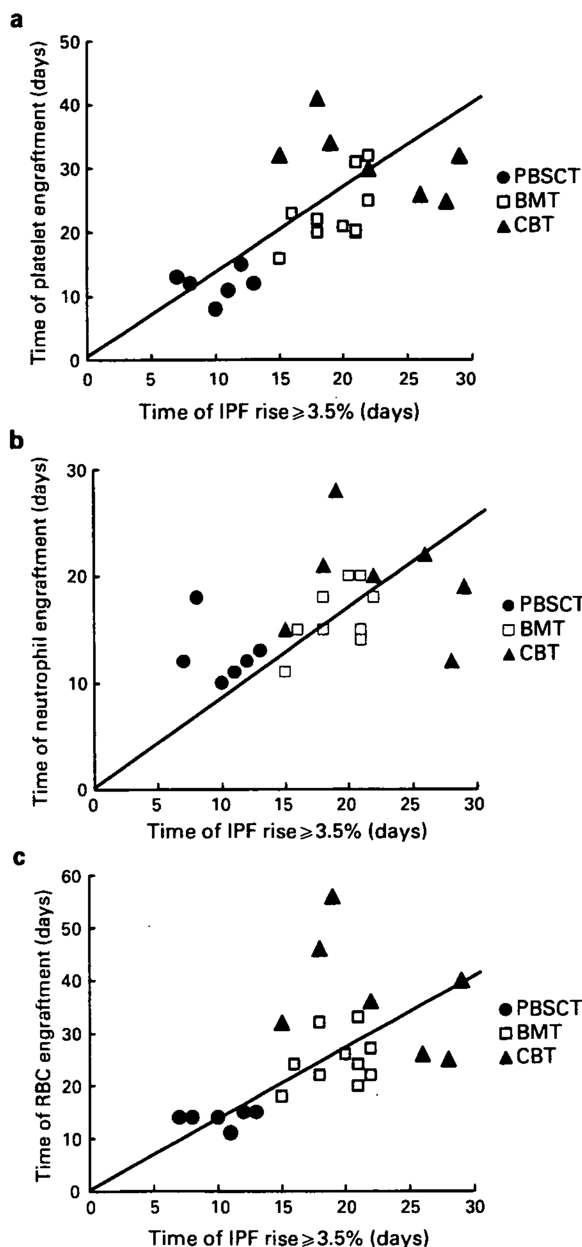

Figure 2 Correlation between IPF rise ⩾3.5% and engraftment of platelets (a), neutrophils (b) and red blood cells (c) in transplant recipients.

Recent advances in technology enabled automatic and immediate measurement of reticulated platelets, expressed as IPF using the Sysmex XE-2100 blood cell counter with upgraded software. This instrument is reportedly useful in the diagnosis and monitoring of thrombocytopenic patients.[8,9]

A reference range for IPF in healthy individuals using this method proved to be 0.5–5.7% in the present study. This seemed comparable to the reference range of 1.1–6.1% reported by a group in London, UK[9] using the same instrument.

In evaluating thrombocytopenia during the early post-transplant period, knowing whether platelet production is developing or not is important. For this purpose, bone marrow aspiration is sometimes used to provide information on platelet production, such as the number of megakaryocytes. However, bone marrow examination is not accepted for routine clinical application, as the procedure is invasive, time-consuming and prone to subjective interpretation.

To examine whether IPF counting can solve this dilemma, we tested IPF as a predictor of the timing of platelet recovery in recipients of allogeneic stem cell transplantation. IPF transiently increased in the early post-transplant period in all engrafted patients, which may reflect an explosive increase in platelet production at the beginning of engraftment. In our study, the IPF ⩾3.0% was the earliest predictor for platelet engraftment, and the IPF ⩾3.5% was the most precise predictor based on the correlation analysis. The IPF rise ⩾3% substantially preceded platelet engraftment after BMT or CBT. No such precedence was evident after PBSCT, although this finding may be attributable to rapid platelet recovery following PBSCT.

Inadequate engraftment following transplantation represents a serious problem with high mortality. Although this event occurs infrequently in patients receiving bone marrow or peripheral blood grafts, engraftment is far slower and graft failure is more likely in patients undergoing CBT.[2,3] Opportunistic infection and organ failure rise exponentially with time to engraftment, and overall long-term survival correlates closely with neutrophil and platelet engraftment after CBT.[3] Early diagnosis or prediction of engraftment failure may allow timely introduction of appropriate treatment.[23]

As far as assessing marrow regeneration is concerned, IPF rise offered a particularly early and sensitive index for platelet and RBC engraftment after BMT and CBT. These findings indicate that automated IPF counting may allow a useful and early evaluation of the re-establishment of hematopoiesis in platelet and RBC lineage following allogeneic BMT and CBT. Notably, IPF rise ⩾3% represented the first sign of hematologic recovery in all six PBSCT patients, six of 12 BMT patients (50%) and six of seven CBT patients (86%), preceding or occurring at the same time as neutrophil engraftment. However, early signs of recovery were not so apparent when considering AIPC that was gradually increased in parallel with platelet recovery. This discrepancy might be due to the fact that small changes are barely perceptible when IPF is converted to absolute numbers like AIPC.

A further advantage of IPF measurement is the ability to make clinical decisions when hematopoietic recovery appears delayed, particularly for patients at highest risk of graft failure, such as those who have received quite low doses of infused CD34-positive cells such as cord blood graft, or recipients of an allograft with some degree of HLA disparity. For such patients, close monitoring to detect any early signs of hematopoietic recovery may be easily accomplished with the IPF counter. Nevertheless, we were unable to define IPF and time points after transplantation predictive of graft failure, as only one of the 26 patients experienced graft failure in the present study.

Rising IRF values, expressed as a percentage of the total reticulocyte count, is reportedly the first sign of hematologic recovery after BMT and PBSCT.[5,6,18,24] The present study showed that the IPF recovered in parallel with the IRF in patients receiving allografts including a cord blood graft. When IPF and IRF were compared with respect to correlation with engraftment, contributions of IPF and IRF are almost equivalent to predict subsequent platelet and RBC engraftment in a patient undergoing BMT or CBT. On the other hand, neutrophil recovery was not predictable from either IPF or IRF, possibly resulting from the early recovery of neutrophils in this study that might be related to the reduced-intensity conditioning regimen used in the majority of the patients.

Previous reports have demonstrated that a transfusion of platelets temporarily reduces the IPF, whereas infection often raises the IPF with no subsequent increase in platelet count.[14,17] In our study, it makes these issues complex that most allograft recipients usually not only experience clinical evidence of sepsis, but receive multiple platelet transfusions in the early post-transplant period. The present study showed no tendency that the patients receiving multiple platelet transfusions or with infection demonstrated a longer period for platelet regeneration or a longer time lag between a rise in the IPF and an increase in the platelet count.

In conclusion, a fully automated, rapid IPF counting using the Sysmex XE-2100 fitted with upgraded software provides clinically useful data on thrombopoietic activity in the immediate post-transplant period. Results are available at the same time as results for full blood counts. In addition, IPF counting can be performed on 0.1 ml of EDTA-anticoagulated blood. Such information will be an important component in determining the necessity and timing of platelet transfusions in patients with thrombocytopenia after allogeneic transplantation. Therefore, measurement of IPF should be considered as routine practice to evaluate and monitor thrombocytopenic patients following stem cell transplantation.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture (KAKENHI 18591049) and from the Ministry of Health, Labor and Welfare, Japan. We are indebted to the patients and their families, the Japan Bone Marrow Donor Program, the Japan Cord Blood Network, the staff at the Division of Hematology and Oncology at Kanazawa University Medical Center, and Kayo Goto and Rie Ohmi for excellent technical assistance.

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Journal of Antimicrobial Chemotherapy (2007) **60**, 350–355
doi:10.1093/jac/dkm217
Advance Access publication 21 June 2007

JAC

Presumptive treatment strategy for aspergillosis in allogeneic haematopoietic stem cell transplant recipients

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Received 2 March 2007; returned 8 April 2007; revised 5 May 2007; accepted 21 May 2007

Background**: The onset of invasive aspergillosis (IA) after allogeneic haematopoietic stem cell transplantation (HSCT) is bimodal. However, IA early after HSCT has become less frequent due to the shortened neutropenic period, and the clinical significance of empirical treatment for aspergillosis based on persistent febrile neutropenia (FN) became less clear. Therefore, we started a presumptive treatment strategy, in which anti-*****Aspergillus*** **agents were started when patients developed positive serum test and/or infiltrates or nodules on X-ray or CT-scan associated with persistent FN, in 2002.**

Methods**: We retrospectively reviewed the records of 114 adult patients who underwent allogeneic HSCT between September 2002 and December 2005 in high-efficiency particulate air-filtered clean rooms. Fluconazole was given as anti-*****Candida*** **prophylaxis. The primary endpoint was the development of early IA, which was defined as probable or proven IA according to the EORTC/MSG criteria that developed between the day of HSCT and 7 days after engraftment.**

Results**: Among 73 patients who experienced persistent FN for 7 days or longer, anti-*****Aspergillus*** **agents were empirically started in 13 patients at the discretion of attending physicians, whereas 60 patients actually followed presumptive treatment strategy. Only 4 of 60 patients received anti-*****Aspergillus*** **agents. Two patients in the presumptive group developed early IA, but were successfully treated with anti-*****Aspergillus*** **agents started after the diagnosis of IA.**

Conclusions**: These findings suggested the feasibility of a presumptive treatment strategy for aspergillosis in HSCT recipients. A randomized controlled trial is warranted to compare empirical and presumptive anti-*****Aspergillus*** **strategy in allogeneic HSCT recipients.**

Keywords: empirical treatment, febrile neutropenia, invasive aspergillosis

Introduction

Invasive fungal infection (IFI) is one of the leading causes of transplant-related mortality and its incidence in allogeneic haematopoietic stem cell transplantation (HSCT) recipients ranges from 8 to 15%.[1–3] Invasive aspergillosis (IA) is the most common IFI after allogeneic HSCT.[1–4] The development of IA after allogeneic HSCT shows bimodal distribution, one in the neutropenic period early after HSCT and the other 2–3 months after HSCT when patients are taking glucocorticosteroid for acute graft-versus-host disease (GVHD).[1,3,5,6] IA early after HSCT, however, has become less frequent because of the shortened neutropenic period due to the use of peripheral blood stem cells (PBSC), granulocyte colony-stimulating factor (G-CSF) and high-efficiency particulate air (HEPA) filtration and/or laminar air flow.[5–12] Therefore, the clinical significance of empirical treatment for aspergillosis based on persistent febrile neutropenia (FN) has become less clear, although it is supported by old evidence and recent guidelines.[11–16]

Our transplantation unit moved to a new building in September 2002. At the same time, we changed the strategy against aspergillosis during the neutropenic period from

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empirical strategy to presumptive strategy, in which anti-*Aspergillus* agents were started based on positive serum test and/or infiltrates or nodules on X-ray or CT-scan associated with persistent FN.[17,18] In this report, we reviewed the outcomes of 114 patients who underwent allogeneic HSCT in the new transplant unit and evaluated the feasibility of the presumptive strategy during the early neutropenic period after allogeneic HSCT.

Materials and methods

Study patients

Medical records of 124 consecutive adult patients who underwent allogeneic HSCT at the University of Tokyo Hospital between September 2002 and December 2005 were reviewed. All patients received prophylactic antifungal agents. Of the 124 patients, 114 who received fluconazole at 200 mg/day as anti-*Candida* prophylaxis were included in this study.[19,20] The remaining 10 patients were excluded from this study, because they had recent IA and prophylactically received anti-*Aspergillus* agents including micafungin and itraconazole. Characteristics of the 114 patients are summarized in Table 1. The median age was 43 years (range, 20–66 years). Patients' underlying diseases included acute myeloblastic leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myelogenous leukaemia (CML), myelodysplastic syndrome (MDS), non-Hodgkin lymphoma (NHL), aplastic anaemia (AA) and so on. Standard-risk diseases were defined as AML/ALL in first complete remission (CR1) or CR2, CML in first chronic phase (CP1) or CP2, chemosensitive NHL, MDS in refractory anaemia or refractory anaemia with ringed sideroblasts and non-malignant haematological disorders. All other diseases were classified as high-risk diseases. Eight patients had received previous autologous or allogeneic stem cell transplantation. Two patients had a previous history of probable IA prior to HSCT.

Transplantation procedure

The stem cell source was bone marrow (BM) from a related donor in 8, BM from an unrelated donor in 47 and PBSC from a related donor in 59. Myeloablative conditioning regimens were used in 74 patients, mainly with total body irradiation plus cyclophosphamide or busulfan plus cyclophosphamide. Fludarabine-based reduced-intensity conditioning regimens were conducted in 40 patients. In these regimens, fludarabine was combined with either busulfan at 8–16 mg/kg in total or melphalan at 140 mg/m^2 in total. In some patients, total body irradiation of 4 Gy in total was added. Therefore, the intensities of regimens were close to the myeloablative conventional regimens. Prophylaxis against GVHD was performed with calcineurin inhibitors (cyclosporine or tacrolimus) with or without short-term methotrexate in the majority of patients. *In vivo* T cell depletion using alemtuzumab or anti-thymocyte globulin was performed in 27 patients, concomitant with cyclosporine and short-term methotrexate.

Neutrophil engraftment was defined as an absolute neutrophil count >500 cells/mm^3 for 3 consecutive days. All patients were housed in double-door HEPA-filtered laminar air flow rooms and provided with low microbial diets until neutrophil engraftment. New quinolones were given prophylactically in all patients. Recombinant G-CSF was routinely administered for patients with non-malignant disease and those with lymphoid malignancies after HSCT. Chest X-ray and non-invasive screening serum tests for IA including galactomannan antigen test (Platelia Aspergillus, Bio-Rad Laboratories, Marnes-la-Coquette, France) and β-D-glucan (BDG) test (β-glucan Test Wako, Wako Pure Chemical Industries, Tokyo, Japan) were performed weekly. Initial empirical antibacterial treatment for FN was started with fourth-generation cephalosporins or carbapenems.[11] For patients with persistent or recurrent FN for 7 days or longer, we did not start anti-*Aspergillus* agents as an early presumptive treatment for aspergillosis until patients developed positive serum test and/or infiltrates or nodules on X-ray or CT-scan (presumptive group). Thirteen patients, however, received

Table 1. Characteristics of the 114 patients who were included in this study

Characteristic	
Median recipient age, years (range)	43 (20–66)
Male/female	66/48
Underlying diagnosis, *n* (%)	
AML	30 (26.3)
ALL	20 (17.5)
AUL	1 (0.9)
CML	13 (11.4)
MDS	13 (11.4)
NHL	16 (14.0)
ATL	4 (3.5)
AA	7 (6.1)
Others	10 (8.8)
IA before HSCT, *n* (%)	2 (1.8)
Disease status, *n* (%)	
Standard-risk	65 (57.0)
High-risk	49 (43.0)
Donor, *n* (%)	
Related	67 (58.8)
Unrelated	47 (41.2)
Stem cell source, *n* (%)	
BM	55 (48.2)
PBSC	59 (51.8)
Number of transplantation, *n* (%)	
1	106 (93.0)
2	7 (6.1)
3	1 (0.9)
HLA mismatches at serological level, *n* (%)	30 (26.3)
HLA mismatches at genetic level, *n* (%)	36 (31.6)
Conditioning regimen, *n* (%)	
Myeloablative conditioning	74 (64.9)
Reduced-intensity conditioning	40 (35.1)
GVHD prophylaxis, *n* (%)	
Cyclosporine alone	4 (3.5)
Cyclosporine and short-term MTX	80 (70.2)
Tacrolimus and short-term MTX	3 (2.6)
In vivo T cell depleted	27 (23.7)
Engraftment, *n* (%)	112 (98.1)
Days of engraftment, median (range)	16.5 (9–43)
Antibacterial prophylaxis, *n* (%)	
Tosufloxacin	110 (96.5)
Ciprofloxacin	4 (3.5)
Use of G-CSF, *n* (%)	68 (59.6)

MTX, methotrexate; G-CSF, granulocyte colony-stimulating factor; AUL, acute unclassified leukaemia; ATL, adult T-cell leukaemia/lymphoma.

Figure 1. Treatment and outcome of patients included in the study. One hundred and fourteen patients were included in the study. One hundred and nine patients experienced FN. Seventy-three patients experienced persistent or recurrent FN for 7 days or longer. Thirteen patients in the empirical group received anti-*Aspergillus* treatment and the remaining 60 patients were included in the presumptive group. Four patients actually received anti-*Aspergillus* treatment presumptively. In total, early IA was observed in two patients in the presumptive group and none in the empirical group (3.3% versus 0%, $P > 0.99$). IPA, invasive pulmonary aspergillosis.

anti-*Aspergillus* agents empirically at the discretion of attending physicians (empirical group, Figure 1).

Definition of IA

The primary endpoint of this study was the development of probable or proven IA, that was diagnosed according to the criteria of 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/MSG).[21] Microbiological criteria included two consecutive positive galactomannan tests using a reduced cutoff of 0.6 optical density index (O.D.I.).[22] IA that occurred between the day of HSCT and 7 days after engraftment was defined as early IA. The cumulative incidences of IA were calculated using Gray's method considering death without IA as a competing risk.[23]

Results

Clinical outcomes after allogeneic HSCT

Engraftment was observed in all patients at a median of 16.5 days (9–43 days) after HSCT, except for two who experienced early death before engraftment. Forty-two patients, 37.5% of those who achieved engraftment, developed grade II–IV acute GVHD at a median of 21 days after HSCT. Fifty-eight patients, 58.6% of those who survived more than 100 days after HSCT, developed chronic GVHD. Thirty-six patients relapsed at a median of 123.5 days after HSCT. Two-year overall survival of all subjects was 52.4% with a median follow-up duration of surviving patients of 822 (range 107–1603) days after HSCT.

Incidence of IA

Sixteen patients developed probable or proven IFI with a cumulative incidence of 15.1%, including 13 IA, 2 mucormycosis and 1 candidiasis (Table 2). The cumulative incidence of IA was 11.6% (Figure 2) and the median onset was 169.5 days (range, 12–531 days) after HSCT. Twelve out of 13 IA patients suffered from invasive pulmonary aspergillosis (IPA), whereas one developed gastrointestinal aspergillosis. No statistically significant risk factor was identified for the incidence of IA except for male sex (17.8% for male patients versus 2.1% for female patients, $P = 0.018$).

FN and early IA

One hundred and nine patients experienced FN with median duration of 12 days (range, 1–39 days). A median of three (range 1–5) antibiotics per patient were used for empirical antibacterial treatment during FN. Seventy-three patients experienced persistent or recurrent FN for 7 days or longer. The median duration of neutropenia was 21 and 20 days in the empirical group and presumptive group, respectively ($P = 0.91$). Thirteen patients in the empirical group received anti-*Aspergillus* treatment at a median of 9 days (range, 3–21 days) after the onset of FN (Figure 1). Amphotericin B was administered empirically in three patients, which was terminated within 2 days because of renal dysfunction. Micafungin was given to the other 10 patients for a median of 16.5 days (range, 3–76 days). Sixty patients followed the presumptive treatment strategy. Of the 60 patients in the presumptive group, 4 patients actually received anti-*Aspergillus* treatment presumptively, triggered by an elevation of BDG in 1 and infiltrates or nodules on chest

Table 2. Incidence of probable or proven IFI after allogeneic HSCT

	No.
Diagnosis of IFI after HSCT	
Proven diagnosis	4
Probable diagnosis	12
Onset of IFI after HSCT	
Early IFI	2
Late IFI	14
Incidence of late IFI	
Patients without FN	6
Empirical group	0
Presumptive group	8
Organisms that caused IFI	
Aspergillus spp.	13
Candida glabrata	1
Mucor spp.	2
Treatment for IFI	
Amphotericin B	5
Itraconazole	2
Micafungin	4
Voriconazole	3
None	2
Outcome	
Improved	8
No change or progression	6

X-ray or CT-scan in 3. One of them was subsequently diagnosed to have probable IA within a week, because galactomannan test became positive. We changed the anti-*Aspergillus* agent from micafungin to voriconazole and IPA was successfully treated (patient no. 4 in Table 3). Another patient in the presumptive group, who did not receive empirical or presumptive anti-*Aspergillus* agents, developed positive galactomannan test and nodules on CT-scan simultaneously, and was diagnosed to have probable IA (patient no. 5 in Table 3). This patient was also successfully treated with micafungin.

In total, early IA was observed in two patients in the presumptive group and none in the empirical group (3.3% versus 0%, $P > 0.99$). There was no significant difference in the duration of FN between the two groups (15.6 days versus 17.7 days, $P = 0.26$). There was no death that was directly associated with early IA in the whole population.

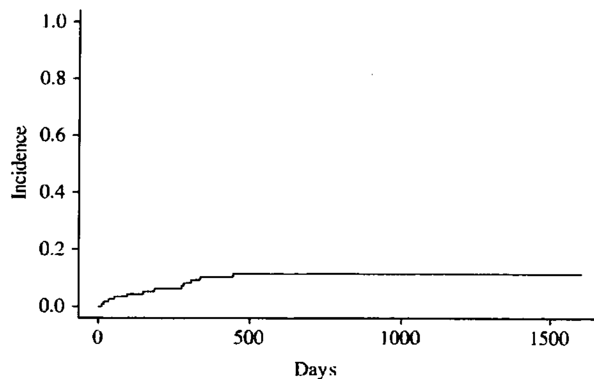

Figure 2. Cumulative incidence of IA after allogeneic HSCT. The cumulative incidence of IA was 11.6% in this study.

Discussion

In this study, the incidences of probable or proven IFI and IA were 15.1% and 11.6%, respectively, which were compatible with other recent studies.[1–3,5,7,8] Only three patients developed IFI other than IA, probably due to the prophylactic use of fluconazole.[5,7,8] Among the 13 patients with IA, only 2 developed IA early after HSCT. Both patients were successfully treated with anti-*Aspergillus* agents and therefore there was no death that was directly related to early IA.

Empirical anti-*Aspergillus* treatment has been recommended for patients with persistent FN.[11,24] However, this strategy is based on two old randomized controlled trials published in the 1980s, before the era of fluconazole prophylaxis.[12,25] Until recently, the standard antifungal agent in this setting has been amphotericin B deoxycholate.[12,25] This approach is limited by the substantial infusion-related toxicity and nephrotoxicity caused by this agent. Recently, lipid formulations of amphotericin B and intravenous itraconazole appeared to have equivalent efficacy compared with conventional amphotericin B with less toxicity.[13,16] Voriconazole and caspofungin were also reported to have similar efficacy.[14,15] However, these alternative agents are very expensive and still more toxic than fluconazole.

A presumptive strategy has been expected to decrease the use of these anti-*Aspergillus* agents by postponing anti-*Aspergillus* treatment until more specific findings are detected in patients with persistent FN. Several findings have been considered specific for IA, such as halo sign on CT-scan in neutropenic patients.[21] In addition, blood tests to detect *Aspergillus* constituents have been investigated, including galactomannan antigen test, BDG test and PCR to detect *Aspergillus* DNA.[22,26,27] Their clinical roles, however, have not been clarified.[26] Previously, we prospectively compared the sensitivity and specificity of these tests and found that the galactomannan test was the most suitable test for the diagnosis of IA with the best cutoff of 0.6 O.D.I.[22] In this study, we included not only blood galactomannan test with this cutoff index and halo sign on CT-scan but also blood BDG test and infiltrates or nodules on X-ray or CT-scan as triggers to start anti-*Aspergillus* treatment to increase sensitivity rather than specificity. By this presumptive strategy, only 2 of the 60 patients with persistent FN developed early IA, both of whom were successfully treated with anti-*Aspergillus* agents after the diagnosis of probable IA. This enabled us to decrease the use of anti-*Aspergillus* agents that are expensive and potentially toxic (4 of 60 in the presumptive group versus 13 of 13 in the empirical group). Considering the low incidence of early IA in the presumptive group, most patients in the empirical group might have been unnecessarily exposed to anti-*Aspergillus* agents. This is a retrospective study and therefore there are several limitations. Especially, we could not exclude the possibility of selection bias that high-risk patients tended to be treated empirically at the discretion of the attending physicians. However, there was no difference in the duration of neutropenia between the two groups. Both patients with a previous history of IA were included in the presumptive group.

Maertens *et al.*[28] recently showed the feasibility of preemptive therapy against IA. They started liposomal amphotericin B

Table 3. Characteristics of patients who received anti-*Aspergillus* agents presumptively (nos. 1–4) and patients who developed early IA (nos. 4 and 5)

No.	Age	Sex	Diagnosis	Prior IA	Triggers to start anti-*Aspergillus* agents	Anti-*Aspergillus* agents	Diagnosis of early IA	Outcome
1	57	Male	AML	—	Elevation of BDG	MCFG	No	Death due to AML progression
2	56	Male	AML	—	XP findings (consolidation)	MCFG	No	Alive
3	35	Male	CAEBV	—	CT findings (small multiple nodules with halo)	MCFG → ITC	No	Alive
4	56	Female	ALL	—	CT findings (nodules with halo) (positive galactomannan test after a week)	MCFG → VRC	Yes	Alive
5	54	Male	MDS	Probable IPA	CT findings (nodules with halo) and positive galactomannan test	MCFG → ITC	Yes	Alive

CAEBV, chronic active Epstein–Barr virus infection; IPA, invasive pulmonary aspergillosis; MCFG, micafungin; ITC, itraconazole; VRC, voriconazole; XP, X-ray photograph.

for patients with two consecutive positive galactomannan tests or with CT findings suggestive of IFI, regardless of the presence or absence of FN. They successfully reduced the use of anti-*Aspergillus* agents and no undetected cases of IA were identified. This approach may be more sensitive than our presumptive strategy to add anti-*Aspergillus* agents only for patients with persistent FN associated with positive serum test and/or radiological evidence. However, frequent galactomannan testing (thrice weekly) is required for this preemptive approach and thus it can be performed in only a limited number of centres.

Recently, prophylactic use of itraconazole, an anti-*Aspergillus* agent, has been evaluated in allogeneic HSCT recipients in two randomized controlled trials.[29,30] The incidence of IA was lower in the itraconazole group than the fluconazole group in both trials. The difference in the incidence of IA appeared 2 or 3 months after HSCT, not in the neutropenic period early after HSCT. Therefore, the prophylactic use of anti-*Aspergillus* agents should be considered for patients at higher-risk for IA, including patients receiving steroid for GVHD or neutropenic patients with a recent history of IA. However, for patients who are receiving anti-*Aspergillus* prophylaxis, another approach other than empirical or presumptive therapy, may be required.

In conclusion, these findings suggested the feasibility of a presumptive strategy for IA in HSCT recipients, provided that they were treated in a HEPA-filtered laminar air flow room. A randomized controlled trial is warranted to compare the efficacy and safety of presumptive and empirical strategy early after HSCT.

Acknowledgements

We thank all clinicians who have assisted with the provision of data for this project.

External funding

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

Transparency declarations

None to declare.

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Journal of Antimicrobial Chemotherapy (2008) **61**, 411–416
doi:10.1093/jac/dkm463
Advance Access publication 30 November 2007

JAC

False-positive *Aspergillus* galactomannan antigenaemia after haematopoietic stem cell transplantation

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Received 2 September 2007; returned 8 October 2007; revised 2 November 2007; accepted 4 November 2007

***Objectives*: Although *Aspergillus* galactomannan (GM) antigen detection is widely applied in the diagnosis of invasive aspergillosis (IA), false-positive reactions with fungus-derived antibiotics, other fungal genera or the passage of dietary GM through injured mucosa are a matter of concern. The aim of this study was to investigate the cumulative incidence and risk factors for false-positive GM antigenaemia.**

***Patients and methods*: The records of 157 adult allogeneic haematopoietic stem cell transplantation (HSCT) recipients were retrospectively analysed. Episodes of positive GM antigenaemia, defined as two consecutive GM results with an optical density index above 0.6, were classified into true, false and inconclusive GM antigenaemia by reviewing the clinical course.**

***Results*: Twenty-five patients developed proven or probable IA with a 1 year cumulative incidence of 12.9%, whereas 50 experienced positive GM antigenaemia with an incidence of 32.2%. Among the total 58 positive episodes of the 50 patients, 29 were considered false-positive. The positive predictive value (PPV) was lower during the first 100 days than beyond 100 days after HSCT (37.5% versus 58.8%). Gastrointestinal chronic graft-versus-host disease (GVHD) was identified as the only independent significant factor for the increased incidence of false-positive GM antigenaemia (PPV 0% versus 66.7%, $P = 0.02$).**

***Conclusions*: GM antigen results must be considered cautiously in conjunction with other diagnostic procedures including computed tomography scans, especially during the first 100 days after HSCT and in patients with gastrointestinal chronic GVHD.**

Keywords: fungal infections, invasive aspergillosis, chronic GVHD, gastrointestinal tract, mucosal damage

Introduction

Invasive aspergillosis (IA) remains one of the leading infectious causes of death after allogeneic haematopoietic stem cell transplantation (HSCT), despite new antifungal agents that have become available in recent years.[1] The high mortality rate of IA was mainly attributed to the difficulty of diagnosis at the early stage of the disease, because histopathological examinations require invasive procedures and fungal cultures have low specificity and sensitivity in detecting IA.

Monitoring of the circulating *Aspergillus* galactomannan (GM) antigen by the sandwich enzyme-linked immunosorbent assay (ELISA) is a feasible non-invasive biological method for early diagnosis of IA.[2] The GM ELISA test has sensitivity of 67% to 100% and specificity of 81% to 99% in neutropenic patients and allogeneic transplant recipients,[3–6] and was introduced as microbiological evidence in the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) criteria for opportunistic invasive fungal infection.[7] However, a concern is the false-positive reactions,

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