Table 1 Dose of CsA and pharmacokinetic parameters during the intravenous and oral administration of CsA

Patient no.	*Day −1*			*Day 0*					*Steady state (Days 3–5)*				
	$DOSE_{IV}$ (mg/day)	C_{mean} (ng/ml)	AUC_{IV} (ng/ml × h)	$DOSE_{PO}$ (mg/day)	C_{max} (ng/ml)	T_{max} (h)	C_{min} (ng/ml)	AUC_{IV-PO} (ng/ml × h)	$DOSE_{PO}$ (mg/day)	C_{max} (ng/ml)	T_{max} (h)	C_{min} (ng/ml)	AUC_{PO} (ng/ml × h)
1	96	590	7110	200	1300	2	370	9525	160	1400	3	550	10 625
2	140	643	7680	280	1600	3	480	10860	250	1000	2	320	7080
3	130	553	6630	260	2700	3	360	12555	160	1200	2	290	7790
4	173	663	7950	360	1900	2	340	11785	360	2500	1	420	12 420
5	192	677	7920	400	1500	3	240	8685	400	1500	2	280	8355
6	125	577	6780	260	1200	2	360	8300	260	1200	3	360	8450
7	80	527	6330	160	650	0	390	5725	160	800	2	280	6105
8	192	717	8730	200	930	2	360	8100	200	990	4	300	7225
9	240	477	5820	500	1600	3	280	9035	500	2400	2	290	11 265
10	125	357	4350	260	840	2	210	5285	260	880	2	210	5310
11	58	257	3090	120	720	2	130	3375	120	360	4	110	2860
12	77	303	3690	160	1100	2	190	6025	160	1000	1	260	6590

Abbreviations: AUC_{IV} = area under the concentration–time curve (AUC) during continuous infusion; AUC_{PO} = AUC during oral administration; $DOSE_{IV}$ = dose of CsA during continuous infusion; $DOSE_{PO}$ = dose of CsA during oral administration.

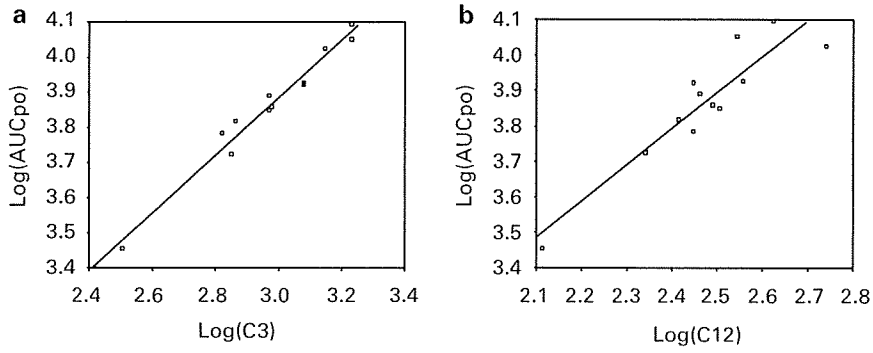

Figure 1 Correlation between the AUC and the CsA peak (**a**: C_3) and trough (**b**: C_{12}) levels.

In three patients (Nos. 1, 2, and 3), the dose of CsA was reduced on day 1 due to the high CsA concentration on day 0 (the day when Neoral was started).

The median AUC value was 6705 ng/ml × h (AUC_{IV}; range, 3090–8730) before the conversion from intravenous to oral administration (day −1), 8493 ng/ml × h (AUC_{IV-PO}; range, 3375–12 555) on day 0, and 7508 ng/ml × h (AUC_{PO}; range, 2860–12420) on days 3–5, respectively. AUC_{PO} was considered to be the AUC of Neoral in the steady state, as AUC_{IV-PO} was affected by the intravenous administration of CsA and at least 3 days are required for the CsA concentration to stabilize after a change in the administration route. As a result, not only AUC_{IV-PO} but also AUC_{PO} was significantly higher than AUC_{IV} ($P = 0.050$), even though the dose of Neoral was reduced in three patients and the conversion ratio was 1:1 in another patient. The median bioavailability of Neoral was 0.685 (range, 0.45–1.04).

Relationship between AUC and the CsA concentration at each measurement point

Although the CsA concentration at each measurement point significantly correlated with AUC_{PO} after logarithmic transformation, the strongest correlation was observed between C_3 and AUC_{PO} (Figure 1a and Table 2, correlation coefficient 0.984, $P < 0.001$). The AUC_{PO} could be predicted from the trough concentration (C_0 or C_{12}), which is widely measured in daily practice, by the following formula based on the linear regression model: $\text{Log}(AUC_{PO}) = 1.020 \times \text{Log}(C_{12}) + 1.344$ (Figure 1b). Accordingly, each trough concentration between 50 and 250 ng/ml corresponds to the CsA concentration during the continuous intravenous infusion of CsA with the same AUC, calculated by dividing the predicted AUC by 12, between 99 and 514 ng/ml (Table 3). Thus, when the continuous intravenous administration of CsA with a target concentration of 500 ng/ml was switched to twice-daily oral administration, the target trough level should be about 250 ng/ml to obtain the same AUC. Also, the target blood concentration of 300 ng/ml during continuous infusion corresponds to the target trough concentration at 150 ng/ml during twice-daily oral administration. This estimation was different from that in kidney transplantation by Nakamura *et al.* (Table 3).[12]

Influence of possible confounding factors on the bioavailability of Neoral

With regard to laboratory data, there were no statistically significant correlations between the bioavailability of Neoral and the serum Cr level, ALT level, and T-bil level

Table 2 Correlation coefficients between the AUC and the cyclosporine concentration at each measurement point

	Correlation coefficient	P-*value*	*Conversion formula*
C0	0.869	<0.001	$Log(AUCPO) = 0.846 \times Log(C0) + 1.747$
C1	0.874	<0.001	$Log(AUCPO) = 0.465 \times Log(C1) + 2.539$
C2	0.953	<0.001	$Log(AUC_{PO}) = 0.718 \times Log(C_2) + 1.693$
C3	0.984	<0.001	$Log(AUC_{PO}) = 0.821 \times Log(C_3) + 1.424$
C4	0.918	<0.001	$Log(AUC_{PO}) = 0.876 \times Log(C_4) + 1.319$
C6	0.961	<0.001	$Log(AUC_{PO}) = 1.314 \times Log(C_6) + 0.258$
C12	0.921	<0.001	$Log(AUC_{PO}) = 1.020 \times Log(C_{12}) + 1.344$

Abbreviation: AUC_{PO} = area under the concentration–time curve during oral administration.

Table 3 Target cyclosporine concentration during continuous infusion to obtain a similar AUC during twice-daily oral administration with each target trough concentration

Trough level of CsA during twice-daily oral administration (ng/ml)	*Corresponding CsA concentration during continuous infusion*	
	Nakamura et al.[12]	*Current study*
50	128	99
100	255	202
150	383	305
200	510	409
250	638	514

Abbreviation: AUC = area under the concentration–time curve.

($P = 0.867$, $P = 0.159$, and $P = 0.770$, respectively). Four patients had developed acute GVHD before the change in the route of CsA administration, but all of them had stage 1 skin GVHD that was successfully controlled by topical steroid. None of the patients had gastrointestinal involvement and thus the influence of gut GVHD on the bioavailability of Neoral could not be evaluated.

With regard to drug interactions, the effects of the following drugs on the bioavailability of Neoral were evaluated; antifungal agents including FLCZ, itraconazole (ITCZ), voriconazole (VRCZ), and MCFG, antibacterial agents including ST, vancomycin, fluoroquinolones (FQ), and cefepime, antiviral agents including ACV and ganciclovir (DHPG), and other drugs including amlodipine, sulpiride, gabapentin, and prednisolone (PSL) (Table 4). FLCZ ($n = 3$), ITCZ ($n = 3$), and VRCZ ($n = 4$) were exclusively administered orally. These agents had been started at least 7 days before the change in the route of CsA administration. By the Mann–Whitney *U*-test, VRCZ, FQ, and ST were shown to have significant effects with at least borderline significance ($P = 0.048$, $P = 0.061$, and $P = 0.100$, respectively). Among these, only VRCZ was identified as an independent significant factor by a multivariate analysis ($P = 0.017$). The median bioavailability of Neoral in patients taking VRCZ was 0.87 (range, 0.76–1.04), whereas it was only 0.54 (range, 0.45–0.94) in those without VRCZ.

Clinical course after the change in the route of CsA administration

One patient (No. 2) developed liver dysfunction with an elevation of ALT from 28 IU/l at baseline to 300 IU/l 2

Table 4 Clinical and laboratory data at the conversion that could influence the cyclosporine pharmacokinetics

Patient no.	AUC_{IV} / $DOSE_{IV}$	AUC_{PO} / $DOSE_{PO}$	*Bioavailability*	*aGVHD*			*Cr (mg per 100 ml)*	*Liver function*		*Concomitant medications*	
				Grade	*Stage*			*ALT (IU/l)*	*T-bil (mg per 100ml)*	*Antifungal agents*	*Others*
					Skin	*Liver*	*Gut*				
1	74	66	0.89	I	(1	0	0)	1.14	40	0.24	VRCZ 400 mg po	VCM, ST, ACV, PPI
2	55	28	0.51	0	(0	0	0)	0.65	28	0.9	ITCZ 200 mg po	ACV, PPI, FQ
3	47	49	1.04	0	(0	0	0)	0.81	182	0.77	VRCZ 400 mg po	ST, ACV, PPI, amlodipine gabapentin
4	46	35	0.76	I	(1	0	0)	0.98	28	1.06	VRCZ 400 mg po	ST, ACV, PPI, PSL
5	41	21	0.51	0	(0	0	0)	0.89	43	0.33	FLCZ 200 mg po	ACV, PPI
6	54	33	0.61	0	(0	0	0)	0.65	92	0.79	ITCZ 200 mg po	DHPG, PPI, amlodipine
7	79	38	0.48	I	(1	0	0)	0.72	85	0.59	ITCZ 200 mg po	DHPG, PPI, amlodipine
8	45	36	0.8	0	(0	0	0)	0.60	78	0.78	FLCZ 200 mg po	ACV, PPI
9	24	23	0.94	0	(0	0	0)	0.66	96	0.65	MCFG 150 mg iv	CFPM, ACV, PPI, amlodipine
10	35	20	0.57	0	(0	0	0)	1.43	46	0.37	FLCZ 200 mg po	CFPM, ACV, PPI
11	53	24	0.45	I	(1	0	0)	0.84	16	0.53	MCFG 150 mg iv	ACV, PPI, FQ, sulpiride
12	48	41	0.85	0	(0	0	0)	1.19	20	0.55	VRCZ 400 mg po	ACV, PPI

Abbreviations: ACV = acyclovir; ALT = alanine aminotransferase; AUC_{IV} = area under the concentration–time curve (AUC) during continuous infusion; AUC_{PO} = AUC during oral administration; CFPM = cefepime; DHPG = ganciclovir; $DOSE_{IV}$ = dose of CsA during continuous infusion; $DOSE_{PO}$ = dose of CsA during oral administration; FLCZ = fluconazole; FQ = fluoroquinolones; ITCZ = itraconazole; MCFG = micafungin; PPI = proton pump inhibitors; PSL = predonisolone; ST = sulphamethoxazole-trimeloprim; VCM = vancomycin; VRCZ = voriconazole.

Table 5 Serial changes in laboratory data and blood pressure after the change in the route of CsA administration

	Mean (minimum–maximum)			
	Serum creatinine (mg per 100 ml)	*ALT (IU/l)*	*Total bilirubin (mg per 100 ml)*	*Blood pressure level (mm Hg)*
Day 0	0.87 (0.60–1.43)	64.4 (16–182)	0.63 (0.24–1.06)	Systolic 130 (114–173) Diastolic 82 (63–103)
Day 3	0.86 (0.32–1.63)	50.1 (10–106)	0.62 (0.27–1.47)	Systolic 124 (109–150) Diastolic 79 (51–103)
Day 7	0.92 (0.69–1.31)	44.6 (10–103)	0.61 (0.30–1.17)	Systolic 122 (109–132) Diastolic 80 (51–103)
Day 14	0.83 (0.67–1.29)	65.8 (10–300)	0.64 (0.27–0.96)	Systolic 121 (113–135) Diastolic 76 (68–89)

Abbreviation: ALT = alanine aminotransferase.

weeks after the conversion. The AUC of CsA was rather lower after conversion, and thus CsA was not considered to be the causative agent of liver dysfunction. Otherwise, no notable changes in laboratory and clinical data were observed (Table 5).

Four patients had developed grade I acute GVHD of the skin before the change in the route of CsA administration. During the 2 weeks after the switch, 3 of the 4 patients had persistent grade I skin GVHD, whereas GVHD was improved in 1 patient. Among the eight patients who did not have acute GVHD at the switch, one patient developed grade I acute GVHD of the skin, which was well controlled by topical steroid, and the other seven patients did not develop acute GVHD during the observation period. No clinically significant changes in vital or biological parameters occurred in the study patients. One patient (No. 9) developed nausea soon after conversion. An excessive increase in the CsA concentration was considered to be the cause of nausea and this symptom was improved after the dose of Neoral was reduced.

Discussion

Neoral is a microemulsion formulation of CsA that has improved bioavailability and reduced variability in pharmacokinetic parameters within and between patients compared with a conventional CsA formulation (Sandimmun).[4] Its bioavailability has been reported to be 0.38 (38%) in healthy volunteers.[13] However, allogeneic HSCT patients have complications that could influence the CsA pharmacokinetics, such as damaged gastrointestinal mucosa and multiple drug interactions. The results of this study showed that the median value of the bioavailability of Neoral was 0.685 (range, 0.45–1.04). Detailed analyses revealed that the oral administration of VRCZ strongly affected the bioavailability of Neoral (0.87 vs 0.54). Therefore, although the switch from intravenous to oral administration of CsA at a ratio of 1:2 seemed to be appropriate in most patients, a lower conversion ratio such as 1:1.1 or 1:1.2 may be better in patients taking oral VRCZ.

The drug interactions between CsA and azole antifungal agents including FLCZ, ITCZ, and VRCZ have been well recognized.[14] Azole antifungal agents are metabolized through the cytochrome P450-3A (CYP3A4) enzyme system, interfere with the metabolism of CsA, and thereby increase the exposure to CsA. Therefore, careful monitoring of the blood CsA concentration is recommended when these agents are added during CsA administration. On the other hand, there are considerable differences among azole antifungals with regard to their ability to inhibit CYP3A4.[14] Interestingly, the concomitant use of oral VRCZ significantly increased the bioavailability of Neoral. We confirmed that VRCZ was started at least 7 days before the switch from intravenous to oral administration of CsA and was continued at the same dose after the switch. Therefore, the drug interaction between CsA and VRCZ seemed to be stronger during oral administration than during the intravenous infusion of CsA. We hypothesized that this stronger interaction can be explained by the presence of the P450 enzyme system in the gastrointestinal mucosa. The CYP3A4 isoenzymes are the most abundant isoforms of CYP and it has been postulated that CsA is also metabolized in the intestine by gut CYP3A4 isoenzymes.[15] The administration of VRCZ might have inhibited the gut metabolism of CsA and increased the bioavailability of CsA. However, a prospective controlled study is required to confirm this hypothesis.

ITCZ, another strong inhibitor of CYP3A4, did not increase the bioavailability of Neoral. As the ratio of $AUC_{IV}/DOSE_{IV}$ was higher not only in patients taking VRCZ but also in patients taking ITCZ compared with other patients (median 47.5, 55, and 41), ITCZ might have inhibited liver CYP3A4 similar to VRCZ, but inhibited gut CYP3A4 less strongly than VRCZ. This might have been affected by the different bioavailable dose of these agents, as the bioavailability of ITCZ is lower than that of VRCZ, in addition to the fact that the dose of ITCZ was lower than that of VRCZ (200 vs 400 mg/day).

With regard to the route of VRCZ, it was exclusively administered orally in this study. Therefore, we could not conclude whether the intravenous administration of VRCZ would similarly affect the bioavailability of CsA. In earlier reports, the extent of drug interaction between CsA and azole antifungals varied according to the route of administration and the dose or kind of antifungal agent. Numerous reports have shown a significant interaction (>84%) between oral FLCZ with a dose of 200 mg/day or greater and oral CsA.[16,17] On the other hand, Osowski *et al.*[18] evaluated the drug interaction between intravenous FLCZ at 400 mg/day and intravenous CsA in HSCT recipients and there was a statistically significant but smaller increase (21%) in the serum CsA concentration.

Mihara *et al.*[19] reported that the mean steady-state whole-blood level of CsA significantly increased after the route of FLCZ administration was switched from intravenous to oral. These data suggest that the drug interaction between CsA and FLCZ was stronger when FLCZ was administered orally. With regard to other azole antifungal agents, not only oral but also intravenous administration of ITCZ significantly affected the blood concentration of CsA.[20–22] Concerning the interaction between VRCZ and CsA, Mori *et al.*[23] reported that the administration of VRCZ to patients receiving CsA resulted in a significant increase in the concentration/dose ratio of CsA, but the route of VRCZ administration did not affect the changes in the concentration/dose ratio. If we consider these findings together, it may be reasonable to suggest that the interaction between azole antifungal agents and CsA is stronger when the antifungals are given orally, but the difference becomes unclear with ITCZ and VRCZ, as the interactions of these agents are stronger than that of FLCZ and can be detected even when they are given intravenously. Therefore, when we interpret pharmacokinetic data of CsA, we must be cautious not only about concomitantly used agents but also the route of administration of both CsA and the other drugs. For example, Parquet *et al.* reported that a ratio of 1:2 in the switch from intravenous to oral administration was appropriate,[5] whereas a 1:1 ratio seemed to be appropriate in the study by McGuire *et al.*[6] In the former study, oral FLCZ was used concomitantly and thus their conclusion was consistent with our data. In the latter study, information on the use of antifungal agents was not described, and thus the data were difficult to interpret.

When we switch the route of CsA administration from continuous infusion to twice-daily oral administration, the target blood concentration should also be changed. Nakamura *et al.*[12] reported that the CsA blood concentration during continuous infusion was estimated to be 2.55 times the trough level during twice-daily oral administration of Neoral to obtain an equal AUC of CsA in kidney transplant patients. In this study, we concluded that the CsA concentration during continuous infusion should be doubled compared with the trough concentration during twice-daily oral administration in allogeneic HSCT recipients. Although the calculation method was different, the conclusion was consistent (mean 2.01) when we applied their methods. Although the reason for the difference between these studies remains unclear, it may have been due to the differences in the use of concomitant drugs or the status of the gastrointestinal tract.

In conclusion, when switching CsA from continuous infusion to oral administration, concomitant medications that could affect the bioavailability of CsA, especially azole antifungal agents, should be taken into account. Although a 1:2 ratio on switching may be appropriate in most patients, a lower conversion ratio is recommended in patients taking oral VRCZ.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan.

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Bone Marrow Transplantation (2009), 1–8

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ORIGINAL ARTICLE

A randomized controlled trial of plasma real-time PCR and antigenemia assay for monitoring CMV infection after unrelated BMT

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Preemptive therapy is the standard strategy for preventing CMV disease after allogeneic hematopoietic SCT. In this study, unrelated BMT recipients were randomly assigned to a plasma real-time PCR group or an antigenemia group to compare the value of these monitoring tools for CMV reactivation. Ganciclovir (GCV) was started at 5 mg/kg/day when PCR reached 300 copies per ml or when antigenemia reached three positive cells per two slides. A total of 88 patients were randomized into the antigenemia group ($n=45$) or the PCR group ($n=43$). A significantly higher number of patients reached the threshold in the antigenemia group than in the PCR group (73.3 vs 44.2%, $P=0.0089$). However, only three patients (one in the antigenemia group and two in the PCR group) developed early CMV disease. These patients exclusively had colitis and were successfully treated with GCV or foscarnet. The median number of antigenemia-positive cells at the start of GCV was 47 in the PCR group. These findings suggest that antigenemia assay with the current cutoff was too sensitive and led to unnecessary use of GCV. However, the appropriateness of the threshold may be different by the methodology used, and therefore, it is difficult to generalize.

Bone Marrow Transplantation advance online publication, 7 December 2009; doi:10.1038/bmt.2009.337

Keywords: CMV; antigenemia; real-time PCR; preemptive therapy

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Received 1 July 2009; revised 1 October 2009; accepted 12 October 2009

Introduction

Cytomegalovirus infection is a frequent complication after allogeneic hematopoietic SCT. Universal prophylaxis with ganciclovir (GCV) did not improve the transplantation outcome because of neutropenia caused by GCV.[1,2] Therefore, the initiation of GCV triggered by the detection of CMV reactivation is currently the standard strategy for preventing CMV disease.[3–5] A CMV antigenemia assay has been widely used to monitor CMV reactivation. However, the details of preemptive therapy still need to be clarified, including the threshold number of antigenemia-positive cells for deciding when to start GCV, the dose and duration of GCV and so on. We previously showed that a risk-adapted preemptive therapy, in which the cutoff number of antigenemia-positive cells for deciding when to start GCV was changed according to the risk for CMV disease, was appropriate in allogeneic SCT recipients, but the incidence of neutropenia was still high.[6] Therefore, in the next study, we evaluated the feasibility of preemptive therapy with low-dose GCV, and the findings showed that the initial dose of GCV could be safely decreased to 5 mg/kg.[7]

The PCR used to detect CMV DNA has also been investigated for its ability to monitor CMV reactivation.[8] PCR using whole blood samples might be too sensitive as a trigger for deciding when to start preemptive therapy compared with an antigenemia assay or PCR using plasma samples.[9,10] However, the recent development of real-time PCR has enabled the quantification of CMV DNA. Several studies have shown the feasibility of preemptive therapy guided by real-time PCR monitoring using either whole blood or plasma samples.[11–14] As for whole blood real-time PCR, Gerna *et al.* performed two randomized controlled trials of PCR and antigenemia, one in young patients (0–25 years old) and the other in older patients (20–67 years old).[12,13] They showed that a threshold value of 10 000

copies per ml for determining when to start GCV by whole blood PCR significantly reduced the use of GCV compared with a threshold in which GCV is started at any level of positive antigenemia. However, the study included heterogeneous patients in terms of donor type, stem cell source and GVHD prophylaxis. In particular, antithymocyte globulin was used in approximately half of the patients, and this may have strongly affected the incidence of CMV reactivation and disease.[15,16] In addition, preemptive therapy guided by antigenemia assay could be more appropriately performed by using a cutoff based on the number of positive cells.

Therefore, we performed a randomized controlled trial of plasma real-time PCR with a cutoff of 300 copies per ml and an antigenemia assay with a cutoff of three positive cells per two slides in a homogenous population of unrelated BMT recipients who received GVHD prophylaxis with a calcineurin inhibitor and MTX.

Patients and methods

Patients

Patients were eligible for the study if they were between 20 and 55 years old, would undergo BMT without *in vivo* or *ex vivo* T-cell depletion from an HLA-matched unrelated donor using a myeloablative conditioning regimen and had a good performance status without significant organ dysfunction, as defined in the protocol. Either the donor, the recipient or both must have been seropositive for CMV. Prophylaxis against GVHD was limited to a combination of CYA and MTX, but a combination of tacrolimus and MTX was allowed after June 2002. Patients were enrolled before starting a conditioning regimen, but randomization was performed between day 10 and day 12 after transplantation to exclude patients who developed significant organ dysfunction early after transplantation. This study was approved by the institutional review board of each participating center and a written informed consent was obtained from each patient (UMIN-CTR C000000347).

CMV monitoring methods

Cytomegalovirus antigenemia assay was performed as described previously.[17] In brief, 1.5×10^5 peripheral blood leukocytes were attached to a slide using a cytocentrifuge and fixed with formaldehyde. The cells were sequentially immunostained with MoAb C10/11 (Clonab CMV; Biotest, Dreieich, Germany) and reacted with goat alkaline phosphatase-labeled anti-mouse Ig (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan). Under a light microscopy, CMV-positive cells were counted and the results are presented as the sum of the number of positive cells per two slides.

Real-time PCR was performed using primers and a TaqMan probe for immediate early genes using serum samples.[18] Briefly, DNA extracted from 100 μl of plasma was subjected to PCR using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA) and the PCR product was detected as an increase in the fluorescent intensity using ABI Prism 7700 (PE Biosystems). Real-time fluorescent measurements were taken and a threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded 10 times the baseline fluorescence. A standard curve was constructed using the CT values obtained from serially diluted DNA extracted from a plasmid that contains the respective region of CMV. The CT values from the clinical samples were plotted on the standard curve and the copy number was calculated automatically using Sequence Detection System version 1.6 (PE Biosystems).

Preemptive therapy against CMV disease

Patients were randomly assigned to the antigenemia group or the PCR group using a random block design. Assignment was stratified by the institute, age and the presence or absence of GVHD at the time of randomization. CMV reactivation was monitored weekly by both the antigenemia assay and PCR in all patients, but only the results of the assigned monitoring method were returned to the physicians. Preemptive therapy with GCV was started at an induction dose of 5 mg/kg/day when three or more CMV-positive cells per two slides were detected in the antigenemia group and 300 or more CMV DNA copies per ml were detected in the PCR group. The dose of GCV was increased to 10 mg/kg/day when a rising CMV load was observed. The dose of GCV was decreased to 5 mg/kg/day when a declining CMV load was observed in patients who were receiving GCV at 10 mg/kg/day. A rising and declining CMV load was defined as an increase and decrease in the CMV load by 50% or more of the previous value, respectively. However, changes in antigenemia-positive cells by less than five cells per two slides and changes in the DNA copy number by less than 500 copies per ml were regarded as a stable CMV load. When the CMV load fell below the threshold to start GCV, the dose of GCV was decreased to 5 mg/kg/day, if the patient was receiving GCV at 10 mg/kg/day, and GCV was discontinued if the patient was receiving GCV at 5 mg/kg/day. The dose of GCV was adjusted according to the renal function.[19] CMV monitoring was continued until all of the following three requirements were fulfilled: (i) More than 100 days had passed after transplantation; (ii) More than 2 weeks had passed after the last administration of GCV; and (iii) Absence of the use of (methyl-)prednisolone at 0.5 mg/kg/day or more.[20]

Definition of CMV disease

All patients with symptoms compatible with CMV disease such as interstitial pneumonia, colitis and gastritis underwent extensive pathological and microbiological examination of biopsy specimens. The diagnosis of CMV disease was made by histopathological examination and immunochemical staining of biopsy specimens. However, CMV retinitis was diagnosed when CMV DNA was detected by PCR using aqueous humor samples associated with characteristic retinal changes by ophthalmoscopy. Early and late CMV diseases were defined as those occurring before and after day 100, respectively.

Statistical considerations

The primary end point of the study was the incidence of early CMV disease. We defined success as the absence of CMV disease before day 100. Noninferiority was predefined as a difference in the success rates between the antigenemia group and the PCR group of no more than 10 percentage points. On the basis of the assumption of a success rate of 95% in the PCR group and 90% in the antigenemia group, 39 patients in each treatment group were required to show noninferiority with an alpha error of 5% and a power of 80%, which permitted a 10% difference in the success rate. On the basis of the assumption of a 20% loss of patients between the enrollment and randomization, a total of 96 patients needed to be enrolled in this study. Comparisons for dichotomous and continuous variables between groups were performed with Fisher's exact test and *t*-test, respectively. Pearson's correlation coefficient was calculated to compare the results of the two monitoring methods after logarithmic transformation.

Results

Incidence of CMV reactivation and the use of GCV

A total of 96 patients were enrolled in the study between January 2002 and March 2007. Among these patients, eight patients were excluded because of the use of tacrolimus as GVHD prophylaxis in one, negative CMV Ab in both the donor and recipient in one and organ dysfunction after the conditioning regimen in six. Therefore, a total of 88 patients were randomized into the antigenemia group ($n=45$) or the PCR group ($n=43$) (Figure 1). There were no differences in age, sex, background disease, CMV serostatus, conditioning regimen or GVHD prophylaxis between the two groups (Table 1). In addition, the incidence of grade II–IV acute GVHD was similar (42 vs 47%, $P=0.67$).

Cytomegalovirus reactivation, defined as a detection of CMV at any level, was more frequently observed in the antigenemia group (40 of 45 patients, 88.9%) than in the

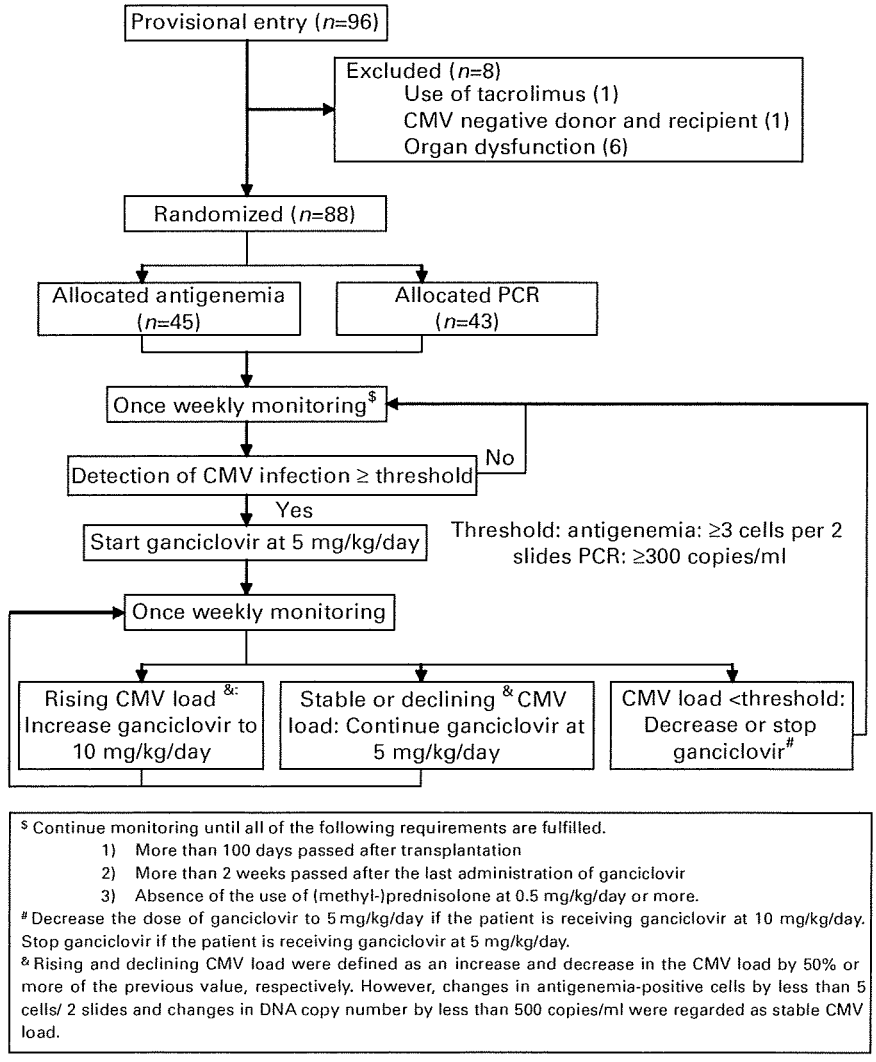

Figure 1 Design of the study.

PCR group (27 of 43 patients, 62.8%) ($P=0.0050$, Table 2). The probability of starting GCV was significantly higher in the antigenemia group than in the PCR group (73.3 vs 44.2%, $P=0.0089$, Figure 2). The results of PCR in the antigenemia group and those of the antigenemia assay in the PCR group were disclosed after the completion of the study. A good correlation was seen between the results of PCR and the antigenemia assay ($P<0.0001$, $r^2=0.38$, Figure 3). Of the 33 patients who received GCV in the antigenemia group, PCR and the antigenemia assay reached the threshold simultaneously in five patients and PCR reached the threshold before starting GCV in only four patients (Figures 4a and 5a). In the other 24 patients, the CMV DNA copy number was persistently below the threshold until GCV was started. On the other hand, in 11 of 19 patients who received GCV in the PCR group, the results of the antigenemia assay reached the threshold earlier in 11 patients and simultaneously in 7 patients (Figures 4b and 5b). The results of the antigenemia assay were persistently below the threshold until GCV was started in only one patient. The median number of antigenemia-positive cells at the start of GCV was 5 (range: 3–102) and 47 (range: 0–2921) in the antigenemia and PCR groups, respectively (Figure 6a, $P=0.0051$). The median CMV DNA copy number was negative (range: 0–4400) and 750 (range: 310–13000) in the antigenemia and PCR groups, respectively (Figure 6b, $P<0.0001$).

Among the 52 patients who received preemptive therapy with GCV at 5 mg/kg/day, only 13 and 7 patients in the antigenemia and PCR groups, respectively, experienced a rising CMV load and required dose-escalation to 10 mg/kg/day, suggesting that the initiation of GCV at 5 mg/kg was appropriate.

Table 1 Patient characteristics

	Antigenemia (n=45)	*PCR (n=43)*	P-*value*
Pre-transplantation factors			
Median age (range)	41 (20–55)	40 (20–53)	0.82
Sex (male/female)	25/20	24/19	>0.99
HLA mismatch	7 (16%)	9 (21%)	0.59
Background disease			
AML	17	18	
ALL	12	12	
CML	6	3	
MDS	5	7	
Others	5	3	0.57
Donor/recipient CMV status			
Pos./Pos.	28	26	
Pos./Neg.	5	4	
Neg./Pos.	8	6	0.74
Conditioning regimen			
TBI	39	36	
Non-TBI	6	7	0.77
GVHD prophylaxis			
CYA–MTX	25	25	
TAC–MTX	16	16	0.59

Abbreviations: MDS = myelodysplastic syndrome; Neg. = negative; Pos. = positive; TAC = tacrolimus.

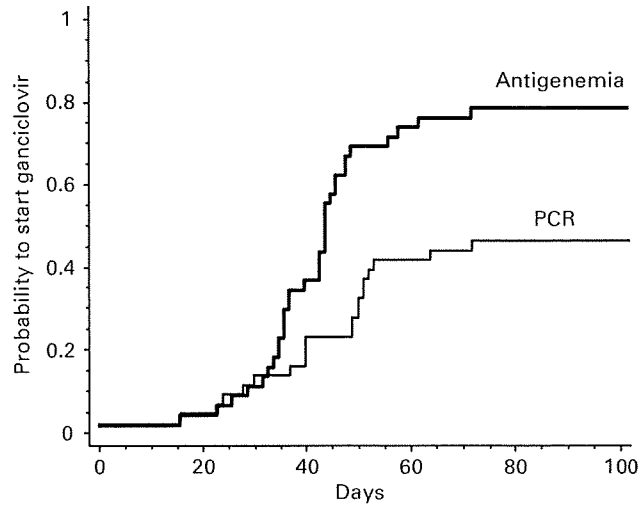

Figure 2 Days to start ganciclovir after transplantation.

Table 2 CMV-related events after engraftment

	Antigenemia (n=45)	*PCR (n=43)*	P-*value*
CMV reactivation[a]	40	27	0.0050
Start ganciclovir	33	19	0.0089
Duration of ganciclovir (days)	23.2 ± 19.4	20.8 ± 14.2	0.64
Total dose of ganciclovir (mg/kg)	140.8 ± 129.7	118.4 ± 91.2	0.51
Dose escalation to level II	13	7	>0.99
Neutropenia <500 per μl	5	3	>0.99
Stop ganciclovir because of neutropenia	1	0	>0.99
Increase in serum creatinine[b]	8	0	0.039
CMV disease			
Early (before day 100)	1	2	0.61
Late (after day 100)	0	1[c]	0.48

[a]Detection of antigenemia or DNA at any level.
[b]Increase in serum creatinine level by 0.5 mg per 100 ml or more from the baseline level.
[c]The patient developed early CMV disease, which was improved by ganciclovir. However, intestinal symptoms recurred after day 100 and CMV colitis was suspected because of positive antigenemia, although it was not confirmed by biopsy.

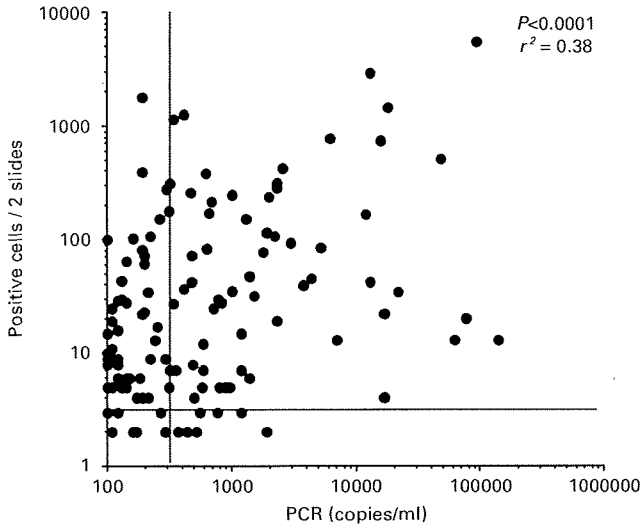

Figure 3 Correlation between the number of positive cells in the antigenemia assay and copy number by PCR.

CMV diseases

Early CMV disease was diagnosed in 1 of the 45 patients (2.2%) in the antigenemia group and 2 of the 43 patients (4.7%) in the PCR group ($P=0.61$). These patients exclusively developed CMV colitis. Another patient in the PCR group showed characteristic retinal changes and was presumptively treated with GCV, although CMV infection was not detected in either the aqueous humor or the peripheral blood. The 95% confidence interval for the difference in the success rate was −10.1 to 5.2%, and thus was just outside the predefined lower limit of −10%. However, as shown in Table 3, the development of CMV disease in the PCR group could not be avoided even if these patients were assigned to the antigenemia group, as either the antigenemia assay and PCR reached the threshold simultaneously (UPN32) or the antigenemia assay did not reach the threshold before the diagnosis of CMV disease (UPN35). All of these patients were successfully treated with GCV or foscarnet, although one patient (UPN35) showed the recurrence of colitis after day 100. None of the other patients developed late CMV disease.

Adverse events during preemptive therapy

The mean duration of preemptive therapy with GCV and the mean total dose of GCV was 23.2 ± 19.4 days and 140.8 ± 129.7 mg/kg in the antigenemia group and 20.8 ± 14.2 days and 118.4 ± 91.2 mg/kg in the PCR group ($P=0.64$ and $P=0.51$), respectively. Neutropenia with a neutrophil count of <500 per μl was observed in 5 of the 33 patients in the antigenemia group and 3 of the 19 patients in the PCR group ($P>0.99$). Only one patient in the antigenemia group required a discontinuation of GCV because of neutropenia. The total dose of GCV was higher in patients who developed neutropenia, but this difference was not statistically significant (163.8 ± 82.5 vs 126.9 ± 121.4, $P=0.42$).

An increase in the serum creatinine level by at least 0.5 mg per 100 ml was observed in 8 of the 33 patients in the antigenemia group and in none of the 19 patients in the PCR group ($P=0.039$). The total dose of GCV was significantly higher in patients who developed renal impairment (255.0 ± 198.0 vs 106.0 ± 45.5, $P=0.0004$).

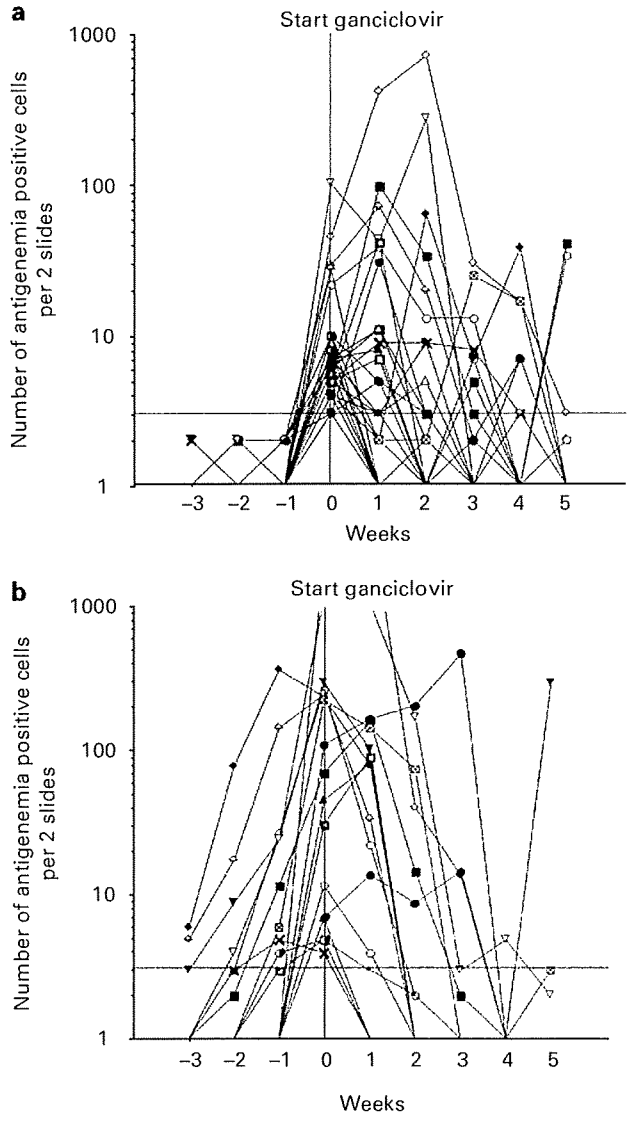

Figure 4 Serial changes in the number of antigenemia-positive cells in patients who received preemptive therapy in the antigenemia group (a) and in the PCR group (b). Week 0 represents the day ganciclovir was started.

Discussion

In this randomized controlled trial, we compared plasma real-time PCR with a cutoff at 300 copies per ml and an antigenemia assay with a cutoff at three positive cells per two slides as a trigger for deciding when to start preemptive therapy with GCV after unrelated BMT. GCV was used significantly less frequently in the PCR group. A comparison of the number of antigenemia-positive cells and the CMV DNA copy number at the start of GCV treatment clearly revealed that plasma PCR was significantly less sensitive than the antigenemia assay, at least with the current cutoff values. Although the 95% confidence

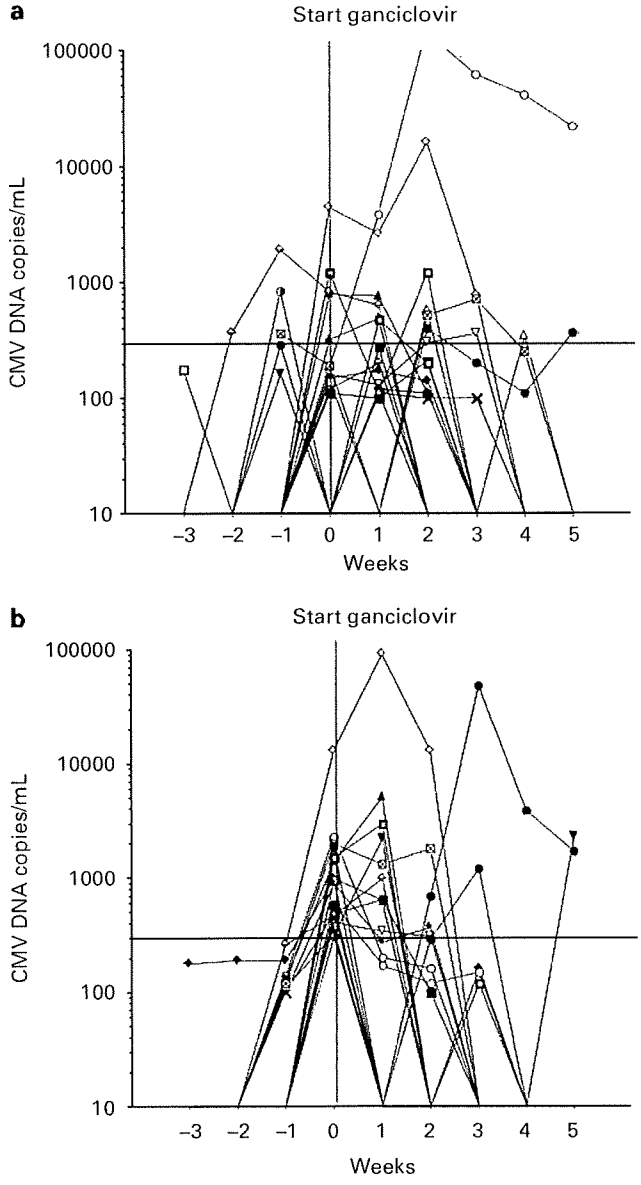

Figure 5 Serial changes in CMV DNA copy number in patients who received preemptive therapy in the antigenemia group (a) and in the PCR group (b). Week 0 represents the day ganciclovir was started.

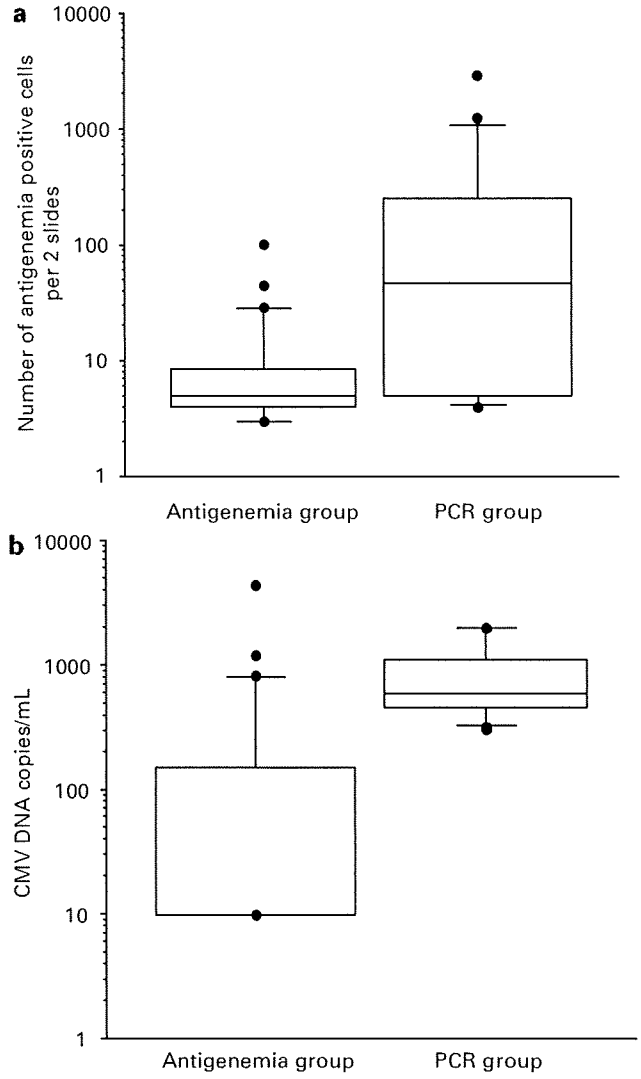

Figure 6 The number of antigenemia-positive cells (a) and the CMV DNA copy number at the start of preemptive therapy (b), grouped according to the randomization arm. The box-and-whisker plot shows 10, 25, 50, 75 and 90 percentile values. Outliers are indicated by dots.

interval for the difference in the successful prevention rate was just outside the predefined lower limit of −10%, and therefore, we could not show the noninferiority of the PCR group, the incidence of CMV disease was limited to two patients even in the PCR group. In addition, prevention of CMV pneumonia, the main aim of preemptive therapy, was completely achieved in both groups. These findings suggest that an antigenemia assay with a cutoff of three positive cells per two slides was too sensitive and resulted in the unnecessary use of GCV.

The unnecessary use of GCV may be reduced if the cutoff value for the antigenemia assay is increased. The antigenemia assay has already been shown to be not sensitive enough for detecting gastrointestinal involvement by CMV even with a low threshold.[21] In this study, the median number of antigenemia-positive cells at the start of GCV treatment was 47 in the 19 patients who received preemptive therapy in the PCR group. Figure 7 shows the serial changes in the number of antigenemia-positive cells in the patients of the PCR group who developed positive antigenemia that reached the threshold, but who did not receive GCV at that time. In about half of the patients, antigenemia spontaneously became negative without GCV treatment. On the other hand, seven patients developed high-grade antigenemia of over 100 positive cells per two slides. However, GCV was started when the number of positive cells was 260 (median, range: 73–1262 cells) and none of these patients developed CMV disease. Although patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or higher experienced high-grade antigenemia more frequently than those who did not

develop grade II–IV acute GVHD and did not receive steroid (Figures 7a and b), the use of GCV was comparable (54.5 vs 40%, $P=0.67$). Thus, although it is difficult to determine the appropriate cutoff value for the antigenemia assay, we thought that it may be worth trying to apply a cutoff value of 20 positive cells per two slides, which we are already safely using in allogeneic hematopoietic SCT from an HLA-matched sibling donor,[20] to transplantation from an unrelated donor.

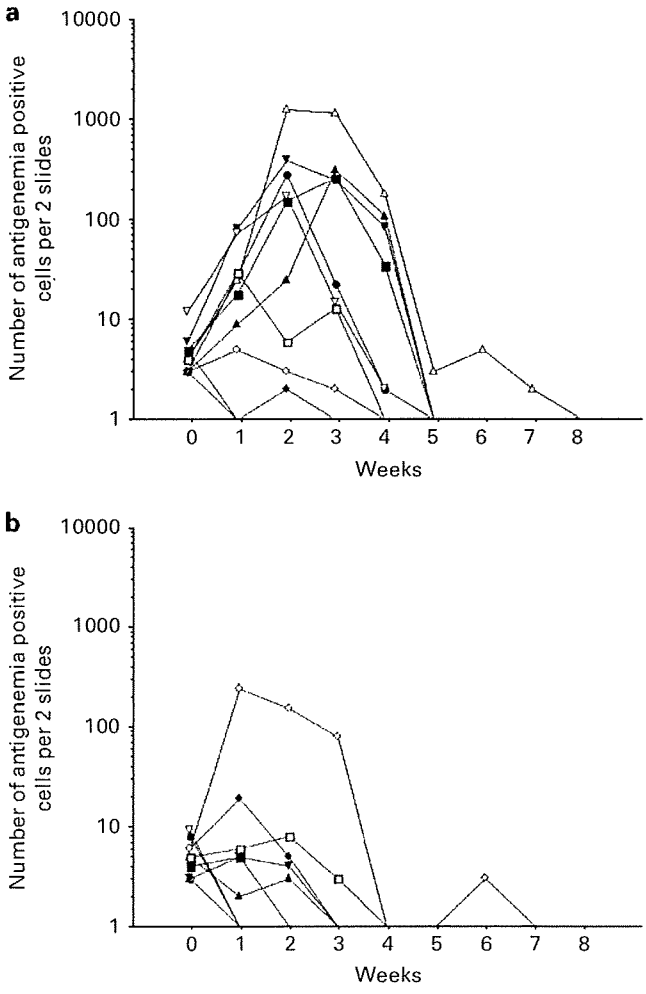

Figure 7 Serial changes in the number of antigenemia-positive cells in the PCR group patients who developed positive antigenemia that reached the threshold, but who did not receive ganciclovir. (a) Patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or more. (b) Patients who did not develop grade II–IV acute GVHD and did not receive steroid.

Although Boeckh *et al.*[3] reported a 14% incidence of early CMV disease using the same cutoff as in the current study, the incidences of positive antigenemia at any level and three or more positive cells per two slides were similar to those in this study (79 and 70% in Boeckh's study and 89 and 73% in the current study). Therefore, the higher incidence of early CMV disease probably resulted from the high incidence (35%) of grade III–IV acute GVHD in their study rather than from the difference in the method used for the antigenemia assay, as acute GVHD is one of the strongest risk factors for CMV disease.

Nevertheless, it is important to note that the sensitivity and specificity of these assays vary depending on the methodology used.[9,22–24] In fact, the unexpected differences in the sensitivities of the two assays in this study could be explained by the difference in the methodology used in the antigenemia assay. The cutoffs used for the antigenemia assay and real-time PCR were determined based on our previous study in which HRP-C7 Ab was used in the antigenemia assay.[18] In this study, however, we used C10/C11 Ab in the antigenemia assay, as this Ab has been used worldwide. Although we did not believe that there are clinical differences between these two antigenemia assays,[6,7,20] we should have tested the correlation between the results of plasma PCR and the antigenemia assay using C10/C11 Ab. Fortunately, the unexpected difference in the sensitivity in these assays contributed to the finding that the antigenemia assay with the current cutoff was too sensitive as a trigger for deciding when to start preemptive therapy. These data are valid only when the same methodology is used, and standardization of the methods is warranted.[25,26]

In conclusion, CMV colitis could not be completely prevented by the current preemptive strategy using the peripheral blood samples, but CMV pneumonia was completely prevented in both groups. The initiation of GCV at 5 mg/kg/day was confirmed to be safe, provided the CMV load continues to be monitored. Plasma PCR with a cutoff at 300 copies per ml seemed to be appropriate for monitoring CMV reactivation after transplantation. The cutoff number of positive cells should be raised above that used here when using an antigenemia assay. However, the appropriateness of the threshold of these assays may be different on the basis of the methodology and patient background, such as the risk of GVHD, and therefore, it is difficult to generalize.

Table 3 CMV load in patients who developed CMV disease

Age/sex	*Acute GVHD*	*Onset/affected organ of CMV disease*		*−3 weeks*	*−2 weeks*	*−1 week*	*Onset*
UPN32 38/M (PCR group)	Grade II	Day 56/colitis	PCR	(−)	260	13 000[a]	93 000
			Ag	(−)	(−)	2921	5467
UPN35 36/M (PCR group)	Grade II	Day 46/colitis	PCR	(−)	(−)	(−)	(−)
			Ag	0	0	2	12
UPN70 38/M (Antigenemia group)	Grade II	Day 50/colitis	PCR	(−)	(−)	110	100
			Ag	2	(−)	5[a]	99

[a]Preemptive therapy was started.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Mrs Aki Tanihara, who was involved in the data management. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

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Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens

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Exposure of offspring to noninherited maternal antigens (NIMAs) during pregnancy may have an impact on transplantations performed later in life. Using a mouse model, we recently showed that bone marrow transplantation (BMT) from NIMA-exposed offspring to the mother led to a reduction of graft-versus-host disease (GVHD). Since offspring can also be exposed to NIMAs by breastfeeding after birth, we tested whether breast milk could mediate the tolerogenic NIMA effect. We found that oral exposure to NIMAs by breastfeeding alone was sufficient to reduce GVHD, and that in utero exposure to NIMAs is required for maximum reduction of GVHD. The tolerogenic milk effects disappeared when donor mice were injected with CD25 monoclonal antibodies during the lactation period, suggesting a $CD4^+CD25^+$ regulatory T cell–dependent mechanism. Our results suggest a previously unknown impact of breastfeeding on the outcome of transplantation. (Blood. 2009;113:1829-1833)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potential curative treatment for malignant hematologic diseases; however, HSCT from haploidentical-related donors is complicated by a high incidence of severe graft-versus-host disease (GVHD).

The fetus and mother must tolerate each other's alloantigens during pregnancy. Fetal and maternal antigens transmitted through the bidirectional transplacental passage during pregnancy might induce tolerance to noninherited maternal antigens (NIMAs) in the offspring and inherited paternal antigens (IPAs) in the mother.[1-4] Exposure of the fetus to allogeneic cells may induce a long-lasting tolerance specific to the alloantigens of the donor cells.[5,6] We recently demonstrated using a mouse model that a "child-to-mother" bone marrow transplantation (BMT) from a NIMA-exposed donor reduces the mortality and morbidity of GVHD, but a "mother-to-child" BMT from a mother donor exposed to IPAs from the fetus does not.[7] We therefore tested the hypothesis that breastfeeding plays an important role in the buildup of the tolerogenic NIMA effect in a mouse model of a "child-to-mother" BMT because breast milk is rich in maternal major histocompatibility complex (MHC) antigens in both soluble and cellular forms.[8-11]

Methods

Mice

Female C57BL/6 (B6, H-$2^{b/b}$) and B6D2F1 (H-$2^{b/d}$) mice were purchased from Charles River Japan (Yokohama, Japan). B6D1F1 (H-$2^{b/q}$) mice were produced by mating a DBA/1 (H-2^q) female (Japan SLC, Shizuoka, Japan) and a B6 male. NIMA-exposed H-2^b mice were produced by mating a B6 (H-2^b) male and a B6D2F1 (H-$2^{b/d}$) female as previously described.[7,9,12] Offspring were typed for the H-2 locus by flow cytometry using monoclonal antibodies (mAbs) specific for H-$2K^b$ and H-$2K^d$ (BD Pharmingen, San Diego, CA) as previously described.[7] The resultant H-$2^{b/h}$ offspring were nursed by either a B6D2F1 mother (NIMA [in utero + oral]) or a B6 foster mother (NIMA [in utero]). Controls were H-2^b mice not exposed to H-2^d. To produce NIMA-exposed mice via breastfeeding, B6 neonates were nursed by a B6D2F1 foster mother (NIMA [oral]). Mice were weaned at 3 weeks after birth. These mice were used as BMT donors at 6 to 10 weeks of age. BMT was performed as previously described.[7] All animal experiments were performed under the auspices of the Institutional Animal Research Advisory Committee at Okayama University.

BMT

Mice underwent transplantation as previously described.[13] In brief, after lethal total body irradiation (TBI; x-ray) was delivered in 2 doses at 3-hour intervals to minimize gastrointestinal toxicity, mice were intravenously injected with 5×10^6 T cell–depleted bone marrow (TCD-BM) cells plus 0.5 to 2×10^6 T cells, $CD4^+$ T cells, or CD25-depleted $CD4^+$ T cells from the donors on day 0. T-cell depletion, CD25 depletion, and splenic $CD4^+$ T-cell isolation were performed using Auto-MACS (Miltenyi Biotec, Tokyo, Japan) as previously described.[7,14] Hybridomas secreting anti-CD25 mAbs (clone PC61) were obtained from the ATCC (Manassas, VA). For in vivo depletion of $CD25^+$ cells,[14,15] mice were injected subcutaneously with 75 μg/g body weight of anti-CD25 or irrelevant mAbs on days 1 and 8 after birth. They were then housed in sterilized microisolator cages, and given autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT and filtered water thereafter. Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system that evaluated changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as previously described.[16]

Submitted May 5, 2008; accepted December 13, 2008. Prepublished online as *Blood* First Edition paper, January 5, 2009; DOI 10.1182/blood-2008-05-155283.

Presented as an abstract at the 49th Annual Meeting of the American Society of Hematology, Atlanta, GA, December 10, 2007.[36]

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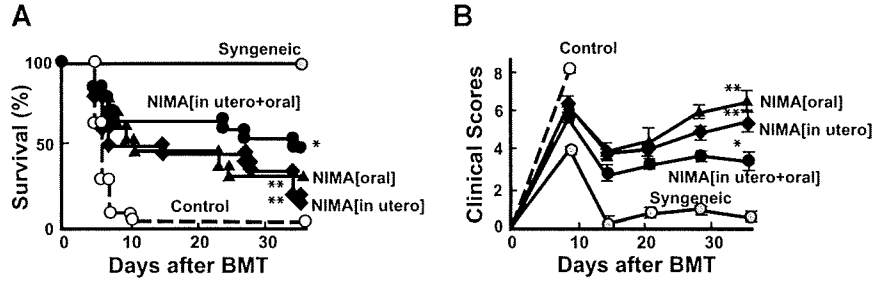

Figure 1. Breastfeeding is required for inducing maximum NIMA effect to reduce GVHD. NIMA-exposed H-2^b offspring were produced by mating a B6 male and a B6D2F1 female, and were fed by either a B6D2F1 mother (NIMA [in utero + oral]) or a B6 foster mother (NIMA [in utero]). NIMA-exposed H-2^b offspring were produced using a B6D2F1 foster mother to nurse newborn B6 mice (NIMA [oral]). Lethally irradiated B6D2F1 mice received a transplant of 5 × 10^6 TCD-BM from controls together with 2 × 10^6 T cells from allogeneic or syngeneic donors. Survival (A) and clinical GVHD scores expressed as mean plus or minus SE (B) after BMT are shown. (A) The results are representative of 3 replicate experiments (n = 17-20/group). (B) Representative data from 1 of the experiments are shown (n = 6/group). *$P < .05$ compared with controls; **$P < .05$ compared with NIMA (in utero + oral).

Flow cytometry

Flow cytometric analysis was performed as previously described.[13] The mAbs used were biotinylated anti–mouse CD25 (clone 7D4); FITC- or PE-conjugated anti–mouse CD4, CD8, H-2K^b, H-2K^d (BD Pharmingen); and Foxp3 (eBioscience, San Diego, CA). For intracellular interferon γ (IFN-γ) staining, splenocytes were incubated for 4 hours with Leukocyte Activation Cocktail and BD GolgiPlug (BD Pharmingen) at 37°C. Then the cells were permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen) and stained with FITC-conjugated anti–IFN-γ mAbs. Dead cells were identified as 7-amino-actinomycin D (BD Pharmingen)–positive cells. At least 5000 live samples were acquired for analysis.

Cell culture

Cell culture was performed as previously described.[17] Splenic CD4$^+$ T cells were cultured at a concentration of 10^5 cells/well with 4 × 10^5 irradiated (30 Gy) splenocytes. After culturing for 72 hours, cells were pulsed with ^{3}H-thymidine (1 μCi [0.037 MBq] per well) for further 18 hours. Proliferation was determined using Topcount NXT (Packard Instruments, Meriden, CT).

Statistical analysis

Mann-Whitney *U* tests were used to analyze cell counts and clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability and the log-rank test was applied to compare the survival curves. We defined a *P* value less than .05 as statistically significant.

Results

Breastfeeding is required for the induction of maximum NIMA effects to reduce GVHD

Lethally irradiated B6D2F1 mice received a transplant of 5 × 10^6 TCD BM from the controls together with 2 × 10^6 T cells from either NIMA (in utero + oral), NIMA (in utero), NIMA (oral), or control mice. NIMA (in utero + oral) T cells produced less severe GVHD than control T cells, as previously reported[7] (Figure 1A). Interestingly, in recipients of NIMA (in utero) and NIMA (oral) T cells, the tolerogenic NIMA effect was partial when considering the mortality and morbidity of GVHD (Figure 1A,B). These results demonstrate that in utero exposure to NIMAs alone could mediate the tolerogenic NIMA effect and that further oral exposure to NIMAs is required for induction of the maximum NIMA effect.

Breastfeeding alone is sufficient to mediate the tolerogenic NIMA effect

We then examined whether NIMA exposure by breastfeeding could induce T-cell hyporesponsiveness to NIMAs. Compared with control T cells, CD4$^+$ T cells isolated from NIMA (oral) mice showed less expansion and IFN-γ production after transfer into irradiated B6D2F1 mice (Figure 2A). Consistent with the reduced donor T-cell responses to NIMAs, NIMA (oral) CD4$^+$ T cells (10^6) mediated the attenuation of GVHD after BMT, compared with control T cells, as assessed by mortality (Figure 2B) and clinical GVHD scores (data not shown).[16] We also observed the tolerogenic NIMA effect in another set of experiments with 2 different T-cell doses (0.25 × 10^6 and 10^6; Table 1). The thymus, which is one of the most sensitive organs to GVHD, shows severe atrophy primarily due to a loss of double-positive thymocytes.[18] Numbers of double-positive thymocytes markedly decreased in allogeneic controls compared with syngeneic controls (Figure 2C). This thymic atrophy was significantly improved in NIMA (oral) T-cell recipients. We then sought to determine whether GVHD could be further reduced by exposing TCD-BM donors to NIMAs in addition to NIMA exposure of T-cell donors. Lethally irradiated B6D2F1 mice received transplants of 2 × 10^6 T cells from NIMA (oral) mice and 5 × 10^6 TCD-BM from NIMA (oral) mice or controls. However, survival (75% vs 70%) and clinical scores (4.0 ± 0.2 vs 3.8 ± 0.4) were not significantly different between the 2 groups. Analysis of donor cell engraftment on day +40 in spleens showed that the recipients of grafts from NIMA-exposed donors had more than 98% donor-derived cells, thus ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

We then evaluated the antigen specificity of milk-mediated tolerogenic NIMA effects. B6D2F1 mice received transplants with T cells from B6 mice nursed by either a B6D2F1 or B6D1F1 (H-$2^{b/q}$) foster mother. GVHD was less severe in recipients of grafts from H-$2^{b/d}$–exposed donors than in those who received grafts from H-$2^{b/q}$–exposed donors, suggesting an antigen specificity of the NIMA effect (Figure 2D).

Breastfeeding-mediated tolerogenic NIMA effect is dependent on CD4$^+$CD25$^+$ cells

We recently demonstrated that CD4$^+$CD25$^+$ regulatory T cells (T_R) play an important role in the tolerogenic NIMA effect.[7] We therefore considered whether these T_R play a role in the induction of tolerogenic milk effects. When B6D2F1 mice received a transplant of CD25-depleted CD4$^+$ T cells from NIMA (oral) mice, the tolerogenic NIMA effect disappeared (Figure 2E), suggesting that T_R in the donor inoculum were involved in the tolerogenic milk effect.

We hypothesized that NIMA-reactive T_R are produced during the lactation period. Newborn B6 mice fed by a B6D2F1 foster mother were

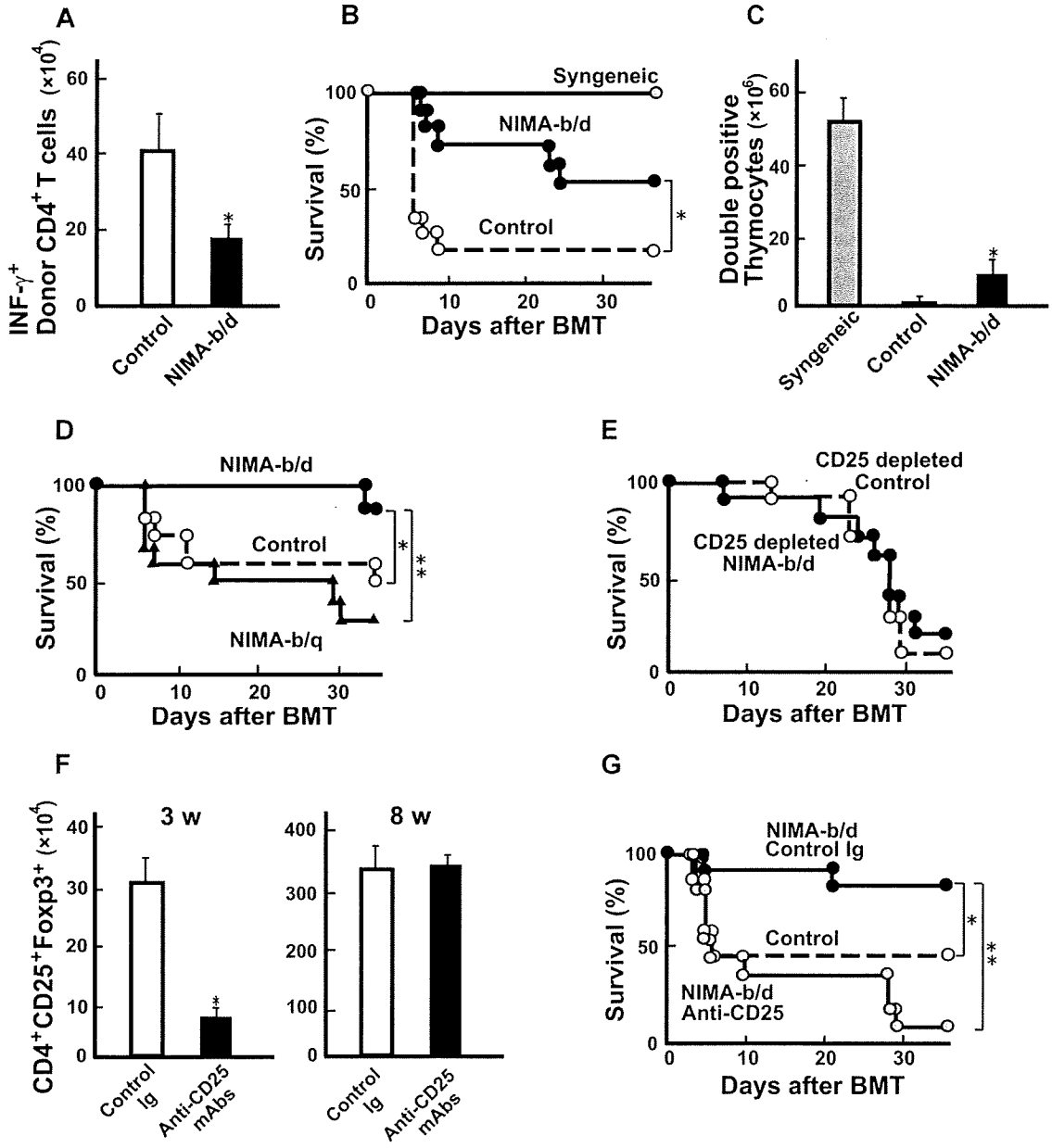

Figure 2. Breastfeeding-mediated tolerogenic NIMA effect is dependent on CD4+CD25+ cells. A newborn B6 mouse was fed by either a B6 mother (control) or a B6D2F1 foster mother (NIMA-b/d). (A) $CD4^+$ T cells (10^6) isolated from spleens of NIMA-b/d mice or controls were adoptively transferred into irradiated B6D2F1 mice. Numbers of IFN-γ^+ donor $CD4^+$ T cells in spleens 5 days after the transfer are shown as mean plus or minus SD (n = 5/group). (B,C) Lethally irradiated B6D2F1 mice received a transplant of 5 × 10^6 TCD-BM from control B6 mice together with 2 × 10^6 T cells from NIMA-b/d, controls, or syngeneic B6D2F1 donors. Survival after BMT (B; n = 11/group) and numbers of double-positive thymocytes at 40 days after BMT (C) are shown. (D) B6D2F1 mice received a transplant as described for panels B and C with T cells from a B6 mouse fed by either a B6D2F1 (NIMA-b/d) or B6D1F1 (NIMA-b/q) foster mother. Survival after BMT is shown (n = 14 /group). (E) BMT was performed as described for panels B and C with 0.5 × 10^6 CD25-depleted $CD4^+$ T cells from NIMA-b/d or control donors. Survival after BMT is shown (n = 10 /group). (F,G) Newborn B6 mice were nursed by a B6D2F1 mother and subcutaneously injected with 75 μg/g body weight of anti-CD25 or irrelevant mAbs 1 and 8 days after birth. The numbers of $Foxp3^+CD4^+CD25^+$ cells in spleens at 3 and 8 weeks after birth (n = 3/group, mean ± SD; F) and survival of B6D2F1 mice that received a transplant of $CD4^+$ T cells from anti-CD25–treated or control-treated NIMA-b/d donors or B6 donors (G) are shown (n = 11/group). (A,F) The results are representative of 3 replicate experiments. (B-E,G) Data from 2 similar experiments are combined. *$P < .05$; **$P < .01$ versus controls.

injected subcutaneously with 75 μg/g body weight of anti-CD25 mAbs on days 1 and 8 after birth to deplete $CD25^+$ T_R in vivo.[15] Injection of anti-CD25 mAbs significantly reduced the numbers of $Foxp3^+$ T_R at 3 weeks of age but this cell population had recovered to normal levels by 8 weeks of age when these mice were used as BMT donors, as reported in previous studies (Figure 2F).[15] NIMA effects disappeared in mice that received a transplant of grafts from CD25-treated donors (Figure 2G). These results suggest that development of T_R during lactation is critical for the induction of the NIMA effect. We then compared the antigen-specificity of T_R isolated from NIMA (oral) mice and T_R from control mice. $CD4^+$ $CD25^+$ T cells were isolated from control and NIMA-exposed mice and added to the culture of B6 T cells and B6D2F1 or DBA/1 stimulators in MLR. However, both T_R equally suppressed T-cell proliferation in response to NIMAs and third-party alloantigens (data not shown).

Finally, we evaluated whether the NIMA effect persists when NIMA-exposed donors are older. B6D2F1 mice received a transplant of T cells from 28-week-old NIMA-exposed mice and age-matched control mice. Again, GVHD was reduced in mice that received a transplant from older NIMA mice (Figure 3).

Table 1. Results of donor T-cell titration experiments

Donor	$CD4^+$ T-cell dose, $\times 10^6$	Survival	Clinical scores at day +14
wt	0	3/3	0.3 ± 0.2
wt	0.25	5/5	3.8 ± 0.1
NIMA (oral)	0.25	5/5	2.8 ± 0.1*
wt	1	2/5	4.7 ± 0.2
NIMA (oral)	1	4/5	4.0 ± 0.2*

A newborn B6 mouse was fed by either a B6 mother (control) or a B6D2F1 foster mother (NIMA [oral]). Lethally irradiated B6D2F1 mice received a transplant of 5×10^6 TCD-BM from control B6 mice together with 0.25×10^6 or 10^6 $CD4^+$ T cells from NIMA (oral) or controls. Survival and clinical scores after BMT are shown.

*$P < .05$.

Discussion

We demonstrated that oral exposure to NIMAs by breastfeeding alone could induce a tolerogenic NIMA effect. Donor T cells from NIMA-exposed mice were hyporesponsive to the corresponding antigens, thereby attenuating GVHD and resulting in an improvement in survival after a "child-to-mother" BMT. We also found that both oral and in utero exposures to NIMAs are required for the maximum reduction of GVHD, as with previous observations in experimental skin allografts.[9,12] Breastfeeding may thus play an additive role in inducing the tolerogenic NIMA effect. These findings are supported by several clinical studies of renal transplantation, which showed that patients who had been breast-fed survived better after maternal and sibling kidney allografts than those who had not been breast-fed.[8,19] These observations may also explain partly why the incidence of GVHD was significantly lower in HSCT from a NIMA-mismatched donor than from an IPA-mismatched donor in mice[7] and humans,[6,20] and why the survival of sibling kidney allografts expressing noninherited paternal antigens is not as good as the survival of those expressing NIMAs.[5] Additional oral exposure to NIMAs by breastfeeding may be required for induction of clinical tolerance.

Breast milk contains maternal MHC antigens in soluble and cellular forms as well as in exosomes.[8-11] The components critical in inducing tolerance in our model remain to be identified. Interestingly, BMT from donors continuously fed with proteins extracted from recipient splenocytes reduces experimental GVHD.[21] Thus, it is possible that oral exposure to host antigens might improve the outcome of allogeneic HSCT. Oral tolerance is characterized by an inhibition of specific immune responsiveness to subsequent parenteral injections of proteins to which the individual has been previously exposed orally.[22] Oral tolerance prevents pathological reactions against environmental and food antigens, and its failure results in an exacerbated inflammation typical of allergies and asthma. Oral tolerance can be mediated by several mechanisms such as deletion, immune deviation, and suppression by T_R.[23] We recently demonstrated that T_R play an important role in the tolerogenic NIMA effect.[7] Similarly, in the mouse model of the NIMA cardiac allografts, graft acceptance is associated with a higher frequency of IL-10– and TGF-β–producing $CD4^+CD25^+$ cells.[24] In this study, we further showed that the tolerogenic NIMA effect mediated by breastfeeding was also dependent upon T_R. The tolerogenic NIMA effect was abrogated by depletion of $CD25^+$ T cells of donor innocula, as well as the in vivo depletion of $CD25^+$ cells in neonates on days 1 and 8 after birth, during the breastfeeding period. Injection of anti-CD25 mAbs significantly reduced generation of $Foxp3^+$ T_R during this period as shown in Figure 2F. However, this cell population had recovered to normal levels by 8 weeks of age when the mice were used as BMT donors, as shown in previous studies[15]; thus, abrogation of tolerogenic NIMA effects is not due to a decrease in the numbers of $Foxp3^+$ T_R. A recent study addressing the kinetics of T_R generation during ontogeny demonstrated that the largest single-day gain in numbers of $Foxp3^+$ thymocytes occurred between days 3 and 4, followed by a steady increase in number over the first 3 weeks. Thereafter, T_R were continuously generated in the thymus.[25] Thus, both de novo generation of T_R in the thymus and peripheral expansion of T_R appear to be responsible for T_R repopulation within 8 weeks. Interestingly, the mechanisms of oral tolerance differ with different antigen doses. A single administration of high-dose antigens causes clonal deletion, whereas repeated administration of low doses of antigens leads to development of T_R.[26] Thus, repeated oral exposure to NIMAs by breastfeeding might help to produce NIMA-specific T_R. However, a single intravenous injection of allogeneic cells can induce T-cell responses against alloantigens in murine neonates.[27] This route of antigen exposure may be critical in determining immunity or tolerance.

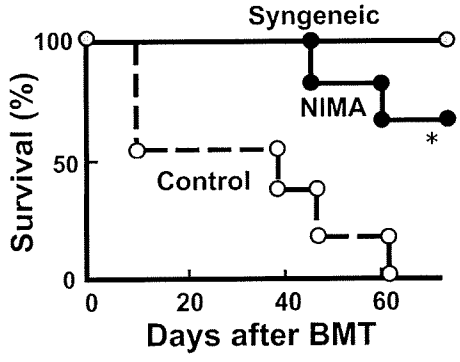

Figure 3. NIMA effects can be mediated by adult donors. Lethally irradiated B6D2F1 mice received a transplant of 5×10^6 TCD-BM from controls together with 2×10^6 T cells from 28-week-old NIMA (in utero + oral) or control donors (n = 5/group). Survival after BMT is shown. *$P < .05$.

Our results suggest the antigen specificity of the NIMA effect in vivo. Oral administration of the self-antigen induces antigen-specific T_R.[28] Breast milk–mediated transfer of an antigen to the neonate from an antigen-exposed mother produces T_R that suppress asthma.[29] Such an antigen specificity may be mediated by "adaptive" T_R that are generated from classical T-cell subsets or "natural" T_R under certain conditions of antigenic stimulation, but not "natural" T_R that develop in the thymus during the early stages of fetal and neonatal T-cell development.[30] In fact, we could not demonstrate antigen-specificity of T_R isolated from NIMA-exposed mice in vitro. Antigen-specific expansion of T_R in vivo may require a T cell–deficient environment and stimulation with the corresponding antigens in the presence of IL-2.[31] Therefore, re-exposure to NIMAs in a lymphopenic environment following pretransplantation conditioning might allow for the generation of "adaptive" T_R, thus contributing to antigen specificity in vivo. Further studies are required to evaluate antigen specificity of the NIMA effect. In addition, it should be noted that oral tolerance can be achieved in the absence of $Foxp3^+CD25^+$ T_R.[23,32] Thus, there could be multiple mechanisms underlying the tolerogenic NIMA effect cause by breastfeeding.

In clinical HSCT with grafts from NIMA-exposed donors, GVHD is mild or absent in some, but not in others.[20] A history of breastfeeding[8,19] as well as the presence of microchimerism[33] and anti-HLA antibodies should be considered. MHC allele mismatch combinations between the donor and recipient could matter.[24] A recent clinical study identified high- and low-risk HLA allele mismatches for severe GVHD.[34] The age of donors may also affect the outcome. In our study, the tolerogenic NIMA effect lasted for at least 28 weeks, suggesting that both child and

adult donors can confer the NIMA effect. Lastly, calcineurin inhibitors used for GVHD prevention inhibit activation and expansion of T_R, thereby suppressing the NIMA effects.[35] The beneficial NIMA effects were observed only if patients were not administered cyclosporine in kidney transplantation.[5] Immunosuppressants such as sirolimus and mycophenolate mofetil, which do not interfere with T_R, may therefore be of use in NIMA-associated HSCT.[35] In conclusion, we demonstrated that breastfeeding plays an important role in the buildup of the tolerogenic NIMA effect, suggesting that novel strategies can be established to induce host-specific tolerance orally in allogeneic HSCT.

Acknowledgments

This study was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology (Tokyo, Japan; no. 20659153; T.T.), Health and Labor Science Research grants (Tokyo, Japan; T.I., T.T.), and Mitsubishi Pharma Research Foundation (Tokyo, Japan; T.T.).

Authorship

Contribution: K. Aoyama conducted the research and wrote the paper; M.K., K.-i.M., D.H., and T.I. conducted the research; K. Akashi, M.H., and M.T. designed the study; and T.T. designed and organized the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells

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Dendritic cells (DCs) can be classified into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can prime antigen-specific T-cell immunity, whereas in vivo function of pDCs as antigen-presenting cells remains controversial. We evaluated the contribution of pDCs to allogeneic T-cell responses in vivo in mouse models of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation by an add-back study of MHC-expressing pDCs into major histocompatibility complex-deficient mice that were resistant to GVHD. Alloantigen expression on pDCs alone was sufficient to prime alloreactive T cells and cause GVHD. An inflammatory environment created by host irradiation has the decisive role in maturing pDCs for T-cell priming but this process does not require Toll-like receptor signaling. Thus, functional outcomes of pDC–T-cell interactions depend on the immunologic context of encounter. To our knowledge, these results are the first to directly demonstrate an in vivo pathogenic role of pDCs as antigen-presenting cells in an antigen-specific T cell–mediated disease in the absence of other DC subsets and to provide important insight into developing strategies for tolerance induction in transplantation. (Blood. 2009;113:2088-2095)

Introduction

The interaction of naive T cells and dendritic cells (DCs) is essential for initiating primary immune responses. DCs can be divided into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) according to their immunophenotype and functional properties.[1-3] pDCs represent a $CD11c^{int}$ $B220^{+}$ DC subset that differs from the $CD11c^{high}$ $B220^{-}$ major histocompatibility complex (MHC) class II^{high} cDCs, commonly viewed as the classic stimulators of naive T cells. One distinctive feature of pDCs is their capacity to rapidly produce high levels of type I interferon (IFN) in response to viral and bacterial stimuli, highlighting the importance of pDCs in innate immune responses.[2-8] pDCs express low levels of surface MHC and classical costimulatory molecules; therefore, they are poor T-cell stimulators.[5-11] In contrast, pDCs matured with CD40 ligands or Toll-like receptor (TLR) ligands are potent antigen-presenting cells (APCs), capable of stimulating naive T-cell proliferation and differentiation to helper, killer, memory, and regulatory T cells in vitro.[7,12,13] In vivo, injection of pDCs activated by synthetic oligodeoxynucleotides containing unmethylated cytosine-guanine motifs (CpG), but not immature pDCs, is capable of eliciting antigen-specific $CD8^{+}$ T-cell responses.[10,14] On the other hand, OVA-pulsed pDCs protected mice against OVA-induced asthma development.[15] pDCs in the tumor-draining lymph nodes express indole 2, 3-dioxygenase, and suppress antitumor T-cell responses.[16] In patients with ovarian cancer, large numbers of pDCs, which induced interleukin 10 (IL10)–producing regulatory T cells, were found in ascites.[17] pDCs mediate tolerance and prolong survival of cardiac allografts.[11,18,19] Several recent clinical observations also suggest that pDCs play important regulatory roles in transplant outcome. An increased ratio of pDCs/cDCs is associated with the successful withdrawal of immunosuppressants after liver transplants.[20] In allogeneic hematopoietic stem cell transplantation (HSCT), low pDC count in the peripheral blood is a risk for graft-versus-host disease (GVHD),[21] while larger numbers of pDCs in donor bone marrow (BM) are associated with increased relapse.[22] Collectively, accumulating data suggest that pDCs are mostly tolerogenic in vivo. However, it remains unclear whether pDCs as APCs have a causative role in antigen-specific T cell–mediated diseases in vivo, although pDCs are involved in the pathogenesis of systemic lupus erythematosus (SLE) and psoriasis through IFN-α production.[23,24]

GVHD, the major obstacle to successful outcome after allogeneic HSCT, is mediated by donor T cells stimulated by recipient DCs.[25-27] MHC class I– or II–deficient ($H2\text{-}Ab1^{-/-}$) mice are resistant to CD8- and CD4-dependent GVHD, respectively.[26-28] When $H2\text{-}Ab1^{-/-}$ mice are repopulated with syngeneic MHC class II–expressing DCs, these mice succumb to acute GVHD.[26,28] Thus, recognition of MHC class II alloantigens on host-derived DCs, alone, is sufficient to prime donor $CD4^{+}$ T cells and cause lethal acute GVHD. CD4-mediated GVHD can develop even in the absence of MHC class II alloantigen expression on GVHD target cells, such as epithelium, endothelium, and parenchyma.[26,28] Thus, this GVHD model system using $H2\text{-}Ab1^{-/-}$ mice presents a stringent test of the allostimulatory capacity of a DC subset when the donor and recipient differ at only MHC class II loci. Using this

Submitted July 14, 2008; accepted December 30, 2008. Prepublished online as *Blood* First Edition paper, January 14, 2009; DOI 10.1182/blood-2008-07-168609.

The online version of the article contains a data supplement.

Presented in abstract form at the 49th Annual Meeting of the American Society of Hematology, Atlanta, GA, December 10, 2007.

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model system with modification, we addressed whether pDCs as APCs have a causative role in antigen-specific T cell–mediated diseases, such as GVHD, or induce tolerance.

Methods

Mice

Female C57BL/6 (B6: H-2^b, CD45.2$^+$), B6D2F1 (H-2^{bd}), and BALB/c (H-2^d) mice were purchased from Charles River Japan (Yokohama, Japan). C3H/HeJ (C3H: H-2^k) and AKR/J (AKR: H-2^k) mice were purchased from Japan SLC (Shizuoka, Japan). B6-Ly5a (H-2^b, CD45.1$^+$), and C3H-background β_2m-deficient ($\beta 2m^{-/-}$: C3.129P2(B6)-*B2m*^{tm1Unc}/Dcr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II$^{-/-}$ mice (*H2-Ab1*$^{-/-}$: B6.129-*Abb*^{tm1} N12) were from Taconic (Germantown, NY). B6-background Toll–IL-1 receptor domain-containing adaptor inducing IFN-β/myeloid differentiation factor 88 double-deficient (TRIF/MyD88 DKO) mice[29] were kindly provided by Dr Kiyoshi Takeda at Kyushu University (Fukuoka, Japan). The age of the mice ranged between 9 and 16 weeks. Mice were maintained in a specific pathogen-free condition and received normal chow and hyperchlorinated drinking water for the first 3 weeks after transplantation. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Kyushu University.

Cell isolation

To expand DCs, we injected mice subcutaneously once daily with 10 μg recombinant human fms-like tyrosine kinase 3 ligand (FL; Amgen, Seattle, WA) for 10 consecutive days, and cDCs, pDCs, and B cells were isolated, as previously described, with a modification.[28,30] Briefly, cDCs were enriched from splenocytes using CD11c microbeads and the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by cell sorting of CD11c^{high} B220$^-$ cells using a BD FACSAria (BD Biosciences, San Jose, CA). B cells were enriched from splenocytes with B220 microbeads and the AutoMACS, and sorted as CD11c$^-$ B220$^+$ cells. pDCs were enriched from BM by depleting CD3$^+$, CD19$^+$, CD11b$^+$, CD49b$^+$, and Ly-76$^+$ cells using a cocktail of biotin-conjugated mAbs, streptavidin-microbeads, and the AutoMACS system, followed by a FACS sorting of CD11c^{int} B220$^+$ cells. CD4$^+$ T cells were negatively selected from splenocytes by depleting CD8$^+$, CD49b$^+$, CD11b$^+$, Ly-76$^+$, and B220$^+$ cells. CD8$^+$ T cells were negatively selected from splenocytes by depleting CD4$^+$, CD49b$^+$, CD11b$^+$, Ly-76$^+$, and B220$^+$ cells, using the AutoMACS system, followed by a FACS sorting of CD4$^-$CD8$^+$ cells. T-cell depletion (TCD) of donor BM cells was performed using CD90 microbeads and the AutoMACS system.

Induction and assessment of GVHD

GVHD was induced as previously described.[28] In brief, mice received 11 Gy total body irradiation (TBI), split into 2 doses separated by 4 hours to minimize gastrointestinal toxicity, and injected intravenously with 2 × 10^6 each APC subset on day −1. On day 0, mice were injected intravenously with 2 × 10^6 CD4$^+$ or CD8$^+$ T cells with or without 5 × 10^6 TCD-BM. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly using a scoring system that evaluated changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10).[31] Acute GVHD was also assessed by detailed histopathologic analysis of the liver and intestine. Slides stained with hematoxylin and eosin were examined systematically using a semiquantitative scoring system.[32] Pictures from tissue sections were taken at room temperature using an ProgRes 3012 mF digital camera (Jenoptik Laser Optik Systeme, Jena, Germany) mounted on an Olympus BX51 microscope (Olympus, Tokyo, Japan) and analyzed using a ProgRes PlugIn for PCI software version 5.0 (Jenoptik Laser Optik Systeme). Images were acquired using an UPlan Apochromat 10×/0.40 numeric aperture (NA) or a Plan Apochromat 40×/0.90 NA WLSM objective, depending on the desired magnification.

Cell culture and enzyme-linked immunosorbent assay

All culture media and incubation conditions have been described previously.[33] CD4$^+$ T cells were cultured at a concentration of 2 × 10^5 cells/well with 10^4 irradiated (20 Gy) APCs. After culturing for 3 days, supernatants were harvested for cytokine measurements, and cells were pulsed with ^{3}H-thymidine (1 μCi per well) for further 16 hours. Proliferation was determined using a Topcount NXT (Packard Instruments, Meriden, CT). In some experiments, 1 μM CpG 1668 (Sigma-Aldrich Japan, Ishikari, Japan) or 100 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich Japan) was added to the culture. To determine secretion of IFN-α and IL-12 p70 from APCs, 10^5 APCs were incubated with 1 μM CpG 2216 (Sigma-Aldrich Japan) for 16 hours. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ (BD Biosciences), IFN-α (PBL Biomedical Laboratories, Piscataway, NJ), and IL-12 p70 (R&D Systems, Minneapolis, MN) was performed according to the manufacturer's protocols with the sensitivity of 31.3 pg/mL, 12.5 pg/mL, and 2.5 pg/mL, respectively.

Flow cytometric analysis

The mAbs used were fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, or allophycocyanin-conjugated anti–mouse TCRβ, CD4, CD8α, CD11c, CD11b, CD19, CD49b, CD45.1, CD45.2, CD90.2, CD86, B220 (CD45R), H-2K^b, H-2K^d, H-2K^k, I-A^b, Ly6C (BD Biosciences), mPDCA-1 (Miltenyi Biotec), and Foxp3 (eBioscience, San Diego, CA). Cells were stained as previously described.[33] Irrelevant IgG$_{2a/b}$ mAbs were used as a negative control. For intracellular IFN-γ staining, splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti–IFN-γ mAbs. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD4$^+$ T cells were stained in phosphate-buffered saline (PBS) with 1 μM CFSE (Molecular Probes, Invitrogen, Eugene, OR), as described earlier.[34] At least 5000 live samples were acquired for analysis. Dead cells were identified as 7-amino-actinomycin D (BD Biosciences) or 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) positive cells. The cells were analyzed using a FACSCalibur or a FACSAria flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Statistical analysis

Mann-Whitney U tests were used to analyze cell counts and clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability and the log-rank test was applied to compare the survival curves. We defined P values of less than .05 as statistically significant.

Results

Isolation and characterization of pDCs

Low frequency of pDCs in vivo has hampered the study of this cell population. To expand DCs in vivo, mice were injected with 10 μg FL for 10 days.[6,28,30] As previously reported,[35,36] FL treatment resulted in an increase in frequency of CD11c$^+$B220$^+$ pDCs to 6.7% in spleen and 18.6% in BM (Figure 1A). pDCs, cDCs, and B cells were then isolated as described in the methods section. The purity of each population was greater than 98% with less than 0.1% contamination by the others (Figure 1A). pDCs appeared plasmacytoid round shaped with excentered nuclei and basophilic cytoplasm (Figure 1B). They expressed low levels of CD86 and MHC class II but high levels of Ly6C and mPDCA-1 (Figure 1C). These cells did not express CD19, CD49b, or CD11b, thus ruling out contamination of B cells, natural killer (NK) cells, and IFN-producing killer