These results imply that proinflammatory cytokines, in particular IL-6 and TNF-α, play a role in the pathogenesis of HHV-6 reactivation after HSCT.

Introduction

Human herpesvirus 6 (HHV-6) was originally isolated from patients with lymphoproliferative disorders and AIDS in 1986.[1] Although primary infection with HHV-6 variant B causes exanthem subitum,[2,3] the clinical features of infection with HHV-6 variant A remain unclear. Like other human herpesviruses, HHV-6 is thought to latently infect the body after a primary infection; it then becomes reactivated in immunosuppressed individuals. HHV-6 has recently been recognized as an opportunistic pathogen in transplant recipients.[4] It has been demonstrated that HHV-6 is associated with fever and a skin rash that resembles acute graft-versus-host disease (GVHD),[5–14] interstitial pneumonitis,[15,16] encephalitis,[17,18] cytomegalovirus (CMV) disease,[19] and bone marrow suppression[20–23] after hematopoietic stem cell transplantation (HSCT). Although it has been demonstrated that HHV-6 reactivation generally occurs approximately 2–3 weeks after transplantation in almost half of the HSCT recipients,[6,13,18] the mechanism of viral reactivation remains unclear.

HHV-6 is a member of the β-herpesvirinae, which contains two additional human herpesviruses: CMV and HHV-7. On the basis of molecular and biological analyses, HHV-6 has been shown to share a number of characteristics with CMV, the prototypic member of the β-herpesvirinae subfamily. Numerous *in vitro* and *in vivo* studies have been performed to elucidate the mechanisms of CMV reactivation, and it has been demonstrated that cytokine production, in particular the production of tumor necrosis factor (TNF)-α, is involved in reactivation.[24,25] TNF-α induces the expression of CMV immediate-early (IE) gene products, which potentially initiate viral replication after latency. Expression of CMV IE genes is controlled by IE promoter/enhancer regions, which contain binding sites for NF-κB, ATF (CREB), and Sp1. The NF-κB and ATF (CREB) binding sites are critical in the regulation of IE gene expression.[26,27] The R3 region of HHV-6 contains multiple putative binding sites for cellular transcription factors, including PEA3, NF-κB, and AP-2. Through interactions with NF-κB, this region strongly enhances the promoter activity of the U95 gene, a potential homolog of the murine CMV IE2 gene.[28] These findings suggest that cytokines may play an important role in HHV-6 reactivation in patients after organ transplant. Thus, we sought to elucidate an association between plasma cytokine levels and HHV-6 reactivation in HSCT recipients.

Materials and methods

Characteristics of the patients

Twenty-four patients received allogeneic HSCTs (22 of bone marrow transplant and 2 of umbilical cord blood transplant) at the Division of Hematology–Oncology at the Children's Medical Center (Japanese Red Cross Nagoya First Hospital) or the Department of Pediatrics at the Nagoya University School of Medicine between October 2002 and July 2004. The patients' guardians consented to their participation in this study. This study was approved by the review boards of the three institutes. Patient characteristics relating to age, gender, HLA matching, granulocyte-colony stimulating factor (G-CSF) treatment after transplantation, and underlying disease and clinical features, including skin rash, liver dysfunction, and acute GVHD, are summarized in Table 1.

Management of patients

Details of the conditioning regimen and GVHD prophylaxis are described elsewhere.[29,30] In brief, patients with hematological malignancies were conditioned with melphalan (180 mg/m^2) plus busulfan (16 mg/kg) or total body irradiation (12 Gy) with high-dose chemotherapy. Patients with severe aplastic anemia were conditioned with cyclophosphamide (50 mg/kg) and anti-thymocyte globulin (ATG; Lymphoglobulin, 15 mg/kg). GVHD prophylaxis consisted of methotrexate with or without cyclosporine. Patients received intravenous γ-globulin preparations weekly during the first 3 months as prophylaxis for CMV infection. Oral acyclovir at a dose of 300–1000 mg was given daily. Pre-emptive therapy against CMV infection using ganciclovir was given following a positive result in an antigenemia assay.

Experimental design

EDTA-treated peripheral blood was collected from the recipients at the time of HSCT, weekly for one month post-transplant, and then biweekly for another month. Clinical data were collected retrospectively and assessed to determine associations with HHV-6 reactivation.

Isolation and identification of HHV-6

The procedures for the isolation and identification of HHV-6 have been described elsewhere.[31] Briefly, peripheral blood mononuclear cells were cocultured with cord blood mononuclear cells in culture medium. Virus isolation was identified primarily by morphologic changes of the cultured cells, which became large and pleomorphic with a balloon-like shape. Virus isolation was confirmed by specific immunofluorescence labeling with monoclonal antibodies against HHV-6 (OHV-3), which were kindly provided by Dr. T. Okuno (Department of Microbiology, Hyogo Collage of Medicine, Hyogo, Japan). Because this monoclonal antibody is specific for variant B, all isolates that were labeled with the antibodies were considered to be HHV-6 variant B.

Serological assay to detect HHV-6 antibodies

IgG antibody titers to HHV-6 were measured with an indirect-immunofluorescence assay as described

Table 1 Characteristics and clinical features of the patients

Categories	Patients with an HHV-6 infection ($n = 14$)	Patients without an HHV-6 infection ($n = 10$)	*P* value
Gender (M/F)	6/8	5/5	>0.9999
Age/range (years)	6.6/0–16	5.7/0–18	0.688[a]
Underlying diseases			
Leukemia/lymphoma	10	4	0.2112
Others	4	6	
HLA matched/miss-matched	9/5	6/4	>0.9999
Conditioning			
Total body irradiation			
Yes	8	7	0.6785
No	6	3	
Anti-thymocyte globulin			
Yes	5	2	0.6529
No	9	8	
G-CSF administration			
Yes	13	7	0.2721
No	1	3	
Clinical events			
Fever	13	8	0.5504
Skin rash	13	3	0.0023
Liver dysfunction	10	5	0.4028
CMV disease	1	1	>0.9999
Acute GVHD	7	2	0.2099
Grade 1	1[b]	0	
Grade 2	3	0	
Grade 3	2	0	
Grade 4	1	2	

[a] Calculated using Student's *t*-test.
[b] Confirmed by skin biopsy.

previously.[32] A representative strain of HHV-6 variant B (FG-1), which was isolated from peripheral blood mononuclear cells obtained from an exanthem subitum patient, was used as the standard antigen. The antibody titer was defined as the reciprocal of the plasma dilution that showed specific fluorescence. Anti-HHV-6 antibody titers were measured in the plasma samples collected pre-transplant and 4 and 8 weeks post-transplant.

Measurement of serum cytokine levels

Plasma samples were processed immediately after collection and stored at −70 °C for the subsequent measurement of cytokine levels. The samples were collected at the time of transplantation (day 0), and 1 week, 2 weeks, and 4 weeks after transplantation. Plasma cytokine levels were determined using a commercially available immunoassay kit that allows the detection of TNF-α, IL-1β, IL-6, and IFN-γ (BioSource). Assays were carried out according to the manufacturer's instructions. All cytokine assays were performed in duplicate and are reported in picograms per milliliter as the mean ± SD.

Statistical analysis

The characteristics and clinical features of the recipients with an HHV-6 reactivation and those without a viral reactivation were compared using Fisher's exact tests. Unpaired comparisons between the recipient's age and the presence or absence of an HHV-6 reactivation were performed using Student's *t*-tests. Mean peak cytokine levels were compared between the recipients with and without an HHV-6 reactivation using the Mann–Whitney *U*-test. Associations between antibody responses and peak cytokine levels were also analyzed using the Mann–Whitney *U*-test. The statistical analysis was performed with StatView software, version J-5.0.

Results

Pattern of HHV-6 reactivation

A total of 24 patients were studied. All recipients were seropositive for anti-HHV-6 antibodies at the time of transplantation. HHV-6 was isolated from 9 of the 24

recipients (37.5%) after transplant; one isolate was recovered at the time of transplantation. Significant increases in anti-HHV-6 IgG antibody titers were observed in five of the nine recipients (55.6%) with viremia during the observation period (from the time of transplant to 6 weeks after transplant). An additional five recipients showed a significant increase in the level of HHV-6 antibodies. HHV-6 IgG antibody titers of γ-globulin preparations are generally ranged between ×16 and ×64. As levels of the antibody titers in all of the post plasma samples collected from the 10 recipients with immune response were ×128 and over, we thought that HHV-6 reactivation occurred in the recipients. If recipients had either HHV-6 viremia or significant increase in HHV-6 antibody titers, we defined that the recipient had HHV-6 reactivation. Therefore, HHV-6 reactivation occurred in 14 of the 24 recipients (58.3%; Fig. 1A). As shown in Fig. 1B, HHV-6 viremia was most commonly observed approximately 2–3 weeks post-transplant.

Demographics and clinical characteristics were compared between the recipients with and without an HHV-6 reactivation (Table 1). No statistically significant differences were observed between these two groups in the demographics and the clinical characteristics except for the skin rash ($P = 0.0023$).

The kinetics of the cytokine concentrations after transplantation

Total of 92 plasma samples serially collected from the recipients were used in this analysis. The kinetics of the concentration of each of the four cytokines is shown in Fig. 2. IL-6 levels were significantly higher in the recipients with an HHV-6 reactivation than in those without an HHV-6 reactivation at 1 week (54.8 ± 86.5 vs. 16.3 ± 51.2 pg/ml; $P = 0.0272$), 2 weeks (44.2 ± 45.6 vs. 0.4 ± 1.0 pg/ml; $P = 0.0002$), and 4 weeks (18.8 ± 33.3 vs. 4.4 ± 13.9 pg/ml; $P = 0.0137$). The IL-6 levels at the time of transplantation were not significantly different between the two groups (7.6 ± 15.8 vs. 0 pg/ml). The TNF-α level was significantly higher in patients with an HHV-6 reactivation than in those without an HHV-6 reactivation at 2 weeks (13.1 ± 13.1 vs. 3.0 ± 4.6 pg/ml; $P = 0.023$). No significant differences in the TNF-α levels were observed between the recipients with and without an HHV-6 reactivation at the time of transplantation (5.5 ± 8.6 vs. 6.5 ± 7.9 pg/ml), 1 week (1.3 ± 4.6 vs. 0 pg/ml) and 4 weeks (14.6 ± 7.9 vs. 9.4 ± 6.8 pg/ml). The levels of both IL-1β (pre-transplant: 0.6 ± 1.6 vs. 0 pg/ml; 1 week: 0 vs. 0.2 ± 0.6 pg/ml; 2 weeks: 0 vs. 0.2 ± 0.6 pg/ml; 4 weeks: 0.1 ± 0.4 vs. 0.1 ± 0.3 pg/ml) and IFN-γ (pre-transplant: 1 ± 3.7 vs. 0 pg/ml; 1 week: 0 vs. 0 pg/ml; 2 weeks: 4.2 ± 10.2 pg/ml; 4 weeks: 0 vs. 0 pg/ml) were not significantly different between the recipients with and without an HHV-6 reactivation at any of the examined time points.

An association between HHV-6 viremia and the kinetics of the cytokine concentrations

In order to clarify the roles of IL-6 and TNF-α in HHV-6 reactivation, the time course of HHV-6 reactivation and kinetics of the concentrations of these two inflammatory cytokines were examined in the seven recipients with HHV-6 viremia from whom the plasma samples were completely collected at each time point during the observation period

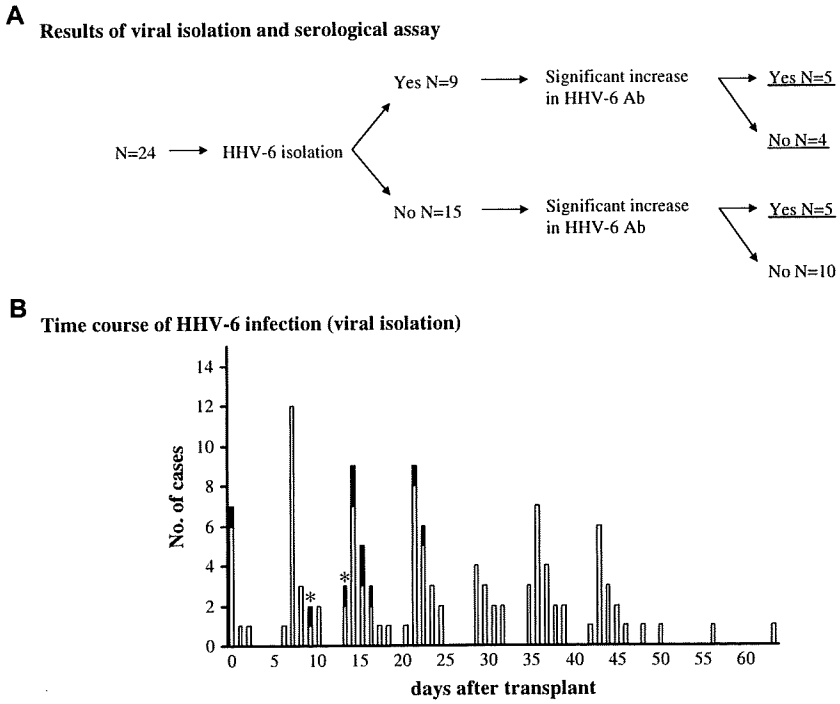

Figure 1 A summary of the virological examinations (A) and the time course of HHV-6 isolation (B). *HHV-6 was repeatedly isolated from same individual. Black boxes indicate positive HHV-6 isolation.

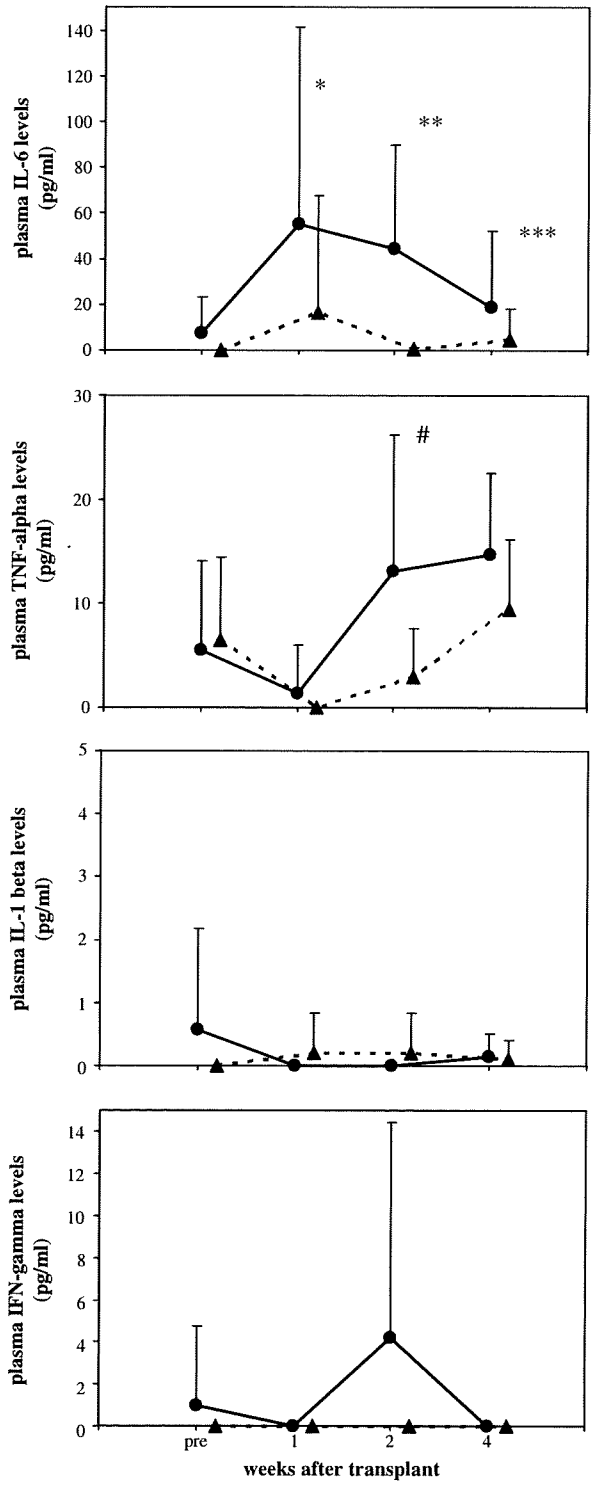

Figure 2 The kinetics of changes in the mean cytokine levels (IL-6, TNF-α, IL-1β, and IFN-γ) in serially collected plasma samples obtained from recipients with or without an active HHV-6 infection. Solid lines indicate cytokine levels in the recipients with an HHV-6 reactivation, whereas dotted lines indicate cytokine levels in the recipients without an HHV-6 reactivation. *$P = 0.0272$; **$P = 0.0002$; ***$P = 0.0137$; #$P = 0.023$.

(Fig. 3) (either the pre-transplant or 1 week sample was not obtained from the remaining two cases). In case 1 and case 2, elevated IL-6 levels were observed after HHV-6 viremia. On the other hand, elevated IL-6 levels were observed before the onset of HHV-6 viremia in cases 2, 4, and 5. Elevated IL-6 levels were concurrently observed with viremia in cases 3, 6, and 7, although subsequently, the cytokine levels significantly decreased in these cases. Slightly increased TNF-α levels were also observed at the time of viremia in cases 1, 3, 4, and 7. Time correlation between HHV-6 viremia and clinical events such as skin rash and acute GVHD was also demonstrated. These clinical features occurred soon after HHV-6 viremia except for case 5. As shown in Fig. 3, although all seven cases had HHV-6 viremia, a significant increase in the anti-HHV-6 antibody titer was only observed in four cases (cases 1, 2, 6, and 7). The peak IL-6 level measured during the observation period was significantly higher in the cases that mounted an antibody response than in the subjects that lacked a response (118.5 ± 4.2 vs. 54.7 ± 16.2 pg/ml; $P = 0.0339$). No statistical difference was observed between the two groups in the peak TNF-α level (15.0 ± 4.8 vs. 19.3 ± 1.5 pg/ml; $P = 0.2845$).

Discussion

In order to uncover an association between HHV-6 reactivation and cytokines, we performed virological examinations with serially collected samples from allogeneic HSCT recipients. As we expected, HHV-6 reactivation occurred in 58% of the recipients around 2–4 weeks after the transplant, which is consistent with previous reports.[6,13,18] Although it was difficult to determine the precise period during which viral reactivation took place in the five recipients who only showed serological responses, we believe that HHV-6 reactivation occurred around 2–4 weeks after the transplants, because significant increases in the anti-HHV-6 antibody titers were observed within the observation period (between 0 and 6 weeks after transplant). HHV-6 was isolated from one patient at the time of transplant. She was a 2-year-old Japanese girl with severe aplastic anemia. She had a high fever and a skin rash of unknown etiology starting 2 weeks prior to the transplant. Furthermore, she was ill when she underwent the transplant. It has been suggested that HHV-6 reactivation can occur in critically ill patients,[33,34] although the precise mechanism remains unclear. Thus, because the patient was ill at the time of her transplant, it is possible that HHV-6 could have reactivated at an earlier time point. Moreover, HHV-6 may have contributed to her symptoms, such as the fever and skin rash, which were observed before the transplantation.

It has been proposed that inflammatory cytokines play important roles in the pathogenesis of many adverse conditions, including acute GVHD in HSCT recipients.[35–38] Because cytokine production is affected by many factors in HSCT recipients, it is difficult to isolate an association between cytokines and HHV-6 reactivation. Although several characteristics of the recipients with and without an HHV-6 reactivation were compared, most of them were not significantly different. This included underlying

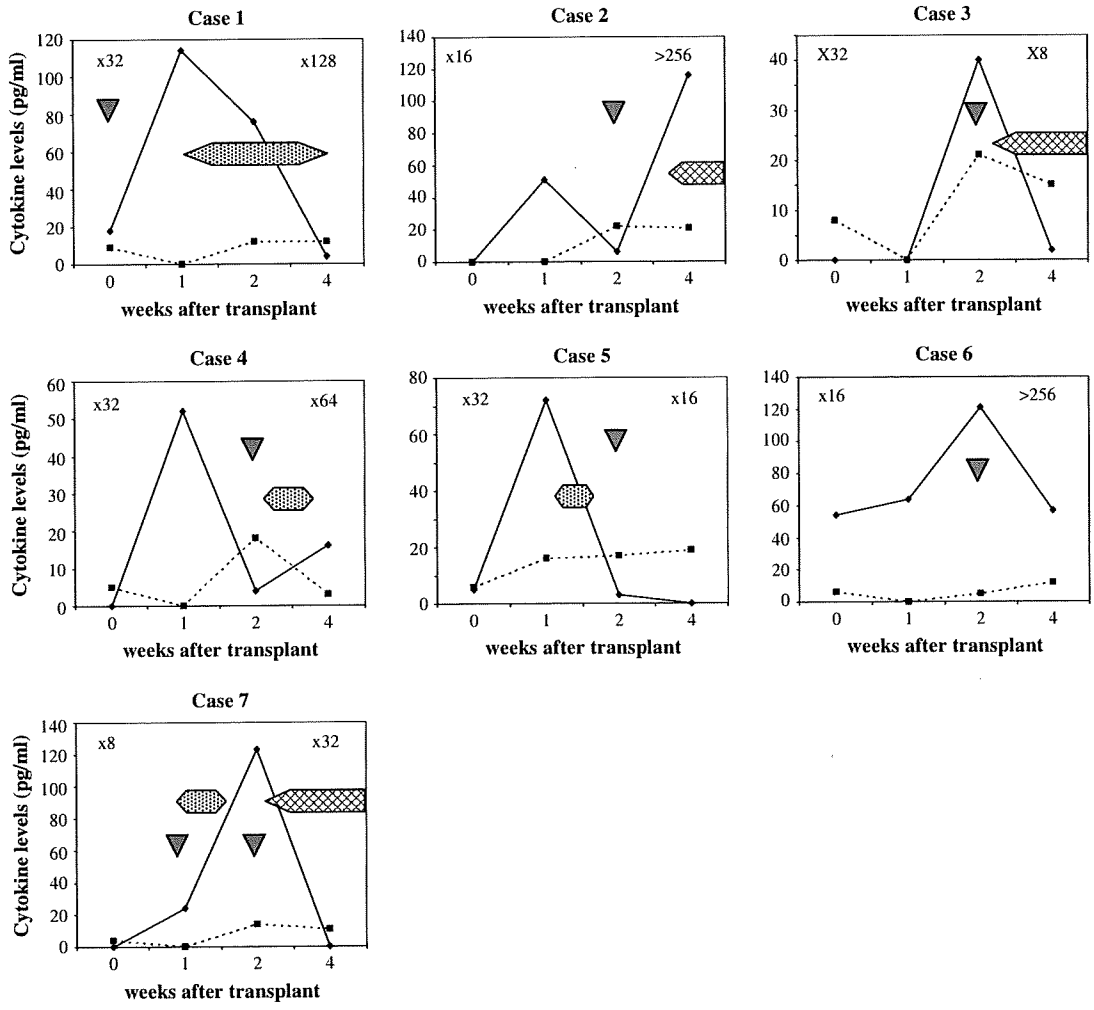

Figure 3 Kinetics of the changes in the levels of IL-6 and TNF-α and the results of virological examinations in the seven patients with HHV-6 viremia from whom plasma samples were collected at each time point during the observation period. Solid lines indicate IL-6 levels, and dotted lines indicate TNF-α levels. The arrowheads indicate the time points at which HHV-6 was isolated from the patient. The results of serological tests were demonstrated in each chart. Dotted bars indicate skin rash. Meshed bars indicate acute GVHD.

diseases, CMV disease, HLA matching, conditioning (total body irradiation and anti-thymocyte globulin), G-CSF administration after transplantation (within 3 weeks after transplantation), and acute GVHD, which may be associated with the production of host cytokines. As we reported previously,[6,12,13] only the skin rash that resembled acute GVHD was closely linked to HHV-6 reactivation. Therefore, we think that the observed increase in the production of the cytokines reflected HHV-6 reactivation and contributed to the skin rashes.

As shown in Fig. 2, plasma IL-6 levels were significantly higher in the recipients with an HHV-6 reactivation than in the recipients without a viral reactivation at 1 week and 2 weeks post-transplant, which was prior to or concurrent with the time at which they were determined to have a viral reactivation. Moreover, the TNF-α level was also higher in the recipients with an HHV-6 reactivation than in the recipients without a reactivation at 2 weeks post-transplant. Frequent HHV-6 reactivation has been suggested to occur in patients in critical condition[34] and in patients with multiple organ failure syndrome.[33] The authors proposed that under those conditions, inflammatory cytokines might have an important role in HHV-6 reactivation. Although the patient backgrounds in the present study and in their study are different, we think that our data support their hypothesis. To our knowledge, the results from this study are the first data that suggest that these two cytokines play a role in HHV-6 reactivation *in vivo*.

It has been suggested that monitoring IL-6 levels is predictive of CMV reactivations.[25] The authors proposed that monitoring cytokine levels could be beneficial for patient management. The measurement of IL-6 level at 1 week post-transplant might be useful for the prediction of HHV-6 reactivation in HSCT recipients. Further prospective analysis is now underway to confirm this hypothesis.

In vitro and *in vivo* experiments have demonstrated that HHV-6 can modulate the expression of host immune factors. The level of IL-6 was significantly higher in the

recipients with an HHV-6 reactivation than in the patients without viral reactivation at 4 weeks post-transplant. Although there was no statistically significant difference, the TNF-α level tended to be higher in the recipients with an HHV-6 reactivation at that time. HHV-6 has been demonstrated to upregulate the production of IL-1β and TNF-α by PBMCs.[39,40] Although IL-6 synthesis does not increase in HHV-6-infected PBMCs,[39] we observed the enhanced production of this cytokine in a virally infected neuroglial cell line.[41] Furthermore, elevated IL-6 levels have been observed in HHV-6-infected patients that exhibit frequent seizures.[42] Recent microarray analyses have demonstrated that the transcription of mRNA encoding inflammatory cytokines was upregulated in virally infected cells.[43] Although the alterations of the cytokine levels were of a lower amplitude than the results seen in *in vitro* analyses, our *in vivo* findings support the hypothesis that HHV-6 reactivation induces cytokine synthesis, which may be involved in the pathogenesis of the skin rashes that resemble acute GVHD in HSCT recipients. It has been suggested that HHV-6 is associated with the induction of acute GVHD.[7,8,10,14] Upregulation of cytokine production by HHV-6 reactivation might cause acute GVHD. As shown in Fig. 3, HHV-6 reactivation appears to be preceded by either skin rash or acute GVHD. This finding also supports the hypothesis that virus reactivation might be involved in the pathogenesis of these two clinical features. Further studies are necessary to confirm this hypothesis, which should allow us to improve the prognoses of these patients.

In order to better understand the association between HHV-6 reactivation and cytokine production, we examined the time course of viral reactivation (viremia) and the kinetics of changes in the concentrations of IL-6 and TNF-α (Fig. 3). Elevated levels of IL-6 or TNF-α that preceded HHV-6 reactivation were observed in cases 2, 4, and 5. HHV-6 isolates were obtained from cases 3, 6, and 7, which also had elevated levels of one of these cytokines. Although these findings support the hypothesis that these cytokines induce HHV-6 reactivation, further large number of case analysis is necessary to confirm the hypothesis, because numerous factors such as conditioning treatments may affect cytokine production in HSCT recipients. On the other hand, a significant increase of the IL-6 concentration was observed after HHV-6 isolation in cases 1 and 2, suggesting that cytokine synthesis was upregulated as a result of the viral reactivation. Four of the seven recipients with HHV-6 viremia had significant increases in their anti-HHV-6 antibody titers during the observation period. Interestingly, the peak IL-6 levels were significantly higher in the four recipients who mounted antibody responses than in the three recipients who did not produce detectable antibody responses (118.5 ± 4.2 vs. 54.7 ± 16.2 pg/ml, $P = 0.0339$). This finding suggests that severe immunosuppression, which impairs host cytokine synthesis, prevented the HSCT recipients from producing an antibody response against viral reactivation.

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References

1. Salahuddin SZ, Ablashi DV, Markham PD, Josephs SF, Sturzenegger S, Kaplan M, et al. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 1986;**234**:596–601.
2. Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, et al. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* 1988;**1**:1065–7.
3. Asano Y, Yoshikawa T, Suga S, Yazaki T, Hata T, Nagai T, et al. Viremia and neutralizing antibody response in infants with exanthem subitum. *J Pediatr* 1989;**114**:535–9.
4. Yoshikawa T. Human herpesvirus 6 infection in hematopoietic stem cell transplant patients. *Br J Haematol* 2004;**124**:421–32.
5. Asano Y, Yoshikawa T, Suga S, Nakashima T, Yazaki T, Fukuda M, et al. Reactivation of herpesvirus type 6 in children receiving bone marrow transplants for leukemia. *N Engl J Med* 1991;**324**:634–5.
6. Yoshikawa T, Suga S, Asano Y, Nakashima T, Yazaki T, Sobue R, et al. Human herpesvirus-6 infection in bone marrow transplantation. *Blood* 1991;**78**:1381–4.
7. Wilborn F, Brinkmann V, Schmidt CA, Neipel F, Gelderblom H, Siegert W. Herpesvirus type 6 in patients undergoing bone marrow transplantation: serologic features and detection by polymerase chain reaction. *Blood* 1994;**83**:3052–8.
8. Appleton AL, Sviland L, Peiris JSM, Taylor CE, Wilkes J, Green MA, et al. Human herpesvirus-6 infection in marrow graft recipients: role in pathogenesis of graft-versus-host disease. *Bone Marrow Transplant* 1995;**16**:777–82.
9. LeCleach L, Joberty C, Fillet AM, Sutton L, Cordonnier C, Frances C, et al. Human herpesvirus 6 infection in patients with exanthema after allogeneic bone marrow transplantation. *Arch Dermatol* 1998;**134**:759–60.
10. Cone RW, Huang MLW, Corey L, Zeh J, Ashley R, Bowden R. Human herpesvirus 6 infections after bone marrow transplantation: clinical and virologic manifestations. *J Infect Dis* 1999;**179**:311–8.
11. Takemoto Y, Takatsuka H, Wada H, Mori A, Saheki K, Okada M, et al. Evaluation of CMV/human herpes virus-6 positivity in bronchoalveolar lavage fluids as early detection of acute GVHD following BMT: evidence of a significant relationship. *Bone Marrow Transplant* 2000;**26**:77–81.
12. Yoshikawa T, Ihira M, Ohashi M, Suga S, Asano Y, Miyazaki H, et al. Correlation between HHV-6 infection and skin rash after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2001;**28**:77–81.
13. Yoshikawa T, Asano Y, Ihira M, Suzuki K, Ohashi M, Suga S, et al. Human herpesvirus 6 viremia in bone marrow transplant recipients: clinical features and risk factors. *J Infect Dis* 2002;**185**:847–53.
14. Zerr DM, Corey L, Kim HW, Huang ML, Nguy L, Boeckh M. Clinical outcomes of human herpesvirus 6 reactivation after hematopoietic stem cell transplantation. *Clin Infect Dis* 2005;**40**:932–40.
15. Carrigan DR, Drobyski WR, Russler SK, Tapper MA, Knox KK, Ash RC. Interstitial pneumonitis associated with human

herpesvirus 6 infection after marrow transplantation. *Lancet* 1991;**338**:147–9.
16. Cone RW, Hackman RC, Huang MW, Bowden RA, Meyers JD, Metcalf M, et al. Human herpesvirus 6 in lung tissue from patients with pneumonitis after bone marrow transplantation. *N Engl J Med* 1993;**329**:156–61.
17. Drobyski WR, Knox KK, Majewski D, Carrigan DR. Brief report: fatal encephalitis due to variant B human herpesvirus 6 infection in a bone marrow-transplant recipient. *N Engl J Med* 1994; **330**:1356–60.
18. Ogata M, Kikuchi H, Satou T, Kawano R, Ikewaki J, Kohno K, et al. Human herpesvirus 6 DNA in plasma after allogeneic stem cell transplantation: incidence and clinical significance. *J Infect Dis* 2006;**193**:68–79.
19. Kadakia MP, Rybka WB, Stewart JA, Patton JL, Stamey FR, Elsawy M, et al. Human herpesvirus 6: infection and disease following autologous and allogeneic bone marrow transplantation. *Blood* 1996;**12**:5341–54.
20. Drobyski WR, Dunne WM, Burd EM, Knox KK, Ash RC, Horowitz MM, et al. Human herpesvirus 6 (HHV-6) infection allogeneic bone marrow transplant recipients: evidence of a marrow-suppressive role for HHV-6 in vivo. *J Infect Dis* 1993;**167**:735–9.
21. Wang FZ, Dahl H, Linde A, Brytting M, Ehrnst A, Ljungman P. Lymphotropic herpesviruses in allogeneic bone marrow transplantation. *Blood* 1996;**88**:3615–20.
22. Ljungman P, Wang FZ, Clark DA, Emery VC, Remberger M, Ringden O, et al. High levels of humanherpesvirus 6 DNA in peripheral blood leukocytes are correlated to platelet engraftment and disease in allogeneic stem cell transplant patients. *Br J Haematol* 2000;**111**:774–81.
23. Imbert-Marcille BM, Tang XW, Lepelletier D, Besse B, Moreau P, Billaudel S, et al. Human herpesvirus 6 infection after autologous or allogeneic stem cell transplantation: a single-center prospective longitudinal study of 92 patients. *Clin Infect Dis* 2000;**31**:881–6.
24. Docke WD, Prosch S, Fietze E, Kimel V, Zuckermann H, Klug C, et al. Cytomegalovirus reactivation and tumor necrosis factor. *Lancet* 1994;**343**:268–9.
25. Humar A, St Louis P, Mazzulli T, McGeer A, Lipton J, Messner H, et al. Elevated serum cytokines are associated with cytomegalovirus infection and disease in bone marrow transplant recipients. *J Infect Dis* 1999;**179**:484–8.
26. Hummel M, Abecassis MM. A model for reactivation of CMV from latency. *J Clin Virol* 2002;**25**:S123–36.
27. Simon CO, Seckert CK, Dreis D, Reddehase MJ, Grzimek NK. Role for tumor necrosis factor alpha in murine cytomegalovirus transcriptional reactivation in latently infected lungs. *J Virol* 2005;**79**:326–40.
28. Takemoto M, Shimamoto T, Isegawa Y, Yamanishi K. The R3 region, one of three major repetitive regions of human herpesvirus 6, is a strong enhancer of immediate-early gene U95. *J Virol* 2001;**75**:10149–60.
29. Azuma E, Kojima S, Kato K, Matsuyama T, Yamada Y, Kondo N, et al. Conditioning with cyclophosphamide/antithymocyte globulin for allogeneic bone marrow transplantation from HLA-matched siblings in children with severe aplastic anemia. *Bone Marrow Transplant* 1997;**19**:1085–7.
30. Matsuyama T, Horibe K, Kato K, Kojima S. Bone marrow transplantation for children with acute myelogenous leukemia in the first complete remission. *Eur J Cancer* 2000;**36**:368–75.
31. Asano Y, Nakashima T, Yoshikawa T, Suga S, Yazaki T. Severity of human herpesvirus 6 viremia and clinical findings in infants with exanthem subitum. *J Pediatr* 1991;**118**:891–5.
32. Yoshikawa T, Suga S, Asano Y, Yazaki T, Kodama H, Ozaki T. Distribution of antibodies to a causative agent of exanthem subitum (human herpesvirus-6) in healthy individuals. *Pediatrics* 1989;**84**:675–7.
33. Desachy A, Ranger-Rogez S, Francois B, Venot C, Traccard I, Gastinne H, et al. Reactivation of human herpesvirus type 6 in multiple organ failure syndrome. *Clin Infect Dis* 2001;**32**: 197–203.
34. Razonable RR, Fanning C, Brown RA, Espy MJ, Rivero A, Wilson J, et al. Selective reactivation of human herpesvirus 6 variant a occurs in critically ill immunocompetent hosts. *J Infect Dis* 2002;**185**:110–3.
35. Imamura M, Hashino S, Kobayashi H, Kubayashi S, Hirano S, Minagawa T, et al. Serum cytokine levels in bone marrow transplantation: synergistic interaction of interleukin-6, interferon-gamma, and tumor necrosis factor-alpha in graft-versus-host disease. *Bone Marrow Transplant* 1994;**13**:745–51.
36. Schwaighofer H, Herold M, Schwarz T, Nordberg J, Ceska M, Prior C, et al. Serum levels of interleukin 6, interleukin 8, and C-reactive protein after human allogeneic bone marrow transplantation. *Transplantation* 1994;**58**:430–6.
37. Nagler A, Or R, Nisman B, Kalickman I, Slavin S, Barak V. Elevated inflammatory cytokine levels in bone marrow graft rejection. *Transplantation* 1995;**60**:943–8.
38. Ferrara JL, Reddy P. Pathophysiology of graft-versus-host disease. *Semin Hematol* 2006;**43**:3–10.
39. Flamand L, Gosselin J, D'Addario M, Hiscott J, Ablashi DV, Gallo RC, et al. Human herpesvirus 6 induces interleukin-1 beta and tumor necrosis factor alpha, but not interleukin-6, in peripheral blood mononuclear cell cultures. *J Virol* 1991; **65**:5105–10.
40. Gosselin J, Flamand L, D'Addario M, Hiscott J, Stefanescu I, Ablashi DV, et al. Modulatory effects of Epstein-Barr, herpes simplex, and human herpes-6 viral infections and coinfections on cytokine synthesis. A comparative study. *J Immunol* 1992; **149**:181–7.
41. Yoshikawa T, Asano Y, Akimoto S, Ozaki T, Iwasaki T, Kurata T, et al. Latent infection of human herpesvirus 6 in astrocytoma cell line and alteration of cytokine synthesis. *J Med Virol* 2002;**66**:497–505.
42. Go T, Nakamura K. Frequent seizures with elevated interleukin-6 at the eruptive stage of exanthema subitum. *Eur J Paediatr Neurol* 2002;**6**:221–3.
43. Mayne M, Cheadle C, Soldan SS, Cermelli C, Yamano Y, Akhyani N, et al. Gene expression profile of herpesvirus-infected T cells obtained using immunomicroarrays: induction of proinflammatory mechanisms. *J Virol* 2001;**75**:11641–50.

bjh research paper

Clinical impact of HLA-DR15, a minor population of paroxysmal nocturnal haemoglobinuria-type cells, and an aplastic anaemia-associated autoantibody in children with acquired aplastic anaemia

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Summary

Aplastic anaemia (AA) is defined as a pancytopenia caused by bone marrow failure, and its pathogenesis is thought to involve autoimmune processes. Several predictive markers of the response to immunosuppressive therapy (IST) have been proposed, which appear to reflect the immune pathophysiology. We prospectively investigated the presence of human leucocyte antigen (HLA)-DR15, a minor population of paroxysmal nocturnal haemoglobinuria (PNH)-type cells, and antibodies to the recently identified autoantigen postmeiotic segregation increased 1 (PMS1) in 103 children with AA enrolled in a multicentre study. In contrast to adults, children with AA did not show an increased frequency of HLA-DR15. In addition, a sensitive flow cytometric assay revealed that children with AA have a much lower prevalence of PNH-type cells (21·4%) than reported for adults with this disease. An immunoblotting assay detected anti-PMS1 antibody in 15 of 103 (14·6%) of the children. Finally, the response rate to IST was not significantly different between patients with and without DR15 (45·5% vs. 54·0%), PNH-type cells (68·2% vs. 53·1%) or anti-PMS1 antibody (40·0% vs. 59·1%). The current study did not confirm a correlation between these markers and the response to IST, suggesting that there is a difference in the pathophysiologies of adult and paediatric AA.

Keywords: acquired aplastic anaemia, children, postmeiotic segregation increased 1, HLA-DR15, paroxysmal nocturnal haemoglobinuria.

Aplastic anaemia (AA) is defined as peripheral blood pancytopenia and bone marrow hypoplasia. Accumulating evidence suggests that immune mechanisms contribute to the pathogenesis of acquired AA (Mathe *et al*, 1971; Young, 1999, 2002a; Maciejewski *et al*, 2002). Immunosuppressive therapy (IST) with anti-thymocyte globulin (ATG) and cyclosporine (CyA) has resulted in response rates of approximately 50–70% for acquired AA (Gluckman *et al* 1992; Bacigalupo *et al*, 1995; Rosenfeld *et al*, 1995, 2003; Kojima *et al*, 2000; Young, 2002b), suggesting that pancytopenia and bone marrow failure are immunologically mediated in at least some AA patients. For the remaining patients, however, IST might even be harmful because of an increased risk of opportunistic infections and a delay in receiving stem cell transplantation. In children, in particular, the choice of an appropriate treatment is influenced by the long-term sequelae of the disease as well as its therapy. The immune pathophysiology of patients should thus be understood at

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diagnosis, and IST should be applied only to those with immune-mediated AA.

Several potential markers of IST that appear to reflect the immune pathophysiology of AA have been suggested. One is human leucocyte antigen (HLA)-DR15 (split of DR2). As in other autoimmune disorders, an inherited predisposition associated with HLA phenotypes has been examined in AA (Albert *et al*, 1976; Dausset *et al*, 1977; Hurtado Monroy *et al*, 1980; Gluckman *et al*, 1981; D'Amaro *et al*, 1983; Chapuis *et al*, 1986; Nakao *et al*, 1994, 1996; Nimer *et al*, 1994; Ihan *et al*, 1997; Chiewsilp *et al*, 2000; Shao *et al*, 2000; Kapustin *et al*, 2001). An increased frequency of HLA-DR2, specifically DR15, has been reported in several (but not all) studies of AA, although these have been mostly studies on adult AA (Gluckman *et al*, 1981; D'Amaro *et al*, 1983; Chapuis *et al*, 1986; Nimer *et al*, 1994; Chiewsilp *et al*, 2000; Shao *et al*, 2000; Fuhrer *et al*, 2007). Some groups have shown that the presence of HLA-DR15 is associated with a good response to IST (Nakao *et al*, 1994; Ilhan *et al*, 1995; Ihan *et al*, 1997; Maciejewski *et al*, 2001; Oguz *et al*, 2002; Usman *et al*, 2004); however, a positive correlation between HLA-DR15 and the response to the combination of ATG and CyA, which is currently the standard for treating acquired AA, has not yet to be clearly demonstrated because the number of patients studied has been relatively small and the drugs used for IST have not been consistent (Nakao *et al*, 1994; Ilhan *et al*, 1995; Ihan *et al*, 1997; Shao *et al*, 2000; Maciejewski *et al*, 2001; Oguz *et al*, 2002; Usman *et al*, 2004; Fuhrer *et al*, 2007).

Another marker closely associated with immune pathophysiology in adult AA is paroxysmal nocturnal haemoglobinuria (PNH)-type cells, a type of glycosylphosphatidylinositol-anchored protein-deficient cell that are very less in number (Young, 1992; Dunn *et al*, 1999; Maciejewski *et al*, 2001; Wang *et al*, 2001; Sauntharajah *et al*, 2002). Two retrospective studies have found a correlation between the presence of PNH-type cells and the response to IST in adults with AA (Maciejewski *et al*, 2001; Sugimori *et al*, 2006), although a third group failed to find this association (Sauntharajah *et al*, 2002).

In organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus and multiple sclerosis, autoantibodies against target proteins are often produced by pathogenic T-cells (Gran & Rostami, 2001; Markovic-Plese & McFarland, 2001; Viglietta *et al*, 2002; Lieberman *et al*, 2003). Because of the dramatic effects of T-cell suppressants including ATG and CyA on *in vivo* haematopoiesis, it has been suggested that autoreactive T-cell responses against target autoantigens play a major role in the development of AA and that these autoantigens also evoke humoral responses. Recently, four different AA-associated autoantigens were identified using the serological identification of antigens by recombinant expression cloning (SEREX) method with a peptide library derived from fetal liver or leukaemic cell lines or through their direct binding to protein in the lysate of a leukaemic cell line (Hirano *et al*, 2003, 2005; Feng *et al*, 2004; Takamatsu *et al*, 2007).

The presence of antibodies to these antigens was proposed as another marker predicting the response to IST. Diazepam-binding inhibitor-related protein 1 (DRS-1) was described as a candidate autoantigen in AA, and among a limited number of adults with AA, there was an association between the presence of antibody to DRS-1 and a good response to IST (Feng *et al*, 2004). Postmeiotic segregation increased 1 (PMS1) was also identified as a possible antigen using SEREX, with an antibody present in the sera of Japanese children with AA (Hirano *et al*, 2005), but the relevance of these antibodies to the pathophysiology of AA and their clinical significance has not yet been determined.

Most of these studies have focused on adults with AA and have been retrospective, and the significance of these markers in paediatric cohorts is largely unknown and needs to be established (Davies & Guinan, 2007). Therefore, in the current study, we prospectively investigated the presence of HLA-DR15, PNH-type cells, and antibodies to PMS1 in a large population of children with AA enrolled in a multicentre study to clarify whether the previously described correlation between these markers and good response to IST in adults could be confirmed.

Materials and methods

Patients

Between July 2001 and September 2005, 103 Japanese children with AA from 57 hospitals were enrolled in prospective multicentre study AA-97 conducted by the Japan Childhood Aplastic Anemia Study Group. Patients with acquired AA were eligible if they met the following criteria: age <18 years, newly diagnosed disease (≤180 d) without specific prior treatment, and moderate to very severe AA. Severity was classified as previously described (Camitta *et al*, 1979; Bacigalupo *et al*, 1993). The disease was considered severe if at least two of the following were noted: a neutrophil count $<0{\cdot}5 \times 10^9$/l, a platelet count $<20 \times 10^9$/l, and a reticulocyte count $<20 \times 10^9$/l with hypocellular bone marrow. AA was considered very severe if the criteria for severe disease were fulfilled and the neutrophil count was $<0{\cdot}2 \times 10^9$/l. Patients were excluded if they had congenital AA or paroxysmal nocturnal haemoglobinuria with positive findings on the Ham test/sucrose test. Allogeneic stem cell transplantation was recommended for patients with severe or very severe disease who had an HLA-matched sibling: these patients were not included in the AA-97 study. Written informed consent was obtained from all parents as well as patients aged 10 years or older. The study was approved by the ethics committee of each participating hospital. The study also conformed to the recently revised tenets of the Helsinki protocol.

IST

All patients with very severe or severe AA were treated with a combination of ATG (Lymphoglobulin; Imtix-SangStat, Lyon, France; 1·5 vials/10 kg/d intravenously for 5 d) and CyA (Novartis, Basel, Switzerland; 6 mg/kg/d per oral). The dose of CyA was adjusted to maintain trough levels between 100 and 200 ng/ml, and the appropriate dose was administered for at least 6 months. Patients with moderate AA were randomized to receive only ATG or a combination of ATG and CyA. Granulocyte colony-stimulating factor (Filgrastim, Kirin, Tokyo, Japan; 400 $\mu g/m^2$ intravenously or subcutaneously) was administered for 3 months to very severe patients (Kosaka *et al*, 2008). Response to IST was evaluated according as previously described (Kojima *et al*, 2000).

HLA typing

At diagnosis, all patients were HLA-typed by DNA techniques and/or by serology. The HLA typing was performed by SRL Inc. (Tokyo, Japan). The frequency of HLA-DR in these patients was compared with that in a control population consisting of 1018 unrelated Japanese individuals (Itoh *et al*, 2005).

Detection of PNH-type cells

To detect PNH-type granulocytes, red blood cells (RBCs) were lysed in ammonium chloride buffer. Leucocyte suspension (50 μl) was incubated with 5 μl of fluorescein isothiocyanate (FITC)-conjugated anti-CD13 monoclonal antibody (mAb) (Beckman Coulter, Miami, FL, USA), phycoerythrin (PE)-labelled anti-CD55 mAb (clone 67, mouse IgG1; Serotec, Oxford, UK), and PE-labelled anti-CD59 mAb (clone p282, mouse IgG2a; Becton Dickinson, Franklin Lakes, NJ, USA) on ice for 30 min. To detect PNH-type RBCs, FITC-labelled anti-glycophorin A mAb (clone KC16; Immunotech, Marseille, France) was included instead of anti-CD13 mAb. Fresh blood was diluted to 3% in phosphate-buffered saline, and 50 μl of diluted blood was incubated with 5 μl of FITC-labelled anti-glycophorin A mAb, PE-labelled anti-CD55 mAb, and PE-labelled anti-CD59 mAb on ice for 30 min. A total of at least 1×10^5 $CD13^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analysed using a FACSCalibur (Becton Dickinson). On the basis of analytic results from 15 healthy individuals, the presence of >0·020% $CD13^+$ $CD55^-$ $CD59^-$ granulocytes and >0·037% glycophorin A^+ $CD55^-$ $CD59^-$ RBCs was defined as abnormal. When the number of PNH-type cells was elevated in granulocytes and/or RBCs, the patient was defined as having a minor PNH clone.

Expression and purification of bacterially expressed fusion proteins

Full-length *PMS1* cDNA was cloned into pET14b vector (Novagen, Madison, WI, USA) for expression as a His-tag fusion protein. Synthesized proteins were purified using a His-bind kit (Novagen) according to the manufacturer's instructions. His-tagged PMS1 protein was induced as previously described (Hirano *et al*, 2003, 2005). Appropriate size and specificity of the expressed protein products were confirmed by Western blot analysis using mouse anti-His mAb (1:3000; Sigma, St Louis, MO, USA) or rabbit anti-PMS1 antibody (1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blot analysis

Plasma samples were obtained from 103 children with AA and 33 healthy volunteers. Samples were stored at −80°C until use. Equal amounts of bacterial lysates expressing His-tagged full-length PMS1 proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and incubated overnight with blocking buffer. Immunoblotting was performed using 1:1000 dilutions of patient plasma, normal plasma, or rabbit anti-PMS1 antibody, followed by 1:10 000 horseradish peroxidase-conjugated anti-human IgG (Amersham Biosciences, Piscataway, NJ, USA) or 1:5000 anti-rabbit IgG secondary antibody (Amersham Biosciences). Immunoreactive bands were detected with chemiluminescence reagents (Amersham Biosciences).

Statistical analyses

Differences in clinical characteristics between patients with and without HLA-DR15, PNH-type cells, or anti-PMS1 antibody and relationships between the presence of these markers and the response to IST were assessed using the Mann–Whitney *U*-test and the Fisher exact probability test. To evaluate the multivariate relationship between IST response and variables, logistic regression modelling was performed. A *P*-value of <0·05 was considered statistically significant.

Results

Patient characteristics and response to immunosuppressive therapy

A total of 103 patients (66 boys and 37 girls) fulfilled the eligibility criteria, and their clinical characteristics are shown in Table I. The median age at diagnosis was 8 years (range, 1–15 years). Of the 103 patients, 82 (79·6%) had idiopathic AA, 18 (17·5%) had hepatitis-associated AA and 3 (2·9%) had AA from other causes. Regarding the severity of AA, 53 patients (51·5%) were rated very severe, 28 (27·2%) were severe and 22 (21·3%) were moderate. All patients with very severe and severe AA were treated with a combination of ATG and CyA, and patients with moderate AA were randomized to receive only ATG (n = 11) or a combination of ATG and CyA (n = 11). A total of 58 of 103 (56·3%) patients improved with IST and achieved partial response (n = 40) or complete response (n = 18) at 6 months.

Table I. Patient characteristics.

No. patients	103
Age at diagnosis, years, median (range)	8 (1–15)
Gender	
Male/female	66/37
Etiology, no. of patients (%)	
Idiopathic	82 (79·6)
Hepatitis	18 (17·5)
Others	3 (2·9)
Severity of AA, no. of patients (%)	
VSAA	53 (51·5)
SAA	28 (27·2)
MAA	22 (21·3)

VSAA, very severe aplastic anaemia; SAA, severe aplastic anaemia; MAA, moderate aplastic anaemia.

Prevalence of HLA-DR15 among children with AA and its relationship with clinical features

The HLA-DR15 types were available for 85 children with AA. The clinical features of these 85 children were similar to those of the remaining children without HLA-DR15 data. HLA-DR15 was present in 22 of 85 children. In our paediatric patient cohort, the HLA-DR15 allelic frequency was 13·5%. This is similar to the frequency in the general population in Japan (15·6%; $n = 1018$) (Itoh *et al*, 2005). Furthermore, the frequency was significantly lower than in Japanese adults with AA (Nakao *et al*, 1996) (13·5% vs. 27·9%; $P = 0·001$). Of the 85 patients whose HLA-DR15 type was available, 44 (51·8%) responded to IST. Ten of 22 HLA-DR15-positive and 34 of 63 HLA-DR15-negative patients achieved a response. The response rate in the DR15-positive group (45·5%) did not significantly differ from that in the DR15-negative group (54·0%; $P = 0·62$). Between these two groups, there was also no significant difference in the age at diagnosis, gender, aetiology, disease severity, presence of PNH-type cells, or presence of anti-PMS1 antibody (Table II).

Detection of PNH-type cells

We found a significant increase in the number of PNH-type cells in 22 of 103 (21·4%) patients. The median percentage of PNH-type RBCs was 0·14% (range, 0·04–0·81%). Notably, the proportions of PNH-type RBCs were below 0·1% in more than 40% of the PNH^+ patients. We further compared the clinical features and treatment responses between PNH^+ and PNH^-

Table II. Clinical features of AA patients with HLA-DR15, PNH-type cells, or anti-PMS1 Ab.

	HLA-DR15		PNH-type cells		Anti-PMS1 Ab	
	positive ($n = 22$)	negative ($n = 63$)	positive ($n = 22$)	negative ($n = 81$)	positive ($n = 15$)	negative ($n = 88$)
Age at diagnosis, years, median (range)	8 (1–15)	9 (1–15)	8·5 (1–15)	8 (1–15)	9 (3–14)	7·5 (1–15)
	$P = 0·93$**		$P = 0·85$**		$P = 0·53$**	
Gender						
Male/female	16/6	42/21	15/7	51/30	10/5	56/52
	$P = 0·79$*		$P = 0·80$*		$P > 0·99$*	
Aetiology, no. of patients (%)						
Idiopathic	18 (81·8)	48 (76·2)	16 (72·7)	66 (81·5)	13 (86·7)	69 (78·4)
Hepatitis	4 (18·2)	12 (19·0)	6 (27·3)	12 (14·8)	2 (13·3)	16 (18·2)
Others	0 (0·0)	3 (4·8)	0 (0·0)	3 (3·7)	0 (0·0)	3 (3·4)
	$P > 0·99$*		$P = 0·37$*		$P = 0·88$*	
Severity of AA, no. of patients (%)						
VSAA	13 (59·1)	33 (52·4)	11 (50·0)	42 (51·9)	6 (40·0)	47 (53·4)
SAA	7 (31·8)	16 (25·4)	8 (36·4)	20 (24·7)	6 (40·0)	22 (25·0)
MAA	2 (9·1)	14 (22·2)	3 (13·6)	19 (23·4)	3 (20·0)	19 (21·6)
	$P = 0·31$*		$P = 0·53$*		$P = 0·49$*	
Response to IST, no. of patients (%)	10/22 (45·5)	34/63 (54·0)	15/22 (68·2)	43/81 (53·1)	6/15 (40·0)	52/88 (59·1)
	$P = 0·62$*		$P = 0·36$*		$P = 0·26$*	
HLA-DR15, no. of patients (%)	–	–	5/18 (27·8)	17/67 (25·4)	2/12 (16·7)	20/73 (27·4)
			$P > 0·99$*		$P = 0·72$*	
PNH-type cells, no. of patients (%)	5/22 (22·7)	13/63 (20·6)	–	–	3/15 (20·0)	19/88 (21·6)
	$P = 0·77$*				$P > 0·99$*	
Anti-PMS1 Ab, no. of patients (%)	2/22 (9·1)	10/63 (15·9)	3/22 (13·6)	12/81 (14·8)	–	–
	$P = 0·72$*		$P > 0·99$*			

VSAA, very severe aplastic anaemia; SAA, severe aplastic anaemia; MAA, moderate aplastic anaemia; PNH, paroxysmal nocturnal haemoglobinuria.
*Fisher exact test.
**Mann–Whitney *U*-test.

patients to determine the clinical significance of the minor population of PNH-type cells. Fifteen of 22 (68·2%) PNH^+ patients and 43 of 81 (53·1%) PNH^- patients improved with IST. These response rates were comparable ($P = 0·36$). In addition, there were no differences in other clinical variables between these two groups (Table II).

Detection of specific antibodies to PMS1

To confirm the presence of antibodies specific to PMS1 and determine its prevalence, we produced recombinant His-tag PMS1 protein and performed Western blot analysis using sera from AA patients and healthy volunteers. The size and specificity of the expressed protein products was confirmed by Western blotting (Fig 1A). Figure 1B shows representative results using the same method for selected patients with AA and healthy individuals. Clear bands indicating the presence of PMS1 antibody were present in the sera of 15 of 103 children with AA (14·6%) but not in any of the sera of healthy volunteers. The difference between these two groups was significant ($P = 0·02$).

We next investigated whether anti-PMS1 antibody titres could be used as a marker for AA disease activity. Serial samples were available for the two patients who had detectable anti-PMS1 antibody. Anti-PMS1 antibody levels persisted in one patient who did not show clinical improvement after IST, and the antibody disappeared after the patient received bone marrow transplantation (BMT; Fig 2A). In another patient who achieved resolution of pancytopenia and clinical improvement following IST, anti-PMS1 antibody became undetectable (Fig 2B). To determine if the presence of anti-PMS1 antibody could be a marker for predicting the response to IST, we then compared anti-PMS1 antibody-positive and -negative patients. Six of 15 (40·0%) anti-PMS1 antibody-positive and 52 of 88 (59·1%) anti-PMS1 antibody-negative patients achieved a response. Similar to other clinical variables, the presence of anti-PMS1 antibody was not significantly associated with a good response to IST (Table II).

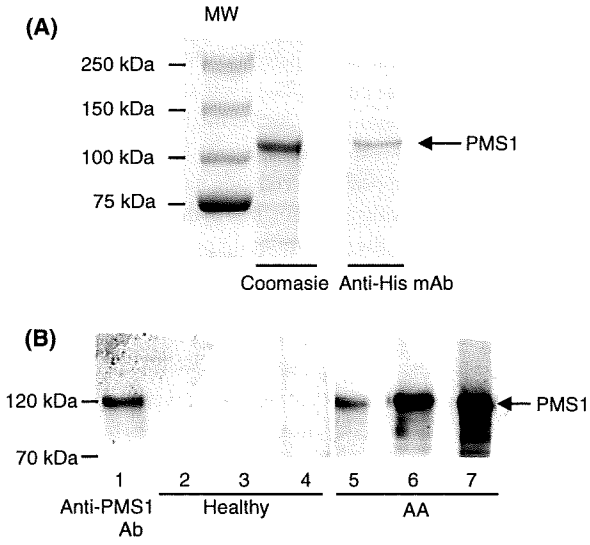

Fig 1. Specific antibody to recombinant postmeiotic segregation increased 1 (PMS1) in sera of AA and healthy controls. (A) Full-length *PMS1* cDNA was cloned into a prokaryotic expression vector and expressed as a His-tagged protein. Equal amounts of bacterial lysates expressing His-tagged full-length PMS1 protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride filters. Blotted membranes were incubated with anti-His mAb, and the size and specificity of the expressed protein were confirmed. (B) Anti-PMS1 antibodies were detected by rabbit anti-PMS1 antibody (lane 1) and in the sera of some AA patients (lanes 5–7) but not in the sera of healthy volunteers (lanes 2–4).

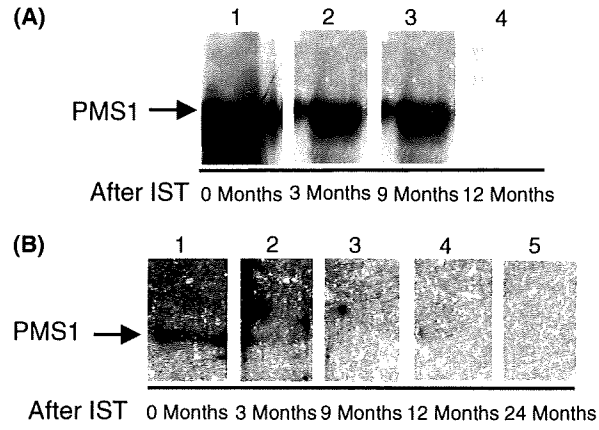

Fig 2. Detection of anti-postmeiotic segregation increased 1 (PMS1) antibody and correlation with disease activity. (A) Anti-PMS1 antibody analysis of serum from an AA patient who did not respond to immunosuppressive therapy (IST) and received bone marrow transplantation (BMT) 10 months after IST. Anti-PMS1 antibody persisted at 3 and 9 months after IST (lanes 1–3) but disappeared after BMT (lane 4). (B) Anti-PMS1 antibody analysis of an AA patient who had clinical improvement following IST. Detected bands became progressively weaker (lane 2) and then undetectable (lane 3–5).

Association of the studied variables and the response to IST in AA

We compared the frequency of the HLA-DR15 allele, the prevalence of PNH-type cells, and the prevalence of the anti-PMS1 antibody between IST responders and non-responders. Univariate analysis indicated that the frequency of these three markers was not significantly different between these two groups. We also performed multivariate logistic regression analysis to assess the simultaneous contribution of each of the three variables in predicting the response. The presence of HLA-DR15, PNH-type cells, and anti-PMS1 antibody were not statistically significant predictors of a response to therapy.

Discussion

Since the first report on HLA-DR antigens in AA by Chapuis *et al* (1986), who found an increased frequency of HLA-DR2

in AA patients, several studies have confirmed the association between the DR15 antigen and AA in adults (Chapuis *et al*, 1986; Nakao *et al*, 1994; Ilhan *et al*, 1995; Shao *et al*, 2000). More recently, a difference in the correlation of this allele in children and adults with AA was described by Fuhrer *et al* (2007). In the current prospective study, we confirmed that there is no association between HLA-DR15 and AA in children. Therefore, the contribution of this allele to the aetiology of AA appears to be limited, at least in children. The frequency of HLA-DR15 varies significantly in different ethnic groups (Ilhan *et al*, 1995; Ihan *et al*, 1997). Our finding is unlikely to be due to a difference in genetic background because the HLA frequency of our Japanese paediatric cohorts was similar to controls with comparable ethnological backgrounds (n = 1018) as reported by Itoh *et al* (2005). Notably, the prevalence of HLA-DR15 in children with AA was significantly lower than the results reported in Japanese adults with AA (13·5% vs. 27·9%; P = 0·001) (Nakao *et al*, 1994).

Although the HLA-DR15 status of the AA patients had also been proposed as a predictive marker of the clinical response to IST, we were unable to obtain a definitive result regarding its predictive value. In some studies, HLA-DRB1*1501 has been shown to be closely associated with a good response to CyA therapy in patients with AA (Nakao *et al*, 1994, 1996). However, the response to ATG does not seem to correlate with DR15 (Nimer *et al*, 1994; Nakao *et al*, 1996; Ihan *et al*, 1997). Conflicting results have been reported for the response to a combination of ATG and CyA (Ilhan *et al*, 1995; Ihan *et al*, 1997; Maciejewski *et al*, 2001; Oguz *et al*, 2002; Saunthararajah *et al*, 2002; Usman *et al*, 2004). The present study investigated a large cohort of children with acquired AA treated with the combination of ATG and CyA or with ATG alone, and no association was found between the presence of DR15 and the response to IST. Unfortunately, high-resolution DNA typing results were not available for our patients and, thus, we could not determine whether there is a specific association with the *HLA-DRB1**1501 allele in children with AA. When the patients who had been randomized to receive only ATG were excluded from the analysis, a negative result was still observed. The discrepancy between the impact of HLA-DR15 status in children and adults with AA and the fact that clinical responses also occurred in DR15-negative subjects suggest that the pathogenesis of AA in children and adults is different because of distinct autoimmune-mediated mechanisms.

Several studies have demonstrated the presence of PNH-type cells in the peripheral blood of AA patients. However, to the best of our knowledge, no study has focused on paediatric AA patients and, to date, no cohorts of children with AA have been prospectively analysed to examine the correlation between the presence of these clones and the response to IST (Davies & Guinan, 2007). This study analysed a large number of paediatric patients with newly diagnosed AA, and found that increased PNH clones were detected in a limited number of patients. Unlike other low-resolution assays, the flow cytometric assay that we employed was able to detect PNH-type granulocytes or RBCs, at percentages <0·01%. These low percentages were comparable with the results of previous studies using high-resolution assays (Wang *et al*, 2001; Sugimori *et al*, 2006). Using this sensitive flow cytometry assay, lower rates of increase in PNH-type cells were detected in children with AA (21·4%) than in adults with AA (68·0%) (Sugimori *et al*, 2006). In retrospective studies of adults with AA, a better response to IST was found in PNH^+ than PNH^- patients (Maciejewski *et al*, 2001; Sugimori *et al*, 2006). In our study, however, the presence of these clones at diagnosis was not associated with an increased response rate after IST or other clinical features, including the frequency of HLA-DR15. These findings support the idea that childhood AA is a type of bone marrow failure distinct from AA in adults.

PMS1 is a DNA mismatch repair gene expressed by many tissues including haematopoietic cells (Horii *et al*, 1994). The PMS1 protein forms heterodimers with MLH1, another DNA mismatch repair protein, and some cases of hereditary non-polyposis colorectal cancer have been found to have mutations in this gene (Nicolaides *et al*, 1994), but the role for PMS1 protein in haematopoiesis and the development of AA has not been clear. Recently, we reported that an IgG antibody response to PMS1 was detected in three of 30 children with AA (Hirano *et al*, 2005). In this study, we prospectively determined the prevalence of this antibody and examined its clinical significance. Fifteen of 103 patients (14·6%) had IgG antibodies directed against PMS1, but no antibody response to PMS1 was observed in healthy donors, suggesting that PMS1 is a specific autoantigen in AA patients. The frequency of anti-PMS1 antibody in the current study was similar to that in our previous study (Hirano *et al*, 2005). In the current study, in one of the patients with detectable anti-PMS1 antibody, the autoantibody disappeared coincidentally with clinical improvement following IST, but in another patient who did not respond to IST, the autoantibody persisted until second-line therapy with BMT. We could not find an association between the presence of this antibody with a good response to IST and other markers, including HLA-DR15 and PNH-type cells. These results suggest that the presence of anti-PMS1 antibody may not be a marker for predicting the response to IST; however, the antibody titre might be a useful surrogate maker for the disease activity of AA.

Diazepam-binding inhibitor-related protein 1 and moesin have been recently identified as possible target antigens in AA, and the correlation between the presence of these antibodies and the response to IST has been examined (Feng *et al*, 2004; Takamatsu *et al*, 2007). These two studies found no significant differences in the rate of response to IST using the combination of ATG and CyA between patients with and without these antibodies. Seven of nine (78%) anti-DRS-1 antibody-positive patients and six of nine (67%) anti-DRS-1 antibody-negative patients achieved a response. Also, nine of 12 (75%) anti-moesin antibody-positive and four of six (67%) anti-moesin antibody-negative patients responded to the combination of ATG and CyA. When both markers were combined, 10 of 14

(71%) patients showing at least one of the two markers improved when treated with ATG and CyA, and three of four (75%) patients not showing either marker responded. Although there was no significant association between the presence of these antibodies and the clinical response, the antibodies to DRS-1 and moesin may reflect the immune mechanism of AA in adults because both correlate with the presence of PNH-type cells, which are thought to participate in the immune pathophysiology of adult AA. A large prospective study is needed to more definitively determine the predictive value of these autoantibodies.

To our knowledge, this is the first prospective study to investigate the clinical relevance of HLA-DR15, PNH-type cells, and antibodies to PMS1 in paediatric AA. Unlike observations in adult AA, we were unable to confirm that there is a correlation between these markers and the response to IST, suggesting that there is a difference in pathophysiology between adult and paediatric AA. We therefore believe that identification of the differences between children and adult AA is important for understanding the pathophysiology of this disease. Nonetheless, the excellent clinical outcomes of IST for AA in both children and adults unequivocally support the idea of an autoimmune pathogenesis of AA. It is conceivable that the differences observed in children and adult AA might reflect distinct key target antigens producing immune response against haematopoietic stem cells between children and adult AA. Future studies are needed to identify appropriate markers that can predict the response to IST, and the significance of any identified markers must be examined in a prospective study consisting of large numbers of adults and children.

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Authorship

NY, HY and SK designed the research protocol; NY, HY, TY, JL, YW and NH performed the research; NY, HY, YT and SK analysed data; HY, YT, MT, AH, NN, RK, NH, KA, AK, TF and SK collected samples and managed clinical data; NY and SK wrote the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

References

Albert, E., Thomas, E.D., Nisperos, B., Storb, R., Camitta, B.M. & Parkman, R. (1976) HLA antigens and haplotypes in 200 patients with aplastic anemia. *Transplantation*, **22,** 528–531.

Bacigalupo, A., Chaple, M., Hows, J., Van Lint, M.T., McCann, S., Milligan, D., Chessells, J., Goldstone, A.H., Ottolander, J., van't Veer, E.T., Comotti, B., Coser, P., Broccia, G., Bosi, A., Locasciulli, A., Catalano, L., Battista, R., Arcese, W., Carotenuto, M., Marmont, A.M. & Smith, E.C.G. (1993) Treatment of aplastic anaemia (AA) with antilymphocyte globulin (ALG) and methylprednisolone (MPred) with or without androgens: a randomized trial from the EBMT SAA working party. *British Journal of Haematology*, **83,** 145–151.

Bacigalupo, A., Broccia, G., Corda, G., Arcese, W., Carotenuto, M., Gallamini, A., Locatelli, F., Mori, P.G., Saracco, P., Todeschini, G., Coser, P., Iacopino, P., van Lint, M.T. & Gluckman, E. for the European Group for Blood and Marrow Transplantation (EBMT) Working Party on SAA (1995) Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. *Blood*, **85,** 1348–1353.

Camitta, B.M., Thomas, E.D., Nathan, D.G., Gale, R.P., Kopecky, K.J., Rappeport, J.M., Santos, G., Gordon-Smith, E.C. & Storb, R. (1979) A prospective study of androgens and bone marrow transplantation for treatment of severe aplastic anemia. *Blood*, **53,** 504–514.

Chapuis, B., Von Fliedner, V.E., Jeannet, M., Merica, H., Vuagnat, P., Gratwohl, A., Nissen, C. & Speck, B. (1986) Increased frequency of DR2 in patients with aplastic anaemia and increased DR sharing in their parents. *British Journal of Haematology*, **63,** 51–57.

Chiewsilp, P., Sujirachato, K., Mongkolsuk, T., Junpong, S., Jootar, S. & Hathirat, P. (2000) Preliminary study of HLA-ABCDR antigens in CML, ANLL, thalassemia and severe aplastic anemia in Thais. *Journal of the Medical Association of Thailand*, **83**(Suppl. 1), S130–S136.

D'Amaro, J., van Rood, J.J., Rimm, A.A. & Bortin, M.M. (1983) HLA associations in Italian and non-Italian Caucasoid aplastic anaemia patients. *Tissue Antigens*, **21,** 184–191.

Dausset, J., Gluckman, E., Lemarchand, F., Nunez-Roldan, A., Contu, L. & Hors, J. (1977) Excess of HLA-A2 and HLA-A2 homozygotes in patients with aplastic and Fanconi's anemias. *Nouvelle revue française d'hématologie; Blood Cells*, **18,** 315–324.

Davies, J.K. & Guinan, E.C. (2007) An update on the management of severe idiopathic aplastic anaemia in children. *British Journal of Haematology*, **136,** 549–564.

Dunn, D.E., Tanawattanacharoen, P., Boccuni, P., Nagakura, S., Green, S.W., Kirby, M.R., Kumar, M.S., Rosenfeld, S. & Young, N.S. (1999) Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Annals of Internal Medicine*, **131,** 401–408.

Feng, X., Chuhjo, T., Sugimori, C., Kotani, T., Lu, X., Takami, A., Takamatsu, H., Yamazaki, H. & Nakao, S. (2004) Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood*, **104,** 2425–2431.

Fuhrer, M., Durner, J., Brunnler, G., Gotte, H., Deppner, C., Bender-Gotze, C. & Albert, E. (2007) HLA association is different in children and adults with severe acquired aplastic anemia. *Pediatric Blood & Cancer*, **48,** 186–191.

Gluckman, E., Papon, L., Hors, J., Devergie, A., Busson, M., Gony, J. & Dausset, J. (1981) HLA markers in patients suffering from aplastic anaemia. *Haematologia (Budap)*, **14,** 165–172.

Gluckman, E., Esperou-Bourdeau, H., Baruchel, A., Boogaerts, M., Briere, J., Donadio, D., Leverger, G., Leporrier, M., Reiffers, J., Janvier, M., Michallet, M. & Stryckmans, P. The Cooperative Group on the Treatment of Aplastic Anemia (1992) Multicenter randomized study comparing cyclosporine-A alone and antithymocyte globulin with prednisone for treatment of severe aplastic anemia. *Blood*, **79,** 2540–2546.

Gran, B. & Rostami, A. (2001) T cells, cytokines, and autoantigens in multiple sclerosis. *Current Neurology and Neuroscience Reports*, **1,** 263–270.

Hirano, N., Butler, M.O., Von Bergwelt-Baildon, M.S., Maecker, B., Schultze, J.L., O'Connor, K.C., Schur, P.H., Kojima, S., Guinan, E.C. & Nadler, L.M. (2003) Autoantibodies frequently detected in patients with aplastic anemia. *Blood*, **102,** 4567–4575.

Hirano, N., Butler, M.O., Guinan, E.C., Nadler, L.M. & Kojima, S. (2005) Presence of anti-kinectin and anti-PMS1 antibodies in Japanese aplastic anaemia patients. *British Journal of Haematology*, **128,** 221–223.

Horii, A., Han, H.J., Sasaki, S., Shimada, M. & Nakamura, Y. (1994) Cloning, characterization and chromosomal assignment of the human genes homologous to yeast PMS1, a member of mismatch repair genes. *Biochemical and Biophysical Research Communications*, **204,** 1257–1264.

Hurtado Monroy, R., Lavalle Montalvo, C., Schmill Lopez, N., Vega Lopez, M.A. & Gonzalez Llaven, J. (1980) The human histocompatibility system (HLA) in aplastic anemia (AA) (author's transl). *Sangre (Barc)*, **25,** 466–470.

Ihan, O., Beksac, M., Arslan, O., Ozcan, M., Koc, H., Akan, H., Gurman, G., Konuk, N. & Uysal, A. (1997) HLA DR2: a predictive marker in response to cyclosporine therapy in aplastic anemia. *International Journal of Hematology*, **66,** 291–295.

Ilhan, O., Beksac, M., Koc, H., Akan, H., Keskin, A., Arslan, O., Gurman, G., Ozcan, M., Konuk, N. & Uysal, A. (1995) HLA-DR frequency in Turkish aplastic anemia patients and the impact of HLA-DR2 positivity in response rate in patients receiving immunosuppressive therapy. *Blood*, **86,** 2055.

Itoh, Y., Mizuki, N., Shimada, T., Azuma, F., Itakura, M., Kashiwase, K., Kikkawa, E., Kulski, J.K., Satake, M. & Inoko, H. (2005) High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics*, **57,** 717–729.

Kapustin, S.I., Popova, T.I., Lyshchov, A.A., Imyanitov, E.N., Blinov, M.N. & Abdulkadyrov, K.M. (2001) HLA-DR4-Ala74 beta is associated with risk and poor outcome of severe aplastic anemia. *Annals of Hematology*, **80,** 66–71.

Kojima, S., Hibi, S., Kosaka, Y., Yamamoto, M., Tsuchida, M., Mugishima, H., Sugita, K., Yabe, H., Ohara, A. & Tsukimoto, I. (2000) Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. *Blood*, **96,** 2049–2054.

Kosaka, Y., Yagasaki, H., Sano, K., Kobayashi, R., Ayukawa, H., Kaneko, T., Yabe, H., Tsuchida, M., Mugishima, H., Ohara, A., Morimoto, A., Otsuka, Y., Ohga, S., Bessho, F., Nakahata, T., Tsukimoto, I. & Kojima, S. (2008) Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. *Blood*, **111,** 1054–1059.

Lieberman, S.M., Evans, A.M., Han, B., Takaki, T., Vinnitskaya, Y., Caldwell, J.A., Serreze, D.V., Shabanowitz, J., Hunt, D.F., Nathenson, S.G., Santamaria, P. & DiLorenzo, T.P. (2003) Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, **100,** 8384–8388.

Maciejewski, J.P., Follmann, D., Nakamura, R., Saunthararajah, Y., Rivera, C.E., Simonis, T., Brown, K.E., Barrett, J.A. & Young, N.S. (2001) Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome. *Blood*, **98,** 3513–3519.

Maciejewski, J.P., Risitano, A., Kook, H., Zeng, W., Chen, G. & Young, N.S. (2002) Immune pathophysiology of aplastic anemia. *International Journal of Hematology*, **76**(Suppl. 1), 207–214.

Markovic-Plese, S. & McFarland, H.F. (2001) Immunopathogenesis of the multiple sclerosis lesion. *Current Neurology and Neuroscience Reports*, **1,** 257–262.

Mathe, G., Amiel, J.L., Schwarzenberg, L., Choay, J., Trolard, P., Schneider, M., Hayat, M., Schlumberger, J.R. & Jasmin, C. (1971) Bone marrow graft in man after conditioning by antilymphocytic serum. *Transplantation Proceedings*, **3,** 325–332.

Nakao, S., Takamatsu, H., Chuhjo, T., Ueda, M., Shiobara, S., Matsuda, T., Kaneshige, T. & Mizoguchi, H. (1994) Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. *Blood*, **84,** 4257–4261.

Nakao, S., Takami, A., Sugimori, N., Ueda, M., Shiobara, S., Matsuda, T. & Mizoguchi, H. (1996) Response to immunosuppressive therapy and an HLA-DRB1 allele in patients with aplastic anaemia: HLA-DRB1*1501 does not predict response to antithymocyte globulin. *British Journal of Haematology*, **92,** 155–158.

Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., Chapelle, A., Vogelstein, B. & Kinzler, K.W. (1994) Mutations of two PMS homologues in hereditary non-polyposis colon cancer. *Nature*, **371,** 75–80.

Nimer, S.D., Ireland, P., Meshkinpour, A. & Frane, M. (1994) An increased HLA DR2 frequency is seen in aplastic anemia patients. *Blood*, **84,** 923–927.

Oguz, F.S., Yalman, N., Diler, A.S., Oguz, R., Anak, S. & Dorak, M.T. (2002) HLA-DRB1*15 and pediatric aplastic anemia. *Haematologica*, **87,** 772–774.

Rosenfeld, S.J., Kimball, J., Vining, D. & Young, N.S. (1995) Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood*, **85,** 3058–3065.

Rosenfeld, S., Follmann, D., Nunez, O. & Young, N.S. (2003) Antithymocyte globulin and cyclosporine for severe aplastic anemia: association between hematologic response and long-term outcome. *The Journal of the American Medical Association*, **289,** 1130–1135.

Saunthararajah, Y., Nakamura, R., Nam, J.M., Robyn, J., Loberiza, F., Maciejewski, J.P., Simonis, T., Molldrem, J., Young, N.S. &

Barrett, A.J. (2002) HLA-DR15 (DR2) is overrepresented in myelodysplastic syndrome and aplastic anemia and predicts a response to immunosuppression in myelodysplastic syndrome. *Blood*, **100,** 1570–1574.

Shao, W., Tian, D., Liu, C., Sun, X. & Zhang, X. (2000) Aplastic anemia is associated with HLA-DRB1*1501 in northern Han Chinese. *International Journal of Hematology*, **71,** 350–352.

Sugimori, C., Chuhjo, T., Feng, X., Yamazaki, H., Takami, A., Teramura, M., Mizoguchi, H., Omine, M. & Nakao, S. (2006) Minor population of CD55-CD59- blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. *Blood*, **107,** 1308–1314.

Takamatsu, H., Feng, X., Chuhjo, T., Lu, X., Sugimori, C., Okawa, K., Yamamoto, M., Iseki, S. & Nakao, S. (2007) Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood*, **109,** 2514–2520.

Usman, M., Adil, S.N., Moatter, T., Bilwani, F., Arian, S. & Khurshid, M. (2004) Increased expression of HLA DR2 in acquired aplastic anemia and its impact on response to immunosuppressive therapy. *Journal of the Pakistan Medical Association*, **54,** 251–254.

Viglietta, V., Kent, S.C., Orban, T. & Hafler, D.A. (2002) GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. *Journal of Clinical Investigation*, **109,** 895–903.

Wang, H., Chuhjo, T., Yamazaki, H., Shiobara, S., Teramura, M., Mizoguchi, H. & Nakao, S. (2001) Relative increase of granulocytes with a paroxysmal nocturnal haemoglobinuria phenotype in aplastic anaemia patients: the high prevalence at diagnosis. *European Journal of Haematology*, **66,** 200–205.

Young, N.S. (1992) The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. *Blood*, **79,** 1385–1392.

Young, N.S. (1999) Acquired aplastic anemia. *The Journal of the American Medical Association*, **282,** 271–278.

Young, N.S. (2002a) Acquired aplastic anemia. *Annals of Internal Medicine*, **136,** 534–546.

Young, N.S. (2002b) Immunosuppressive treatment of acquired aplastic anemia and immune-mediated bone marrow failure syndromes. *International Journal of Hematology*, **75,** 129–140.

bjh research paper

Risk factors for early death in neonates with Down syndrome and transient leukaemia

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Summary

Transient leukaemia (TL) in neonates with Down syndrome (DS) is characterized by the transient appearance of blast cells in the peripheral blood that resolves spontaneously. Some TL patients die at an early age due to organ failure. Seventy DS patients with TL were studied retrospectively to identify clinical and laboratory characteristics associated with early death (<6 months of age). Sixteen of 70 patients (22·9%) died early. The main causes of death were organ failure, particularly hepatic and cardiopulmonary failure. On univariate analysis, early gestational age (EGA), high white blood cell (WBC) count ($\geq 100 \times 10^9$/l), percentage of peripheral blasts, elevated aspartate transaminase (AST), elevated direct bilirubin (DB), and low Apgar score were significantly associated with poor survival. On multivariate analysis, EGA, WBC count, and DB were independent predictors of poor outcome. A simple risk stratification system combining EGA and WBC count was devised to predict poor outcome. Term infants (EGA ≥ 37 weeks) whose WBC count was lower than 100×10^9/l had the best outcome [7·7% (3/39) died early], while preterm infants (EGA < 37 weeks) whose WBC count was higher than 100×10^9/l had the worst outcome [54·5% (6/11) died early]. This stratification system may be useful for identifying high-risk patients who need early therapeutic interventions.

Keywords: transient leukaemia, Down Syndrome, prognostic factors, estimated gestational age, white blood cell count.

Transient leukaemia (TL), also known as transient myeloproliferative disorder (TMD) or transient abnormal myelopoiesis (TAM), occurs in approximately 10% of infants with Down syndrome (DS) (Doyle, 1994). This disorder is characterized by the appearance of blast cells with megakaryoblastic and/or erythroblastic characteristics in the peripheral blood. While most TL patients have a favorable clinical course, and the blast cells disappear spontaneously, some develop vital organ failure and die at an early age (Miyauchi *et al*, 1992; Hoskote *et al*, 2002). In this group of patients, liver failure with hepatic fibrosis and cardiopulmonary failure are the major causes of death (Massey *et al*, 2006).

Recently, low-dose cytosine arabinoside (Ara-C) therapy has been reported to be effective for improving the clinical outcome in TL patients with severe liver or cardiopulmonary disease (Al-Kasim *et al*, 2002; Dormann *et al*, 2004). However, because the disease resolves spontaneously in most instances, it is not generally recommended that all patients receive antileukaemic therapy. It is not known which patients with TL should be treated with chemotherapeutic agents. The present study aimed to identify the group at high risk for early death that would potentially benefit from treatment with antileukaemic agents.

Materials and methods

A retrospective chart review of patients admitted to 14 hospitals located in the Tokai area of Japan between August 1985 and August 2006 was undertaken to identify all DS neonates with TL. The eligibility criteria for the analysis were DS infants who were younger than 3 months and had circulating blast cells in the peripheral blood. Patients with leucocytosis and peripheral blast cells as a result of infections or blood group incompatibility were excluded. The study was

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Table I. Clinical characteristics and laboratory findings at diagnosis of patients with transient leukemia and Down syndrome.

	Normal range	Low risk group (n = 39)	Intermediate risk group (n = 19)	High risk group (n = 11)	Total patients (n = 70)
Estimated gestational age, weeks		38 (37–41)	36 (30–39)	36 (28–36)	37 (28–41)
Birth weight, kg		2·86 (1·98–3·78)	2·16 (1·71–3·01)	2·28 (1·21–2·97)	2·55 (1·21–3·78)
Apgar score at 1 min		8·5 (1–10)	6·5 (1–9)	7 (3–9)	8 (1–10)
Apgar score at 5 min		9 (5–10)	8 (4–10)	9·5 (5–10)	9 (4–10)
Onset of TL, days		0 (0–63)	0 (0–4)	0 (0–2)	0 (0–63)
Peak value of WBC, $\times 10^9$/l	3·8–8·5	36 (0·6–99)	51 (0·7–187)	170 (103–1033)	49·3 (0·6–1033)
Peripheral blasts, %		26 (0·5–90)	54 (0·9–92)	75 (60–96)	43 (0·5–96)
Peak value of AST, IU/l	13–33	63 (18–463)	74 (22–1401)	296 (33–5038)	74 (18–5038)
Peak value of ALT, IU/l	6–30	37 (7–669)	43 (8–219)	138 (8–495)	43 (7–669)
Peak value of T.Bil, μmol/l	5–20	198 (7–407)	192 (68–742)	215 (55–527)	203 (7–742)
Peak value of D.Bil, μmol/l	0–7	45 (5–270)	70 (12–503)	52 (13–307)	50 (5–503)

Values are given as median (range).
TL, transient leukemia; WBC, white blood cell count; AST, aspartate transaminase; ALT, alanine transaminase; T.Bil, total bilirubin; D.Bil, direct bilirubin.

approved by the ethics committee of each participating hospital, and written informed consent was obtained from all parents.

A total of 70 patients (46 male, 24 female) were identified. Their clinical characteristics and laboratory findings at the time that TL was diagnosed are summarized in Table I. Of the 70 patients, 43 had congenital heart disease.

The probability of overall survival and the development of subsequent leukemia were calculated using the Kaplan-Meier method. The correlations between patients' covariates and early death (<6 months of age) were evaluated using univariate and multivariate Cox proportional hazards regression model. The following covariates were evaluated: gender, presence of congenital heart disease, estimated gestational age, birth weight, Apgar score, white blood cell counts, total and direct bilirubin, alanine aminotransferase, aspartate aminotransferase, and the results of cytogenetic study. All P values reported are two-sided, and P values <0·05 were considered statistically significant.

Results

Eighteen of the 70 patients (26%) died. These 18 patients' clinical and laboratory findings are summarized in Table II and Table SI. Early death occurred in 16 of 70 patients (23%); the median age at death was 33 d (range: 1–143 d). In all but one patient with early death, the cause of death was vital organ failure due to blast cell infiltration (11 patients died of hepatic failure, three of cardiopulmonary failure, and one of renal failure). These patients eventually suffered from multi-organ failure with disseminated intravascular coagulation. One patient with a ventricular septal defect (VSD) and an atrial septal defect (ASD) whose family refused active treatment died of cardiac failure at 136 d of age.

Table II. Clinical and laboratory data on all deaths, both early and late, in the study group.

Patient no.	Age of death, days	EGA, weeks	Peak value of WBC, $\times 10^9$/l	Peak value of D.Bil, μmol/l	Main cause of death	Antileukemic agent
1	2	37	50·7	–	CF	–
2	6	30	12·7	27	CF	–
3	7	28	260	25	HF	–
4	7	34	1033	146	HF	–
5	17	32	197·7	13	RF	–
6	19	36	174·2	83	HF	–
7	31	37	109·5	42	CF	–
8	35	36	154·4	178	HF	–
9	48	36	97·3	472	HF	–
10	55	35	46·6	487	HF	–
11	56	35	7·7	393	HF	–
12	85	38	186·8	503	HF	Low-dose Ara-C
13	118	36	399	307	HF	Vincristine
14	121	37	11·3	270	HF	–
15	136	39	18·9	113	CF	–
16	143	35	27·8	438	HF	–
17	441	37	36·7	165	CF	–
18	887	38	137·7	15	AMKL	–

EGA, early gestational age; WBC, white blood cell count; D.Bil, direct bilirubin; CF, cardiopulmonary failure; HF, hepatic failure; RF, renal failure; CHD, congenital heart disease; AMKL, acute megakaryoblastic leukemia; Ara-C, cytosine arabinoside.

The covariates that were significantly correlated with early death were examined further. On univariate analysis, the following covariates were identified: early gestational age (EGA), low Apgar score at 1 min and at 5 min, peak value of white blood cell (WBC) count ≥100 × 10^9/l), percentage of peripheral blasts, elevated aspartate transaminase (AST),

Table III. Clinical and laboratory covariates and early death in patients with transient leukemia and Down syndrome (univariate analysis).

	Normal range	Patients with early death (n = 16)	Total patients (n = 70)	Hazard ratio (95% CI)	P value
Sex, n (%)					
Male		9 (56)	46 (66)	1	0·2309
Female		7 (44)	24 (34)	1·830 (0·681–4·917)	
Congenital heart disease, n (%)		10 (62)	43 (61)	1·086 (0·394–2·988)	0·8737
Term vs. Preterm, n (%)					
Term (≥37 weeks)		5 (31)	45 (65)	1	0·0025
Preterm (<37 weeks)		11 (69)	24 (35)	5·121 (1·774–14·788)	
Not LBW vs. LBW, n (%)					
Not LBW (≥2·5 kg)		5 (31)	37 (53)	1	0·0565
LBW (<2·5 kg)		11 (69)	33 (47)	2·799 (0·972–8·060)	
Apgar score at 1 min (mean)		4·9	7·2	0·678 (0·564–0·815)	<0·0001
Apgar score at 5 min (mean)		7·3	8·6	0·607 (0·460–0·802)	0·0004
Onset of TL, days (mean)		0·4	3·8	0·813 (0·572–1·156)	0·2498
Peak value of WBC, $\times 10^9$/l (mean)	3·8–8·5	174	86	1·004 (1·002–1·006)	0·0002
WBC < 100×10^9/l vs. WBC ≥ 100×10^9/l, n (%)					
WBC < 100×10^9/l		8 (50)	53 (76)	1	0·0062
WBC ≥ 100×10^9/l		8 (50)	17 (24)	3·956 (1·477–10·592)	
Peripheral blasts, % (mean)		52·3	44·0	1·054 (1·005–1·107)	0·0314
Peak value of AST, IU/l (mean)	13–33	669	249	1·001 (1·000–1·001)	0·003
Peak value of ALT, IU/l (mean)	6–30	138	102	1·002 (0·999–1·101)	0·1407
Peak value of T.Bil, μmol/l (mean)	5–20	375	232	1·004 (1·002–1·006)	0·0005
Peak value of D.Bil, μmol/l (mean)	0–7	233	108	1·005 (1·003–1·008)	0·0002
D.Bil < 83 μmol/l vs. D.Bil ≥ 83 μmol/l, n (%)					
D.Bil < 83 μmol/l		11 of 18 (61)	34 of 49 (69)	1	0·0020
D.Bil ≥ 83 μmol/l		7 of 18 (39)	15 of 49 (31)	6·150 (1·943–19·469)	
Additional chromosomal abnormality, n (%)		2 of 14 (14)	5 of 58 (9)	1·777 (0·398–7·944)	0·4517

LBW, low body weight; TL, transient leukemia; WBC, white blood cell; AST, aspartate transaminase; ALT, alanine transaminase; T.Bil, total bilirubin; D.Bil, direct bilirubin.

elevated total and direct bilirubin (DB) levels (Table III). On multivariate analysis, it was confirmed that EGA, peak value of WBC count ≥100×10^9/l, and DB levels were independent risk factors for early death (Table IV).

To classify the risk groups simply, the 70 patients were stratified into three groups according to their WBC counts and EGA: low risk (WBC < 100×10^9/l and term, n = 39); intermediate risk (WBC < 100×10^9/l and preterm or WBC ≥ 100×10^9/l and term, n = 19); and high risk (WBC ≥ 100×10^9/l and preterm, n = 11). One patient could not be classified due to incomplete data. The 1-year overall survival was 92·1% ± 4·4% for the low-risk group, 63·2% ± 11·1% for the intermediate-risk group, and 45·5% ± 15·0% for the high-risk group (P = 0·001) (Fig 1).

Table IV. Clinical and laboratory covariates and early death in patients with transient leukemia and Down syndrome (multivariate analysis).

Covariates	Hazard ratio (95% CI)	P value
Preterm vs. Term	3·630 (1·133–11·624)	0·030
WBC ≥ 100×10^9/l vs. WBC < 100×10^9/l	3·309 (1·157–9·468)	0·026
D.Bil ≥ 83 μmol/l vs. D.Bil < 83 μmol/l	5·461 (1·667–17·886)	0·005

WBC, white blood cell; D.Bil, direct bilirubin; CI, confidential interval.

Twelve of the 54 patients (22%) who survived more than 6 months after birth subsequently developed acute myeloid leukaemia at a median age of 19 months (range: 13–25 months). All 12 patients were diagnosed as having acute megakaryoblastic leukaemia (AMKL) and received low intensity chemotherapy specific for AMKL in DS patients (Kojima *et al*, 1993, 2000; Kudo *et al*, 2007). All patients achieved a complete remission and are alive and well, except for one patient who relapsed and died at 887 d of age. None of the covariates analyzed was significantly correlated with the later development of acute leukaemia (data not shown). At the time when TL was diagnosed, cytogenetic data were obtained in 58 of 70 patients (83%). Fifty-three patients had only trisomy 21. One patient had 46, XY, t(21q21q), and four patients had additional chromosomal abnormalities ((47, XY, inv(9)(p12q13), +21), (47, XY, inv(2)(8p11.2q13), +21), (47,XY,

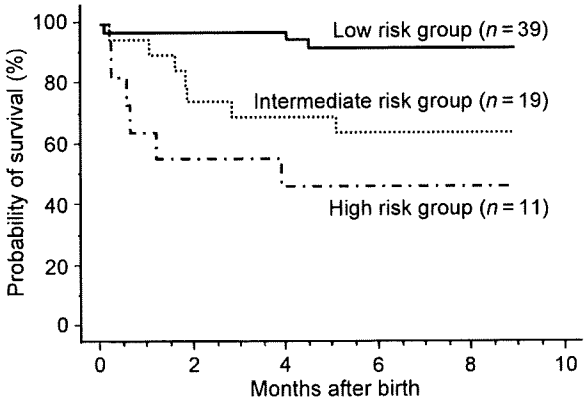

Fig 1. Kaplan–Meier curves showing the overall survival of patients in each risk group: low-risk group (WBC count $<100 \times 10^9$/l and term, $n = 39$); intermediate-risk group (WBC count $<100 \times 10^9$/l and preterm or WBC count $\geq 100 \times 10^9$/l and term, $n = 19$); and high-risk group (WBC count $\geq 100 \times 10^9$/l and preterm, $n = 11$). The probability of survival differed significantly among groups ($P = 0{\cdot}001$).

der(14;21)(q10;q10), +21), (47, XY, 15p+, +21) for each case). An additional chromosomal abnormality was not associated with early death or with the later development of AMKL.

Three of the 70 patients received antileukaemic therapy. One patient received vincristine (0·02 mg/kg per day) shortly before his death (grouped as high-risk), and two received low-dose cytosine arabinoside (Ara-C). Of the two patients who received low-dose Ara-C, one received Ara-C (0·7 mg/kg per day) for 5 d, starting at 30 d of age (grouped as intermediate-risk). His peripheral blast cells disappeared rapidly, but his clinical condition did not improve, and he died at the age of 85 d. The other patient (grouped as low-risk) received Ara-C (10 mg/m^2 per dose twice a day) for 10 d soon after his diagnosis; his clinical condition improved rapidly, and he is currently alive and well.

Discussion

Transient leukaemia in DS neonates was first described in two cases reported in 1956 (Schunk & Lehman, 1954). One of these patients died on the 11th day of life due to gastrointestinal bleeding, and the other patient survived following spontaneous remission. TL was considered to be "self-limiting"; the prognosis of TL was favorable, except for the risk of the subsequent development of acute leukaemia. However, deaths due to vital organ failure were occasionally reported (Hayashi *et al*, 1988; Miyauchi *et al*, 1992; Schwab *et al*, 1998; Hoskote *et al*, 2002). Homans *et al* (1993) reviewed the literature published before 1993 and reported that 10 of 95 DS patients with TL (11%) died early.

Recent clinical trials have reported that the prognosis of AMKL in DS patients improved with the use of the Cooperative Study Group protocol (Kojima *et al*, 1993, 2000; Gamis *et al*, 2003; Creutzig *et al*, 2005; Abildgaard *et al*, 2006; Kudo *et al*, 2007). Thus, in order to improve the long-term outcome of DS patients, the prevention of early death has received much attention. Several reports advocate the use of low-dose Ara-C in patients with severe forms of TL; Ara-C treatment may decrease mortality if started early in the clinical course (Al-Kasim *et al*, 2002; Dormann *et al*, 2004). However, the efficacy of Ara-C treatment has not been confirmed by a prospective clinical trial. There is still controversy as to which patients should be treated with low-dose chemotherapy and which patients should receive supportive care only.

The present retrospective analysis is one of the largest studies of TL in DS neonates. There have been only a few studies that examined the risk factors associated with early death in DS neonates (Massey *et al*, 2006). We devised a simple risk stratification system based on the EGA and the peak WBC count. The high-risk group (HR) was defined as preterm infants with WBC $\geq 100 \times 10^9$/l, the intermediate-risk group (IR) was defined as preterm infants with WBC $< 100 \times 10^9$/l and term infants with WBC $\geq 100 \times 10^9$/l, and the low-risk group (LR) was defined as term infants with WBC $< 100 \times 10^9$/l. In the LR group, only three of 39 patients (7·7%) died early. One of them died of heart failure secondary to an untreated VSD; thus, the cause of death was not directly related to TL. Based on the data of the present study, patients in the LR group should receive no interventions. However, since the probability of early death in patients in the HR group exceeded 50%, active intervention including low dose Ara-C should be tried in the context of a clinical trial for these patients.

In patients with acute myeloid leukaemia (AML), hyperleucocytosis increases the risk of fatal complications, such as intracerebral hemorrhage and respiratory failure (Bunin & Pui, 1985). In patients with a severe form of TL, the main causes of death in early life are progressive hepatic fibrosis, cardiopulmonary failure, and disseminated intravascular coagulation. These complications may be caused by blast cell infiltration into vital organs. Thus, it is understandable that hyperleucocytosis was found to be a strong risk factor for early death in both the study reported by the Children's Oncology Group (COG) (Massey *et al*, 2006) and the present study.

According to the COG report, higher WBC counts, higher AST and alanine transaminase (ALT) levels, and failure to clear peripheral blasts were significant predictive factors for poor outcome. In the COG study, EGA was not a statistically significant predictor. In contrast to the COG study, the present study found that EGA was strongly associated with early death. In the COG study, EGA data were available for only 26 of 47 patients; this may have reduced the statistical power of the study. The immaturity of vital organs, such as the liver and kidneys, compared to those of full-term neonates, may be one of the reasons that premature infants cannot tolerate blast cell infiltration. In fact, TL itself might be a cause of premature delivery. In patients with a severe type of TL, the fetus may

