other interleukin molecules (IL-2, -4, -7, -9, -15 and -21) [32-34]. Without curative HST therapy, patients with X-SCID will generally not survive more than a year due to severe, uncontrollable infectious episodes. Therefore, HST will be an immediate consideration as soon as an X-SCID diagnosis is made because there is no alternative therapy available similar to the PEG-ADA replacement for ADA deficiency. The survival rate after HLA-matched HST is over 90%, whereas that of haploidentical HST is only 70-78% [35, 36]. Even in the majority of successful cases, reconstitution of normal B cells is not fully complete; therefore patients should continue with IVIg replacement therapy afterwards [37]. In addition, HST still carries a potential risk of graft verses host disease (GVHD), which cannot be prevented or fully controlled by present techniques. These circumstances point to gene therapy providing an alternative treatment for X-SCID patients. After a large number of basic studies on X-SCID stem cell gene therapy [38-40], a French group started the first gene therapy clinical trial for X-SCID in 1999 [41]. They performed the same therapy in 11 patients up until the present, and have obtained gene corrections in 9 patients. It is of note that the serum IgG levels in most cases, which remain low in many successful HST cases, were improved in these 9 patients. [42]. However, approximately 3 years after the therapy, leukemia-like episodes were detected in two patients [43]. The precise clinical features and the suspected mechanisms causing these events will be discussed in the later section.

3. Chronic Granulomatous Disease (CGD)

Chronic granulomatous disease (CGD) is an X-linked or autosomal recessively inherited PID with defects in the plasma membrane associated enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in phagocytes [44]. Deficiency of this oxidase causes a notable reduction or absence of the phagocytic respiratory burst and superoxide radicals, resulting in defective killing of catalase positive bacteria and fungal infections. The disease results from mutations in any one of 4 subunits of NADPH oxidase; gp91-phox, p22-phox, p47-phox and p67-phox. Although HST is the only curative therapy for CGD, most patients do not receive HST because of the possible risks associated with the therapy. In addition, palliative therapy with antibiotics, gamma interferon and surgical procedures is in part effective for most patients. However, the long-term prognosis of CGD patients is not good mainly due to chronic and progressive fungal infections, in those cases HST cannot be performed under the optimal conditions. Due to these factors, gene therapy studies were started using the patient's cell lines [45, 46] and CGD model mice [47]. Then, a series of clinical trials involving hematopoietic stem cell gene therapy for two types of CGD; X-CGD (defective gp-91phox) and p47-phox defective CGD were finally performed at NIH, USA [6, 48]. These results indicated that gene-corrected cells were detectable at low levels but only for a short time periods after treatment, and failed to show any sustained clinically beneficial effects. Gene-corrected cells in CGD patients fail to gain any selective advantage over non-corrected cells, therefore, more innovative strategies are required for effective CGD gene therapy. Depending on the results of HST under non-myeloablative conditioning, which was recently performed on CGD patients [49, 50], the current enthusiasm for CGD gene therapy might wane.

4. Other Diseases

Clinical gene therapy trials involving other PID include a case of SCID harboring a Jak3 deficiency and two cases with leukocyte adhesion deficiency (LAD) [51-53]. Neither trial has, as yet, shown any clear clinical effects. In the case of the Jak3 deficiency, it seems that the same results, which were obtained in X-SCID trials, might be expected in this disease. The gene-corrected cells are expected to gain a selective advantage over the other cells. This is due to the fact that the Jak3 molecule is directly involved in signaling *via* the γc, it therefore appears that the X-SCID and Jak3 deficiencies share a common pathological mechanism. Indeed, patients with a Jak3 deficiency are clinically indistinguishable from X-SCID patients except for inheritance patterns [54]. In this respect, serious adverse events should be anticipated and carefully monitored even after successful gene therapy has been performed. In contrast, the LAD results were predictably poor because the gene corrected cells do not gain any selective advantage similar to those in the CGD gene therapy. As mentioned for CGD gene therapy, new strategies are needed to increase the effectiveness of LAD gene therapy.

SERIOUS ADVERSE EVENT IN TWO X-SCID PATIENTS AFTER GENE THERAPY

The French group reported two cases with X-SCID, who received hematopoietic stem cell gene therapy and showed sustained therapeutic effects, but who developed T cell leukemia-like diseases in September and December of 2002, respectively [43]. They were two of a 9 patient series who showed good clinical effects from 11 patients receiving gene therapy. There were similar characteristic features observed in both patients and they are listed below (see Table 2).

CASE REPORT

Case 1: the case 1 was the forth patient treated by the French clinical gene therapy trial. Two of his family members developed medulloblastoma in their infancy. Gene therapy was performed in October 1999 when he was just one month old. He received $18x10^6$ CD34+ γc+ cells per kg of body weight without any conditioning before the infusion. A few months after the therapy, a reconstitution of his T and a part of his B cells was observed. He suffered a varicella infection in April 2002, and during that period increased γδT cells were detected. The γδT cells rapidly increased to 2-3 $x10^5/\mu l$ in August and were associated with splenomegaly and thrombocytopenia. It was demonstrated that this increase in γδT cells was monoclonal in origin (Vγ9/Vδ1) and that it expressed the γc protein derived from the vector used. The insertion site of the vector in the abnormal cell was studied by linear amplification-mediated (LAM) PCR. It was shown that the vector was inserted into intron 1 of the *LMO2* proto-oncogene, and *LMO2* was expressed in all these abnormal cells. He received multiple chemotherapies with vincristine, daunorubisine and steroids, and HST was sequentially performed. Although his long-term prognosis is as yet unknown, he is currently in good in health.

Table 2. Characteristic Feature of Two X-SCID Patients with Leukemia Like Events; Comparison with Other X-SCID Patients Receiving Gene therapy (French Group)

	Case 1	Case 2	Others
Age at the therapy	1 month (the youngest)	3 month (the second youngest)	
Total gene-transduced CD34+ cells received	$18x10^6$/kg	$20x10^6$/kg	Median;$4.3x10^6$/kg
T cell development early after the therapy	Rapid and intense	Rapid and intense	Slower
Intervals of post-therapy to leukemia event	30 months	34 months	
Family history of childhood cancer	Yes*	No	No
Character of abnormal cells	One clone; γδ T cell	3 clones; αβ T cell (different β)	No
Vector insertion site in abnormal cells	1 intron of *LMO2* (reverse)	3kb upstream of *LMO2* (the same site)	Detected within *LMO2* in one patient's normal cells

*: Two of his family mumbers developed medulloblastome in their infancy.

Case 2: the case 2 was the fifth patient of the same French group's trial. He had no specific family history and received gene therapy at 3 months old. He received $20x10^6$ CD34+ γc+ cells per kg of body weight. He also showed a good immune reconstitution after his therapy. Thirty-four months after the therapy, however, abnormal lymphocyte proliferation, splenomegaly, anemia and thrombocytopenia were noted. The abnormal cells consisted of 3 different αβT clones, but LAM-PCR revealed that the vector insertion site was identical in all three clones. It was notable that the vector had been inserted into a 5' end of *LMO2* exon 1, and that this gene was also expressed. The patient showed a good response to the chemotherapy and the abnormal cells subsequently disappeared.

POSSIBLE MECHANISMS CAUSING SERIOUS ADVERSE EVENTS OBSERVED IN THE X-SCID PATIENTS

As mentioned in the introduction, it had been considered that the risk of causing an insertional mutation from gene therapy using retroviral vectors, which may cause activation of some oncogenes, resulting in the development of uncontrollable cell proliferation, would be wiped out by the initial gene therapy trial. This was thought to be because large numbers of retroviral vector-introduced cells had been repeatedly introduced to patients without any serious adverse events. It was therefore concluded that these putative, serious adverse events might theoretically be possible, but would practically be negligible. However, this conclusion has since proved to be incorrect.

In response to the serious adverse events reported by the French group, the NIH, USA has reviewed all its previously planned clinical trials using retroviral-mediated gene transfer to hematopoietic stem cells [55]. They checked the outcomes of 232 cases in 40 trials completed by April 2003, but no serious adverse events had been reported although their gene transduction efficiency was not good as French group's trial.

We must therefore ask ourselves; how did these adverse events occur in the two X-SCID patients of the French group's trial? The reason lies in the vector insertion site in/near the *LMO2* gene in both of the patients' abnormal cells. As *LMO2* was abnormally expressed in these cells, it seems reasonable that non-physiological *LMO2* expression due to vector insertion might be related to these events. *LMO2* encodes a transcription factor expressed only in hematopoietic stem/ progenitor cells, and it is known that its continuous, abnormal activation can result in the development of T cell leukemias in both clinical and experimental models [56, 57].

Previously, the activation of some proto-oncogenes by the insertion of a retroviral vector was estimated at the rather low rate between 10^{-6} to 10^{-8} insertion event [58]. It is estimated that the area of retroviral insertion interference in the human host gene is restricted to less than 10kb. Therefore, considering that the human genome consists of $3x10^9$ base pairs, the risk of a single vector insertion event within 10 kb of a proto-oncogene; for example *LMO2*, can be estimated as 10^{-5} to 10^{-6} per insertion event. In the French gene therapy trial group, the median of gene-introduced hematopoietic stem cell number was $4.3x10^6$/kg [43]. Moreover, in some hematopoietic stem cell gene therapy trials reviewed by NIH/USA above, more than 10^6 gene-transduced cells were introduced into patients [55]. Thus, the retroviral vector integration, which activates the *LMO2*, as well as other proto-oncogenes, can occur relatively frequently during retrovirus mediated gene therapy clinical trials. However, no such cases have been reported in previous gene therapy trials, and thus far only two of 11 X-SCID cases in the French group's trial have shown any leukemia-like adverse events. In general, a single event is not sufficient for the development of malignancy, but somewhere between 4 to 6 genetic hits seem to be required. Therefore, we should expect there to be several other promoting factors, besides LMO2 activation, γc vector insertion, in the two X-SCID patients who developed the leukemia-like disorders.

Firstly, the nature of the transgene construct used to be important. There may exist two broad categories of transgenes; safe and unsafe gene. The vector insertion in/near the *LMO2* gene might well have occurred in previous non X-SCID clinical trials, but similar adverse events may not have

been reported. Both LMO2 and γc induce T cell proliferation, but are not expressed simultaneous under normal physiological conditions. Thus, it appears likely that unregulated expression of γc together with the *LMO2* gene expression can together cause promotion towards leukemia-like development. Secondly, the immaturity of the target cell might be important, because in the gene therapy trials targeting peripheral mature T cells, more than 10^{12} cells were transduced and infused without any insertional mutagenesis episodes. In addition, as most leukemia cells generally originate from immature cells, immature transduction may have the potential to lead to a predisposition to malignancy. Thirdly, the target disease itself might be important. It is well known that the immune surveillance system for cancer, such as NK cell function, is defective in X-SCID patients, which in turn may be one of promoting factors for malignancy. In this regard, other types of SCID may have a similar level of risk. Indeed, most PID cases are frequently complicated by malignancy.

Thus far, similar hematopoietic stem cell gene therapy trials for X-SCID patients have been performed in 11 patients in France, and 5 patients in UK, and two patients among them showed leukemia-like episodes. Moreover, using LAM-PCR the French group has indicated that they found a third X-SCID patient, who exhibited some gene-transduced cells with insertions in *LMO2* although this patient has shown no abnormal cell proliferation, as yet (unpublished, personal communication). Thus, in addition to LMO2 activation due to γc vector insertion, same patients may share the common factors promoting malignancy more than others. It is notable that both the patients are the youngest and the second youngest (one moth and 3 months, respectively at the time of gene therapy), and the amount of infused gene-corrected cells in these patients were above the mean number for other X-SCID patients in the French group. In fact, early T cell development after the therapy was more rapid and intense in both patients when compared to the other patients treated [43]. It was also speculated that in the first patient, a positive family history of two siblings suffering medulloblastoma, and a concomitant episode of varicella infection might be related to these development of malignancy. These data may suggest a limitation both in a patient's age and number of gene corrected cells infused in similar gene therapy trials in the future.

FUTURE GENE THERAPY FOR PID

There are various problems associated with future PID gene therapy that need to be solved. The main aim of current PID gene therapy is to develop a first line therapy of choice instead of relying on allogenic HST, especially because HLA-identical donors are not always available. To achieve this purpose, both the efficacy and safety issues associated with gene therapy need to be improved.

Most importantly, the safety issues related to the gene therapy should be immediately re-evaluated. It is necessary to know the frequency of similar leukemia-like episodes that are associated with this kind of gene therapy, and which individuals are at the most real risk to develop malignant episodes, carefully examining all the suspected factors including; the nature of the gene introduced, the immaturity of target cells, a patient's age (not less than 3 months), the abundance of infused cells, any familial predisposition to cancer and any concomitant episodes of infection. Until then, we must balance the risk and benefits of this type of gene therapy and we should carefully consider each patient on a case-by-case basis. To avoid uncontrolled proliferation of a gene-introduced cells by insertional mutagenesis in retroviral gene therapy, ideas for the modifying the retroviral vector must be proposed, for example; a vector containing a suicide gene for self-inactivation, a vector with insulator sequences to prevent activation of an adjacent gene at the insertion site by the upstream, inserted vector promoter sequences [59], a vector capable of insertion into any specific safety sites on specific chromosomes, would also be beneficial.

As a second safety issue, regulation of the transduced-gene expression is also important, because it is not physiologically regulated in the present gene therapy technology. Therefore, aberrant or uncontrolled gene expression might cause unexpected adverse effects for patients with PID. To establish the physiological expression of the transduced-gene, studies for promoter sequences of a vector gene are necessary.

Although there seem to be numerous of obstacles to be cleared, mutation repair gene therapy strategies [60], may satisfy many of the safety issues mentioned above.

In PID (excluding X-SCID and ADA deficiency), no curative effects have yet been obtained by gene therapy. Therefore, efforts to amplify the efficacy of gene transduction and its expression similar to previous attempts should be maintained without interruption. Especially in some PID like CGD and LAD in which the gene corrected cells do not obtain a selective advantage over the others, major breakthroughs in technology, which also satisfy all of the safety issues, will be of the utmost importance.

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Successful Report of Reduced-Intensity Stem Cell Transplantation from Unrelated Umbilical Cord Blood in a Girl with Chronic Active Epstein-Barr Virus Infection

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Summary: We describe an 8-year-old girl with chronic active Epstein-Barr virus (EBV) infection (CAEBV) who was treated successfully by reduced-intensity stem cell transplantation (RIST) from unrelated cord blood (CB). She had been suffering from fever, abdominal pain, and interstitial lymphadenopathy, and CAEBV was diagnosed. After chemotherapy that included etoposide, the amount of EBV decreased transiently below the detection level. However, the disease due to CAEBV worsened despite the chemotherapy, and she finally needed chemotherapy every week. Therefore, instead of conventional myeloablative transplantation, we performed CB transplantation with reduced-intensity conditioning regimens consisting of low-dose total body irradiation, fludarabine, and etoposide. CB, for which human leukocyte antigen (HLA) was 2-loci mismatched on the DR loci from an unrelated donor, was infused after conditioning. Although grade III acute graft-versus-host disease (GVHD) in the gut and chronic GVHD in the lung developed, the symptoms of GVHD disappeared with immunosuppressive therapy. After 15 months, the patient remained a complete chimera, with undetectable levels of EBV in peripheral blood and bone marrow. We conclude that RIST from unrelated CB can be indicated for some cases of CAEBV who are refractory to chemotherapy and have no HLA-matched related and unrelated donors as the source of bone marrow or peripheral blood stem cells.

Key Words: chronic active Epstein-Barr virus infection (CAEBV), reduced-intensity stem cell transplantation (RIST), cord blood (CB)

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Chronic active Epstein-Barr virus (EBV) infection (CAEBV) is characterized by chronic or recurrent infective mononucleosis-like symptoms lasting more than 6 months, such as fever, hepatosplenomegaly, and lymphadenopathy. In patients with CAEBV, there are abnormal high titers of anti-EBV antibodies, no evidence of prior immunologic abnormality, and detection of the EBV genome in biopsy samples, such as the lymph node.[1] Patients with severe CAEBV die from hemophagocytic syndrome, malignant lymphoma, and/or multiple organ failure within several years after the onset. EBV infects T or natural killer (NK) cells by latency I or II in most patients, and cells infected by EBV cannot be eradicated by patients' cytotoxic T cells (CTLs).[2–3] Although several therapies including antiviral agents and chemotherapy, such as with etoposide (VP-16), have been reported,[4–5] they appear to have only a transient effect and are unable to control CAEBV.

Stem cell transplantation (SCT) is the only curative treatment for CAEBV.[6–8] The effects of SCT are considered cytotoxic with preconditioning chemoradiotherapies and the infusion of normal-donor mature CTLs. In this context, myeloablative conditioning and allogenic transplantation from bone marrow or peripheral blood stem cells have been recommended. Recently, nonmyeloablative SCT for CAEBV has been reported as an alternative therapy for patients who are apprehensive about the regimen-related toxicity of myeloablative SCT.[9,10] However, use of cord blood (CB) as a source of stem cells in nonmyeloablative SCT for CAEBV has not been reported because the majority of T cells derived from CB seem to be composed of immature or null T cells. Therefore, SCT from CB has been considered ineffective for CAEBV therapy, and there have been no successful reports of reduced-intensity stem cell transplantation (RIST) from cord blood for curative treatment of CAEBV. Here we report the first successful allogenic RIST rescue from unrelated CB for treatment of severe CAEBV.

CASE REPORT

An 8-year-old girl presented with abdominal pain, high fever, systemic lymphadenopathy, and hepatosplenomegaly in July 1999. She did not have any allergy to mosquito bites. The EBV serology was VCA-IgG 1:5260, VCAIgM < 1:10 (negative), EADRIgG 1:1020, and EBNA 1:10. Although histology of the interstitial lymph node revealed nonspecific inflammation, EBV-DNA was detected in the lymph node and peripheral

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blood T cells by polymerase chain reaction (PCR). Therefore, her diagnosis was CAEBV.

Soon after diagnosis, the patient's symptoms due to CAEBV disappeared without medication; however, EBV-DNA was consistently detected by PCR in peripheral blood and lymph nodes. In January 2002, she presented with symptoms similar to those in 1999, such as high fever, systemic lymphadenopathy, and hepatosplenomegaly, and her symptoms consequently became uncontrollable without medication. Therefore, she was treated with chemotherapy, including VP-16, cytosine arabinoside, vincristine, cyclophosphamide, doxorubicin, and prednisolone. However, the effects of these chemotherapies were transient, and the disease due to CAEBV worsened. Finally the patient needed chemotherapy every week. Although we intended to perform allogenic SCT, she did not have any HLA-matched related or unrelated volunteer donors. Moreover, she also exhibited hepatic and renal dysfunction (creatinine clearance, 55–62 mL/min/1.73 m^2; GOT, 71–110 IU/L; GPT, 122–160 IU/L) as a result of the disease and frequent chemotherapies. Therefore, we decided to perform CB transplantation (CBT) from an unrelated donor with a nonmyeloablative conditioning regimen.

After conditioning with total body irradiation (TBI), 2 Gy × 2 on day −8 (total dose, 4 Gy), fludarabine, 30 mg/m^2 from day −7 to −3 (total dose, 150 mg/m^2), and VP-16, 30 mg/kg on day −2, she received intravenous infusion of CB cells (a total of 2.45×10^7/kg cells, 1.07×10^5/kg as CD34-positive cells), in which HLA was mismatched for 2-loci at the DR loci (patient, A: 11.1, B: 51, DR 4 and 8; donor, A: 11.1, B: 51, DR 9 and 12, respectively). Cyclosporine from day −1 and methylprednisolone from day +7 were used for graft-versus-host disease (GVHD) prophylaxis. Granulocyte colony stimulating factor (G-CSF) was administered from day +5 after CBT to engraftment. The neutrophil count reached 0.5×10^9/L on day +22, and the platelet count exceeded 5×10^{10}/L on day +44.

Although grade III acute GVHD involving the gastrointestinal tract developed on day +25 and chronic GVHD involving the lung developed on day +141, these symptoms disappeared after administration of immunosuppressive agents, such as tacrolimus, prednisolone, and azathioprine. Beyond day +52 after SCT, chimerism analysis of peripheral blood and bone marrow mononuclear cells showed that these cells were 100% donor-derived. The EBV DNA was undetectable in peripheral blood mononuclear cells with real-time PCR after SCT (Fig. 1).

The patient has been doing well for 15 months after SCT, although steroid and azathioprine are necessary for the skin involvement of chronic GVHD.

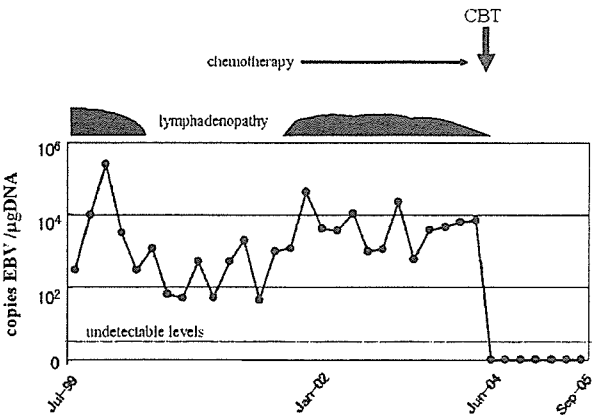

FIGURE 1. Clinical course of the patient. Epstein-Barr virus (EBV)-DNA levels in the peripheral blood mononuclear cells were examined by real-time polymerase chain reaction. The detection limit was estimated to be 20 copies per μg total DNA. Note that after CBT (↓), the EBV-DNA became undetectable, and systemic lymphadenopathy also disappeared.

DISCUSSION

This is the first successful report of RIST from unrelated CB for the treatment of CAEBV. This treatment controlled EBV-infected cells and safely and effectively cured CAEBV. The effects of SCT are considered cytotoxic with the preconditioning chemoradiotherapies and infusion of normal-donor mature CTLs. However, the use of CB as a source of stem cells in nonmyeloablative SCT for CAEBV has not been reported because the majority of T cells derived from CB seem to be composed of immature or null T cells. Moreover, there have been no established clinical effects in nonmyeloablative conditioning in CAEBV. Therefore, RIST from an EBV-seronegative donor such as CB has been considered ineffective for CAEBV therapy.

Although our patient urgently needed SCT as a curative treatment because her symptoms could not be controlled by conventional chemotherapy alone, she had no HLA-matched related or unrelated donors. Furthermore, chemotherapy, such as with VP-16 and prednisolone, was finally required every week, and hepatic and renal dysfunction caused by the disease and frequent chemotherapies were evident. Therefore, we performed RIST from unrelated CB for the patient, although it was uncertain whether the therapy was appropriate for CAEBV. In our case, the patient underwent conditioning with low-dose TBI, fludarabine, and VP-16. Although coadministration with both fludarabine and alkylating agents such as melphalan[9,10] had demonstrated particularly strong immune suppression in RIST, our patient received VP-16 instead of alkylating agents, because it had been demonstrated that VP-16 inhibits EBV nuclear antigen synthesis[4] and VP-16 had been used as one of the key drugs in the conditioning of SCT for CAEBV.[6–8]

After transplantation, EBV-DNA was not detected, and 100% donor chimerism was achieved for 15 months. Systemic lymphadenopathy also disappeared after transplantation. It was considered that conditioning chemoradiotherapies and transplantation of HLA-mismatched CB might both have contributed to the effectiveness of the treatment. It is also possible that provision of normal immune-competent cells in the CB may be effective in terminating EBV infection. The mismatched HLA loci were considered a target of CTLs, and the immunological effects of CTLs might contribute to both disease control and development of GVHD.

We propose that RIST from unrelated CB could be considered for some patients with severe CAEBV who are

refractory to chemotherapy and who have no HLA-matched related or unrelated volunteer donor as a source of bone marrow or peripheral blood stem cells.

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Epitope Mapping of Anti-α-Fodrin Autoantibody in Juvenile Sjögren's Syndrome: Difference in Major Epitopes Between Primary and Secondary Cases

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ABSTRACT. ***Objective.*** Juvenile Sjögren's syndrome (SS) is an early-onset type of SS. Autoantibody against the N-terminal 120 kDa form of α-fodrin is a specific and sensitive disease marker for both juvenile and adult SS. We investigated the initial and major determinants of α-fodrin in SS.

Methods. Sera were obtained from patients with juvenile SS, 10 with primary SS and 10 with secondary SS. Epitope specificities of IgG antibodies were examined by dot-blot analyses using overlapping fusion proteins of the N-terminal part (561 amino acid residues) of α-fodrin as antigens.

Results. All sera from patients with primary SS reacted with amino acid residues 1 to 98 and 36 to 150, but not with 91 to 199. Epitope mapping using fusion proteins with subfragments, each consisting of about 50 amino acid residues, showed reactivity with amino acid residues 27–80 and 79–132, suggesting that at least 2 epitopes are contained in the first 150 amino acid residues. All 3 cases with neurological complications had additional epitope specificities. Sera from patients with secondary SS showed more diversified specificities and strongly reacted with amino acid residues 1–98 and 334–432, whereas the reactivities to 36–150, a major epitope in primary SS, were minimal.

Conclusion. Major and initial B cell epitopes specifically reside in N-terminal amino acids 36–132 and could be used as a diagnostic tool for primary SS. The epitope subsequently expands to other regions of α-fodrin in association with the development of neurological complications or disease progression. Secondary SS has distinct epitope specificities. (J Rheumatol 2006;33:1395–400)

Key Indexing Terms:
SJÖGREN'S SYNDROME α-FODRIN AUTOANTIBODY
EPITOPE MAPPING EPITOPE SPREADING

Sjögren's syndrome (SS) is an autoimmune disease characterized by a progressive infiltration of lymphocytes and plasma cells to the exocrine glands, predominantly salivary and lachrymal glands, with varying degrees of systemic involvement[1]. SS is classified into 2 groups: primary SS, which occurs alone, and secondary SS, which occurs in association with other collagen vascular diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or scleroderma. Whereas SS commonly affects middle-aged women, juvenile onset SS is not so rare as has been estimated before[2–8]. Most cases of juvenile SS lack sicca symptoms, but share similarity with adult cases in both pathological and laboratory features, suggesting that juvenile SS is an early-onset type of the disease[2–8]. Recent studies using immunoblot analyses have shown that IgG-class autoantibody against the N-terminal portion of the 120 kDa form of α-fodrin, an actin-binding protein, is highly sensitive and specific to both adult and juvenile SS[9–12]. Anti-α-fodrin IgG antibodies appear before either anti-SSA/Ro or SSB/La antibodies become positive, and could be an early diagnostic marker of the disease[10]. In addition, critical roles of α-fodrin-specific T cells in the initiation or progression of the disease have been suggested in both murine models and human primary SS, particularly with short duration from onset of the disease[9,13,14]. Thus, autoimmunity to α-fodrin could be involved in the development of primary SS, although the pathological role of the antibodies remains unknown. On the other hand, anti-α-fodrin antibodies are detected by ELISA in less than 10% to 64% of patients with primary SS, whereas 26% of patients with SLE are positive for the antibodies[15–20]. As well, the antibodies are detect-

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ed in 29% of primary SS and 47% of SLE by immunoprecipitation assay[21]. The discrepancy in the sensitivity and specificity could be attributed to the differences in assay systems[9-12,15-21], classification criteria[12], treatment[19], and possibly age groups[10,11]. Antigens are usually denatured in the Western blot system and differ in structure from those bound to ELISA plates[17]. These facts raise the possibility that the autoantibodies preferentially recognize linear and hidden epitopes of α-fodrin in primary SS. Thus, the identification of initial and major epitopes of the protein could provide insight into both the mechanisms of the disease and the development of more sensitive and specific diagnostic systems. Because anti-α-fodrin antibodies normalize within 3 months of treatment, the antibodies should be assessed in an untreated group[20]. In this study, to identify the initial and major epitopes, we constructed overlapping fusion proteins of α-fodrin and used them for epitope analyses of IgG autoantibodies in untreated juvenile SS.

MATERIALS AND METHODS

Patients. Ten patients with primary SS, aged 5 to 15 years, (Group I) and 10 patients with secondary SS, aged 7 to 15 years, (Group II) classified according to the Japanese criteria[22] were included in the study (Table 1). All the primary cases had positive findings in both sialography and lip biopsy and were finally classified as having SS according to the American-European criteria for SS[23]. Sera were obtained before treatment with corticosteroids, antimalarials, or immunosuppressive agents, and were stored at –20°C until use. Sera were additionally obtained from a primary SS case (Patient I-5) upon diagnosis of aseptic meningitis and 2 years after remission. Some cases have been described previously[5,10,24]. Control sera were obtained from 6 patients with SLE alone, 7 with juvenile idiopathic arthritis (JIA), 7 with juvenile dermatomyositis, and 30 with no connective tissue diseases.

Production of fusion proteins. Ten nanograms of plasmid DNA (pGEX-JS-1), which encodes the N-terminal 561 amino acids of α-fodrin[25], were used as template for 50 μl of polymerase chain reaction (PCR). Complementary DNA coding 11 overlapping fragments and 5 subfragments of α-fodrin were amplified using the GenAmp 2400 PCR system (Applied Biosystems, Foster City, CA, USA) using primer pairs shown in Table 2. After an initial denaturation at 94°C for 5 min, each fragment was amplified by 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 7 min. PCR products were subcloned into TA cloning vector pCR2.1 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Each clone was examined for nucleotide sequence by ABI Prism GenAnalyzer 310 (Applied Biosystems). DNA fragments with correct sequence were digested with EcoRI, purified by gel extraction, and then subcloned into EcoRI site of glutathione-S-transferase (GST)-fusion protein expression vector, pGEX4T-2 or 4T-3, in-frame. Fusion proteins were expressed in *E. coli*, TB1, in the presence of 1 mM isopropylthiogalactoside (Amersham Pharmacia, Buckinghamshire, UK), and purified with glutathione-Sepharose beads (Amersham Pharmacia) according to the manufacturer's protocol.

Western blotting and dot blotting. One hundred nanograms of recombinant GST-JS-1 or fusion proteins with 11 overlapping fragments or 5 subfragments were electrophoresed on 7.5% or 12.5% sodium dodecyl sulfate-polyacrylamide gels and electrically transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia). For dot-blot analyses, 100 ng of each fusion protein were put onto the nitrocellulose membrane. After blocking with 5% nonfat milk at 4°C for 16 h, membranes were incubated with 1000-fold diluted goat anti-GST antibody or 400-fold diluted human sera at 4°C for 16 h as primary antibody. To prevent cross-reactivity with *E. coli* components or GST, human sera were diluted with the extract of *E. coli* expressing GST

Table 1. Clinical and serological findings of primary and secondary SS cases.

Patient	Sex	Age at Disease Onset, yrs	Dry Mouth	Dry Eye	Associated CVD	Complications	ANA	RF	SSA	SSB
Primary SS										
I-1	F	5	No	No	Primary	RTA	1:1280	+	+	+
I-2	F	9	No	No*	Primary		1:320	+	+	+
I-3	F	9	No	No*	Primary		1:2560	+	+	+
I-4	F	10	No	No	Primary	RTA	1:40	+	–**	–
I-5	F	8	No	No	Primary	Meningitis	1:1280	+	–**	–**
I-6	F	14	No	No	Primary		1:320	–	+	+
I-7	F	14	No*	No*	Primary	Neuropathy	1:160	+	+	+
I-8	F	7	No	No	Primary		1:2560	+	+	–
I-9	F	14	No	No	Primary	Meningitis	1:320	+	–	–
I-10	F	10	Yes	No	Primary		1:320	+	–	–
Secondary SS										
II-1	F	11	No	No	SLE	Pericarditis	1:2560	–	+	+
II-2	F	10	No	No	MCTD	Hashimoto	1:1280	+	+	–
II-3	F	7	Yes	Yes	SLE	GN	1:640	+	+	–
II-4	F	14	Yes	Yes	SLE	GN	1:2560	+	+	+
II-5	M	11	No	No	JDM	Meningitis	1:1280	+	+	+
II-6	F	11	No	No	SLE		1:1280	–	+	–
II-7	F	14	No	No	SLE		1:1280	–	+	–
II-8	F	14	No	No	SLE		1:1280	+	+	–
II-9	F	12	No	No	JIA		—	+	+	–
II-10	F	15	No	No	JIA		1:320	+	+	+

* Sicca symptoms and ** autoantibodies appeared later. CVD: collagen vascular disease; SLE: systemic lupus erythematosus; MCTD: mixed connective tissue disease; JDM: juvenile dermatomyositis; JIA: juvenile idiopathic arthritis; RTA: renal tubular acidosis; GN: glomerulonephritis; ANA: antinuclear antibodies; RF: rheumatoid factor.

Table 2. Forward and reverse primer pairs for PCR amplification.

Primers	Nucleotide Sequences
Fp1-98F	ATG GAC CCA AGT GGG GTC AAA GT
Fp1-98R	GGC TCC TGA GTT GGC CTG CAC
Fp36-150F	GGC GTC AGA AGC TGG AAG ATT CCT
Fp36-150R	GTC CAT CAC GTC CTC ACA TTC TCG
Fp91-199F	GTG CAG GCC AAC TCA GGA GCC
Fp91-199R	AGC CAT ATC TGT TTG AAA CTC TTC
Fp148-236F	CAG AAC TTG GTG CAG TAC TTA CGA
Fp148-236R	GGC TGC ATT GAC TTC ATC CTG
Fp191-298F	GAA GAG TTT CAA ACA GAT ATT GGC T
Fp191-298R	GTG CTT CCG AAG CAG AGC CTG
Fp236-334F	TGG CAG CGG CTG AAG GCC TGG CT
Fp236-334R	CAC TTG AAT CTG TGT TGC ACT CAG
Fp290-399F	CAG GCT CTG CTT CGG AAG CA
Fp290-399R	CAG GGC TTC AGC CCC AGC CAC ACT
Fp334-432F	AAG CGA GAG GAA CTG ATT ACA AAC TGG
Fp334-432R	GTG ACC AGC AGC AAG CAG TGC CTG
Fp372-480F	AGT GAT GTG GCT GGG GCT GAA GCC CTG
Fp372-480R	GTC CAC CTG CTC AGT GTC CGG TAG AAG AG
Fp432-545F	CAC TAT GCC TCA GAT GAA GTG AGG
Fp432-545R	GGC CAC ATC TTC CAT TGC ATA GTG
Fp470-561F	CTC TTC TAC CGG GAC ACT GAG CAG GT
Fp470-561R	CCG TCA TCA CCG AAA CGC
Fp1-52F	ATG GAC CCA AGT GGG GTC AAA GTG
Fp1-52R	GCA TCT CTT TGA AAG AAC TGG
Fp27-80F	CCG CTT CAA GGA ACT CTC AAC CCT
Fp27-80R	TCC CTG CAA GTT GGT TGG GTC
Fp52-107F	GAT GCT GAA GAG CTG GAG AAA TGG
Fp52-107R	GGT TTC CAG TTT CAT CCA GCT
Fp79-132F	GGG AAA GCT TCA GAA GCA TCA
Fp79-132R	ATT CCC ACT GGC GGT GCA GCT CCA
Fp106-150F	GGA AAC CTG ATG ATC TCA GAA GGA
Fp106-150R	GTT CTG GGC CTG CAG CAA TTT GA

alone. For preabsorption analyses, human sera were preincubated with 10 μg of fusion proteins at 4°C for 1 h and then used as the primary antibody. The membranes were washed 4 times with Tris-buffered saline containing 0.1% Tween-20 (TBST), and then were incubated with 30,000-fold diluted horseradish peroxidase-conjugated (HRP) anti-goat antibody or 25,000-fold diluted HRP-goat anti-human IgG (Biosource, Camarillo, CA). After washing 5 times with TBST, signals were detected by ECL Plus Western blotting detection reagents (Amersham Pharmacia).

RESULTS

Epitope mapping in primary SS. Sera from all 10 primary SS, 9 of the 10 secondary SS, and one of the 6 SLE subjects reacted with GST-JS-1 and were tested for epitope mapping (data not shown). None of the cases of JIA, dermatomyositis, or non-collagen vascular diseases was positive for antibody against GST-JS-1 (data not shown). Each overlapping fusion protein had a molecular weight on SDS-PAGE consistent with the calculated one and was used for the following epitope mapping as an antigen (data not shown). Western blot analyses showed that sera from Patients I-1, I-2, and I-3 reacted with both fusion proteins with amino acid residues 1 to 98 (Fp1–98) and Fp36–150, but not with any other fragments (data not shown). Because only the predicted bands were detected by Western blotting, further analyses were carried out by a dot-blot method. Dot-blot analysis showed that all the sera from 10 patients with primary SS reacted with both Fp1–98 and Fp36–150 (Figure 1). Sera from Patients I-7, I-9, and I-10 showed reactivity to additional fragments but not to Fp91–199. The serum from Patient I-5 showed specificity to Fp1–98 and Fp36–150 at her first visit, and to additional fragments upon the diagnosis of meningitis and later (Figure 2).

To confirm that Fp1–98 and Fp36–150 contain dominant determinants, a preabsorption study was performed. The reactivity of case I-1 serum with GST-JS-1 was partially inhibited by preabsorption of the sera with excess amount of either Fp1–98 or Fp36–150, and was completely diminished by preabsorption with a mixture of both fragments, suggesting that the N-terminal portion of α-fodrin contains at least 2 dominant epitopes (data not shown). To determine the dominant epitopes of α-fodrin more precisely, we constructed 5 subfragments of the N-terminal portion of α-fodrin consisting of about 50 amino acid residues (Table 2). All the sera from primary SS reacted with Fp27–80 and Fp79–132, and weakly with Fp52–107, but not with Fp1–52 (Figure 3). Considering these data together, multiple dominant epitopes are localized to amino acids 36 to 132.

Epitope mapping in secondary SS and SLE. Nine of the 10 secondary SS sera and one of the 6 SLE sera (SLE-3) reacted with recombinant GST-JS-1 (data not shown) and were used for dot-blot analysis. All the sera from secondary SS and SLE-3 showed reactivity with most of the overlapping fragments, including Fp91–199, which was scarcely reactive to sera from

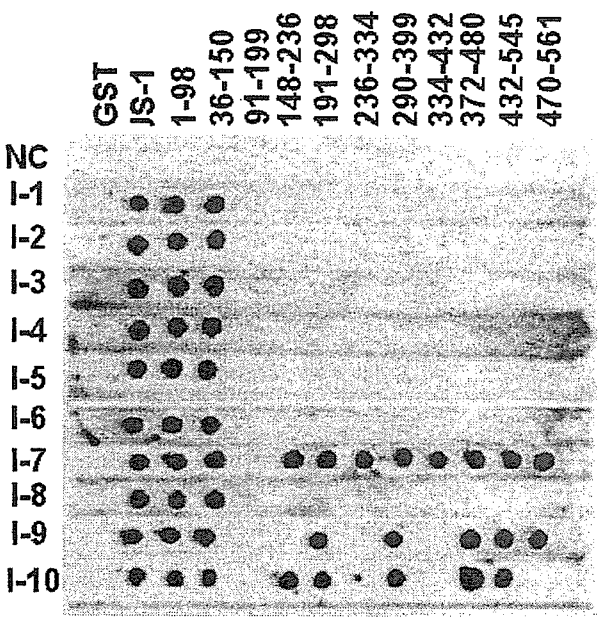

Figure 1. Dot-blot analysis of sera from patients with primary SS using fusion proteins of α-fodrin. Reactivity of each fragment, consisting of about 100 amino acid residues (indicated across the top), was tested with sera from cases of primary SS. GST-JS-1 and GST were used as positive and negative controls, respectively.

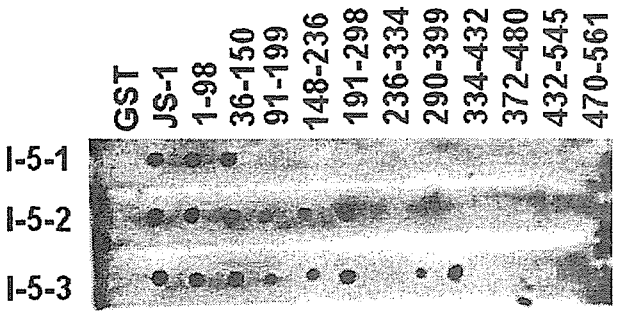

Figure 2. Epitope spreading in a case of primary SS (Patient I-5). Sera were obtained at 3 points: 2 years before the diagnosis of SS (I-5-1), at the time of diagnosis of SS complicated with meningitis (I-5-2), and 2 years after remission of meningitis (I-5-3).

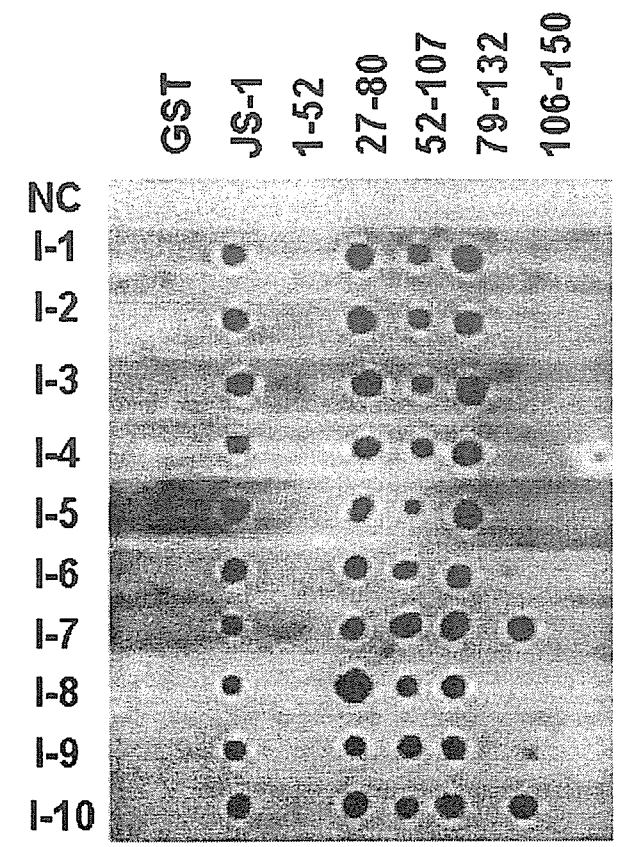

Figure 3. Epitope mapping analyses using subfragments of N-terminal portion of α-fodrin. Reactivity of each subfragment, consisting of about 50 amino acid residues (indicated across the top), was tested by dot-blotting with sera from cases with primary SS. GST-JS-1 and GST were used as positive and negative controls, respectively.

primary SS (Figure 4). In particular, the strongest reactivity was observed with Fp1–98 and Fp334–432.

DISCUSSION

The sensitivities of antibodies to the N-terminal 120 kDa form

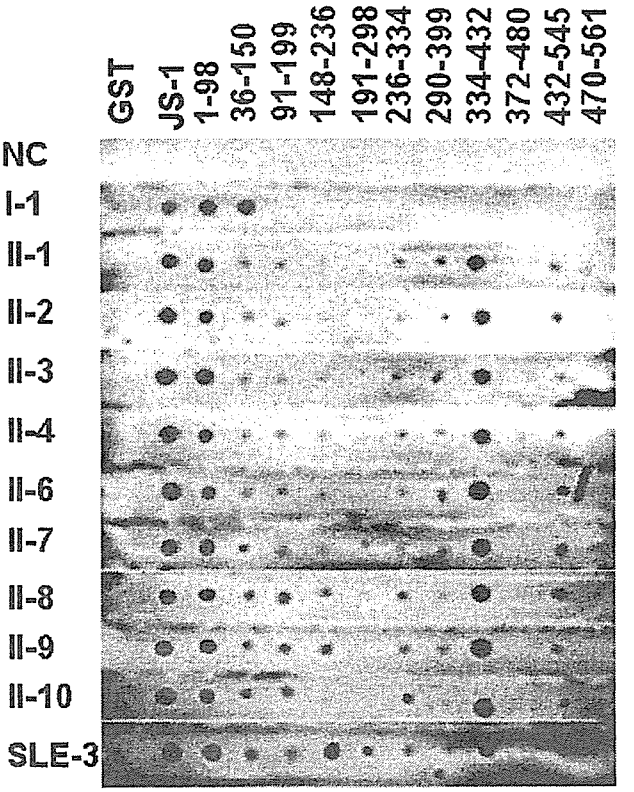

Figure 4. Epitope mapping analysis in cases of secondary SS and SLE. Reactivity of each fragment, consisting of about 100 amino acid residues (indicated across the top), was tested by dot-blotting with sera from secondary SS and a case of SLE (SLE-3). Sera from a primary SS patient (I-1) and a healthy individual were used as positive and negative control (NC) of antibody, respectively. GST-JS-1 and GST were used as positive and negative controls of antigen, respectively.

of α-fodrin in untreated juvenile primary and secondary SS were 100% and 90%, respectively, which are higher than those in adult cases, confirming previous reports in juvenile SS[9-12]. Most cases of juvenile SS lack apparent sicca symptoms and are initially suspected from nonspecific extraglandular symptoms or enlargement of salivary glands associated with elevated erythrocyte sedimentation rate, hypergammaglobulinemia, and autoantibodies, such as antinuclear antibodies, rheumatoid factor, or anti-SSA or SSB antibodies[2-8]. Furthermore, sicca symptoms developed in some of the patients in our study during the followup period (Table 1). Thus, it is possible that juvenile SS is an early stage of the disease, which develops on a strong autoimmune background and accordingly tends to be positive for anti-α-fodrin autoantibodies.

We observed that the initial and major epitopes in primary SS reside in amino acid residues 36–132 of α-fodrin. This portion likely contains at least 2 epitopes, because the reactivity of sera with GST-JS-1 fusion protein was completely inhibited by preabsorption with mixtures of both Fp1–98 and

Fp36–150, but partially inhibited with each of them. This was confirmed by the finding that primary SS sera strongly reacted with 2 separate subfragments, Fp27–80 and Fp79–132. Two of the 3 cases that showed additional specificities, I-7 and I-9, were complicated by peripheral neuropathy and meningitis, respectively[10,24]. The other case, I-10, had apparent sicca symptoms at presentation. Further, Patient I-5 showed progressively diversified epitope specificities with the development of meningitis. Thus, diversified epitope specificities may be associated with neurological complications or disease progression. Our findings are consistent with the high prevalence of the autoantibody in adult primary SS with neurological complications[26,27]. Up to 20% of cases of primary SS have neurological complications similar to multiple sclerosis (MS)[28]. In addition, anti-α-fodrin antibodies are detected in 13% of patients with MS[26]. Thus, the epitope analyses of α-fodrin in MS may clarify the epitopes related to neurological complications of SS. Greidinger, *et al* have reported dominant epitopes of autoantigens such as SmD1, SSA/Ro, and SmB'/B antigens in human autoimmune diseases, and demonstrated the intermolecular epitope spreading of anti-U1-RNP antibody by a large-scale prospective study[29]. Similar mechanisms may be involved in the production of autoantibody against ß-fodrin, the other component of fodrin in SS[30]. On the other hand, intramolecular epitope spreading has been reported mainly in experimental animals, because in human autoimmune disease the autoantibodies usually appear preceding the onset of the disease[31-35]. Our results suggest the presence of intramolecular epitope spreading associated with neurological complications or progress of the disease in human SS.

In contrast to primary SS, all the sera from secondary SS cases reacted with most of the fragments. Particularly strong reactivity of the sera was observed with Fp1–98 and Fp334–432. It is noteworthy that Fp334–432 was not reactive with the primary SS sera, except for 2 cases with neurological complication (cases I-5-3 and I-7). On the other hand, all sera from secondary SS cases showed only minimal reactivity with Fp36–150, a major epitope in primary SS, suggesting that Fp36–150 is a candidate for a primary SS-specific antigen in the ELISA system. Recent studies have suggested that apoptosis induced by stimulation such as infection triggers an autoimmune reaction against autoantigens[36]. α-fodrin is cleaved to the N-terminal 120 kDa form by apoptosis-activated caspase-3 and calpain, and then acquires antigenicity[13,37-39]. After the cleavage, α-fodrin is translocated to the cell membrane[40,41]. It is possible that the enzymatic cleavage induces conformational change of the antigen and subsequent exposure of hidden epitope(s) at the cell surface of the salivary and possibly the lachrymal gland in primary SS. The different epitope specificity in secondary SS may reflect different degradation pathways from those in primary SS. Cleavage product of α-fodrin is also present in neuronal cells undergoing apoptosis, and is localized to plaques in the central nervous system (CNS) of patients with MS[42]. Additional epitope specificities in primary SS with neurological complications raise the possibility that the degradation pathways are different among the tissues. Indeed, the expression of calpain and its intrinsic inhibitor, calpastatin, is different in a salivary gland cell line, HSY, and a T cell line, Jurkat[13]. Given that α-fodrin is ubiquitously distributed, it is possible that the antibodies detected by ELISA or immunoprecipitation assay in SLE[15-21] are developed against the intact or partly cleaved α-fodrin that has leaked from cells undergoing destruction in other organs. One case of SLE positive for these autoantibodies in our study was complicated by CNS disease[43]. Because this case showed epitope specificities similar to those of secondary SS, secondary SS may develop in the future. Otherwise, anti-α-fodrin antibodies detected in this case could be associated with the neurological complication rather than the underlying SLE.

We found the N-terminal amino acid residue 1–98 reacted with sera from cases of both primary and secondary SS. Major and specific epitopes in primary SS initially reside in amino acid residues 36–132 of α-fodrin and might subsequently expand to other regions of the protein in association with the development of neurological complications or progression of SS disease. The autoantibodies showed distinct epitope specificities in secondary SS, suggesting that secondary SS is different from primary SS in regard to the degradation of the autoantigen. Detection of antibodies against the major determinants, amino acid residues 36–132, rather than intact molecules could be a specific diagnostic test for primary SS, at least in the early phase of the disease.

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bjh research paper

Outcome in patients with Wiskott–Aldrich syndrome following stem cell transplantation: an analysis of 57 patients in Japan

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Summary

A total of 57 patients with Wiskott–Aldrich syndrome (WAS) were studied after undergoing stem cell transplantation (SCT) in Japan between January 1985 and December 2004. Eleven patients received transplants from human leucocyte antigen (HLA)-matched related donors, 10 from HLA-mismatched related donors, 21 from unrelated bone marrow donors, and 15 from unrelated cord blood donors. Nine of the 57 patients rejected the initial graft. The overall 5-year survival rate was 73·7% and the 5-year failure-free survival rate was 65·7% (failure was defined as rejection or death). The overall 5-year survival rates for patients receiving bone marrow and cord blood from unrelated donors were both 80·0%. Based on univariate analysis, the factors associated with poor survival were: transplantation from an HLA-mismatched related donor, patient age of more than 5 years at the time of transplantation, and a conditioning regimen other than busulfan and cyclophosphamide (BU-CY) or busulfan, cyclophosphamide and antithymocyte globulin (BU-CY-ATG). In a multivariate analysis, a conditioning regimen other than BU-CY and BU-CY-ATG was the only independent factor associated with transplantation failure. Given the improved outcome for WAS patients following transplantation from an unrelated donor, we conclude that patients with WAS should receive SCT as soon as possible after diagnosis.

Keywords: Wiskott–Aldrich syndrome, stem cell transplantation, immunodeficiency, unrelated donor.

Wiskott–Aldrich syndrome (WAS) is an X-linked disorder of haematopoietic cells characterised by thrombocytopenia with small platelets, eczema and progressive immunodeficiency (Wiskott, 1937; Aldrich *et al*, 1954; Perry *et al*, 1980). The condition is caused by mutations in the *WASP* gene at Xp11.22 (Derry *et al*, 1994), and has a worldwide distribution with an estimated incidence of 4 per one million live male births (Somerville & Forsyth, 1993). Diagnostic measures for WAS patients have progressed markedly since identification of the responsible gene (*WASP*) and its product. We have established a method of flow cytometric analysis of intracellular WASP (Yamada *et al*, 2000), which can be used for WAS screening and is potentially useful for the clinical evaluation of WAS patients after stem cell transplantation (SCT) (Yamaguchi *et al*, 2002). A suspected diagnosis of WAS diagnosis following screening can be confirmed by subsequent mutation analysis;

thus, early diagnosis of WAS is now possible, whereas it was previously difficult for younger children.

Untreated patients with typical WAS have a poor prognosis, with the major causes of death being infection, bleeding and lymphoproliferative disorders. Although splenectomy generally increases platelet count and reduces the risk of major haemorrhage (Lum *et al*, 1980; Mullen *et al*, 1993; Litzman *et al*, 1996), the risk of death from sepsis is increased. Allogeneic SCT has been recognised as an effective method for curing WAS patients who have HLA-matched siblings (Rimm and Rappeport, 1990; Bortin *et al*, 1994), but the number of WAS patients with such a sibling is only small. Although patients who do not have an HLA-matched sibling can undergo SCT from an HLA-mismatched related donor or an unrelated donor, the results to date have been extremely poor (Brochstein *et al*, 1991; Fischer *et al*, 1991). In particular, low survival rates are likely for patients receiving transplants from related donors other than HLA-identical siblings, or from unrelated donors for boys older than 5 years, according to the National Marrow Donor Program (Filipovich *et al*, 2001). The use of cord blood stem cell transplantation (CBSCT) has become common for many diseases, but only a few examples of this procedure for patients with WAS have been reported (Knutsen & Wall, 2000; Kaneko *et al*, 2003; Knutsen *et al*, 2003; Tsuji *et al*, 2006). Herein, we report the outcomes and prognostic factors of 57 WAS patients who underwent SCT in Japan since 1985.

Patients and methods

Between January 1985 and December 2004, 57 WAS patients who were registered with the Committee for Stem Cell Transplantation of the Japanese Society of Pediatric Haematology underwent a total of 64 SCTs. The age of the patients at transplantation ranged from 3 months to 19 years, and the median age was 1·6 years. The origin of the stem cells was bone marrow (BM), peripheral blood (PB) stem cells, and cord blood (CB) stem cells for 40, 2 and 15 patients respectively. The donors were HLA-matched siblings (in eight cases), siblings with a single locus mismatch (5/6 matched, two cases), HLA-matched parents (three cases), a parent with a single locus mismatch (5/6 matched, one case), parents with two loci mismatches (4/6 matched, four cases), parents with three loci mismatches (3/6 matched, two cases), an uncle with a single locus mismatch (5/6 matched, one case), HLA-matched unrelated donors (25 cases), unrelated donors with one HLA locus mismatch (5/6 matched, 10 cases), and an unrelated donor with two loci mismatches (4/6 matched, one case). One patient received transplantation using CD34-positive selection, and transplantation using T-cell depletion was not performed in any patients. A conditioning regimen of busulfan, cyclophosphamide and anti-thymocyte globulin (BU-CY-ATG) was used in 20 patients, and a BU-CY regimen was employed in 13 patients. Radiation-containing regimens (total body irradiation, thoraco-abdominal irradiation and total lymphoid irradiation) were used for 14 patients. Prophylaxis for graft-*versus*-host disease (GVHD) was performed using ciclosporin A and short term methotrexate in 28 patients, ciclosporin A alone in seven patients, ciclosporin A and methyl prednisolone in five patients, tacrolimus and short term methotrexate in eight patients, methotrexate alone in four patients, tacrolimus and methyl prednisolone in three patients, and tacrolimus alone in one patient.

Statistical analysis

The data were analysed as of December 1, 2005. Failure-free survival (FFS) was defined as survival with treatment response, whereas death, rejection and secondary malignancy were considered to be treatment failures. Analyses of overall survival (OS) and FFS were performed using the Kaplan–Meier method, with differences compared by log-rank test. Multivariate stepwise regression analysis was performed to explore the independent effects of variables that showed a significant influence on outcome in univariate analysis. Statistical analyses were performed using Dr SPSS II for Windows (release 11.0.1J, SPSS JAPAN Inc., Tokyo, Japan).

Results

Nine of the 57 patients who underwent SCT rejected their graft; six of these patients received a second transplantation. An additional patient received a second transplantation because of mixed chimaerism. Fourteen patients died, and the causes of death were infection (nine patients), GVHD (three patients), bleeding (one patient) and thrombotic microangiopathy (one patient). Four patients died following rejection, but no patients suffered a secondary malignancy. Acute GVHD ≥ grade II developed in 36·8% of all patients, and in 27·3% of HLA-matched related SCT recipients, 40·0% of HLA-mismatched related SCT recipients, 33·3% of unrelated bone marrow transplantation (BMT) recipients and 46·7% of unrelated CBSCT recipients. Chronic GVHD occurred in 40·4% of all patients, and in 36·4% of HLA-matched related SCT recipients, 60·0% of HLA-mismatched related SCT recipients, 47·6% of unrelated BMT recipients and 33·3% of unrelated CBSCT recipients (Table I). Seven patients received a second transplantation: six for failure to engraft or graft failure, and one for mixed chimaerism, and the interval between the two transplantations ranged from 1 to 36 months. Two of the seven patients had the same donor for both transplantations, three patients received a transplantation from an HLA-mismatched related donor following graft failure after an initial transplantation from an unrelated donor, and one patient who had mixed chimaerism had different unrelated donors for each transplantation; all six patients are still alive without graft failure. The seventh patient underwent a second transplantation from an HLA-matched sibling following graft rejection after the first transplantation from an HLA-mismatched related donor; however, this patient died from sepsis.

The OS rate 5 years after transplantation was 73·7 ± 6·1% and the 5-year FFS rate was 65·7 ± 6·6% (Figs 1 and 2).

Table I. Patient profile by transplant type.

	Matched related BMT-PBSCT unrelated BMT	Mismatched related BMT-PBSCT	Unrelated BMT	Unrelated CBSCT
Patients number	11	10	21	15
Age at transplantation (median)	4·3*	5·7*	1·6*	1·1*
≥5 years	4	4	5	0
Body weight at transplantation (median)	12·4*	13·9*	10·0*	8·2*
Conditioning regimen				
BU + CY	6	1	4	2
BU + CY + ATG	2	1	11	6
Radiation-containing	1	3	6	4
Others	2	5	0	3
GVHD prophylaxis				
CsA + MTX	7	3	11	7
CsA + PSL	0	2	0	3
CsA	2	2	0	3
MTX	2	0	0	1
FK506 + MTX	0	1	7	0
FK506 + PSL	0	1	1	1
FK506	0	1	0	0
Others	0	0	2	0
Acute GVHD(≥gradeII)	3	4	7	7
Chronic GVHD	4	6	10	5
Rejection	3	2	2	2
Dead	2	5	4	3

BU, busulfan; CY, cyclophosphamide; ATG, antithymocyte globulin; GVHD, graft-*versus*-host disease; CsA, ciclosporin A; MTX, methotrexate; PSL, prednisolone; FK506, tacrolimus; CBSCT, cord blood stem cell transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.
*$P < 0.05$.

Univariate analyses of OS and FFS grouped by patient characteristics and transplant variables are shown in Table II. Statistically significant univariate associations were noted for poorer OS with HLA-mismatched related donors and children aged more than 5 years at the time of transplantation, and for poorer OS and FFS with use of a conditioning regimen other than BU-CY and BU-CY-ATG. The overall 5-year survival rates for patients who received BM and CB from an unrelated donor were 80·0 ± 9·0% and 80·0 ± 10·3% respectively. Using multivariate analysis, a conditioning regimen other than BU-CY and BU-CY-ATG was found to be the only factor examined that was associated with transplantation failure (Table III).

Discussion

Our review of the outcomes of haematopoietic SCT for treatment of WAS in Japan since 1985 showed that in 57 WAS patients who underwent SCT, the overall 5-year survival rate

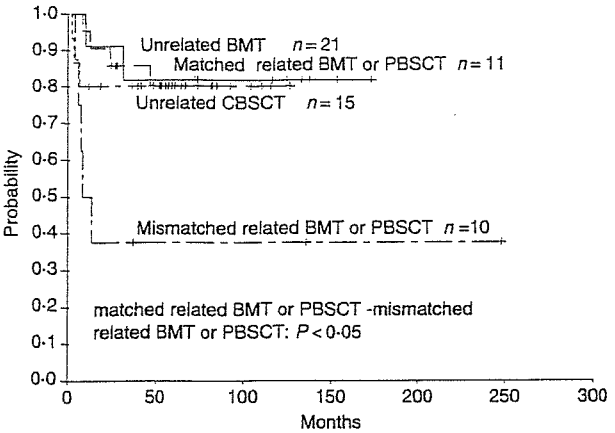

Fig 1. Overall survival (OS) rate of Wiskott–Aldrich syndrome (WAS) patients after transplantation. Kaplan–Meier estimates of OS for patients with WAS who received a transplant from a related matched donor (matched related), a related mismatched donor (mismatched related), an unrelated bone marrow donor (unrelated BMT), and an unrelated cord blood donor (unrelated CBSCT).

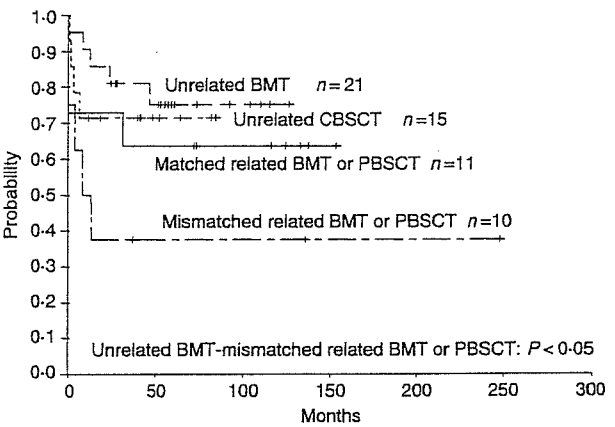

Fig 2. Failure-free survival (FFS) rate of Wiskott–Aldrich syndrome (WAS) patients after transplantation. Kaplan–Meier estimates of FFS for patients with WAS who received a transplant from a related matched donor (matched related), a related mismatched donor (mismatched related), an unrelated bone marrow donor (unrelated BMT), and an unrelated cord blood donor (unrelated CBSCT).

for those with an HLA-matched related donor was 81·8%, and the 5-year FFS rate was 64·3%. Surprisingly, the overall survival of patients who underwent transplantation from an HLA-matched unrelated BM or CB donor was 80·0%, and therefore did not differ from that of patients who received a transplant from a related HLA-matched donor. This result is quite different from past reports, in which transplant from an unrelated donor has been associated with a poor outcome (Fischer *et al*, 1994). The availability of a detailed HLA-matching system for unrelated donors for BMT may be a reason for the improved outcome in transplantation from HLA-matched unrelated donors. In CB transplantation, graft rejection is a common complication in many patients with

Table II. Survival and failure-free survival 5 years after stem cell transplantation for Wiskott–Aldrich syndrome (univariate analysis).

	n	OS at 5 years (%)	FFS at 5 years (%)
All patients	57	73·7 ± 6·1	65·7 ± 6·6
Age			
<5 years	41	79·8 ± 6·4*	72·0 ± 7·3
≥5 years	13	53·8 ± 13·8*	46·2 ± 13·8
Source and donor			
Related matched BMT, PBSCT	11	81·8 ± 11·6*	64·3 ± 14·5
Related mismatched BMT, PBSCT	10	37·5 ± 17·1*†	37·5 ± 17·1*
Unrelated BMT	21	80·0 ± 9·0†	75·2 ± 9·7*
Unrelated CBSCT	15	80·0 ± 10·3	71·4 ± 12·1
Conditioning regimen			
BU-CY and BU-CY-ATG	32	86·9 ± 6·1*	83·8 ± 6·7*
Other	23	55·8 ± 10·5*	40·4 ± 10·6*
Year of transplantation			
Till 1999	30	73·3 ± 8·1	60·0 ± 8·9
2000 and after	25	74·1 ± 9·2	72·9 ± 9·6

BU, busulfan; CY, cyclophosphamide; ATG, antithymocyte globulin; OS, overall survival; FFS, failure-free survival; CBSCT, cord blood stem cell transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.
*†$P < 0·05$.

Table III. Multivariate analysis of failure-free survival after stem cell transplantation for Wiskott–Aldrich syndrome.

Factor	RR of failure	*P*	CI
Conditioning (except BU-CY and BU-CY-ATG)	7·455	0·003	1·996–27·834
Age (≥5 years)	2·410	0·243	0·550–10·559
Donor (mismatched related donor)	1·774	0·516	0·315–9·994

non-malignant diseases, but the incidence of graft rejection in our subjects was lower than that for other transplant patients; this result suggests that CBSCT may be preferable in immunodeficiency disorders. On the other hand, patients receiving transplants from HLA-mismatched related donors had poorer OS, consistent with data in past reports. Our results suggest that WAS patients without an HLA-matched sibling should receive BM or CB transplantation from an unrelated donor, but not from an HLA-mismatched related donor. Boys older than 5 years also showed shorter survival and, because WAS patients become increasingly susceptible to episodes of infection and haemorrhage with aging, immediate transplantation should be performed after WAS diagnosis. Therefore, prompt diagnosis of WAS is extremely important. In addition, a benefit of CBSCT is that the period from registration to the transplant is short, suggesting that CBSCT should be actively considered for WAS patients.

For patients receiving transplantation from an HLA-matched sibling, a conditioning regimen including BU (16 mg/kg) and CY (200 mg/kg) has been shown to give relatively high survival rates, whereas total body irradiation plus cyclophosphamide has been associated with poorer survival (Fischer *et al*, 1994). In our study, patients who underwent a conditioning regimen other than BU-CY and BU-CY-ATG had poorer OS and FFS, and use of a BU-CY regimen was favourable for WAS patients for transplantation from both an HLA-matched sibling and from an unrelated donor. CY (200 mg/kg) was used in all 13 patients who received a BU-CY conditioning regimen, whereas a CY dose of 120 mg/kg was used in five of the 20 patients who received BU-CY-ATG. As all five of these patients are alive and did not have graft rejection, reduction of drug doses in conditioning may be possible. Recently, non-myeloablative transplantation for WAS patients has been reported (Longhurst *et al*, 2002), and several patients in our study received transplantation using a fludarabine-containing regimen. However, because this was used as conditioning in the second transplantation for most patients, we were unable to perform an accurate evaluation of the fludarabine-containing regimen. As it is important to evade conditioning-related sequelae, evaluation of the efficacy of reduced-intensity transplantation in WAS patients through the accumulation of cases will be of importance.

Unexpectedly, six out of seven patients who underwent second transplantation are alive. Moreover, all three patients receiving transplantation from a HLA-mismatched related donor are alive with complete donor chimaerism. These findings might justify the use of reduced-intensity conditioning regimens for the initial transplantation of patients with WAS, especially as the prognosis of the second transplantation of these patients was better than expected.

In conclusion, we recommend that patients diagnosed with WAS receive SCT as soon as possible after diagnosis. As donor

sources, unrelated but HLA-matched BM or CB donors are appropriate for transplantation, with a low associated risk similar to that with a related HLA-matched donor.

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Human Tyrosine Kinase 2 Deficiency Reveals Its Requisite Roles in Multiple Cytokine Signals Involved in Innate and Acquired Immunity

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