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Plenary paper

High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism

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In allogenic hematopoietic stem-cell transplantation, an effect of HLA locus mismatch in allele level on clinical outcome has been clarified. However, the effect of each HLA allele mismatch combination is little known, and its molecular mechanism to induce acute graft-versus-host disease (aGVHD) remains to be elucidated. A total of 5210 donor-patient pairs who underwent transplantation through Japan Marrow Donor Program were analyzed. All HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles were retrospectively typed in all pairs. The impacts of the HLA allele mismatch combinations and amino acid substitution positions in 6 HLA loci on severe aGVHD were analyzed. A total of 15 significant high-risk HLA allele mismatch combinations and 1 HLA-DRB1-DQB1 linked mismatch combinations (high-risk mismatch) for severe aGVHD were identified, and the number of high-risk mismatches was highly associated with the occurrence of severe aGVHD regardless of the presence of mismatch combinations other than high-risk mismatch. Furthermore, 6 specific amino acid substitution positions in HLA class I were identified as those responsible for severe aGVHD. These findings provide evidence to elucidate the mechanism of aGVHD on the basis of HLA molecule. Furthermore, the identification of high-risk mismatch, that is, nonpermissive mismatch, would be beneficial for the selection of a suitable donor. (Blood. 2007;110:2235-2241)

Introduction

Allogenic hematopoietic stem-cell transplantation (HSCT) from an HLA-matched unrelated (UR) donor has been established as a treatment for hematologic malignancies, when an HLA-identical sibling donor is unavailable.[1,2] When a matched unrelated donor was not found in the donor registry, a partially HLA-matched unrelated donor was one of the candidates for alternative donor. But the higher risk of immunologic events, especially graft-versus-host disease (GVHD), was an important drawback. Extensive recent research has accumulated evidence of the role of each HLA locus mismatch on clinical outcome for UR-HSCT,[3-9] which has made it easy to search and select a partially matched donor. To further expand options for donor selection, our next challenge is to identify permissive and nonpermissive mismatch combinations of each HLA allele. Although there were some divisional trials with small populations,[10,11] a large-scale cohort is essential for comprehensive analysis to identify nonpermissive mismatch combinations that are significant risk factors for severe acute graft-versus-host disease (aGVHD).

In this study, we identified nonpermissive HLA mismatch allele combinations of all major 6 HLA loci, and their responsible positions of amino acid substitution for aGVHD.

Patients, materials, and methods

Patients

A total of 5210 donor-patient pairs who underwent transplantation through the Japan Marrow Donor Program (JMDP) with T-cell–replete marrow from a serologically HLA-A, -B, and -DR antigen-matched donor between January 1993 and January 2006 were analyzed in this cohort study. Patients who received a transplant of harvested marrow outside Japan (n = 51) or were unavailable for blood sample (n = 428) were not eligible for this study of a total of 5689 consecutively registered patients.

Patient characteristics are shown in Table S1, available on the *Blood* website (see the Supplemental Materials link at the top of the online article). The final clinical survey of these patients was completed by June 1, 2006. Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki, and approval of the study was obtained from the Institutional Review Board of Aichi Cancer Center and JMDP.

HLA typing of patients and donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified by the methods described previously.[4,5] Six HLA locus alleles were typed in all 5210 pairs. HLA genotypes of HLA-A, -B, -C, -DQB1, and -DPB1 allele of patient and donor were reconfirmed by the Luminex microbead method (Luminex 100 System; Luminex, Austin, TX). For convenience, we showed the frequency of HLA alleles that existed with

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more than a 5% allele frequency in the current Japanese data set and less than a 1% allele frequency in white populations[12] in Table S2.

Matching of HLA allele between patient and donor

For the analysis of aGVHD, HLA allele mismatch among the donor-recipient pair was scored when the recipient's alleles were not shared by the donor (GVH vector). We also used GVH vectors for the analysis of overall survival (OS) to indicate OS of aGVHD high-risk or low-risk group.

Evaluation of acute GVHD

Occurrences of aGVHD were graded with grade 0, I, II, III, and IV according to established criteria.[13] Grades III and IV were defined as severe aGVHD.

Definitions of amino acid substitution

Amino acid sequences of HLA-A, -B, -C, -DR, -DQ, and -DP molecules were obtained from IMGT/HLA sequence database.[14] For example, Tyr9A-Phe9A indicated amino acid substitutions of position 9 in HLA-A molecule at which the donor had tyrosine and the patient phenylalanine. Substituted amino acids in HLA class I were summarized in Tables S3-S5.

Definition of nonpermissive HLA combinations

We defined the nonpermissive HLA allele combination as a significant risk factor for severe aGVHD, because severe aGVHD was a solid marker for alloreactivity in HSCT and was the main contributor to transplantation-related mortality.[15,16]

Definition of hydropathy scale

The hydropathy scale proposed by Kyte and Doolittle[17] evaluates the hydrophilicity and hydrophobicity of 20 amino acids to estimate the protein structure. Hydrophobic amino acid has a plus value and hydrophilic amino acid a minus value, and their absolute value indicates the grade of each property.

Statistical analysis

Cumulative incidences of aGVHD were assessed by the method described elsewhere to eliminate the effect of competing risk.[18,19] The competing event regarding aGVHD was defined as death without aGVHD. A log-rank test was applied to assess the impact by the factor of interest. Multivariable Cox regression analyses[20] were conducted to evaluate the impact of HLA allele mismatch combination, and the positions and types of amino acid substitution (for example, alanine, arginine, asparagines) of HLA molecules.

The HLA mismatch combination was evaluated for each locus separately, and the HLA match and HLA one-locus mismatch in every locus were analyzed. For example, A0206-A0201 mismatch combination meant that the donor has HLA-A*0206, recipient has HLA-A*0201, and another HLA-A allele of each donor and recipient was identical. This mismatch was compared with the HLA-A allele match. The mismatch combination of which the number of pairs was less than 10 was lumped together as "other mismatch." This is because, according to the computer simulation by Peduzzi et al,[21] it is generally accepted that regression analysis for a variable having fewer than 10 events might give an unreliable estimation. The model was constructed with mismatch combinations, mismatch status in other loci (match, 1 locus mismatch, and 2 locus mismatches as ordinal variable), and potential confounders. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (cyclosporine [CSP] vs FK 506 [FK]), ATG (ATG vs no ATG), and preconditioning (total body irradiation [TBI] vs non-TBI). We used these confounders in all analyses in this paper to keep results comparable.

The impact of positions and types of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 separately. The amino acid positions we analyzed were all those at which amino acid was substituted in each locus. We analyzed the impact of each amino acid substitution on each position separately. Multivariable Cox models including positions and types of amino acid substitution, mismatch status in other loci (match, 1 locus mismatch, and 2 locus mismatches as ordinal variable), and confounders described in "Statistical analysis" were constructed.

We applied a P value of less than .005 as statistically significant to eliminate false-positive associations. All the analyses were conducted by STATA version 9.2 (Stata, College Station, TX).

Validation of statistical analysis

We validated the statistical analysis using 2 methods, traditional training-and-test method and bootstrap resampling method, in HLA-A analysis to confirm the usability of bootstrap resampling. In the traditional training-and-test method, donor-recipient pairs were divided at random in 2 equally scaled groups, group A and group B. When consistent results were obtained in both analyses, we considered the results as validated. In the bootstrap resampling method,[22] we estimated the measure of association with the resampled data repeatedly drawn from the original data. Although around 100 to 200 bootstrapped samplings are generally sufficient,[23] we explored 500, 1000, 5000, 10 000, and 50 000 bootstrappings in analysis of HLA-A mismatch combinations. We confirmed that an analysis using more than 5000 bootstrappings made the results stable. Because there was high concordance between these 2 methods (Table S6), we adopted bootstrap resampling using 10 000 bootstrap samples for all analyses in this paper as the method for validation. This is because traditional training-and-test methods do not work efficiently when small subgroups are considered as in this paper. Only when the results of base analysis and validating analysis using bootstrap resampling were significant concurrently were the results of the analysis judged to be statistically significant. When the result of base analysis was significant but the result of validating analysis using bootstrap resampling was not, we indicated this by adding an asterisk next to the P value of the base analysis.

Results

Impact of HLA allele mismatch combinations on severe aGVHD

Hazard ratios (HRs) of HLA allele mismatch combinations in HLA-A and -C on severe aGVHD are shown in Table 1 (HLA-B, -DR, -DQ, and -DP are available in Table S7).

In HLA-A locus mismatch combinations, A*0206-A*0201 (HR: 1.78; CI, 1.32-2.41), A*0206-A*0207 (HR: 3.45; CI: 2.09-5.70), A*2602-A*2601 (HR: 3.35; CI: 1.89-5.91), and A*2603-A*2601 (HR: 2.17; CI: 1.29-3.64), were significant risk factors for severe aGVHD.

In HLA-C locus mismatch combinations, 7 combinations were significant risk factors for severe aGVHD; those were as follows: Cw*0401-Cw*0303 (HR: 2.81; CI: 1.72-4.60), Cw*0801-Cw*0303 (HR: 2.32; CI: 1.58-3.40), Cw*0303-Cw*1502 (HR: 3.22; CI: 1.75-5.89), Cw*0304-Cw*0801 (HR: 2.34; CI: 1.55-3.52), Cw*1402-Cw*0304 (HR: 3.66; CI: 2.00-6.68), Cw*1502-Cw*0,304 (HR: 3.77; CI: 2.20-6.47), and Cw*1502-Cw*1402 (HR: 4.97; CI: 3.41-7.25). To summarize, high-risk HLA allele mismatch combinations for severe aGVHD, that is, nonpermissive mismatch combinations, of all major 6 HLA loci were listed in Table 2. A total of 15 nonpermissive HLA allele mismatch combinations (4 in HLA-A, 1 in HLA-B, 7 in HLA-C, 1 in HLA-DRB1, and 2 in HLA-DPB1) and 1 HLA-DRB1-DQB1 linked mismatch combination (Table 2 legend) were identified.

We divided donor-recipient pairs into 4 groups according to the number of nonpermissive mismatches: (1) full match (in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1) group; (2) zero nonpermissive mismatch (with mismatches other than nonpermissive mismatches)

Table 1. Multivariable analysis of impact of mismatch pairs for sever aGVHD in HLA-A and -C

Mismatch combination, donor-patient	N	HR (95% CI)	P
A locus match	4510	1	NA
A0201-A0206	138	1.23 (0.87-1.73)	.223
A0206-A0201	131	1.78 (1.32-2.41)	< .001
A0201-A0207	28	0.83 (0.34-2.03)	.699
A0207-A0201	20	1.12 (0.42-3.02)	.809
A0201-A0210	11	1.57 (0.58-4.23)	.367
A0206-A0207	27	3.45 (2.09-5.70)	< .001
A0207-A0206	22	0.71 (0.23-2.24)	.571
A2402-A2420	60	0.64 (0.32-1.30)	.225
A2420-A2402	30	1.18 (0.56-2.49)	.66
A2601-A2602	24	0.64 (0.26-1.58)	.34
A2602-A2601	21	3.35 (1.89-5.91)	< .001
A2601-A2603	34	1.37 (0.73-2.57)	.326
A2603-A2601	35	2.17 (1.29-3.64)	.003
A2602-A2603	10	1.23 (0.30-4.98)	.763
A2603-A2602	12	1.50 (0.48-4.68)	.485
A other mismatch	97	1.47 (1.00-2.15)	.047
C locus match	3685	1	NA
C0102-C0303	30	2.83 (1.50-5.32)	.001*
C0303-C0102	38	1.05 (0.47-2.36)	.899
C0102-C0304	12	1.85 (0.59-5.81)	.287
C0304-C0102	19	0.89 (0.28-2.79)	.854
C0102-C0401	14	1.87 (0.77-4.55)	.164
C0102-C0803	24	1.97 (0.87-4.42)	.099
C0803-C0102	10	1.66 (0.53-5.19)	.383
C0102-C1402	16	3.86 (1.98-7.51)	< .001*
C1402-C0102	13	0.46 (0.06-3.33)	.45
C0303-C0304	83	1.08 (0.63-1.85)	.761
C0304-C0303	62	0.83 (0.41-1.68)	.614
C0303-C0401	31	1.73 (0.89-3.36)	.103
C0401-C0303	42	2.81 (1.72-4.60)	< .001
C0303-C0702	25	1.16 (0.52-2.62)	.706
C0702-C0303	18	2.16 (0.96-4.85)	.062
C0303-C0801	76	1.07 (0.63-1.84)	.782
C0801-C0303	80	2.32 (1.58-3.40)	< .001
C0303-C1502	25	3.22 (1.75-5.89)	< .001
C0304-C0401	15	3.02 (1.34-6.79)	.007
C0401-C0304	12	6.22 (3.07-12.5)	< .001*
C0304-C0702	26	2.35 (1.16-4.76)	.017
C0702-C0304	33	1.22 (0.58-2.59)	.59
C0304-C0801	69	2.34 (1.55-3.52)	< .001
C0801-C0304	47	1.64 (0.98-2.76)	.057
C0304-C1402	28	3.06 (1.68-5.60)	< .001*
C1402-C0304	23	3.66 (2.00-6.68)	< .001
C0304-C1502	53	1.82 (1.08-3.05)	.023
C1502-C0304	27	3.77 (2.20-6.47)	< .001
C0801-C0102	10	2.88 (0.92-9.03)	.068
C0801-C0803	27	1.55 (0.69-3.48)	.284
C0803-C0801	26	2.04 (1.04-3.99)	.037
C0801-C1502	36	1.59 (0.79-3.21)	.19
C1502-C0801	23	2.28 (1.07-4.85)	.031
C1402-C1502	55	1.67 (1.01-2.77)	.043
C1502-C1402	50	4.97 (3.41-7.25)	< .001
C other mismatch	347	1.69 (1.34-2.14)	< .001

A0206-A0201 mismatch combination meant that the donor has HLA-A*0206, recipient has HLA-A*0201 and another HLA-A allele of each donor and recipient was identical. Each mismatch pair in HLA-A was compared with the HLA-A allele match, and each mismatch pair in HLA-C was compared with the HLA-C allele match. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high and diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs non-TBI).

HR denotes hazard ratio; CI, confidence interval; NA, not applicable.

*The result of base analysis was significant, but the result of validating analysis using bootstrap resampling was not. The results of the analysis were thus judged not to be statistically significant.

Table 2. Nonpermissive allele mismatch combinations for severe aGVHD

Mismatch combination, donor-patient	N	HR (95% CI)	P
A0206-A0201	131	1.78 (1.32-2.41)	< .001
A0206-A0207	27	3.45 (2.09-5.70)	< .001
A2602-A2601	21	3.35 (1.89-5.91)	< .001
A2603-A2601	35	2.17 (1.29-3.64)	.003
B1501-B1507	19	3.34 (1.85-5.99)	< .001
C0303-C1502	25	3.22 (1.75-5.89)	< .001
C0304-C0801	69	2.34 (1.55-3.52)	< .001
C0401-C0303	42	2.81 (1.72-4.60)	< .001
C0801-C0303	80	2.32 (1.58-3.40)	< .001
C1402-C0304	23	3.66 (2.00-6.68)	< .001
C1502-C0304	27	3.77 (2.20-6.47)	< .001
C1502-C1402	50	4.97 (3.41-7.25)	< .001
DR0405-DR0403	53	2.13 (1.28-3.53)	.003
(DR1403-DQ0301)-(DR1401-DQ0502)	19	2.81 (1.44-5.51)	.002
DP0301-DP0501	49	2.41 (1.49-3.89)	< .001
DP0501-DP0901	71	2.03 (1.30-3.16)	.002

Analysis method is the same as in Table 1. We surveyed specific linked mismatches between nonpermissive mismatches elucidated. As a result, obvious specific linked mismatches exist only between DRB1*1403- DRB1*1401 and DQB1*0301- DQB1*0502. Therefore, we could not evaluate which mismatch combination impacted aGVHD, and we considered this linked mismatch did so. On the other hand, because other nonpermissive mismatch combinations had no specific link with the others, we judged other than DRB1*1403- DRB1*1401 and DQB1*0301- DQB1*0502 nonpermissive mismatches solely impacted aGVHD. (DR1403-DQ0301)-(DR1401-DQ0502) linked mismatch meant that the donor has HLA-DRB1*1403-HLADQB1*0301 and the recipient has HLA-DRB1*1401-HLADQB1*0502.

HR indicates hazard ratio; CI, confidence interval.

group; (3) 1 nonpermissive mismatch (with or without mismatches other than nonpermissive mismatches) group; and (4) 2 or more nonpermissive mismatches (with or without mismatches other than nonpermissive mismatches) group, and analyzed for association with severe aGVHD. This analysis excluded pairs with 2 locus mismatches in the same locus. Patient characteristics according to the number of nonpermissive mismatches are shown in Table 3. The curve of cumulative incidence of severe aGVHD is shown in Figure 1A. Multivariable analysis revealed that severe aGVHD occurred with almost equal frequency between the full match group and zero nonpermissive mismatch group, and was significantly associated with the number of nonpermissive mismatches (Table 4). Relative risk of significant factor for aGVHD and OS is shown in Table S8. In terms of the mortality due to aGVHD according to the number of nonpermissive mismatches, one nonpermissive mismatch group and 2 or more nonpermissive mismatch groups showed higher mortality (19.7% and 15.8%, respectively) than full match group and zero nonpermissive mismatch group (8.5% and 11.4%, respectively).

Impact of positions and types of amino acid substitutions of HLA molecules for severe aGVHD

One specific amino acid substitution at position 9 in HLA-A molecule and 6 specific amino acid substitutions at positions 9, 77, 80, 99, 116, and 156 in HLA-C molecule were significant risk factors for severe aGVHD: Tyr9A-Phe9A (HR: 1.66; CI: 1.19-3.32), Tyr9C-Ser9C (HR: 1.66; CI: 1.23-2.25), Asn77C-Ser77C (HR: 1.87; CI: 1.46-2.39), Lys80C-Asn80C (HR: 1.87; CI: 1.46-2.39), Tyr99C-Phe99C (HR: 1.64; CI: 1.21-2.22), Leu116C-Ser116C (HR: 3.40; CI: 2.20-5.25), and Arg156C-Leu156C (HR: 1.48; CI: 1.15-1.90) (Table 5). The amplitude of hydropathy scales were 4.1, 0.5, 2.7, 0.4, 4.1, 4.6, and 8.3, respectively. Although all

Table 3. Patient characteristics according to number of nonpermissive mismatches

Group	Total	Full match	Zero nonpermissive mismatch	One nonpermissive mismatch	Two or more nonpermissive mismatches
Total	4050	712	2670	602	66
Patient age, median y	30	32	30	29	29
Sex, donor/patient, no. patients					
Male/male	1673	312	1096	237	28
Male/female	785	134	518	119	14
Female/male	769	115	524	117	13
Female/female	823	151	532	129	11
Disease, no. patients					
ALL	981	162	668	139	12
ANLL	1075	196	698	158	23
CML	703	119	453	115	16
Hereditary disease	85	14	56	15	0
MDS	476	91	304	72	9
Malignant lymphoma	349	69	229	48	3
Multiple myeloma	42	8	29	4	1
Severe aplastic anemia	247	33	175	37	2
Other disease	92	20	58	14	0
Risk of leukemia relapse,* no. patients					
Standard risk	1308	249	857	181	21
High risk	1451	228	962	231	30
Diseases other than leukemia	1291	235	851	190	15
GVHD prophylaxis, no. patients					
Cyclosporin-based	2198	402	1444	319	33
Tacrolimus-based	1852	310	1226	283	33
ATG, no. patients					
ATG	323	48	215	53	7
Non-ATG	3727	664	2455	549	59
Preconditioning, no. patients					
TBI regimen	3117	539	2071	449	58
Non-TBI regimen	933	173	599	153	8

ALL indicates acute lymphoblastic leukemia; ANLL, acute non-lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; and TBI, total body irradiation.

*Standard risk for leukemia relapse was defined as the status of the 1st CR of AML and ALL and the 1st CP of CML at transplant, while high risk was defined as a more advanced status than standard risk in AML, ALL, and CML, and diseases other than leukemia was defined as other than ALL, ANLL, and CML.

amino acid positions substituted in each HLA locus were analyzed, amino acid substitutions of any other HLA-A and -C positions were not significant risk factors. As for HLA-B, DRB1, DQB1, and DPB1, there was no significant association between the positions of amino acid substitution and severe aGVHD. Impact for OS about positions and types of amino acid substitutions that were significant risk factors for aGVHD was shown in Table S9.

Discussion

Extensive recent research has accumulated evidence of the role of each HLA locus mismatch on clinical outcome for UR-HSCT.[3-9] Our next concern is identifying the combinations of HLA allele mismatch and the positions of amino acid substitution of the HLA molecules responsible for aGVHD. In the present study, multivariable analysis revealed that 15 combinations of HLA allele mismatch and 1 HLA-DRB1-DQB1 haplotype mismatch significantly increase the occurrence of severe aGVHD (Table 2), and most of them increased the mortality rate after transplantation (data not shown). Thus, these mismatch combinations of HLA allele might be called nonpermissive clinically. We speculated that the effect of HLA locus mismatch was a reflection and summation of these HLA allele mismatch combinations. Discrepancies of responsible HLA locus for aGVHD between ethnically diverse transplantations might be explained by the proportions of nonpermissive mismatch combinations in each HLA locus. The same study in other populations would be needed to clarify this question as well as the severity of aGVHD. Interestingly, the full match group and zero nonpermissive mismatch group showed an almost equal occurrence of severe aGVHD, though pairs in zero nonpermissive mismatch group had one or more mismatches other than nonpermissive mismatches. And HR was elevated with the increase in the number of nonpermissive mismatches (Figure 1A; Table 4), while the number of nonpermissive mismatches also had a significant effect on OS after transplantation (Figure 1B; Table 4). These findings indicated at least that nonpermissive mismatches should be avoided in donor selection for UR-HSCT, and that the order of donor selection based on this nonpermissive mismatch would be useful, instead of that based on HLA locus mismatch. We also speculated that there are permissive mismatches in mismatches other than nonpermissive mismatches. It is therefore an important task in the future to identify permissive mismatches for partially HLA-matched donor selection. On the other hand, we do not deny the possibility that some mismatch combinations not classified as nonpermissive may actually be potential nonpermissive ones. Misclassification might happen because of insufficient statistical power due to the relatively small number of subjects in subcategories.

At present, there have been only a few reports indicating that the transplant-related immunologic reactions and clinical outcomes were caused by the HLA allele mismatch combinations. Macdonald

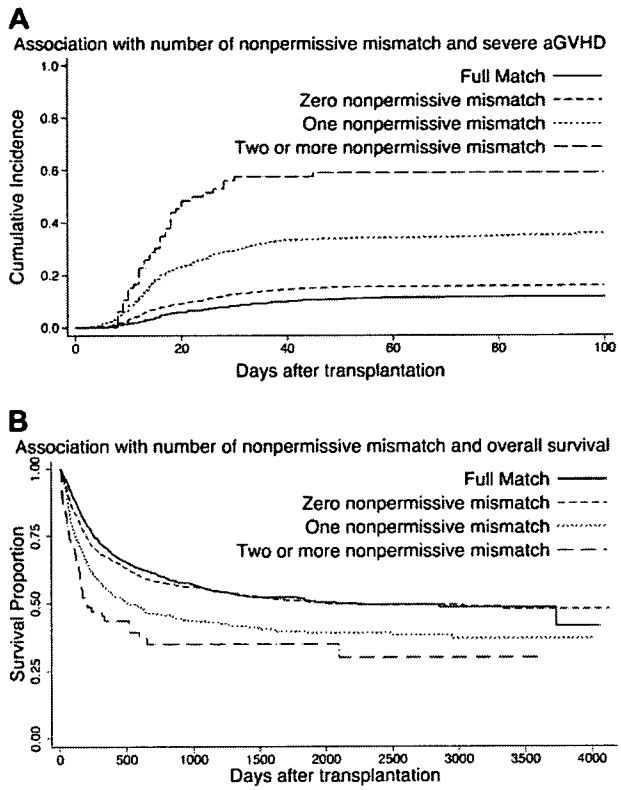


Figure 1. Impact of number of nonpermissive mismatches on severe aGVHD and overall survival. (A) Cumulative incidence of severe aGVHD according to number of nonpermissive mismatches. — indicates full match (in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1) group; ----, zero nonpermissive mismatch (with mismatches other than nonpermissive mismatches) group; · · · ·, one nonpermissive mismatch (with or without mismatches other than nonpermissive mismatches) group; and – – –, 2 or more nonpermissive mismatches (with or without mismatches other than nonpermissive mismatches) group. (B) Kaplan-Meier estimates of survival according to number of nonpermissive mismatches. Each group was divided as described for panel A.

et al[24] reported that cytotoxic T lymphocytes (CTLs) discriminate between HLA-B*4402 and HLA-B*4403, and induce strong alloresponses, but the stronger T-cell alloreactivity is observed toward HLA-B*4403 compared with HLA-B*4402 in vitro. Zino et al[10] and Fleischhauer et al[11] attempted to develop an algorithm for prediction of nonpermissive HLA-DPB1 mismatches. The present report is the first to provide far more precise and detailed evidence for numerous HLA allele mismatch combinations for severe aGVHD.

Table 5. Multivariable analysis of impact of amino acid substitution on HLA class I molecules for severe aGVHD

Posisiton and kind of amino acid substitution, donor-recipient	HS	N	Event†	HR (95% CI)	P
HLA-A locus					
Tyr9A-Phe9A	4.1	163	64	1.66 (1.19-2.32)	.003
Asn116A-Asp116A	0	32	15	2.25 (1.26-4.01)	.005*
HLA-C locus					
Tyr9C-Ser9C	0.5	146	59	1.66 (1.23-2.25)	.001
Asn77C-Ser77C	2.7	205	90	1.87 (1.46-2.39)	< .001
Lys80C-Asn80C	0.4	205	90	1.87 (1.46-2.39)	< .001
Tyr99C-Phe99C	4.1	146	59	1.64 (1.21-2.22)	.001
Leu116C-Ser116C	4.6	53	30	3.40 (2.20-5.25)	< .001
Arg156C-Leu156C	8.3	251	88	1.48 (1.15-1.90)	.002

HLA-B. -DRB1, -DQB1 -DPB1 locus had no significant substitutions. The impact of positions and types of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 separately. For example, Tyr9A-Phe9A indicated amino acid substitutions of position 9 in HLA-A molecule at which donor had tyrosine and patient phenylalanine. The impacts of positions and kinds of amino acid substitutions in each HLA molecule were evaluated in pairs with HLA one locus mismatch in each HLA locus separately. Pairs which substituted specific amino acid at each position were compared with amino acid matched pairs at that position.

HS indicates hydropathy scale; HR, hazard ratio; CI, confidence interval; Tyr, tyrosine; Phe, phenylalanine; Asn, asparagine; Asp, asparatic acid; Ser, serine; Lys, lysine; Leu, leucine; and Arg, arginine.

*Result of base analysis was significant but result of validating analysis using bootstrap resampling was not. Results of analysis were thus judged not to be statistically significant.

†Measured in number of occurrences of severe acute GVHD.

In this study, substitutions of specific amino acids at positions 9, 77, 80, 99, 116, and 156 were elucidated as a significant risk factor for severe aGVHD. We speculated that the responsibility of positions 77 and 80 in HLA-C for severe aGVHD was associated with ligand matching of NK-cell receptor (KIR2DL). Although the role of KIR2DL in acute GVHD has been controversial,[25] a recent JMDP analysis demonstrated that KIR2DL ligand mismatched pairs in GVH vector induced severe aGVHD in UR-HSCT with T-cell–replete marrow.[9] The ligand of KIR2DL is located at positions 77 and 80, which are completely linked in HLA-C molecule. And almost all pairs in this study with Asn77C-Ser77C and Lys80C-Asn80C substitutions have a KIR2DL mismatch in GVH vector.

Except for positions 77 and 80, which are associated with KIR2DL ligand in HLA-C, positions 9, 99, 116, and 156 were elucidated. Positions 9, 99, and 116 are located in the beta-plated

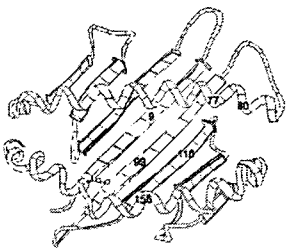
Table 4. Multivariable analysis of impact of number of nonpermissive mismatches on severe aGVHD and overall survival

			Univariate analysis		Multivariate analysis		Bootstrap (10000)	
	N	Event*	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
For severe aGVHD								
Full match group	972	129	1.00	NA	1.00	NA	1.00	NA
Zero nonpermissive mismatch group	2446	411	1.21 (0.95-1.54)	.111	1.00 (0.75-1.32)	.996	1.00 (0.74-1.33)	.996
One nonpermissive mismatch group	571	211	2.88 (2.20-3.78)	< .001	2.22 (1.62-3.04)	< .001	2.22 (1.63-3.02)	< .001
Two or more nonpermissive mismatch group	61	36	5.62 (3.77-8.39)	< .001	3.68 (2.33-5.80)	< .001	3.68 (2.33-5.80)	< .001
For overall survival								
Full match group	972	400	1.00	NA	1.00	NA	1.00	NA
Zero nonpermissive mismatch group	2446	1021	1.10 (0.98-1.23)	.091	1.06 (0.94-1.20)	.315	1.06 (0.94-1.20)	.299
One nonpermissive mismatch group	571	309	1.55 (1.34-1.78)	< .001	1.51 (1.30-1.76)	< .001	1.51 (1.29-1.77)	< .001
Two or more nonpermissive mismatch group	61	39	2.12 (1.54-2.90)	< .001	2.25 (1.65-3.08)	< .001	2.25 (1.65-3.08)	< .001

Each group was compared with Full match group. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, highand diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs. non-TBI).

HR indicates hazard ratio; CI, confidence interval; Boot strap (10000), bootstrap resampling using 10000 bootstrapping.

*For severe aGVHD, "Event" refers to number of occurrences; for overall survival, number of deaths.



	beta-plate sheet			alpha helix
Position of HLA class I	9	99	116	156
Peptide-binding pocket	B C	A B D	F	D E
Amino acid substitution				
HLA-A	Tyr-Phe		Asn-Asp*	
HLA-C	Tyr-Ser	Tyr-Phe	Leu-Ser	Arg-Leu

Figure 2. Schematic presentation of HLA class I molecule and summary of features of significant amino acid substituted positions. Numbers in schema of HLA molecule indicate substituted amino acid positions that were elucidated as significant risk factor for severe aGVHD. Positions 9, 99, and 116 are located in the beta-plated sheet and positions 77, 80, and 156 in the alpha helix of HLA class I molecule (left). Positions 77 and 80 are associated with KIR2DL ligand in HLA-C molecule. Position 9 constitutes peptide-binding pockets B and C; position 99 constitutes A, B, and D pockets; position 116 constitutes F pocket; and position 156 constitutes D and E pockets (right). For example, Tyr-Phe indicated amino acid substitution at indicated position in HLA molecule at which donor had tyrosine and patient phenylalanine. Tyr indicates tyrosine; Phe, phenylalanine; Asn, asparagine; Asp, asparatic acid; Ser, serine; Lys, lysine; Leu, leucine; and Arg, arginine. *Result of base analysis was significant but result of validating analysis using bootstrap resampling was not. Results of analysis were thus judged not to be statistically significant.

sheet, and position 156 is in the alpha helix of HLA class I molecule (Figure 2).[26,27] Position 9 constitutes peptide-binding pockets B and C, position 99 constitutes A, B, and D pockets, position 116 constitutes F pocket, and position 156 constitutes D and E pockets.[28] As a result, all amino acid positions elucidated in this study were important positions for peptide binding and T-cell recognition, although all substituted positions including positions at which residues are not accessible in the vicinity of peptide binding sites were analyzed.

To our knowledge, amino acid substitutions at position 9 (Tyr9A-Phe9A and Tyr9C-Ser9C) and position 99 (Tyr99C-Phe99C) were newly identified in the present study as responsible for severe aGVHD.

Ferrara et al reported that the amino acid substitution at position 116 in HLA class I molecule increased the risk for aGVHD, although the substituted amino acid was not taken into consideration.[29] In our study, specific amino acid substitution at position 116 had a significant effect in HLA-C (Leu116C-Ser116C) and a marginal effect in HLA-A (Asn116A-Asp116A) for severe aGVHD (Table 5).

Position 156 of HLA molecule was certified to modify T-cell alloreactivity in vitro in HLA-A2,[30-32] HLA-B35,[33] and HLA-B44.[24] For example, in contrast to Asp156B in HLA-B*4402, the nonpolar nature of substituted Leu156B in HLA-B*4403 lost many interactions such as hydrogen bonds and van der Waals interactions with the other amino acid residues that constructed binding pockets. As a result, this substitution made the significant conformation change for alloreactivity.[24] In the HLA-B*3501 and HLA-B*3508 combination, Leu156B in HLA-B*3501 with nonpolar residue was substituted for Asp156B in HLA-B*3508 with polar residue, and induced strong alloreactivity.[33] In our study, the magnitude of the polar change of each substituted amino acid was calculated by "hydropathy scale,"[17] because the influence of this scale on the amino acid interaction was much greater than the influence of the isoelectric point.[34] Specific amino acid substitutions at position 9, 99, 116, and 156, which were not associated with KIR2DL ligand, were found to induce great polar changes except for Tyr9C-Ser9C. Generally speaking, the 3 major physicochemical properties of amino acids that play important roles in protein structure are the hydropathy scale, isoelectric point, and molecular weight, and molecular weight is reflected in the size of amino acids.[34] Indeed, although tyrosine and serine in Tyr9C-Ser9C show few differences in hydropathy scale and isoelectric point, their molecular weights are quite different and may well induce an important conformation change in the HLA molecule. Thus, the change in the conformation by the polar change of the HLA molecule might be one of the mechanisms inducing alloreactivity. These data serve to clarify the mechanisms of aGVHD based on the HLA molecule.

The analysis of HLA-B, -DRB1, -DPB1, and -DQB1 mismatch for the substitution of amino acid elucidated no responsible position for severe aGVHD, and the analysis of HLA-A elucidated only one position. We speculate that the reason for the above result in HLA class I was that in this population there were fewer HLA-mismatched pairs in HLA-A and -B than in HLA-C. Although the findings are due mainly to the HLA-C molecule, specific amino acid substitution at positions 9, 99, 116, and 156 on the HLA class I molecule may induce strong alloreactivity because the structures of HLA class I molecules are quite similar.[29] Indeed, position 9 is selected in HLA-A and -C concurrently, and position 116 had a significant effect on HLA-C and a marginal effect on HLA-A (Figure 2). In HLA class II, we speculated that the molecular base of aGVHD caused by the HLA class II mismatch might be different from that in HLA class I.

In conclusion, we clarified nonpermissive mismatch combinations of all major 6 HLA loci. These data would be beneficial for the selection of suitable donors and international donor exchange for UR-HSCT. Furthermore, we identified the positions and types of amino acid substitutions responsible for severe aGVHD and presented speculations for alloreactivity on the basis of the conformation change of the HLA molecule. These findings provide evidence to elucidate the mechanism of aGVHD on the basis of the HLA molecule.

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Authorship

Contribution: T.S., Y.M., T.K., T.J., and Y.K. participated in the conception of this study; K.K., H.I., and H.S. performed the execution for histocompatibility; Y.M. and S.K. performed the execution for transplantation; T.K. and K.M. performed statistical

data analysis; T.K. and Y.M. wrote the paper; all authors checked the final version of the paper.

A complete list of the institutions participating and registering patients through the Japan Marrow Donor Program for the present study is available on the *Blood* website; see the Supplemental Appendix link at the top of the online article.

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

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Unification of Hematopoietic Stem Cell Transplantation Registries in Japan and Establishment of the TRUMP System

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Abstract

There are 4 registries of hematopoietic cell transplantation in Japan; the Japan Society for Hematopoietic Cell Transplantation (JSHCT), Japanese Society of Pediatric Hematology, Japan Marrow Donor Program, and Japan Cord Blood Bank Network; each play an important role in society by reporting the number and outcomes of transplantations and contributing new findings obtained from studies on individual topics. However, there have been a number of issues with the difficulty of analyzing data in overlapping registries and multiple databases at centers affiliated with each of the 4 registry organizations. JSHCT was pivotal in orchestrating the computerization and unification of hematopoietic stem cell transplantation registries for the purpose of resolving these issues and providing a more accurate awareness of hematopoietic stem cell transplantations being performed in Japan. JSHCT played a central role in developing the "Transplant Registry Unified Management Program (TRUMP)" to enable transplantation institutes to manage patient information with emphases on convenience to institutes, safety of patient information, and quality of data management. While enhancing domestic registries, the program seeks to coordinate with other hematopoietic cell transplantation registries around the world to contribute to the development of registries throughout Asia.

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Key words: Hematopoietic stem cell transplantation; Registry; Unification

1. Introduction

To date, there have been 4 hematopoietic cell transplantation registries in Japan. These main 4 organizations are the Japan Society for Hematopoietic Cell Transplantation (JSHCT; the JSHCT Registry Office consists of the Adult Office and the Child Office), Japanese Society of Pediatric Hematology (JSPH; same as the JSHCT Child Office), Japan Marrow Donor Program (JMDP), and Japan Cord Blood Bank Network (JCBBN). The 4 registries developed and have been operating independently of one another. The results of data aggregation and analyses from each database were made public, thus providing clinicians with important feedback [1,2].

JSHCT (changed from the Japan Society for Bone Marrow Transplantation in December 1995) has been conducting nationwide surveys since 1993. The survey, which uses a paper questionnaire, is a voluntary registration of hematopoietic stem cell transplantations (HSCT) performed during the previous year. JSHCT also conducts follow-up surveys with registered patients who survive until the following year. Cumulatively, 19,118 transplantations have registered with the Adult Office through fiscal 2005 [2005]. To avoid duplicate registration,

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unrelated allogeneic bone marrow transplantations through JMDP have been registered only with JMDP since the 1999 survey. JSHCT issues an annual report based on its nationwide survey, integrating it with JSPH (JSHCT Child Office) and JMDP registry data. JSHCT publishes the aggregate number of transplantations performed by departments at each transplantation center and the survival analyses by disease, type of transplant, and donor. JSHCT also publishes this information on its website. There has also been a large number of studies on the present status of HSCT in Japan [3], as well as retrospective studies by individual topic [4-10].

In the pediatric field, the JSPH Bone Marrow Transplantation Committee has been conducting paper-based nationwide surveys since 1983. The survey is a voluntary registration of HSCTs performed during the previous year. Cumulatively, 8354 transplantations have registered with the Child Office through fiscal 2005 [1]. Reports on the present state of pediatric HSCT in Japan are published regularly [11-15].

Since its first transplantation in 1993, as of December 2005, the number of unrelated bone marrow transplantations through JMDP has reached 7017 [2]. JMDP conducts an initial survey on day 100 posttransplantation and follow-up surveys each subsequent year on transplantations performed at accredited centers throughout Japan, each of which is paper-based. Transplantation outcomes are reported regularly based on registry data [2], as well as many detailed analyses by disease and other topics [16-20].

There are 11 cord blood banks in Japan. Although each bank has operated individually since 1995, they formed a collective in 1999, establishing the JCBBN. Using different paper questionnaires, each of the 11 cord blood banks had collected clinical information after transplantation, but the standardized questionnaire was created in 2001. In November 2004, the number of unrelated cord blood transplantations through the cord blood banks eclipsed 2000. Results of aggregate analyses using the integrated JCBBN database are published on the JCBBN website. In addition, there have also been retrospective studies by topic [21,22].

The 4 registries played important roles in each field. On the other hand, however, the fact that these 4 registries are separate from one another makes it difficult to have a comprehensive understanding of all hematopoietic cell transplantations performed and increases the workload of multiple registrations at transplantation centers (Figure 1). Because all 4 organizations conducted paper-based surveys until 2005, there exists a disadvantage that no database was left at the institutions. Furthermore, there have still been few analyses that incorporated data from multiple databases due to the fact that the codes for each database were distinct from one another. This also led to difficulty in compiling data on the annual status of transplantations performed. As a result, we decided to develop a program named "Transplant Registry Unified Management Program (TRUMP)," which enabled registrants to manage transplantation patient information at their institutions.

2. Materials and Methods

The JSHCT Data Management Committee formed a working group in March 2004 for unification and computerization of data management on HSCT performed in Japan. We basically integrated survey items with those used by the 4 existing registries. As for common items, the choices were standardized. Table 1 summarizes survey items. There are approximately 700 survey items including all those specific for each disease. To leave the databases in the institutions, we decided to develop a program that enabled registrants to manage transplantation patient information. In developing the TRUMP system, we emphasized convenience of registrants (transplantation institutions), safety of patient information, and quality of data management. Entered data would be stored in electronic storage media as the registry data set and sent by mail from each institution to the JSHCT Data Center (Figure 2).

3. Results

3.1. Development of the TRUMP System

3.1.1. Convenience to Registrants at Transplantation Institutions

This program enables transplantation institutes to manage data of transplantation patients using patients' personal information including names and medical record numbers. On the screen with an all patients list, clinicians can see at a glance the list and outcome of patients who underwent transplantations at their institutions (Figure 3). The "Export" button enables one to produce a registry data set. With a single click of the "Export the data set" button on the data export screen, the program can automatically remove identifiable patient personal information, anonymize after assigning concatenation numbers, and then save the encrypted data set in the registrant's computer. Furthermore, using the "Export general data" button enables one to output the data set in CSV format and utilize them at the transplantation institutions for data aggregation and analysis.

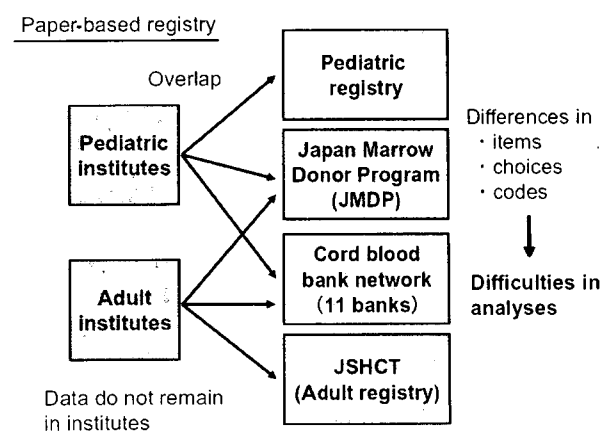

Figure 1. Hematopoietic stem cell transplant registry system before 2005. Previous system required multiple registrations from transplantation institutes to each registry. Because of the paper-based surveys, there exists a disadvantage that no database was left at the institutions. Furthermore, mismatch in the codes resulted in difficulties in compiling and analyzing the data.

Table 1.
Overview of TRUMP Survey Items*

Survey Overview	Survey Items
Disease information	
Basic information on disease	Name of disease
	Past medical history
	Underlying conditions
Detailed information on disease	Classification
	Stage at transplantation
	Chromosomal abnormalities, etc
Transplantation information	
Basic information	Patient age, weight, and PS at transplantation
	Medical history/transfusion history
	Past (previous) transplant history
Source of stem cells/donor type	Source of stem cells
	Donor information
	Bank management number, etc.
Blood type/HLA	Patient/donor blood type
	Patient/donor HLA (serotype/genotype)
	Degree of HLA match
Preparative regimens	Chemotherapeutic agents and dose
	Irradiation technique and dose
GVHD prophylaxis	Type of medication used
Stem cell collection	Quantity and cell count collected
Cell infusion	Presence of graft manipulation and type
	Infused cell count
Engraftment/hematological recovery	Engraftment and date of engraftment
	Date of recovery of trilineage
Cytokine/transfusion	Use of cytokine
	Transfusion quantity and date of last transfusion
Chimerism/secondary graft failure	Analysis results of posttransplantation chimerism
	Presence of secondary graft failure
Acute GVHD	Severity
	Therapeutic regimen and outcome
Chronic GVHD	Type and severity
	Region
	Therapeutic regimen and outcome
CMV prevention and early treatment	Presence of prophylaxis and regimen
	Information on CMV antigenemia and early treatment
CMV infection	Infected region
	Therapeutic regimen and outcome
Other viral infections	Name of virus/therapeutic regimen/outcome
Candida/aspergillus/other fungal infections	Name of fungus/infected region/outcome
Other infections/complications	Date of diagnosis, causative organism and outcome
	Information on complications
Outcome	Posttransplantation relapse
	Presence of secondary malignancy and diagnosis
	Donor lymphocyte infusion
	Late complications

*GVHD indicates graft-versus-host disease; CMV, cytomegalovirus.

The program includes various input support functions. Several tables have been provided to display definitions and severity scales and so forth on the input screen so that registrants can use them as reference. When a score on the appendix table screen is selected, the severity and score is automatically entered to the data base. There is a built-in program that automatically determines degree of HLA match. After entering a patient's HLAs, a "Same as the patient" button alleviates the registrant's task of having to re-enter a donor's HLAs.

In terms of patient information entered at each institution, we also provided a convenient function for creating backup files, along with an additional function that restores files that have already been backed up.

3.1.2. Quality of Data Management

Inquiries for items left blank and contradictory responses on paper-based surveys required a tremendous amount of work from data centers and transplantation institutes in the past. Consequently, we designed TRUMP surveys with as many response selections as possible and minimized the number of direct input fields. Furthermore, we installed a logical check function that checks for both fields left blank and contradictory data. After all information has been entered and the "Register" button is pressed, all error messages appear as an "Error list" on the screen that can then be printed or saved as a file.

Once the "Register" button is pressed, all modifications to patient information are saved as a history. The computer account stores "how" and "when" and "who" changed "which items". This modification history is included in the data sets for submission. For example, in the event where acute graft-versus-host disease was reported as "Present" and "Grade II" at the previous registration and then changed to "Absent," further inquiry would be required because of a possibility of some kind of input error. This modification history is also utilized at the data center for data management.

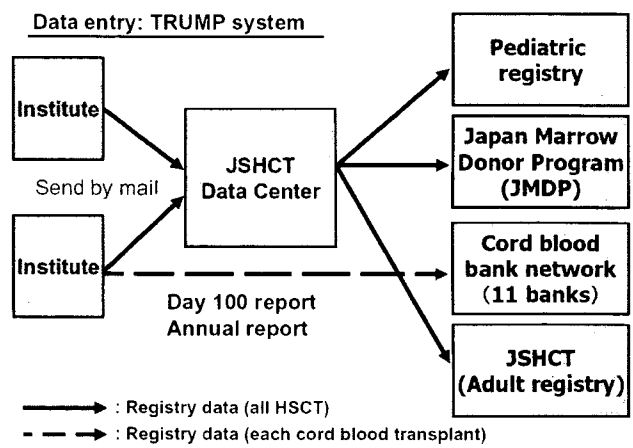

Figure 2. Destination and method of registry data after initiation of transplantation registry unified management. After the unification, data from each institute are sent to the Japan Society for Hematopoietic Cell Transplantation Data Center by postal mail. Only the Code Blood Bank Network requires direct data submission from the institutes. Each institute can use and analyze their original data under the new system.

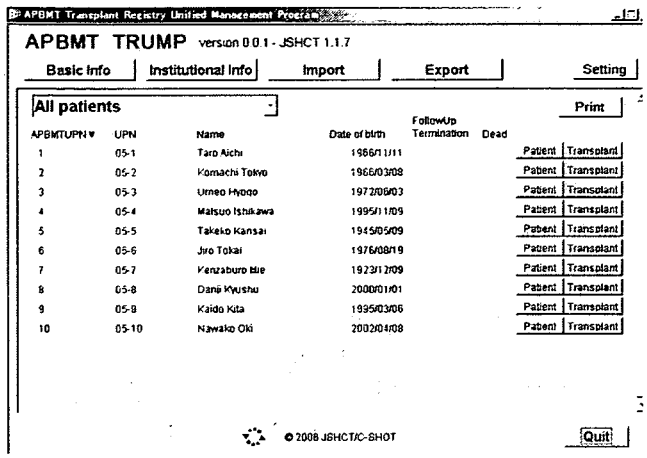

Figure 3. "Transplant Patient List" screen for the "TRUMP." In the TRUMP system, data can be managed in each institute using personal information including patient's name and medical record number. Data can be managed using patient's name, the most accurate identification item, in the institutes. By clicking the "Export" button, an anonymized and encrypted data file is produced.

3.1.3. Safety of Patient Information

We designed the program so that identifiable patient information would remain only at transplantation institutes. It was decided that transplantation patient information would be managed using the TRUMP system on transplantation institutes' computers not connected to a network. Each institute is responsible for individual information of their patients and data sets for submission are anonymized with an anonymous number automatically assigned by the program. The patients' anonymized clinical information is encrypted into a file for sent. The registry manager at each institute stores this file into an electronic storage device and sends it by mail to the JSHCT Data Center. Thus patient information is entered into the registry. An encrypted file cannot be opened even if it is mistakenly sent to another institute with the same program installed.

Each transplantation institute is accountable for the management of patient information. Access to the program can further be restricted through a password. Because data files that have been entered are encrypted and can only be opened using the TRUMP, any unauthorized person, even in the institute, cannot browse or change the transplantation data by using this function.

3.2. Operation of the TRUMP System

This program is a joint project between the 4 registry organizations and is run according to the following operational methods to ensure the smooth implementation of the program.

3.2.1. Basic Registration

In January of each year, we request "basic registration" data on all HSCTs during the previous one-year period. Registration items are minimum essentials to identify the number of transplantations performed, which includes date of transplantation, patient age at transplantation, number of transplantations, type of stem cell, donor type, and disease. The TRUMP contains a page for basic registration. Files are to be sent to the JSHCT Data Center.

3.2.2. Detailed Registration

In July of each year, transplantation institutes enter all information regarding HSCTs performed during the previous one-year period onto all relevant pages of the TRUMP. The institutes then deliver data sets for submission to the JSHCT Data Center. In January and July of each year, transplantation institutes deliver the anonymized and encrypted data sets to the JSHCT Data Center (Figure 2).

3.3.3. Bank Program Registry

With paper-based registrations up until fiscal 2005, bone marrow and cord blood banks had requested institutes to submit reports on day 100 after each transplantation. Because both banks provide bone marrow or cord blood for transplantations, they can identify the total number of transplantations as well as the exact reporting rate. The 100-day reporting rate for JMDP through the last fiscal year exceeded 99% due to extensive follow-up efforts. In order to maintain this excellent reporting rate, JMDP added a procedure to submit a registry data set to the JSHCT Data Center 100 days after each transplantation. The JSHCT Data Center then confirms the submitted data set and informs the JMDP ID to JMDP. JMDP confirms the reported ID and follows up with the clinicians at centers late in submitting reports.

The cord blood banks adopted a procedure by which transplantation institutes report patient data directly to the JCBBN. As shown in Figure 2, institutes are required to submit data to both the JSHCT Data Center and the JCBBN. Consistency between data sets in the JSHCT Data Center and the JCBBN is maintained and the data comparison can be done because data were entered at the same institute using the TRUMP and the modification history is saved in the submitted data.

3.3.4. Ethical Issues

To start the JSHCT TRUMP from fiscal 2006, the JSHCT Ethics Committee, comprising outside legal and nonmedical committee members, reviewed and approved the "Nationwide Survey of Hematopoietic Cell Transplantation." The review focused on research proposals, informed consent documents, and patient consent forms. Paper-based informed consent is obtained from each patient that the patient's anonymized clinical information are used for further clinical research purpose. The sheet is stored in institutes, and the information of informed consent is recorded and reported through the TRUMP. Because patients who have not given their consent bias a total number of transplantations and the registry data consists of anonymized clinical information, patient consent is not required at the initial registration. However, patients who have not given their consent are to be

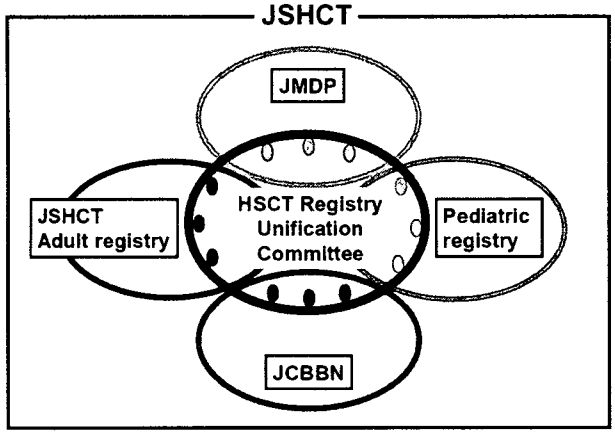

Figure 4. Organizational chart of data management and approval committee. Data management and approval committee of the unified transplantation registry consists of members from Japan Society for Hematopoietic Cell Transplantation, Japanese Society of Pediatric Hematology, Japan Marrow Donor Program, and Japan Cord Blood Bank Network.

excluded from the follow-up surveys and inquiries to the institutes using the anonymous numbers.

3.2.5. Agencies for Data Use Approval

Each registry has a committee for data use approval, such as a "Data Management Committee" or its equivalent. After implementation of the TRUMP, the JSHCT Hematopoietic Cell Transplant Registry Unified Management Committee gives approval to data use for research that requires data sets in multiple registries (Figure 4).

4. Discussion

We reported on the unification of the HSCT registries in Japan and establishment of the TRUMP system. There are two large-scale hematopoietic cell transplantation registries in the world. One is the Center for International Bone Marrow Transplant Registry (CIBMTR) in the United States and the other is the European Group for Blood and Marrow Transplantation (EBMT).

IBMTR was established in the Medical College of Wisconsin, the United States, as a voluntary registry for HSCT information from transplantation centers in 1972. Not limited to centers within the United States, transplantation centers worldwide participate in the registry, including several centers in Japan. According to a 2005 report, over 400 transplantation centers from 47 countries participate. Over 300 studies have been published using this registry data in over 30 years since establishment [22-30]. Collaborating with the research wing of the National Marrow Donor Program, a bone marrow bank in the United States, the CIBMTR was built in July 2004, and has conducted research activities on registries and prospective clinical studies.

EBMT is a nonprofit organization based in Maastricht, The Netherlands, founded in 1974 in order to allow scientists and physicians involved in HSCT to conduct independent research activities. EBMT has working parties on diseases such as acute and chronic leukemia, as well as other research topics such as total body irradiation. Each working party is responsible to its data registration. In addition to these working parties, national registries participate in the building of the definitive EBMT database. EBMT publishes annual reports [31-33] as well as many analysis results from each working party [34-37].

CIBMTR and EBMT have agreed to share the same basic survey items since 1999 (information from EBMT data center). At the time of deciding survey items of TRUMP, we investigated the forms of CIBMTR and EBMT, as well as those of the 4 Japanese registries. With small number of exceptions on the items of patients' background diseases, all of their basic survey items are covered in our program.

It is important that establishment and maintenance of a system to capture the accurate number and outcomes of HSCTs performed in Japan. These can be accomplished through improving the rationality of both transplantation centers and registry organizations and improving the data quality and registration rate. Based on the current status of hematopoietic cell transplantation registries around the world, we must contribute to the development of registries throughout the Asia-Pacific region. Also, we seek to coordinate with IBMTR and EBMT, by participating in the discussion on survey items and to perform transplantation activity survey in collaboration.

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Bone Marrow Transplantation (2007) 40, 165–174

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ORIGINAL ARTICLE

The HLA-A*0201-restricted minor histocompatibility antigen HA-1^H peptide can also be presented by another HLA-A2 subtype, A*0206

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HA-1^H is one of the most attractive minor histocompatibility antigens (mHA) as a target for immunotherapy of hematopoietic malignancies, but HLA-A*0201 and HLA-B60 molecules capable of presenting HA-1^H-derived peptides are less common in eastern Asian populations when compared with Caucasian populations. Therefore, an attempt was made to search for novel epitopes presented by HLA alleles other than those previously reported by generating CTL lines from patients undergoing HLA-identical, HA-1 disparate hematopoietic stem cell transplantation (hematopoietic SCT) by stimulation with a 29-mer HA-1^H peptide spanning a central polymorphic histidine (His). Two CTL clones established were found to be restricted by HLA-A*0206, which is the second or third most common HLA-A2 subtype worldwide. Epitope mapping revealed that the clones recognized the same nonameric peptide as A*0201-restricted HA-1^H, VLHDDLLEA. This epitope was unexpected, since it does not contain any preferred anchor motifs for HLA-A*0206. However, an HLA peptide binding assay revealed stronger binding of this peptide to A*0206 than to A*0201. Interestingly, HLA-A*0206-restricted CTL clones could lyse both HLA-A*0206$^+$ and HLA-A*0201$^+$ targets (including leukemic blasts) that express HA-1^H peptide endogenously, whereas an HLA-A*0201-restricted, HA-1^H-specific CTL clone failed to lyse HLA-A*0206$^+$ targets. This finding will expand the patient population who can benefit from HA-1^H-based immunotherapy.

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Keywords: minor histocompatibility antigen; graft-versus-leukemia; HLA-A*0206; cytotoxic T lymphocyte; HA-1

Introduction

Minor histocompatibility antigens (mHAs) are MHC-bound peptides derived from cellular proteins, which are immunogenic because of their differential expression between the donor and recipient, most often due to a single nucleotide polymorphism (SNP).[1,2] To date, significant efforts have been made to identify mHAs, particularly those specific for hematopoietic cells, since such mHAs have been speculated to contribute to the GVL effect following allogeneic stem cell transplantation (allogeneic SCT). Among these, HA-1, whose immunogenicity is controlled by a nonsynonymous SNP (dbSNP cluster ID: rs1801284) on the *HMHA1* gene (GeneID: 23526),[3] has been studied most extensively since it can be used for immunotherapy of recurring hematological malignancies after HA-1-mismatched SCT.[4] The originally reported HA-1 mHA is an HLA-A*0201-restricted nonameric peptide carrying His (HA-1^H) but not Arg (HA-1^R) at position 3.[3] In an effort to expand the patient population for HA-1-based immunotherapy, a decameric peptide containing His at position 6 from the same polymorphic region has been identified as an mHA presentable by the HLA-B60 molecule.[5] The only other mHAs with selective expression in hematopoietic cells described to date are HA-2,[6,7] BCL2A1 (ACC-1 and ACC-2),[8,9] HB-1[10,11] and PANE1,[12] the latter two of which are B cell lineage-specific. Thus, identification of novel hematopoietic system-specific mHAs is warranted to facilitate the development of effective immunotherapy to induce GVL reactions.

HLA-A*0201 is the most common allele in HLA-A2 subtypes and is distributed worldwide, especially among the general Caucasian populations (according to unpublished data from the 12th International Histocompatibility Workshop (IHW)). The next most common HLA-A2 subtypes appear to be HLA-A*0206 and/or -A*0207, which are found frequently in the eastern Asian populations, including Chinese and Japanese populations.[13,14] To date, it has not been examined whether an HLA-A*0201-restricted HA-1^H mHA epitope can be presented by HLA-A*0206, which possesses a single amino-acid substitution (9Phe-9Tyr) in the B-pocket compared with HLA-A*0201,[15] probably owing to the rarity of the HLA-A*0206 allele in

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Caucasian populations. While attempting to identify HLA alleles capable of presenting HA-1 mHAs other than the previously reported HLA-A*0201 and HLA-B60,[5] we unexpectedly found that HLA-A*0206 was able to present the nonameric peptide originally described as the A*0201-restricted HA-1^H mHA (VLHDDLLEA). These findings may be clinically beneficial for SCT recipients expressing HLA-A*0206, which accounts for 20–40% of HLA-A2 alleles in eastern Asia, by broadening the potential clinical relevance of an epitope that can be used for HA-1 mHA-based immunotherapy.

Materials and methods

Patients, cell culture and clones

This study was approved by the Institutional Review Board of Aichi Cancer Center according to the Declaration of Helsinki. All blood samples were collected after written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque (Pharmacia, Uppsala, Sweden). CD4$^+$ cells were isolated from donor PBMCs using anti-human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), expanded with 5 μg/ml PHA-L (Roche, Mannheim, Germany), 20 U/ml interleukin (IL)-2 (Chiron, Emeryville, CA, USA) and 20 ng/ml IL-7 (R&D Systems, Minneapolis, MN, USA) as previously reported,[16] and used as antigen presenting cells (APCs). CD3$^+$ cells were isolated from recipient PBMCs using anti-human CD3 microbeads (Miltenyi Biotec) and used as responder cells. CD34$^+$ leukemic cells were isolated from bone marrow specimens from acute myelocytic leukemia patients using anti-human CD34 microbeads (Miltenyi Biotec) and used as target cells. Epstein–Barr virus-transformed B lymphoblastoid cell lines (LCL) derived from donors, recipients and normal volunteers and the HLA class I-negative, LCL, 721.221 cell line[17] were maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (IBL, Takasaki, Japan). LCLs were transduced with retroviral vectors carrying individual HLA cDNAs by a method described previously.[18] HLA cDNA-transfected cell lines are referred to by their 'cell line name/HLA allele' designation (e.g. 721.221/A*0201). EH6-CTL is a CD8$^+$ clone previously generated from an HLA-A*0201$^+$ and HA-1^R healthy individual.[18]

Synthetic peptides and in vitro *CTL induction*

The peptide used for CTL induction consisted of 29 amino acids from the HA-1 polymorphic region with the histidine (underlined) in the middle (RFAEGLEKLKECVLHDDL-LEARRRRAHEC). All peptides were synthesized using standard Fmoc chemistry and dissolved in 100% DMSO.

Donor-derived PHA-activated CD4$^+$ cells were pulsed with the 29-mer peptide (50 μg/ml) for 2 h at room temperature in AIM-V medium (Invitrogen, Carlsbad, CA, USA). CTL lines were generated from post-SCT CD3$^+$ cells (5×10^4) by coculturing with irradiated (35 Gy) peptide-pulsed CD4$^+$ cells (5×10^4) in 0.2 ml RPMI 1640 supplemented with 6% pooled human serum, 2 mM L-glutamine and penicillin/streptomycin (referred to as CTL medium) in the presence of recombinant human IL-6 (20 ng/ml, R&D Systems) and IL-12 (20 ng/ml, R&D Systems) in a 96-well round-bottomed plate. IL-2 (10 U/ml) was added on day 3. Individual wells were similarly restimulated weekly with irradiated, peptide-pulsed (10 μg/ml) donor CD4$^+$ cells without cytokines except for IL-2 (10 U/ml) on day 1. After the fourth stimulation, T cells in culture wells showing lytic activity to both recipient LCL and 29-mer peptide-pulsed donor LCL but not unpulsed donor LCL were combined and cloned by limiting dilution. Putative CTL clones were expanded as previously described[19] and frozen until use. The cytotoxic activity of CTL lines and clones was evaluated by standard ^{51}Cr release assays. Percent specific lysis was calculated as follows: Experimental c.p.m.−spontaneous c.p.m./(maximum c.p.m.−spontaneous c.p.m. × 100. For tetramer blocking experiments, 0.25×10^6 CTL clones were incubated with 40 μg/ml of individual tetramers in PBS containing 2% fetal calf serum at room temperature for 15 min, then washed twice before coculturing with either 721.221/A*0201 or 721.221/A*0206 target cells.

Genotyping of HA-1 polymorphisms

Genomic DNA was isolated from each B-LCL with a DNA blood kit (QIAGEN, Valencia, CA, USA) and amplified by PCR. The primer sequences and amplification conditions used to amplify *HMHA1* gene encoding the HA-1 polymorphic region were (obtained from http://snp.ims.u-tokyo.ac.jp/cgi-bin/SnpInfo.cgi?SNP_ID = IMS-JST118551) as follows:

sense, 5′-CTGACCTCTGGCCTTTGACC-3′
antisense, 5′-ATCTGAGCCTCCCTCCCTTC-3′

PCR products were purified and directly sequenced with the same primer and a BigDye Terminator kit (ver. 3.1; PE Applied BioSystems, Foster City, CA, USA) using an ABI PRISM 3100 (PE Applied Biosystems).

Epitope reconstitution assay

^{51}Cr-labeled HA-1^R LCLs from either HLA-A*0201$^+$ or -A*0206$^+$ individuals were incubated for 30 min in medium containing 10-fold serial dilutions of test peptides and then used as target cells in standard 4 h cytotoxicity assays.

HLA peptide binding assay

A quantitative ELISA-based assay capable of measuring the affinity of the interaction between peptide and HLA was carried out as described previously,[20] with some modifications. In brief, purified recombinant HLA molecules in 8 M urea, 10 mM EDTA, 25 mM 2-(*N*-morpholino)-ethanesulfonicacid and 0.1 mM dithiothreitol were diluted to 4 μg/ml in refolding buffer containing 400 mM arginine, 100 mM Tris pH 8.0, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.2 mM phenylmethyl sulfonyl fluoride (all from Sigma-Aldrich) and 2 μM purified β2-microglobulin (β2m) on ice. Ten-fold dilutions of each peptide were made with 100% DMSO in 96-well round-bottomed polypropylene plates, then 1 μl individual aliquots were transferred into new plates and 99 μl of the above HLA-β2m mixture was added to each

well (i.e. 100-fold dilution for each peptide solution). The plates were incubated on a shaker at 4°C for 48–72 h. One day before ELISA analysis, 96-well ELISA plates (Costar, Cambridge, MA, USA) were coated with 50 μl/well W6/32 MoAb (10 μg/ml) in 50 mM carbonate–bicarbonate buffer, pH 9.6 (Sigma), and kept overnight at 4°C. After washing thrice with washing buffer containing 0.05% Tween 20 (Sigma-Aldrich) in PBS, the wells were blocked for 1 h. Just before the ELISA analysis, the reaction volume was diluted 10 times by PBS at 4°C, and 50 μl/well of aliquots were transferred in duplicate to the W6/32 MoAb-coated plates. The plates were incubated for 2 h at room temperature and then washed six times. To detect properly refolded complexes, plates were incubated for 2 h at room temperature with 100 μl/well of a horseradish peroxidase-conjugated anti-human β2m MoAb (1:1000 dilution; DAKO, Copenhagen, Denmark), followed by washing as above. Finally, color development was performed with 3,3′,5,5′-tetramethyl-benzidine as substrate (Sigma-Aldrich).

Tetramer construction and flow cytometric analysis
HLA-A*0201 or HLA-A*0206 tetramers incorporating the HA-1^H peptide (VLHDDLLEA) were produced as described previously.[21] For staining, cells were incubated with the tetramers at a concentration of 20 μg/ml at room temperature for 15 min followed by FITC-conjugated anti-CD3 (Becton-Dickinson, San Diego, CA, USA) and Tricolor anti-CD8 MoAb (Caltag, Burlingame, CA, USA) on ice for 15 min. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson).

Determination of TCR variable (V)-gene usage
TCR V-gene usage was assessed by RT-PCR using primers covering the entire families of functional TCR alpha (TCRAV) and beta (TCRBV) chains. Briefly, total RNA was extracted from individual CTL clones and cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and primer $p(dT)_{15}$ (Roche) and used for PCR. For TCRBV usage determination, each reaction was carried out with an optimal concentration of sense primers specific for four or five different BV families and a primer specific for the constant region of TCRB.[22] For TCRAV usage determination, each reaction was carried out with a single sense primer specific for individual alpha families and a primer specific for the constant region of TCRA.[23] The PCR products were size-fractionated on 2% agarose gels. Subsequently, the complementarity-determining region 3 (CDR3) of each positive PCR product was sequenced with corresponding antisense primer as above. TCR V-gene usage was determined by the international ImMuno GeneTics information system (IMGT) software, IMGT/V-QUEST (http://imgt.cines.fr).[24]

Results

Induction of CTL lines reactive to 29-mer HA-1^H peptide
A cohort of 28 patients were enrolled into our clinical study to generate mHA-specific T cells. Three of the 28 patients were found to have received an HA-1 disparate (namely, from HA-1$^{R/R}$ to HA-1$^{H/H}$ or HA-1$^{H/R}$) transplant. We chose the two patients negative for HLA-A*0201 since the presence of HLA-A*0201 was expected to induce T cells mostly specific for the immunodominant A*0201-restricted HA-1^H mHA. The two patients (designated by unique patient numbers (UPN)) and their corresponding HLA types were UPN012 (A*1101/2402, B*3901/5101, Cw*0702/1402, DRB1*0803/1302) and UPN022 (A*0206/3303, B*4006/4403, Cw*0801/−, DRB1*0901/1302). Both patients were typed as HA-1$^{H/R}$ and received HA-1$^{R/R}$ marrow from their donors. Peripheral blood CD3$^+$ cells obtained at days 102 and 196 from UPN012 and at days 28 and 99 from UPN022 were stimulated with peptide-pulsed, activated CD4$^+$ APCs prepared from their respective donor PBMC. Out of the four sets of cultures, two of 18 T-cell lines induced from day 99 CD3$^+$ cells of UPN022 preferentially lysed the recipient LCL and 29-mer HA-1^H peptide-pulsed donor LCL (Supplementary Figure 1). After the two lines were combined and cloned by limiting dilution, four putative CD8$^+$ CTL clones with the same specificity as the original T-cell lines were obtained (Table 1).

To test the HLA restriction of these putative clones, HLA class I-deficient 721.221 LCL were transduced with individual HLA class I cDNA of UPN022 and used as targets because this cell line was found to be positive for the HA-1^H allele. All four putative CTL clones demonstrated an identical pattern of HLA restriction, which was HLA-A*0206. Interestingly, these clones also lysed 721.221/A*0201 efficiently (Table 1). In contrast, EH6-CTL, a CD8$^+$ clone previously generated from an HLA-A*0201$^+$ and HA-1^R healthy individual,[18] was only able to lyse 721.221/A*0201 but not 721.221/A*0206, suggesting a higher degree of specificity. Out of the four putative clones from UPN022, 3B11-CTL and 4B1-CTL were found to be clonal by TCRBV usage analysis. Since the latter 4B1-CTL grew better, further experiments were carried out with it in comparison with EH6-CTL.

HLA restriction of 4B1-CTL was further tested using a panel of HLA-typed LCLs. As shown in Table 2, 4B1-CTL lytic activity was dependent on the presence of the HA-1^H allele and either the HLA-A*0206 or HLA-A*0201 allele. UR6 LCL carrying the HA-1^H allele and HLA-A*0207 was not lysed.

*Identification of the minimal epitope for the HLA-A*0206-restricted 4B1-CTL clone*
It has been shown that HLA-A*0206 has a single dominant anchor motif of Val at position 2 (P2), whereas A*0201 has two dominant anchor motifs, Leu at P2 and Val at P9.[25,26] Because HLA-A2 molecules incorporate nonameric peptides preferentially, we searched for such nonamers with Val at P2 in the 29-mer peptide used in the stimulation. One peptide, CVLHDDLLE, which had a Leu at P3 as a strong anchor motif for A*0206 as well, was the only candidate to fulfill the requirement. We conducted peptide reconstitution assays using 4B1-CTL and LCL from an HLA-A*0206$^+$, HA-1^R individual by pulsing the following peptides: CVLHDDLLE, its allelic variant CVLRDDLLE,

Table 1 HLA restriction element of putative CTL clones isolated from UPN022

Targets	*Putative CTL clones*				
	1E3	*2C3*	*3B11*	*4B1*	*EH6*[a]
% Specific lysis (E:T = 10:1)					
UPN022 Recipient LCL	73	68	70	66	−4
Donor LCL	3	2	−2	−1	−3
29-mer peptide[b] + donor LCL	72	73	71	71	3
721.221 LCL transfected with[c]					
HLA-A*0206	82	67	69	70	2
HLA-A*3303	2	−1	−1	−2	1
HLA-B*4406	5	0	−1	0	1
HLA-B*4403	5	−1	−1	1	1
HLA-Cw*0801	5	2	4	4	−1
HLA-A*0201	82	69	72	74	56
Clonality assessed by TCR usage[d]	No	No	Yes	Yes	Yes
Cell surface phenotype	CD8$^+$	CD8$^+$	CD8$^+$	CD8$^+$	CD8$^+$

Abbreviations: CTL = cytotoxic T lymphocyte; HLA = human leukocyte antigen; LCL = lymphoblastoid cell lines; TCR = T cell receptor for antigen.
[a]EH6, HLA-A*0201-restricted, HA-1^H-specific CTL clone.[18]
[b]Peptide used for induction (see Materials and methods).
[c]HLA class I-deficient LCL retrovirally transduced with individual HLA cDNA as reported previously.[18]
[d]The complementarity-determining region 3 of T-cell receptor β chain was sequenced by reverse transcription-PCR as previously reported.[22]

Table 2 Detailed restriction analysis of CTL clone 4B1 for HLA-A2 subtypes

Target[a]	*HLA-A*[b]	*HA-1 status*	*% Specific lysis*[c]
UPN022 recipient	0206, 3303	H/R	58
UPN022 donor	0206, 3303	R/R	0
UR1	0206, 2402	H/H	64
UR2	0206, 2402	R/R	2
UR3	0206, 0201	R/R	3
UR4	0201, 1101	H/R	57
UR5	0201, 2601	R/R	6
UR6	0207, 2402	H/R	4
UR7	2402, —	H/R	0
UR8	3101, —	H/R	0

Abbreviations: CTL = cytotoxic T lymphocyte; HA = histocompatibility antigen; HLA = human leukocyte antigen; LCL = lymphoblastoid cell lines.
[a]UR, LCL derived from unrelated individuals.
[b]HLA alleles shared with the UPN022 are underlined.
[c]A standard cytotoxicity assay was carried out at the E:T ratio of 30:1.

the original HA-1/A*0201 peptides (VLHDDLLEA and VLRDDLLEA) and decameric peptides of HA-1/A*0201 with a C-terminus extension of Arg (Figure 1a). Unexpectedly, 4B1-CTL recognized the HA-1^H/A*0201 peptide at half maximal lysis of ~1 nM and to lesser extent, its C-terminal extended decamer but could not recognize the predicted CVLHDDLLE nonamer at all.

To confirm that the HA-1^H/A*0201 peptide is indeed incorporated into the HLA-A*0206 molecule, an HLA peptide binding assay was carried out (Figure 1b). Surprisingly, the HA-1^H/A*0201 peptide was incorporated into HLA-A*0206 10-fold better than into HLA-A*0201 as assessed by means of quantifying peptide–MHC–β2m complexes refolded successfully.

Next, recognition of the HA-1^H/A*0201 peptide in the context of the two HLA-A2 subtypes was assessed by cytotoxicity assay. As expected from the results shown in Tables 1 and 2, 4B1-CTL recognized the HA-1^H/A*0201 peptide when pulsed onto either HLA-A*0206$^+$ or HLA-A*0201$^+$ LCL generated from HA-1^R individuals although recognition of HLA-A*0201-presented peptide was 10-fold lower (30 pM for HLA-A*0206$^+$ LCL versus 300 pM for HLA-A*0201$^+$ LCL by half maximal lysis; Figure 1c). In marked contrast, EH6-CTL recognized the HA-1^H/A*0201 peptide only when pulsed onto LCL generated from an HLA-A*0201$^+$ HA-1^R individual with a similar efficiency to that for 4B1-CTL. In addition, recognition of the HA-1^H/A*0201 peptide endogenously generated and presented by the two HLA-A2 subtypes was tested using the two CTL clones. As shown in Figure 1d, 4B1-CTL recognition of HA-1^H peptide presented by HLA-A*0206 was consistently better over the wide range of E:T ratio than both 4B1-CTL and EH6-CTL recognition of peptide presented by HLA-A*0201. These results indicate that the HA-1^H/A*0201 peptide, VLHDDLLEA, is presentable by both HLA-A2 subtypes and is sufficiently immunogenic. 4B1-CTL generated from an HLA-A*0206$^+$ SCT recipient showed less fine specificity in terms of scaffold molecules presenting VLHDDLLEA than EH6-CTL generated from an HLA-A*0201$^+$ individual.

Phenotypic and functional analyses using tetramers

We prepared HLA-A*0206 and HLA-A*0201 tetramers incorporating the HA-1^H/A*0201 peptide, VLHDDLLEA, and stained two CTL clones. As expected from the results shown in Table 2, 4B1-CTL was stained not only with A*0206 tetramer but also with A*0201 tetramer, although mean fluorescence intensity with the latter tetramer was nearly three-fold lower (67.5 versus 194.5; Figure 2, upper panels). In contrast, EH6-CTL was stained weakly with A*0206 tetramer (12.1%), while it was strongly stained with the cognate A*0201 tetramer (Figure 2, lower panels).

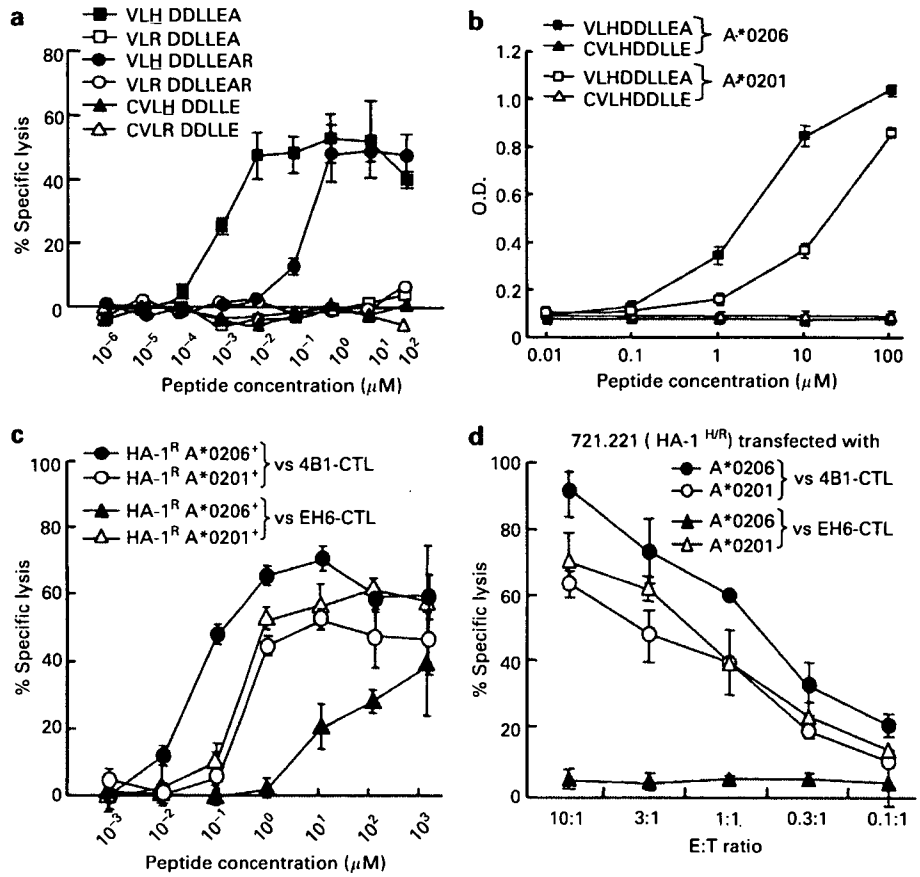

Figure 1 Identification of minimal epitope by epitope reconstitution and peptide-HLA binding assays. (a) UPN022 donor LCL were labeled with ^{51}Cr and distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 min at room temperature, and then used as targets for HLA-A*0206-restricted 4B1-CTL in a standard ^{51}Cr release assay (E:T ratio of 10:1). Data shown are representative of two independent experiments and the experiments were performed in duplicate. All values with error bars indicated in all figures applicable are shown as mean ± s.d. VLHDDLLEA was originally described as an HLA-A*0201-restricted *HMHA1*-derived mHA.[3] (**b**) Peptide-HLA binding assays were carried out as previously described[20] with some modifications (see Materials and methods). Purified HLA-A*0206 or HLA-A*0201 molecules were folded in folding buffer containing β2m and the serially diluted peptides indicated for 48–72 h. Amounts of properly folded HLA-A*0206 or HLA-A*0201 molecules were assessed by ELISA using plate-coated, conformation-dependent anti-HLA class I (clone W6/32) and horseradish peroxidase-tagged anti-β2m MoAbs. Folding efficiency is expressed in optical density (OD) at 630 nm. Data shown are representative of three independent experiments and the experiments were performed in duplicate. (**c**) Epitope reconstitution assay with VLHDDLLEA peptide for 4B1-CTL and HLA-A*0201-restricted, HA-1^H-specific EH6-CTL. HA-1^R-homozygous LCL positive for either HLA-A*0206 or HLA-A*0201 were pulsed with serial dilutions of VLHDDLLEA peptide for 30 min at room temperature and lytic activity by either 4B1-CTL or EH6-CTL was plotted. Data shown are representative of four independent experiments and the experiments were performed in duplicate. (**d**) Lytic activity of HLA-A*0206-restricted 4B1-CTL and HLA-A*0201-restricted EH6-CTL against HA-1^H-positive 721.221 LCLs transduced with either HLA-A*0206 or HLA-A*0201. A standard 4 h ^{51}Cr-release assay was carried out at the various E:T ratios indicated. Data shown are representative of two independent experiments and the experiments were performed in duplicate.

Next, we examined whether these tetramers could specifically block the recognition of target cells by the CTL clones (Figure 3a). The lysis of 721.221/A*0206 LCL by A*0206-restricted 4B1-CTL was efficiently inhibited by the cognate A*0206 tetramer, whereas inhibition with A*0201 tetramer was less significant. Similarly, the lysis of 721.221/A*0201 LCL by A*0201-restricted EH6-CTL was more efficiently blocked with the cognate A*0201 tetramer. Interestingly, A*0206 tetramer appeared to be able to block the recognition of EH6-CTL as well as A*0201 tetramer at the highest E:T ratio tested, suggesting that crossreactivity can be observed under certain conditions (consistent with the low degree of lysis by EH6-CTL of A*0206$^+$ LCL seen at high peptide concentrations in Figure 1c).

TCR variable region usage by HA-1^H-specific CTL clones

It has been shown previously that all HLA-A*0201-restricted clones specific for HA-1^H peptides from three individuals use the conserved TCRBV gene, TCRBV6S9,[27,28] corresponding to TCRBV7-9 by the IMGT classification.[24] We analyzed the variable and CDR3 usage of CTL clones, EH6, 4B1 and 3B11 (Table 3, and data not shown). Interestingly, not only HLA-A*0201-restricted EH6 but also the HLA-A*0206-restricted 4B1 and 3B11 CTL clones used the TCRBV7-9, although TRAV usage was different. The CTL clones 4B1 and 3B11 were found to have identical TCRAV, TCRBV and CDR3 sequences (data not shown). It is to be noted that HLA-A*0206-restricted 4B1-CTL had the Leu–Val amino-acid pair in the TCRB *N*-region (Table 3, underlined) which was

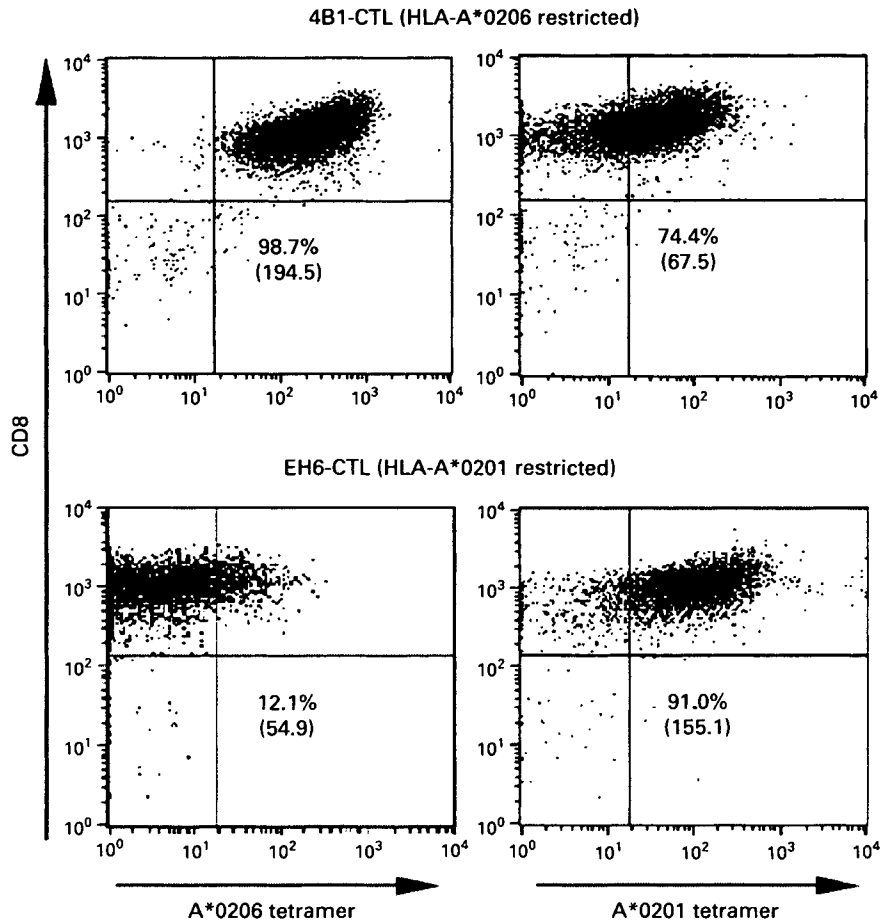

Figure 2 Representative staining profiles of CTL clones by tetramers. 4B1-CTL (upper panels) and EH6-CTL (lower panels) were stained with VLHDDLLEA peptide-incorporated HLA-A*0206 (left column) and HLA-A*0201 (right column) tetramers for 15 min at room temperature. Cells were washed and counterstained with CD3 and CD8 MoAbs, as described in Materials and methods. The percentage of T cells binding to the tetramer among all CD8$^+$ population is indicated in each panel and mean fluorescence intensity for tetramer$^+$ cells located in the upper right quadrant is shown in parentheses. Data shown are representative of three independent experiments.

also shown to be shared by HLA-A*0201-restricted HA-1^H-specific CTL clones.[27]

In vivo *relevance of the HLA-A*0206-restricted HA-1^H peptide-specific CTL*

Since the *HMHA1* gene is highly expressed in hematopoietic cells including hematopoietic malignancies,[3] we sought to examine whether 4B1-CTL could lyse leukemic CD34$^+$ cells carrying HA-1^H. As shown in Figure 3b, the CTL clone lysed not only HLA-A*0206$^+$ blasts but also HLA-A*0201$^+$ blasts as efficiently as UPN022 recipient-derived LCL. In contrast, the EH6-CTL recognized HLA-A*0201$^+$ but not HLA-A*0206$^+$ blasts (data not shown).

Finally, we examined the *in vivo* presence of HLA-A*0206$^+$-restricted, HA-1^H-specific T cells by means of tetramers. To this end, we stained PBMCs obtained at day 98 after SCT from a patient (UPN011) who was positive for both HLA-A*0201 and HLA-A*0206 and received HA-1 disparate marrow transplant from an unrelated, HLA-A, -B, -DR-matched donor (UPN022 PBMCs were no longer available). As shown in Figure 3c, minor populations of unstimulated CD8$^+$ cells were stained by HLA-A*0206 and HLA-A*0201 tetramers (0.06 and 0.18%, respectively) and with very low fluorescence intensity (compared to staining of the 4B1-CTL clone in Figure 2, upper left panel). To ascertain that these minor tetramer$^+$ populations were indeed HA-1^H specific, PBMCs were stimulated with peptide-pulsed APCs and assayed. After stimulation, individual tetramers detected around 15% tetramer$^+$ CD8$^+$ cells with subpopulations of cells (9.5 and 5.7%, boxed in Figure 3c) staining as brightly as the 4B1-CTL and EH6-CTL clones, respectively. It is speculated that these populations might represent HA-1^H-specific CD8$^+$ T cells restricted by HLA-A*0206 and HLA-A*0201 molecules, respectively.

Discussion

Among human mHAs identified to date, HA-1^H is one of the most promising mHAs involved in GVL and graft-versus-tumor effects due to its limited expression in hematopoietic cells[3] and some epithelial cancers.[29,30]