In conclusion, this study indicated that the PD-1/B7-H1 signaling pathway is required for the induction of peripheral tolerance in a mouse liver transplant model. In the liver allograft infiltrating $CD8^+$ T cells underwent apoptosis, which was dependent on the B7-H1 expression on the liver graft tissue cells. Blockade of the PD-1/B7-H1 signal by administration of anti-B7-H1mAb or liver donor deficient in B7H1 resulted in the acute rejection of the allograft, associated with an increase of infiltrating CTLs and inflammation-related cytokines mRNA expression. This study confirms, for the first time, the role of PD-1/B7-H1 signaling in the induction of spontaneous liver tolerance, thereby suggesting that selectively sparing the PD-1/B7-H1 signal may be beneficial in the development of a tolerance strategy in organ transplantation. Furthermore, the implications of these findings may pertain not only to the mouse spontaneous acceptance of liver transplants, but also to the immunopathogenesis of chronic viral hepatitis.

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Prolongation of Rat Major Histocompatibility Complex–compatible Cardiac Allograft Survival During Pregnancy

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Background: It has been suggested that pregnancy-related hormones play a critical role in mediating selective immune tolerance during pregnancy. An understanding of why a woman's body normally does not reject the fetus is highly relevant to the prevention of transplant rejection.

Methods: The hearts of female inbred F344 rats (*RT-1*^{lvl}) were transplanted into naive Lewis (*RT-1*^{l}; nLewis) or pregnant (pLewis; Day 6, 12 and 18 of pregnancy) rats. The mean survival time (MST) of the cardiac allografts between the nLewis and pLewis rats was compared. We determined the rate of proliferation of the T cells isolated from nLewis and pLewis rats in response to concanavalin A (ConA), anti-CD3 and -CD28 antibody and alloantigen stimulation ex vivo. mRNA expression of several cytokines in these T cells was analyzed using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, the effect of estriol on the cardiac allograft was tested.

Results: The pLewis rats with transplant on Day 12 of pregnancy had the most significantly prolonged F344 cardiac graft survival (MST 13.3 days) as compared with nLewis recipients (MST 8 days). pLewis T-cell proliferation was stimulated by alloantigen and antibody but ConA was reduced, whereas Th1/Th2 cytokine mRNA profiles in the T cells were similar for nLewis and pLewis rats. Likewise, estriol also significantly prolonged survival of cardiac allografts.

Conclusions: The results of this study demonstrate that pregnancy hormones not only appear to play a critical role in maternal acceptance of the fetus, but also have therapeutic potential for prolonging the survival of major histocompatibility complex (MHC)-compatible allografts during pregnancy. J Heart Lung Transplant 2009;28:176-82. Copyright © 2009 by the International Society for Heart and Lung Transplantation.

Cardiac transplantation has become a widely accepted treatment for end-stage heart diseases. The survival rate has improved because of improvements in organ preservation and immunosuppressive treatments[1]; however, allograft rejection remains a major complication after transplantation.[2] It is evident that factors such as the degree of HLA mismatching, donor age and cold ischemia time significantly influence outcome of organ transplantation.[3] Nevertheless, there are a number of parameters that may be considered to have a marginal influence on graft outcome, contributing to the deterioration of allograft function. An area that has remained topical is the effect of donor and recipient gender on graft outcome.[4] A number of studies have reported that female recipients usually have a greater propensity for rejection than do male recipients and are less likely to tolerate withdrawal of maintenance steroids.[5,6]

During pregnancy, women usually have a decrease in autoimmune and certain infectious diseases. Many theories have been proposed to explain the disease-modifying influence of pregnancy. The most popular of these is a generalized Th2 shift in cytokine secretion, perhaps induced by the high circulating levels of estrogens or progesterone.[7] Doses of estradiol typical of levels during pregnancy have been shown to significantly enhance interleukin (IL)-10 secretion from antigen-stimulated proteolipid protein-specific T-cell clones isolated from multiple sclerosis (MS) patients.[8] Furthermore,

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peripheral blood mononuclear cells (PBMC) from normal pregnant women secrete higher amounts of IL-10 when stimulated with phytohema g glutinin (PHA) when compared with non-pregnant controls.[9]

In rodents, females exert a stronger and longer lasting immune response against foreign antigens than do males. Female rat recipients of rat cardiac allografts have more frequent and more severe cellular rejections and, consequently, shorter graft survival than males.[10] These sex differences have been attributed to many factors, the most prominent being the sex hormones.[11]

The purpose of this study was to examine the effect of different gestational stages on cardiac allograft survival and to explore the immunologic mechanisms of pregnancy-related hormones operative at each stage. We studied the effect of pregnancy on acute rejection of cardiac allograft survival in Lewis rats.

METHODS

Animals and Estriol

Adult male and/or female Lewis (*RT-1^l*) and F344 (*RT-1^{lvl}*) rats were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). For mating, a Lewis pro-estrous rat was transferred to a cage with Lewis male rats and left overnight. The vaginal plug was checked the next morning to ensure that successful copulation had occurred, then the pregnant rats were obtained on Days 6, 12, and 18 of gestation and individually housed in standard cages and used as recipients.

The animals were maintained under standard conditions and fed rodent food and water according to the laboratory animal care principles and the guide for the care and use of laboratory animals in our institution. This investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Estriol was synthesized in our laboratory according to the conventional method. Pellets containing 10 mg estriol were hand-pressed using the Pellet Presser (Parr Instrument Co., Moline, IL). Each pellet (weighing 25 mg) contained 5 mg of sodium chloride and a combined 20 mg of estriol plus cholesterol. Dry crystals of sodium chloride, cholesterol and estriol were mixed thoroughly by grinding the mixtures to fine powers. The pellets were made by the same person applying approximately the same pressure.

Heterotopic Cardiac Transplantation

Heterotopic cardiac transplantation at the neck was performed between an F344 donor and a naive Lewis (nLewis; $n = 14$), or pregnant Lewis (pLewis) rat, on Day 6 ($n = 8$), 12 ($n = 12$) or 18 ($n = 6$) post-conception (pc) of recipient rats, as described elsewhere.[12] In brief, donor hearts were first isolated under ether anesthesia and blood was washed out from the aorta and pulmonary artery with 20 ml of saline. End-to-end anastomoses were performed using the cuff technique between the donor ascending aorta and the recipient right common carotid artery and between the donor pulmonary artery and the recipient right jugular vein. Graft rejection was determined by the cessation of cardiac contraction by palpation every 8 hours.

Lymphocytes Isolation and Lymphocyte Proliferation Assay

Spleens from nLewis and pLewis recipient rats were harvested on Day 6 after transplantation, and single-cell suspensions were prepared by passing the tissue through a 70-μm cell strainer (Falcon, Franklin Lakes, NJ). The cell suspension was overlaid on Ficoll Isopaque (Lympholyte Rat; Cedarlane Lab, Ontario, Canada) and centrifuged at 3,000 rpm for 20 minutes at 22°C. The cells of the interface layer were harvested and washed twice in phosphate-buffered saline (PBS). Erythrocytes were lysed by hypotonic shock, and the remaining cells were washed with RPMI 1640 medium (Gibco-BRL, Grand Island, NY).[13] For alloantigen stimulation, lymphocytes (1×10^5) from the Lewis rat as the responder and 20-Gy-irradiated spleen antigen-presenting cells (APC; 5×10^2 to 1×10^5) from the F344 rats as the stimulator were incubated in a 96-well microtiter plate (Costar, Corning, NY) at a final volume of 200 μl/well in a humidified atmosphere at 37°C for 5 days.

To evaluate and assess the response to mitogenic stimulations, purified lymphocytes were cultured at 37°C for 3 days with concanavalin A (ConA) at 1 mg/10^6 cells or anti-CD3 and -CD28 monoclonal antibody (MAb) at 2 μg/ml. The proliferation of lymphocytes in response to alloantigen and mitogenic stimulators was then measured with cell-proliferation enzyme-linked immunoassay (ELISA) kits (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. Fluorescence intensity was analyzed by a chemiluminescence reader (Wallac ARVO SX; PerkinElmer, Inc., Wellesley, MA) and WALLAC1420 manager software (PerkinElmer), as described elsewhere.[14]

RNA Isolation and Quantitative Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

We isolated total RNA from lymphocytes from the recipient spleen using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) and then treated it with DNase (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. The concentration of total RNA was determined by measuring the optical density at 260 nm. The quality of total RNA was checked a ratio of 260/280 nm. Each milligram of RNA was reverse-transcribed to cDNA using oligo-(dT) primer and PRIMESCRIPT reverse transcriptase (Takara-bio Co., Shiga, Japan) according to the manufacturer's protocol. Quantitative RT-PCR was per-

formed using the TaqMan system with Prism 7700 (Applied Biosystems Co., Tokyo, Japan) sequence detection. The target-specific primers and probes were purchased from Applied Biosystems. Data were expressed as the comparative cycle threshold (Ct). The normalized Ct value of each gene was obtained by subtracting the Ct value of 18S rRNA. The fold change versus one sample of the control group was calculated as described previously.[15]

Histologic Studies

We harvested cardiac grafts at 6 days after transplantation from the nLewis and pLewis Day 12 pc recipients. The samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin, and their 6-μm-thick sections were stained with hematoxylin-eosin for standard microscopy.[16]

Statistical Analysis

Statistical evaluation for graft survival was performed using the Kaplan-Meier test. $p < 0.05$ was considered statistically significant using the Breslow-Gehan-Wilcoxon test.

RESULTS

Pregnancy Prolonged Cardiac Allograft Survival in Major Histocompatibility Complex (MHC)-compatible Rat Cardiac Allografts

To develop an understanding of the effects of pregnancy on allograft rejection, we first examined the effect of pregnancy on MHC-compatible rat cardiac allograft survival. Our investigation was designed to include multiple gestational stages. We studied three time-points during pregnancy (early, middle and late) to evaluate the degree of protection offered throughout pregnancy. Non-overlapping, discrete days after pregnancy were chosen (e.g., early pregnancy = Day 6 pc, mid-pregnancy = Day 12 pc, late pregnancy = Day 18 pc), enabling a distinct characterization of each stage. In this combination, all allografts in the control group (nLewis) were rejected approximately 8 days after transplantation (Figure 1), whereas pregnant recipients (pLewis) exhibited significantly prolonged cardiac graft survival ($p < 0.05$). The respective mean survival time (MST) of the pregnant recipients at 6, 12 and 18 days after pregnancy was 10, 13.5 and 11 days, respectively. These results suggest that each gestational stage has its own unique effect on graft survival.

Histologic Studies After Cardiac Grafting

The cardiac graft of the nLewis recipients exhibited extensive perivascular infiltration of mononuclear cells and myocardial structure damage (Figure 2A-C). In contrast, grafts from the pLewis (Day 12 pc) recipients revealed relatively little infiltration of mononuclear

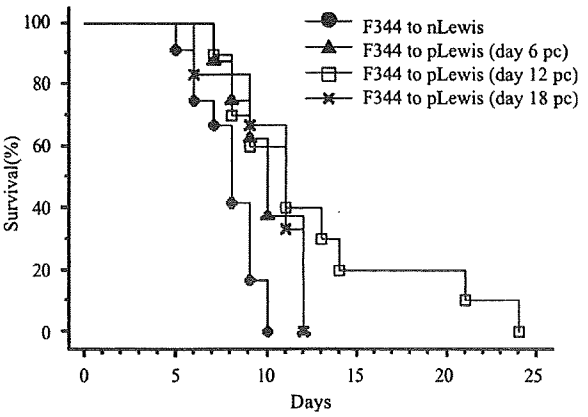

Figure 1. Pregnancy prolonged survival of cardiac allografts in rats. All allografts in the control group (naive Lewis; nLewis) were rejected at approximately 8 days after transplantation, whereas pregnant recipients (pregnant Lewis; pLewis) exhibited significantly prolonged cardiac graft survival ($p < 0.05$). The respective mean survival times (MSTs) of the pregnant recipients at Days 6, 12 and 18 after pregnancy were 10, 13.5 and 11 days, respectively (nLewis, $n = 14$; Day 6 pc, $n = 8$; Day 12 pc, $n = 12$; Day 18 pc, $n = 6$).

cells, and almost normal tissue architecture was observed (Figure 2D-F). Furthermore, the original heart of the nLewis rat exhibited normal tissue architecture without infiltration of mononuclear cells (Figure 2G-I). These results suggest that pregnancy-related factor(s) during pregnancy suppress the alloresponse and inhibit graft rejection, thereby prolonging allograft survival.

Hyporesponsiveness of In Vitro Proliferation Response in Lymphocytes Isolated From Cardiac-allografted Pregnant Rats

To examine the cause of prolonged cardiac allograft survival, we tested the T-cell proliferation response to alloantigens using an in vitro MLR assay system. We used splenic lymphocytes derived from pLewis (Day 12 pc) or nLewis of F344 cardiac allografts on Day 6 after transplantation. T-cell proliferation was assessed by uptake of 5-bromo-2′-deoxyuridine (BrdU) for 2 hours at the end of the culture period. The lymphocytes isolated from pLewis rats grafted with F344 hearts exhibited less proliferative response than nLewis rats after coculturing with irradiated F344 splenocytes (Figure 3). Furthermore, T-cell proliferation responded to anti-CD3 and -CD28 MAb, but ConA decreased more in lymphocytes isolated from cardiac-allografted pLewis recipients than nLewis recipients (Figure 3).

Rats Receiving Cardiac Allografts During Pregnancy Have Normal Cytokine Profiles

The possibility of a shift in cytokine expression profile conferring prolonged allograft survival during pregnancy was tested by examining the amounts of

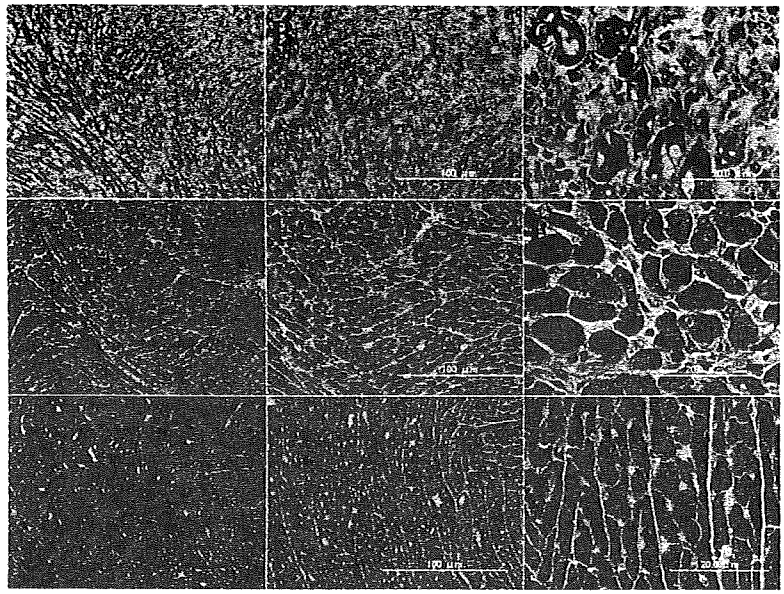

Figure 2. Cardiac allograft pathologic changes in pregnant and naive Lewis rats. Extensive perivascular accumulations of mononuclear cells with clear evidence of architecture damage and myocytic necrosis were seen in heart grafts of nLewis rats (A–C) on Day 6 after transplantation. In contrast, normal tissue architecture with little infiltration of mononuclear cells was observed in heart grafts of pLewis rats on Day 12 of pregnancy (D–F). The original hearts of nLewis recipients exhibited normal tissue architecture without infiltration of mononuclear cells (G–I). (A), (D) and (G): ×40; (B), (E) and (H): ×100; (C), (F) and (I): ×400.

IL-2, −4 and −10, interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), granzyme B, perforin and inducible nitric oxide synthase (iNOS) in lymphocytes from the spleen. No difference in the amount of IL-2, −4 and −10, IFN-γ, TGF-β, granzyme B, perforin or iNOS was seen in rats grafted with allogeneic hearts during pregnancy as compared with controls (Figure 4).

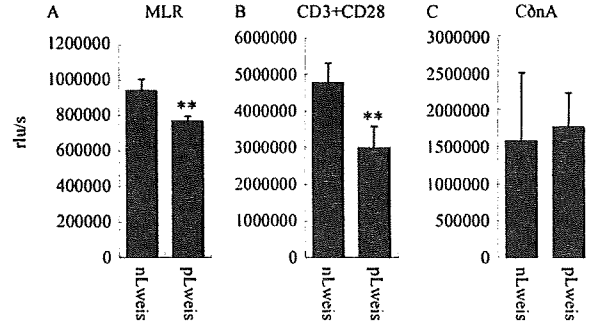

Figure 3. Proliferation of lymphocytes from the pregnant recipients was reduced. The lymphocytes isolated from pLewis rats grafted with F344 hearts exhibited less proliferative response than nLewis induced by allogeneic stimulator cells (A), anti-CD3$^+$CD28 (B) and ConA (C). Data are representative of two independent experiments and indicate the mean ratio of triplicate results in each experiment. $^{**}p < 0.05$ vs control.

Estriol Has Immunosuppressive Potential in MHC-compatible Rat Cardiac Allografts

The effect of estriol on MHC-compatible rat cardiac allograft survival was also examined. A pellet containing 10 mg of estriol was implanted in naive Lewis rats for 10 days, and the rats then received F344 heart transplants. As depicted in Figure 5, treatment with estriol prolonged allograft survival significantly (MST 10.5 days, $p < 0.05$) compared with that of the control group (MST 8 days). This result suggests that estriol may have therapeutic potential for suppression of allograft rejection.

DISCUSSION

In this study, survival of the cardiac allograft was prolonged after it was transplanted into pregnant rats, and the effect was most pronounced when the heart was transplanted during the middle stage of pregnancy, with slight histologic inflammatory infiltrates compared with the control groups (Figure 2). These results suggest that pregnancy-related factor(s) during pregnancy suppress the alloresponse and inhibit graft rejection, thereby prolonging allograft survival. However, these findings are somewhat at odds with observations made using the experimental autoimmune encephalomyelitis (EAE) model, where it was reported that immunization during the latter half of pregnancy (Day 18 pc) was

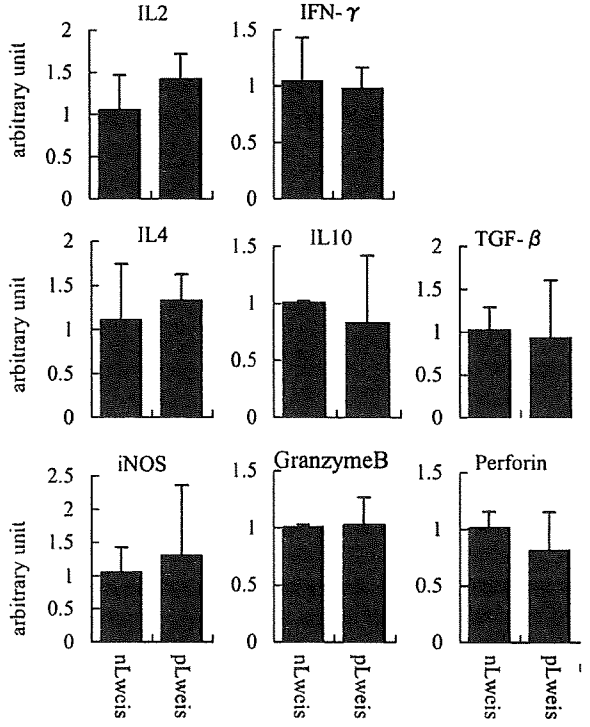

Figure 4. Cytokine expression of lymphocytes from pregnant and naive cardiac allograft recipients. Cytokine mRNA expression in the spleen lymphocytes from pregnant and naive cardiac allograft recipients on Day 6 after transplantation were detected by quantitative RT-PCR and had no change. Data are representative of three independent experiments and indicate the mean ratio of triplicate results in each experiment.

more protective than immunization during the earlier period of gestation.[17,18]

To delineate the mechanism(s) by which the different gestational stages mediated the observed effects on cardiac allografting, we have explored three alternatives. First, we examined whether cardiac allografting during the middle stage of pregnancy resulted in lymphocyte activation. Some evidence has indicated that certain aspects of immunity are suppressed during pregnancy. In our model, lymphocytes from animals with a cardiac allograft during pregnancy exhibited a lower proliferation rate in response to the alloantigen as well as anti-CD3 and -CD28 MAb, but not ConA stimulation. A previous study indicated that regulatory T cells expand during pregnancy and that these regulatory T cells suppress the alloresponse.[19] Therefore, our results suggest that the decline in lymphocyte proliferation may be due to expansion of regulatory T cells, although further investigation is needed to identify lymphocyte subsets.

We also explored the alternative that, when cardiac allografting is performed during pregnancy, there is a shift in cytokine production away from pro-inflammatory Th1 cytokines and toward Th2 cytokines.[20] To determine whether a Th2 shift occurred, we compared the production of pro-inflammatory cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4, TGF-β and IL-10) between naive, non-pregnant, allografted recipients and those allografted during pregnancy. The two groups exhibited similar levels of IFN-γ and IL-2, and the expression levels of IL-4, IL-10 and TGF-β were similar. Thus, no differences were observed in the typical Th1 and Th2 cytokine profiles. Many metabolic pathways are known to be affected by pregnancy. Although metabolic changes, except cytokine profiles of lymphocytes, were not examined in the present study, the possibility also exists that alterations in metabolic pathways, as well as in some of the products of these metabolic pathways, may contribute significantly to the prolongation of allograft survival seen during pregnancy. These observations will help focus the search for a pregnancy-related therapeutic agent on one with immunosuppressive properties rather than on one that induces Th2 cytokines. Further investigation into which factor it may be will lead to the development of a potent disease-alleviating medication for patients with multiple sclerosis (MS) and other autoimmune diseases and allograft rejection.

Although we could not find any specific elevation of estrogens, including estrone, estradiol and estriol, in mid-term pregnant Lewis rats (data not shown) for examining the effect of pregnancy-related hormones in the cardiac allograft model, we administered estriol to naive rats with cardiac allografts because previous studies reported that estriol ameliorates autoimmune demyelinating disease.[21,22] In contrast, another previous study demonstrated that estradiol enhances murine cardiac allograft rejection in the presence of cyclosporine and can be antagonized by the anti-estrogen tamox-

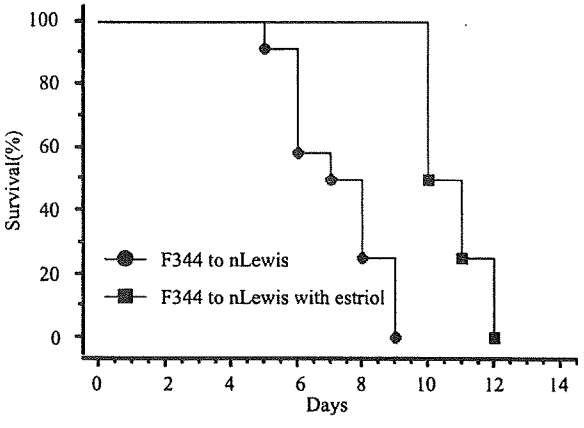

Figure 5. Estriol prolonged survival of cardiac allograft in rat. Estriol treatment significantly prolonged allograft survival (MST 10.5 days) compared with the control group (MST 8 days, $p < 0.05$) (without estriol, $n = 14$; with estriol, $n = 4$).

ifen.[23] In the present study, estriol was shown to prolong cardiac allograft survival. This observation was in accordance with a study using the feline renal allograft model.[24] In that study, animals given estriol did not show fibinoid reactions, tubular or glomerular necrosis or typical graft rejection-related changes in the vessels, and there was very little interstitial nephritis compared with control animals. Furthermore, we found that estriol significantly ameliorated the clinical severity of EAE, an animal model of MS in rats (unpublished data). These findings suggest that estriol may be useful for suppressing allograft rejection as well as autoimmune disease. One study demonstrated that estriol enhances secretion of antigen- and anti-CD3 MAb-stimulated IL-10 and IFN-γ in a dose-dependent fashion and has a biphasic effect on TNF-α and -β secretion, with low-concentration stimulatory and high-dose inhibitory findings.[25] In our study, the inhibitory effects of estriol may have played a major role in prolongation of cardiac allograft.

In this study we have characterized the cardiac allograft in MHC-compatible rats in which the effect of pregnancy, particularly mid-pregnancy (12 days pc), prolonged allograft survival in Lewis rats. The prolongation of allograft survival in the pregnant recipients was associated with a dramatic reduction in the amount of lymphocyte infiltration and presence of normal tissue architecture in the grafts as compared with naive controls. Although cytokine profiles were similar in both pregnant and naive recipients, lymphocytes isolated from pregnant rats proliferated less than those of naive controls. Estriol also prolonged survival of cardiac allografts in rats. These results suggest that intragraft suppression of allogeneic T cells, or a combination of suppression and graft protection, is responsible for prolonging graft survival during pregnancy. Although further studies are needed to determine the effect of estriol in other allograft models and to clarify the role of estriol in immunosuppression, the present results may provide a significant clue for studying the mechanisms of immunoregulation by sex-related hormones, especially during pregnancy.

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Japan Elaprase® Treatment (JET) study: Idursulfase enzyme replacement therapy in adult patients with attenuated Hunter syndrome (Mucopolysaccharidosis II, MPS II)

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ABSTRACT

This open-label clinical study enrolled 10 adults with attenuated Mucopolysaccharidosis II and advanced disease under the direction of the Japan Society for Research on Mucopolysaccharidosis Disorders prior to regulatory approval of idursulfase in Japan. Ten male patients, ages 21–53 years, received weekly intravenous infusions of 0.5 mg/kg idursulfase for 12 months. Significant reductions in lysosomal storage and several clinical improvements were observed during the study (mean changes below). Urinary glycosaminoglycan excretion decreased rapidly within the first three months of treatment and normalized in all patients by study completion (−79.9%). Liver and spleen volumes also showed rapid reductions that were maintained in all patients through study completion (−33.2% and −31.0%, respectively). Improvements were noted in the 6-Minute Walk Test (54.5 m), percent predicted forced vital capacity (3.8 percentage points), left ventricular mass index (−12.4%) and several joint range of motions (8.1–19.0 degrees). Ejection fraction and cardiac valve disease were stable. The sleep study oxygen desaturation index increased by 3.9 events/h, but was stable in 89% (8/9) of patients. Idursulfase was generally well-tolerated. Infusion-related reactions occurred in 50% of patients and were mostly mild with transient skin reactions that did not require medical intervention. Two infusion-related reactions were assessed as serious (urticaria and vasovagal syncope). One patient died of causes unrelated to idursulfase. Anti-idursulfase antibodies developed in 60% (6/10) of patients. In summary, idursulfase treatment appears to be safe and effective in adult Japanese patients with attenuated MPS II. These results are comparable to those of prior studies that enrolled predominantly pediatric, Caucasian, and less ill patients. No new safety risks were identified.

Introduction

Mucopolysaccharidosis type II (MPS II, Hunter syndrome, OMIM #309900) is an X-linked recessive, lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase (IDS, EC3.1.6.13). This lysosomal enzyme catalyzes the first step in the degradation of the glycosaminoglycans (GAG), dermatan sulfate and heparan sulfate [1]. Iduronate-2-sulfatase deficiency leads to the accumulation of GAG within the lysosomes of virtually every cell in the body and is excreted in excessive amounts in the urine. MPS II encompasses a wide phenotypic spectrum that includes severe and attenuated forms. The severe form has onset of symptoms by 2–4 years old, progression of somatic symptoms and severe cognitive impairment during childhood, and death by 10–15 years of age. The attenuated form has a later onset in childhood, slower and milder progression of somatic disease, little to no cognitive impairment, and survival into adulthood. (Fig. 1) Common clinical features include coarse faces, upper airway obstruction, cardiac valve regurgitation, restrictive lung disease, hepatosplenomegaly, hernias, joint contractures, poor endurance, and reduced quality of life [2,3]. *IDS* gene mutations are heterogeneous, but some show genotype–phenotype correlations: deletions and gross rearrangements of the *IDS* gene are associated with the severe form, whereas missense

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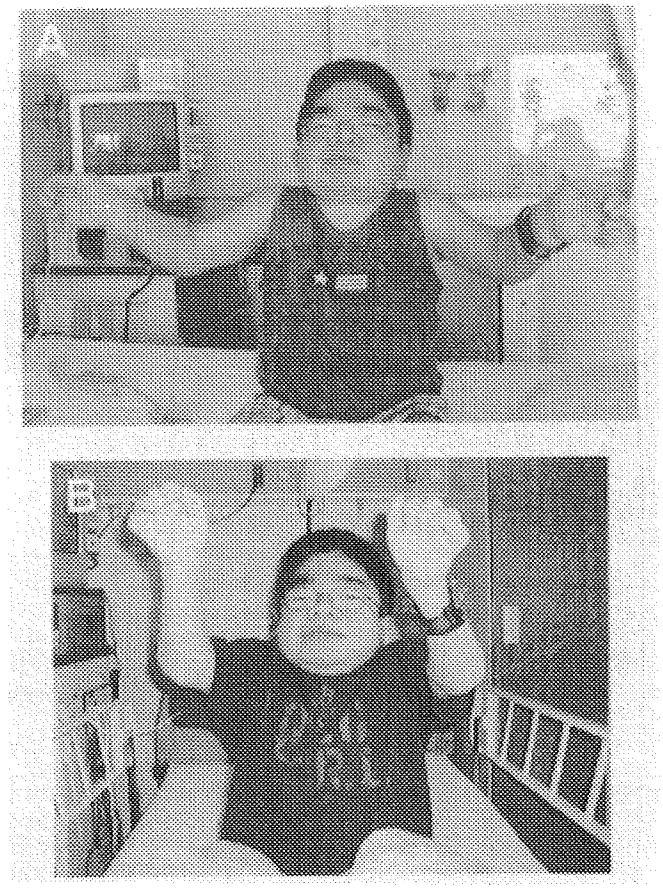

Fig. 1. A 23-year-old Japanese male study patient with MPS II. (A) Before treatment. (B) After 12 months of idursulfase treatment. Note the coarse facial features characteristic of MPS II. At baseline, the patient had severely limited shoulder range of motion (flexion and abduction), which improved following treatment.

mutations are more often associated with attenuated disease [4–10]. No racial or geographic differences have been observed. Females are only rarely affected, most often through skewed X-inactivation [1]. MPS II is the most prevalent MPS disorder in Asia, accounting for >50% of all MPS patients in Japan [10]. The annual incidence of all MPS disorders in Japan is estimated to be 1/50,000–1/60,000, and approximately half of the cases are due to MPS II. The estimated birth incidence of MPS II in Japan is, therefore, 1/90,000–1/100,000 [11], similar to the 1/92,000 to 1/162,000 incidences reported for predominantly Caucasian countries [12–15].

Until recently, treatment of MPS II was mainly palliative and focused on alleviating clinical symptoms through a variety of surgeries, medical devices, therapies, and medications. Several patients have undergone hematopoietic stem cell transplant (HSCT) as a source of iduronate-2-sulfatase, but unlike for MPS I, cognitive decline is not halted and the long-term effects on somatic disease are not well-documented [16,17]. Therefore, most centers consider the risk–benefit profile unfavorable and do not recommend HSCT for patients with MPS II.

Idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) is a recombinant human form of iduronate-2-sulfatase that is produced in a human cell line. Preclinical studies carried out in an MPS II knockout-mouse model [18] and in a Phase 1/2 dose-ranging study of MPS II patients [19] indicated that idursulfase was effective at reducing lysosomal GAG. The safety and efficacy of idursulfase was confirmed in a Phase 2/3 double-blind, placebo-controlled clinical study that randomized 96 MPS II patients to one of three treatment arms for 52 weeks: 0.5 mg/kg idursulfase weekly, 0.5 mg/kg idursulfase alternating with placebo every other week, or placebo weekly [20]. The primary efficacy endpoint was a composite of changes in percent predicted forced vital capacity (FVC) and the 6-Minute Walk Test (6MWT). Patients who received weekly idursulfase showed a greater difference in the composite endpoint compared to placebo ($p = 0.005$) than did the every other week idursulfase group ($p = 0.042$). The weekly idursulfase arm showed a mean 44.3 m increase in 6MWT distance (37 m difference from placebo, $p = 0.013$) and a mean 3.45 percentage point increase in percent predicted FVC (2.7 percentage point difference from placebo, $p = 0.065$). These clinical changes were associated with significant reductions versus placebo in urinary GAG level (−52.5%, $p < 0.0001$), liver volume (−25.3%, $p < 0.0001$), and spleen volume (−25.1%, $p < 0.0001$). Idursulfase was well-tolerated, with infusion-related reactions being the most common drug-related related adverse events, occurring in 69% (22/32) of patients in the weekly idursulfase arm.

Idursulfase was approved for the treatment of MPS II by the United States Food and Drug Administration (FDA) in July 2006 and by the European Medicines Agency (EMEA) in January 2007. Due to the life-threatening nature of the disease and the small number of patients, the Japanese Ministry of Health, Labour, and Welfare (MHLW) Committee for the Use of Unapproved Drugs recommended that idursulfase be approved based on ethical grounds and the results of overseas clinical trials, which included four Japanese patients. The committee also requested that idursulfase be made available to the most seriously ill MPS II patients prior to approval, which occurred in October 2007. Consequently, the Japan Elaprase Treatment (JET) study was initiated under the direction of the Japan Society for Research on MPS Disorders. Here, we present the results of this study.

Materials and methods

Patients

To be eligible for the study, patients had to meet all of the following inclusion criteria: (1) Documented deficiency of iduronate-2-sulfatase enzyme activity of <10% of the lower limit of normal with a normal enzyme activity level of one other sulfatase. (2) Male and above 20 years of age. (3) Clinically advanced disease status with <80% predicted FVC and New York Heart Association Class II–IV. (4) Capable of showing improved quality of life. (5) Able to complete study assessments.

Patient exclusion criteria included: (1) Previous bone marrow or cord blood transplant. (2) Known hypersensitivity to one of the components of idursulfase. (3) Previous treatment with idursulfase. (4) Unable to receive weekly infusions of idursulfase at the patient's local hospital. All patients provided signed informed consent prior to enrollment.

Study design

This was a multi-center, open-label study that enrolled 10 adult males with MPS II at 5 clinical sites in Japan. The study adhered to the guidelines set forth in the Declaration of Helsinki. Idursulfase was manufactured by Shire Human Genetic Therapies, Inc. and distributed by Genzyme Corporation (Cambridge, MA, USA). Genzyme Corporation performed all statistical analyses, and Genzyme Japan KK (Tokyo, Japan) provided data management support.

Idursulfase

Patients were administered 0.5 mg/kg idursulfase diluted in saline to a final volume of 100 cc intravenously over 3 h on a weekly

basis (±3 days) for up to 12 months. Infusions rates were ramped up over the first hour as described in the Phase 2/3 study [20]. Patients were monitored during each infusion and were discharged 1 h after completing the infusion, if clinically stable.

Efficacy assessments

Urinary GAG level was determined as the concentration of uronic acid normalized for creatinine (mg/g creatinine) and was measured using the carbazole reaction at a central laboratory (SRL Medisearch, Tokyo, Japan) or at Osaka City University Hospital. Liver and spleen volumes were quantitated by computerized tomography (CT), with the upper limits of normal being 2.5% and 0.2% of body weight, respectively. Percent predicted FVC and the 6MWT were performed according to American Thoracic Society guidelines [21,22]. Cardiac structure and function were evaluated by echocardiography (two-dimensional and M-mode). Left ventricular mass index (LVMI) was calculated as the left ventricular mass normalized for body surface area, with normal values defined as <131 g/m^2. Active joint range of motion was measured by goniometry, and included the shoulder (flexion, extension, and abduction), elbow (flexion and extension), hip (flexion and extension), and knee (flexion and extension). Left and right joint ranges of motion for each were averaged for each patient. The sleep study oxygen desaturation index (ODI) was assessed by pulse oximetry and defined as the number of desaturations (<89% oxygen saturation or ⩾4% decrease in oxygen saturation from baseline lasting ⩾10 s) per hour of sleep. A normal ODI was considered to be <5 events/h [23].

Safety assessments

Safety evaluation included continuous monitoring of adverse events and periodic clinical laboratory and physical examination evaluations. Adverse events were reported by severity (mild, moderate, severe, life-threatening) and by relatedness to idursulfase. An infusion-related reaction was defined as any adverse event occurring during or following an infusion (i.e., within 24 h of infusion initiation) that was reported by the investigator as related to idursulfase. Antibodies to idursulfase were measured by an enzyme-linked immunosorbant assay (ELISA; Shire Human Genetic Therapies).

Statistics

Efficacy results are reported as the mean ± standard error of the mean (SEM). For missing data at 12 months, the last observation carried forward method was used for values obtained at 6 months or later. The number of evaluable patients was at least nine for each endpoint, except for LVMI ($n = 6$, primarily due to missing baseline data) and the 6MWT ($n = 7$, primarily due to the inability to perform the test). The Wilcoxon signed rank test was used to evaluate changes in efficacy endpoint from baseline to 12 months, and p-values <0.05 were considered statistically significant. Percent change was tested for pharmacodynamic parameters (i.e., urinary GAG level and liver and spleen volumes), whereas absolute change was tested for clinical endpoints.

Results

Patient disposition

Ten adult Japanese males with attenuated MPS II were enrolled in the study and received idursulfase treatment. Nine patients completed the 12-month study; one patient died of causes unrelated to idursulfase after receiving 41 of 44 scheduled infusions (see Safety Section). Compliance with treatment was excellent, with all 10 patients receiving >93% of scheduled infusions; 80% (8/10) of patients did not miss a single scheduled infusion.

Patients

The mean patient age was 30.1 years (range 21.1–53.9). All patients had been diagnosed during mid-childhood or adolescence with MPS II (mean age 7.9 years), and all had advanced disease burden at the time of enrollment into the study. All patients had short stature (height <3rd percentile for Japanese adult males). Past medical history was significant for the following MPS II-related features (n = number of patients): valvular heart disease consisting mainly of aortic and/or mitral valve insufficiency (10), joint contractures (7), hepatomegaly (7), deafness (6), retinal degeneration (5), sleep apnea (5), otitis media

Table 1
Summary of efficacy changes after 12 months of treatment with idursulfase.

	N	Baseline	12 months	Change	% Change	p-Value
Urinary GAG (mg/g creatinine)	9	106.4 ± 7.8	21.2 ± 2.9	−85.2 ± 7.1	−79.9 ± 2.2	0.004†
Liver volume (cc)	10	1491.2 ± 92.9	993.2 ± 75.0	−498.0 ± 70.2	−33.2 ± 4.0	0.002†
Spleen volume (cc)	10	210.2 ± 22.5	138.1 ± 12.5	−72.1 ± 15.7	−31.0 ± 5.5	0.002†
6-Minute Walk Test (m)	7	286.0 ± 53.4	340.5 ± 49.6	54.5 ± 27.0	37.4 ± 18.1	0.109
Forced vital capacity (% predicted)	9	39.9 ± 6.6	43.7 ± 6.0	3.8 ± 2.8	15.0 ± 8.0	0.250
Forced vital capacity (L)	9	1.4 ± 0.3	1.5 ± 0.2	0.1 ± 0.1	16.3 ± 8.0	0.250
Left ventricular mass index (g/m^2)	6	139.9 ± 25.1	133.2 ± 38.9	−6.7 ± 15.5	−12.4 ± 11.1	0.563
Left ventricular ejection fraction (%)	10	67.0 ± 5.2	64.3 ± 6.0	−2.8 ± 2.5	−6.1 ± 5.7	0.244
Joint range of motion (degrees)					NA	
Shoulder flexion	10	93.8 ± 4.9	109.8 ± 7.1	15.0 ± 7.3		0.066
Shoulder extension	10	44.1 ± 4.1	43.8 ± 3.8	−0.3 ± 4.1		0.945
Shoulder abduction	10	76.3 ± 3.9	95.3 ± 8.1	19.0 ± 8.8		0.125
Knee flexion	9	103.7 ± 8.5	114.4 ± 5.2	10.7 ± 10.3		0.461
Knee extension	9	−11.1 ± 4.5	−10.3 ± 5.0	0.8 ± 2.5		0.875
Hip flexion	9	89.2 ± 8.1	103.3 ± 7.6	14.2 ± 5.1		0.031
Hip extension	9	3.1 ± 5.0	1.9 ± 6.7	−1.3 ± 1.8		0.750
Elbow flexion	10	120.9 ± 4.0	121.8 ± 3.7	0.9 ± 2.5		0.828
Elbow extension	10	−43.1 ± 4.2	−35.0 ± 4.2	8.1 ± 3.4		0.063
Oxygen desaturation index (events/h)	9	18.5 ± 6.1	22.3 ± 7.4	3.9 ± 3.5	NA	0.426

The last observation carried forward (LOCF) method was used to replace a missing value at the 12-month timepoint.
All values are the observed means ± SEM. All p-values are based on the Wilcoxon signed rank test for change from baseline to the 12-month timepoint.
NA, not applicable. Some patients had values of 0 at baseline that precluded calculation of percent change.
† The p-value is based on the Wilcoxon signed rank test for % change from baseline to the 12-month timepoint.

(4), macroglossia (3), umbilical hernia (2), carpal tunnel syndrome (2), heart failure (2), and left ventricular hypertrophy (1).

Urinary glycosaminoglycan (GAG)

All nine evaluable patients had elevated urinary GAG levels at baseline (mean 106.4 mg/g creatinine, approximately 8 times the upper limit of normal); one patient lacked an appropriate baseline value (Table 1). Following idursulfase treatment, urinary GAG levels decreased rapidly within the first three months of treatment and remained low for the remainder of the study (Fig. 2A). There was a statistically significant mean decrease in the urinary GAG level of −79.9 ± 2.2% from baseline to 12 months ($p = 0.004$). All nine evaluable patients showed a >70% decrease in urinary GAG levels and had normal values by the end of the study.

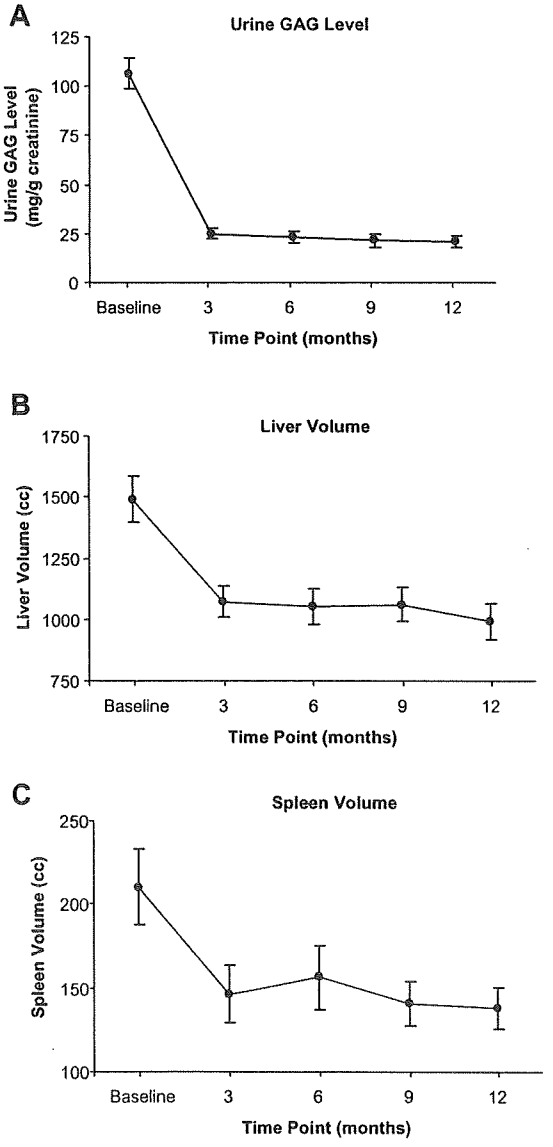

Fig. 2. The effects of idursulfase treatment on lysosomal storage over 12 months. (A) Urinary GAG level. (B) Liver volume. (C) Spleen volume. All changes are reported as mean ± SEM.

Liver and spleen volumes

At baseline, 9 (90%) patients had hepatomegaly (mean 1.3 MN, multiples of normal) and all 10 (100%) patients had splenomegaly (mean 2.4 MN) by CT. After 12 months of treatment, mean liver volume decreased by −33.2 ± 4.0% and mean spleen volume decreased by −31.0 ± 5.5% (Fig. 2B and C; Table 1), and both changes were statistically significant ($p = 0.002$). Most of the reductions occurred within the first three months of treatment. By the end of the study, all patients had liver volumes within the normal range and spleen volumes that were <2 MN, demonstrating efficient reduction of lysosomal GAG storage.

6-Minute Walk Test (6MWT)

At baseline, the mean 6MWT distance was 286.0 m for the seven patients who could perform the test (Table 1). All but one patient walked <399 m, the lower limit of normal for healthy adult men in the United States [24]. Three patients could not perform the 6MWT: one patient broke his leg just prior to the start of the study; one patient was wheelchair-bound secondary to shortness of breath and muscle weakness; and one patient was obese and could only walk a few steps with assistance. By the end of the study, the mean 6MWT distance had increased by 54.5 ± 27.0 m (Fig. 3A). This change represents a relative increase of 37.4%, and included one patient whose 6MWT distance increased by 131%. Four patients (57%) showed a clinically meaningful improvement of ⩾54 m [25], while the one patient with a normal 6MWT at baseline showed a decline (−71 m).

Percent predicted forced vital capacity (FVC)

Nine patients underwent spirometry at baseline and all showed a restrictive lung disease pattern: three were classified as having a severe defect (<50% predicted FVC) and five had a very severe defect (<34% predicted FVC) [26]. At baseline, mean percent predicted FVC was 39.9% (Table 1), and after 12 months it increased by 3.8 ± 2.8 percentage points (Fig. 3B). This improvement corresponds to a relative increase of 15.0% over baseline, which is considered clinically meaningful (⩾15% relative change) [25] and was achieved by four (44%) patients. Similarly, mean FVC increased by 16.3% over the baseline of 1.4 L. The mean forced expiratory volume in 1 s (FEV_1):FVC ratio remained unchanged at 0.70 during the study.

Cardiac

All patients had valve disease that remained stable during the study. The mean ejection fraction (EF) was normal at baseline and showed little change over 12 months (67.0–64.3%, change of −2.8 ± 2.5%) (Table 1). One patient with pre-existing cardiac failure showed gradual worsening during the study (EF 27–14%). At baseline, mean LVMI was slightly elevated at 139.9 g/m^2 (normal <131 g/m^2), and 50% (3/6) of evaluable patients had an elevated LVMI. After 12 months, mean LVMI decreased by −12.4%, with four patients showing a clinically meaningful improvement of >10% [27]. The patient with the largest LMVI at baseline showed a further increase (254.1–312.9 g/m^2).

Joint range of motion

Fig. 4 and Table 1 show the changes in joint range of motion observed during the study. At baseline, patients had significant joint contractures involving the shoulder (flexion, extension, and abduction), knee (flexion and extension), hip flexion and extension), and elbow (flexion and extension). Following 12 months of treatment, several joints showed increased range of motion, including mean

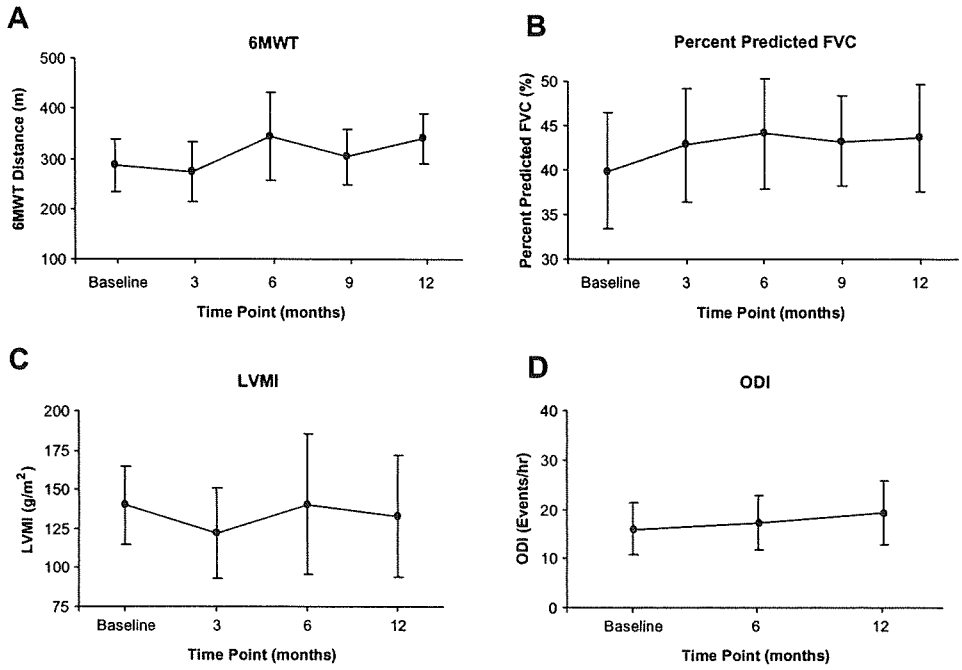

Fig. 3. The effects of idursulfase treatment on clinical endpoints over 12 months. (A) 6-Minute Walk Test. (B) % Predicted forced Vital Capacity. (C) Left Ventricular Mass Index. (D) Oxygen Desaturation Index. All changes are reported as mean ± SEM.

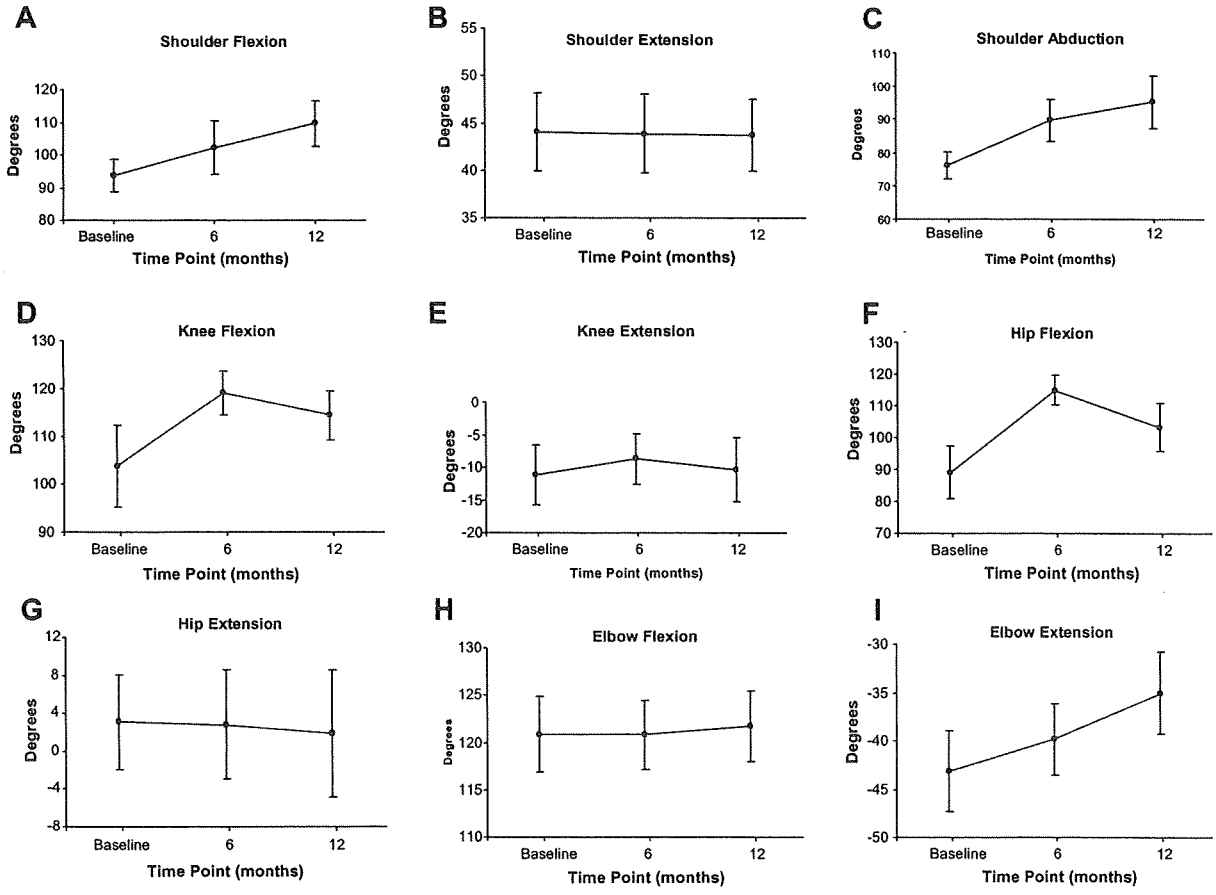

Fig. 4. The effects of idursulfase treatment on joint range of motion over 12 months. (A) Shoulder flexion. (B) Shoulder extension. (C) Shoulder abduction. (D) Knee flexion. (E) Knee extension. (F) Hip flexion. (G) Hip extension. (H) Elbow flexion. (I) Elbow extension. All changes are reported as mean ± SEM.

shoulder flexion (15.0 ± 7.3 degrees), shoulder abduction (19.0 ± 8.8 degrees), knee flexion (10.7 ± 10.3 degrees), hip flexion (14.2 ± 5.1 degrees; $p = 0.031$), and elbow extension (8.1 ± 3.4 degrees). However, most of the changes did not achieve statistical significance. Shoulder extension (−0.3 ± 4.1 degrees), elbow flexion (0.9 ± 2.5 degrees), knee extension (0.8 ± 2.5 degrees), and hip extension (−1.3 ± 1.8 degrees) showed little change during the study. Fig. 1 shows a 23 year-old study patient with severely limited shoulder range of motion (abduction and flexion), which improved following one year treatment with idursulfase.

Oxygen desaturation index (ODI)

At baseline, the mean oxygen desaturation index (ODI) was 18.5 events/h ($n = 9$), which is moderately abnormal [23]. Three patients had a normal ODI (<5 events/h), two had a mildly abnormal ODI (5–15 events/h), and four had a moderately to severely abnormal ODI (>15 events/h). During the study, the mean ODI increased by 3.9 ± 3.5 events/h, which was largely due to a single patient with an increase of 26.8 events/h. The other seven patients had stable ODI values (changes $\leqslant$ 10 events/h).

Safety

Idursulfase was well-tolerated over the course of the study. Adverse events were mainly mild, unrelated, and attributable to expected symptoms of MPS II disease. Fifty percent (5/10) of patients experienced a total of 11 drug-related adverse events. Urticaria was the most frequent event (five events in two patients), followed by erythema (two events in the same patient). Similarly, 50% (5/10) of patients experienced infusion-related reactions (i.e. adverse events assessed as drug-related and occurring within 24 h of the infusion). The highest patient incidence involved skin reactions, i.e. urticaria and erythema (three patients each), while dyspnea, abdominal pain, and vasovagal syncope also were observed in one patient each. Except for one patient who experienced several episodes of urticaria between 9 and 12 months, the other four patients had infusion-related reactions only once or twice during the first three months of treatment. Management of infusion-related reactions included antihistamine therapy and temporary interruption of the infusion, and all events were followed by a successful patient recovery. There were no clinical laboratory abnormalities reported as related to idursulfase.

Two patients experienced serious adverse events, including one death, in the study. A 26 year-old male experienced an infusion-related reaction involving diffuse urticaria, flushing, and numbness of the tongue 1 h after initiation of the fifth infusion. The patient was pre-medicated with antihistamines without further events. A 42 year-old male had an infusion-related reaction reported by the investigator as vasovagal syncope, which consisted of hypotension, vomiting, weak pulse, and decreased consciousness and occurred 30 min into the first infusion. Subsequent infusions were preceded by corticosteroid pre-medication administration without further infusion-related reactions. The patient had a history of cardiac valve incompetence and cardiac failure requiring medications, including furosemide. Later in the study, he experienced an increase in leg edema secondary to worsening congestive heart failure. He was depressed and attempted suicide by drug overdose (not idursulfase). Upon arrival at the hospital, the patient went into cardiac arrest. Subsequent resuscitation measures were unsuccessful, and he died due to hypoxic encephalopathy, pneumonia and renal failure.

Antibodies

Anti-idursulfase IgG antibodies were detected in 60% (6/10) of patients, two of who became seronegative later in the study. No IgE antibodies were detected in patients who underwent testing for infusion-related reactions. The mean reductions in urinary GAG levels did not differ between patients who were seropositive at any time (−80.9% ± 3.8%; $n = 5$) and those who remained seronegative throughout the study (−78.6% ± 1.8%; $n = 4$). Although hypersensitive reactions or infusion-related adverse reactions tended to occur in the antibody-positive patients (four antibody-positive patients versus one antibody-negative patient), there was no correlation between the presence of antibodies and other adverse events. Furthermore, the frequency of hypersensitivity reactions did not correlate with antibody titer.

Discussion

The most remarkable difference between this and previous clinical studies of idursulfase [19,20] relates to the patient demographics and characteristics. The purpose of the JET study was to provide access to treatment for the most seriously ill MPS II patients while awaiting regulatory approval of idursulfase in Japan, which occurred in October 2007. Patients in the JET study had a mean age of 30.1 years, all were Japanese, and all were seriously ill (mean percent predicted FVC 39.9% and mean 6MWT distance 286.0 m). By comparison, MPS II patients in the Phase 1/2 and Phase 2/3 studies of idursulfase were younger (mean ages 13.9 years and 14.2 years), predominantly Caucasian (100% and 83%, respectively), and less severely affected (mean percent predicted FVC 55.1% and 55.4%; mean 6MWT distance 397 m and 395 m) [19,20]. Despite these patient differences, the JET study has shown that idursulfase is a safe and effective (Table 1) treatment for Japanese patients with MPS II and its risk–benefit profile is similar to that reported in previous studies.

In this study, idursulfase efficiently reduced GAG storage, as evidenced by the statistically significant reductions in urinary GAG levels ($p = 0.004$) and hepatosplenomegaly ($p = 0.002$) (Fig. 2; Table 1). These pharmacodynamic changes appeared to translate into clinical benefit, as evidenced by trends towards improvement in functional capacity (mean 54.5 m increase in 6MWT), respiratory function (mean 15.0% relative increase in percent predicted FVC), joint range of motion (mean increases ranging from 8.1–19.0 degrees for several joints), and LVMI (mean −12.4% decrease). Cardiac EF and valve disease remained mostly stable, although one patient with severe congestive heart failure showed progressive worsening and one patient with a greatly elevated LVMI showed a further increase. The mean ODI increased slightly by 3.9 events/h, but importantly 89% (8/9) of patients showed no clinically significant changes.

The safety profile of idursulfase in the JET study was similar to that of previous studies with no new or unexpected adverse events despite the older and more seriously ill patient population. Most adverse events were considered by investigators to be disease-related and unrelated to idursulfase. The most common drug-related adverse events were infusion-related reactions, occurring in 50% of patients. The most common infusion-related reactions were skin reactions consisting of urticaria and erythema. There were two related serious adverse events that occurred during the infusions—one involving urticaria, flushing, and numbness of the tongue, and the other involving vasovagal syncope. The one patient death was attributed to suicide from a drug overdose and was not related to idursulfase.

MPS II is a progressive and debilitating multisystem disease that is associated with a shortened lifespan, primarily from cardiorespiratory compromise [28]. Therefore, it is noteworthy that in this one-year study, cardiac and respiratory functions were improved or stable in most patients. Decreasing lung volumes are known to be associated with increased morbidity and mortality [26];

given the low percent predicted FVC values at baseline in study patients (mean 39.9%), a relative increase of 15% is of particular importance. The American Thoracic Society defines a >15% relative change in FVC occurring over a one-year period as being clinically meaningful [26]. Similarly, the 54.5 m mean increase in 6MWT distance also is considered to be a clinically meaningful improvement, based on a study of adult men with chronic obstructive pulmonary disease [25]. The 6MWT is a sub-maximal exercise test that is a composite assessment of cardiac, respiratory, and musculoskeletal function. Because all three of these organ systems are involved in the MPS disorders, walking tests have been widely used as primary efficacy endpoints in clinical trials of enzyme replacement therapy for other MPS disorders, including MPS I [29,30] and MPS VI [31].

We observed no evidence for an effect of race on immunogenicity or safety. IgG antibodies were detected in 60% (6/10) of patients treated with idursulfase, which is similar to the 49.6% rate seen in the Phase 2/3 study that enrolled predominantly Caucasian and other non-Asian patients [20]. In addition, the adverse event profile was similar in all respects; infusion-related reactions occurred in 50% of patients in the current study compared to 69% of patients receiving weekly idursulfase in the Phase 2/3 study [20].

Limitations of this study include its open-label treatment, lack of control group, and small sample size. Other aspects of the study design, however, including the treatment dose and regimen, study duration, and efficacy and safety assessments were identical or very similar to those used in the Phase 2/3 study [20]. A placebo effect in this study cannot be excluded, especially for effort-dependent assessments such as the 6MWT and active joint range of motion. Nevertheless, the magnitude of change in the 6MWT distance was similar to those observed in previous studies of idursulfase [19,20]. Determination of FVC by spirometry is less susceptible to a placebo effect given the requirement for test–retest reproducibility at each assessment [21]. This study enrolled only 10 patients, which may not have had sufficient power to detect a statistically significant clinical response even if clinical improvements were present. On the other hand, the biomarkers of lysosomal GAG clearance, i.e. liver and spleen volumes and urinary GAG level, did have sufficiently large effect sizes (change/standard deviation of change) to show statistically significant differences. Finally, the study involved only adult males, all of whom had a substantial pre-existing disease burden. This study shows that many disease features of seriously ill patients, including diminished cardiorespiratory function, restricted joint range of motion, and hepatosplenomegaly can improve with idursulfase treatment. An even better response is expected in young children prior to final organ maturation and the development of chronic tissue damage. In this regard, a study in MPS II patients ⩽5 years of age is underway.

Conclusions

Idursulfase was generally well-tolerated and produced clinical improvements in adult Japanese patients with attenuated MPS II treated with the labeled dose, 0.5 mg/kg administered intravenously once weekly. Treatment with idursulfase also resulted in substantial reductions in hepatosplenomegaly and urinary GAG excretion, indicating efficient clearance of lysosomal GAG. The safety profile and immunogenicity of idursulfase appear to be similar between Japanese and previously studied Caucasian patients.

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X-linked agammaglobulinemia in a 10-year-old boy with a novel non-invariant splice-site mutation in *Btk* gene

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ABSTRACT

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease caused by mutations in the gene coding for Bruton's tyrosine kinase (Btk). Most XLA patients have severely reduced or absent peripheral blood B cells and serum immunoglobulins, since the expression or function of Btk, critical for the maturation of B cell lineages at pro-B and pre-B cell stages, is deficient. Early and accurate diagnosis of XLA is important, since the affected patients suffer from severe and recurrent infections unless they receive intravenous immunoglobulin (IVIG) replacement therapy. However, the diagnosis of XLA is not always easy because some patients have detectable (~2%) B cells in the peripheral blood and have significant levels of serum immunoglobulins. In this study, we report on a patient who was diagnosed with XLA at the age of 10 years. The diagnosis was delayed due to near-normal levels of serum immunoglobulins, although he presented with severe and recurrent bacterial infections since the age of 1 year. He was demonstrated to have a novel non-invariant splice-site mutation in intron 10 (IVS10 −11C→A) of the *Btk* gene, which was not detected by the standard PCR-based mutation analysis. This mutation resulted in no detectable Btk expression. This case suggests that patients suffering from severe or recurrent bacterial infection should be suspected to have XLA even though they may have significant levels of serum immunoglobulins. Furthermore, significant levels of serum immunoglobulins in XLA patients do not necessarily mean less severe phenotype.

Introduction

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease first described by Bruton in 1952 [1]. It is characterized by marked reduction in serum levels of all immunoglobulin isotypes, defective B cell development at pro-B and pre-B cell stages [2,3] and severely decreased numbers of circulating B cells. Affected males have susceptibility to severe bacterial infections unless they receive appropriate therapy, including intravenous immunoglobulin (IVIG) replacement therapy. Since the gene responsible for XLA was identified as coding for a cytoplasmic tyrosine kinase named Bruton's tyrosine kinase (Btk), various mutations have been reported [4–7]. However, the direct detection of *Btk* mutations by sequence analysis is time-consuming as a diagnostic procedure. Furthermore, some XLA patients have detectable (~2%) B cells in the peripheral blood and/or show significant levels of serum IgG (~800 mg/dl), which makes the diagnosis of XLA difficult. Flow cytometric analysis of Btk expression (FCM-Btk) is one of the simple and useful methods for the diagnosis of XLA [7]. In this study, we report on a patient who was given the diagnosis of XLA at the relatively advanced age of 10 years. The delay in diagnosis was apparently due to the presence of near-normal levels of serum immunoglobulins, although he presented with severe and recurrent infections since the age of 1 year. He was demonstrated to have a novel non-invariant splice-site mutation in intron 10 (IVS10 −11C→A) of the *Btk* gene, which was not detected by the standard PCR-based mutation analysis. Although splice-site mutations sometimes result in partial ("leaky") expression of Btk, this mutation resulted in no detectable Btk expression.

Materials and methods

Subjects

Samples of three unrelated XLA patients confirmed by *Btk* mutations were used as Btk-deficient controls. The patient, W.I., had suffered from recurrent episodes of acute otitis media, sinusitis, lymphadenitis with abscess, and colitis since the age of 1 year. He also had repetitive episodes of high fever with marked leukocytosis and elevation of CRP (~20 mg/dl). Every time, he was suspected to have bacterial septicemia or meningitis and was treated with intravenous antibiotics effectively, although focus of infection was not identified. He had no family history of severe infections or immunodeficiency. His recent

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Table 1
Primers used for *Btk* RT-PCR and intron 10 PCR studies.

Primers for *Btk* RT-PCR	Sequence	Tm (°C)	Corresponding exon (s)
Forward			
Btk1F	5′- gac tgt cct tcc tct ctg ga -3′	54	1
Btk2F	5′- ctt caa gaa gcg cct gtt tct -3′	52	2
Btk3F	5′- cat tga aag att ccc tta tcc c -3′	51	4
Btk4F	5′- atg ggc tgc caa att ttg gag -3′	52	6
Btk5F	5′- tga tta cat gcc aat gaa tgc -3′	49	8
Btk6F	5′- gct cat taa cta cca tca gca c -3′	53	12
Btk6F-2	5′- cat tga aga agc caa agt c -3′	47	14
Btk7F	5′- ccg gaa gtc ctg atg tat ag -3′	52	17
BtkNoF	5′- agc aag agg gga aag aag gag	54	10–11
Reverse			
Btk1R	5′- acc ctt ctt act gcc tct tc -3′	52	3
Btk2R	5′- aat cca ccg ctt cct tag ttc -3′	52	5
Btk3R	5′- tca ggc gtt ggg gga aga ggc -3′	60	7
Btk4R	5′- cta gga atg tag cct tcc tgc -3′	54	9
Btk5R	5′- gca gtg gaa ggt gca ttc ttg -3′	54	13
Btk6R	5′- ggc cga aat cag ata ctt t -3′	47	16
Btk7R	5′- agc ttg gga ttt cct ctg aga -3′	59	19
Primers for *Btk* intron 10 PCR	Sequence	Tm (°C)	Corresponding exon/intron
Intron 10F1	5′- gag caa ctg cta aag caa gag -3′	52	exon 10
Intron 10F2	5′- gtc tcg aac tcc tga cct cag -3′	56	intron 10
Intron 10R1	5′- ctg tgg att tag caa aca cag -3′	50	exon 11

serum immunoglobulin levels were: IgG 400–800 mg/dl (normal range: 608–1572 mg/dl), IgA of 4–5 mg/dl (normal range: 33–236 mg/dl), and IgM of 64–102 mg/dl (normal range: 52–242 mg/dl). However, he failed to develop specific IgG antibodies against all the immunizations he received (rubella, measles and mumps), and had no antibodies against Epstein–Barr virus (EBV) and Cytomegalovirus (CMV). He had not received further laboratory studies because of near-normal levels of immunoglobulins. The proportion of his peripheral blood lymphocytes that were CD19+ cells (B cells) at the age of 8 years and 10 years was significantly reduced to be 0.6% and 0.2%, respectively. He was referred to our hospital for further evaluation of B cell deficiency at the age of 10 years.

Flow cytometric analysis of intracellular Btk expression (FCM-Btk) in monocytes

We performed FCM-Btk, originally established by Futatani et al. [7], basically following the WASP detection protocol established for the diagnosis of Wiskott–Aldrich syndrome [8]. Briefly, heparinized blood samples were collected from a patient and a normal individual under the same conditions. Peripheral blood mononuclear cells (PBMC) isolated by standard Ficoll-Hypaque gradient centrifugation methods were washed with phosphate-buffered saline (PBS) containing 1% fetal bovine serum. Cell pellets were resuspended with 100 μl of Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA) and incubated at 4 °C for 20 min. After three washes with Perm/Wash solution, they were incubated with 1 μg/ml of anti-Btk antibody (48-2H) [9] or MOPC21 mouse IgG1 control (Sigma, Saint Louis, MO) at 4 °C for 30 min and washed three times. They were then reacted with 1 μg/ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) at 4 °C for 30 min and washed three times. Samples were then analyzed on a FACSCalibur (BD Biosciences). A total of 20,000 events of monocytes, which were gated based on forward and side scatter, were analyzed.

PCR, RT-PCR, and Btk sequence analysis

SepaGene (Sanko Junyaku, Tokyo, Japan) and TRIzol Reagent (Invitrogen, Carlsbad, CA) were used for the isolation of DNA and total RNA from PBMC, respectively. *Btk* exons, adjacent intronic regions, and a putative promoter region were amplified by PCR with primers

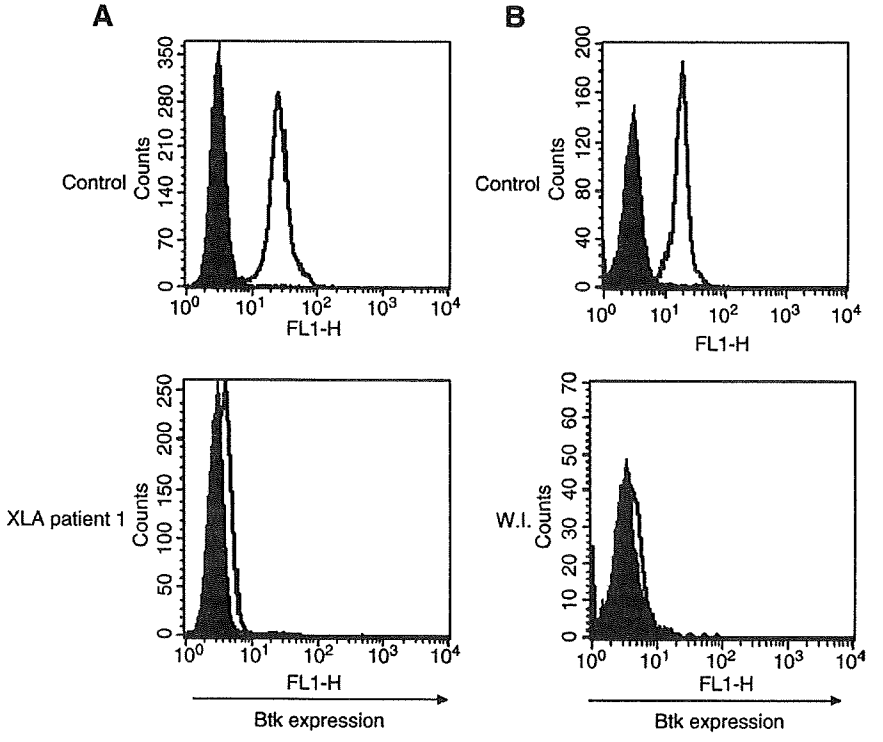

Fig. 1. Flow cytometric analysis of intracellular Btk expression (FCM-Btk) in monocytes ■: isotypic control, ■: anti-Btk antibody (48-2H). A: FCM-Btk result of a normal control (Control) and a known XLA patient (XLA patient 1). B: FCM-Btk result of a normal control (Control) and the patient, W.I.

described by Vorechovský et al. [10]. For the analysis of the whole sequence of intron 10, we performed PCR with a combination of Intron 10F1 and Intron 10R1 primers and a combination of Intron 10F2 and Intron 10R1 primers (Table 1). Intron 11 full sequences were studied with a combination of exon 10 forward and exon 11 reverse primers described by Vorechovský et al. [10]. RT-PCR was performed using the primers described in Table 1 after the preparation of complementary DNA (cDNA) from total RNA using 1st Strand cDNA Kit (GE Healthcare Buckinghamshire, UK). The primer BtkNoF was designed to match the exon 10–exon 11 junction to specifically amplify only wild-type transcripts (Table 1). PCR and RT-PCR products isolated by Gel-purification kit (Invitrogen) were then directly sequenced using BigDye Terminator Cycle Sequencing Kits v1.1 (Applied Biosystems, Carlsbad, CA). "Splice Site Prediction by Neural Network" (www.fruitfly.org/seq_tools/splice.html) was used for the prediction of splice sites.

Western blot analysis of Btk expression

PBMC pellets from normal controls and XLA patients were resuspended in cytoplasmic extract buffer containing 0.5% Nonidet P-40, 250 mM NaCl, 10 mM HEPES pH 7.9, 0.1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, pH 8.0 with the addition of the recommended volume of dissolved protease inhibitor cocktail tablets (Roche, Basel, Switzerland). After incubation on ice for 15 min, the supernatant after centrifugation at maximum speed for 5 min at 4 °C was saved as cytoplasmic extract. Protein concentration was measured by Protein Assay (Bio-Rad, Hercules, CA). Thirty micrograms of cytoplasmic extract after addition of SDS sample buffer was separated by 8% polyacrylamide gels and transferred to Hybond-P PVDF membranes (GE Healthcare). Two anti-Btk monoclonal antibodies were used: 48-2H [9] and 8E5.A10.E5 [11] corresponding to SH3 and PH domains of Btk, respectively. Anti-actin antibody (AC40) was purchased from Sigma. All the primary antibodies were used at the final concentration of 1 μg/ml. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibodies (GE Healthcare) were used at 1:2000 dilution. The blots were then visualized by ECL plus (GE Healthcare).

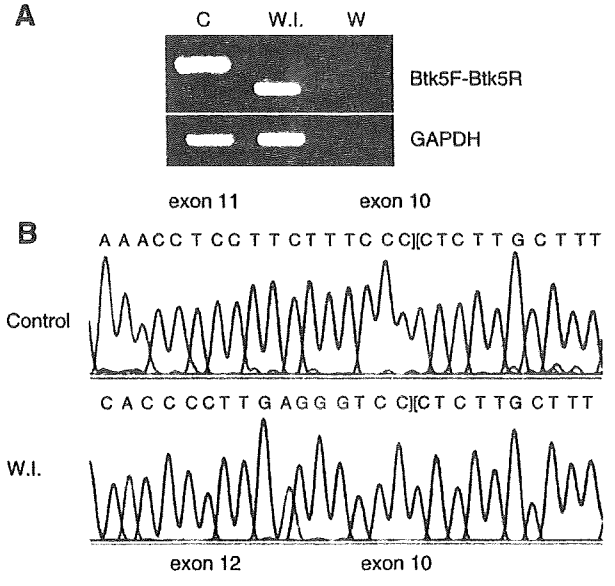

Fig. 2. RT-PCR analysis of *Btk* followed by sequence analysis. A: Samples of a control (C), the patient, W.I., and water (W) after RT-PCR with Btk5F and Btk5R primers were separated on agarose gel. The housekeeping gene GAPDH was used as a positive control for the presence of mRNA in the samples. B: Direct sequence analysis of RT-PCR products from a control (Control) and W.I. Reverse sequence was shown.

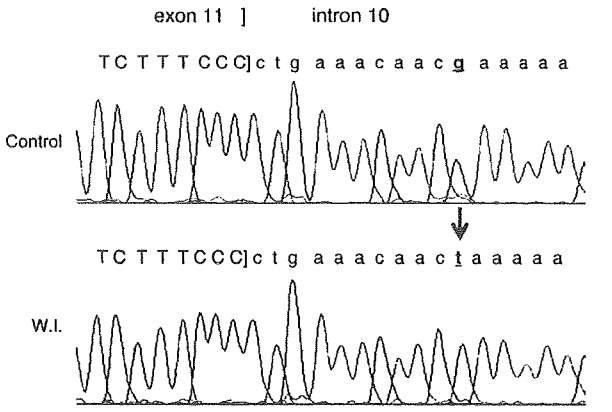

Fig. 3. Direct sequence analysis of samples from a normal control (Control) and W.I. after PCR with Intron 10F2 and Intron 10R1 primers. Reverse sequence was shown.

Results

First of all, we performed flow cytometric analysis of intracellular Btk protein expression (FCM-Btk) in monocytes from the possible XLA patient, W.I., who had severe and recurrent infections in spite of consistently showing near-normal levels of serum IgG. Patient, W.I, as well as 3 XLA patients confirmed by detection of *Btk* mutations was demonstrated to have deficient Btk expression, indicating he has XLA (Fig. 1 and data not shown). We then proceeded to a molecular study of *Btk* gene using the standard PCR-based mutation analysis with primers as described by Vorechovský et al. [10]. Unexpectedly, no mutations were detected in direct sequence analysis of all the exons, exon–intron boundaries and a putative promoter region of this gene. However, the study of the Btk mRNA expression by RT-PCR and the subsequent direct sequence analysis of these products demonstrated that he had a shorter RT-PCR product with Btk5F (a forward primer in exon 8) and Btk5R (a reverse primer in exon 13), which resulted from skipping of exon 11 (Fig. 2). RT-PCR products amplified from other parts of the mRNA were normal in size and sequences. To identify the mechanism of exon 11 skipping, we performed direct sequence analysis of the whole intron 10 and intron 11 as described in Materials and methods. The results demonstrated that he had wild-type sequences except a base change in intron 10, 11 bases upstream of exon 11 (Fig. 3). This base change was found to be included in the middle of the forward primer used for sequence analysis of the intron 10–exon 11 boundary [10]. His mother showed c + a signals at this site (data not shown), indicating that his mother is heterozygous for this base change. "Splice Site Prediction by Neural Network" analysis of the sequence of the intron 10–exon 11 junction gave a significantly lower score for the sequence with the patient's mutation (0.80), compared with that of wild-type sequence (0.93) (Fig. 4). These

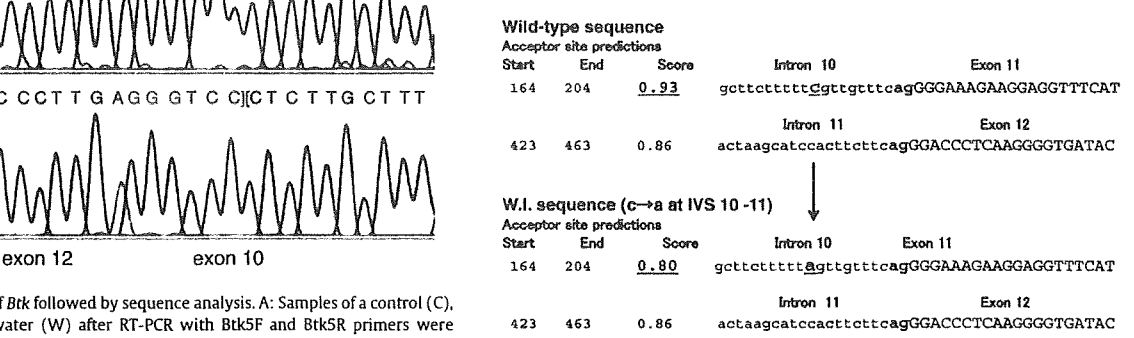

Fig. 4. The results of predicted splice sites with "Splice Site Prediction by Neural Network" (http://www.fruitfly.org/seq_tools/splice.html) were shown.

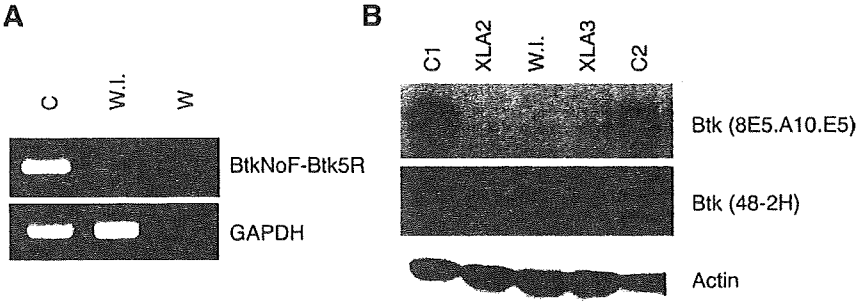

Fig. 5. Study of possible leaky expression of normal *Btk* transcripts and Btk expression. A: Control (C), W.I., and water (W) samples after RT-PCR with BtkNoF and Btk5R primers were separated on agarose gel. The housekeeping gene GAPDH was used as a positive control for the presence of mRNA in the samples. B: Western blot analysis of Btk expression in PBMC from two controls (C1 and C2), two XLA patients (XLA2 and XLA3), and the patient, W.I. Actin expression was used as a loading control.

results suggest that the base change of IVS10 −11C→A is a novel splice-site mutation leading to skipping of exon 11, which was predicted to cause frameshift and premature termination of protein translation, 23 codons downstream from the end of exon 10. Since the mutation is not within the invariant GT or AG splice site (the first two or the last two base pairs of an intron), it was possible that low levels of normal Btk message could be transcribed due to a leaky splicing defect. To study this possibility, we performed RT-PCR with a primer corresponding to the exon 10–exon 11 junction (BtkNoF) and Btk5R to specifically amplify only wild-type transcripts. The result, however, demonstrated that W.I. had no wild-type transcripts of *Btk* gene (Fig. 5A). Consistent with this result, Western blot analysis of PBMC also demonstrated that expression of normal Btk protein was not detectable with the use of two monoclonal anti-Btk antibodies: 8E5.A10.E5 and 48-2H (Fig. 5B, Materials and methods). Since his mutation was predicted to affect the mRNA sequence downstream of exon 10, these two antibodies, 8E5.A10.E5 (corresponding to PH domain, exon 2–5) and 48-2H (corresponding to SH3 domain, exon 8–9) should detect the truncated Btk protein if it exists. However, truncated Btk expression was undetectable in his PBMC with these two antibodies (data not shown).

Discussion

In this study, we report on a patient, W.I., who was diagnosed with XLA at the age of 10 years. In spite of normal or near-normal levels of serum IgG, he presented severe and recurrent infections since the age of 1 year. He was demonstrated to have deficient Btk expression in monocytes which was caused by a novel non-invariant splice-site mutation in intron 10 (IVS10 −11C→A) of the *Btk* gene. At first, sequence analysis of the *Btk* gene using primers previously reported [10] demonstrated no mutations in all the exons, exon–intron boundaries and a putative promoter region of *Btk* gene. Based on deficient Btk expression, we considered the possibilities that he had a splice-site mutation located outside of the sequences studied or a mutation in an unknown region regulating Btk expression. RT-PCR studies revealed a skipping of exon 11 (Fig. 2), indicating a splice-site mutation in intron 10 or 11. Subsequent sequence analysis of the whole intron 10 and 11 demonstrated that he has a base change in intron 10, 11 bases upstream of exon 11 (Fig. 3), which is a non-invariant site for splice mutation. This result explains why we could not detect his mutation by the original PCR-based mutation analysis, because the mutation was in the middle of the forward primer used for sequence analysis of the intron 10–exon 11 boundary, not because it was located outside of the sequences studied. These results suggest that a combination studies at the DNA, RNA and protein levels are important to avoid overlooking a mutation of this gene that is responsible for XLA. Although no strong genotype–phenotype correlation has been established in XLA patients [12–15], some mutations in *Btk*, particularly mutations that allow Btk protein expression are likely to be associated with less severe phenotype. As for splicing defects, a few patients with mild disease were shown to have some Btk expression due to leaky splicing [16–18]. In contrast to these patients, the patient, W.I., who presented severe phenotype, was demonstrated to have absent expression of wild-type Btk both at mRNA and protein levels (Figs. 1B and 5B). He also had no truncated Btk protein expression due to skipping of exon 11. Therefore, this study indicated that the splice-site mutation of IVS10 −11C→A could be one of the mutations causing the severe, typical XLA phenotype. Some XLA patients have been shown to have significant levels of serum immunoglobulins, similar to the present case with near-normal IgG and normal IgM. This might be explained by the presence of small numbers of "leaky" mature B cells possessing the ability to proliferate, undergo class switching, and differentiate into antibody-producing cells, through unknown mechanisms independent of Btk function, as demonstrated by Nonoyama et al. [19]. It is not clear whether there is special significance to the low IgA level observed in the present case. Considering that serum IgA level is more likely to be low or undetectable even in XLA patients with normal or near-normal level of serum IgG [9,16,17,19,21], IgA production might be more dependent on Btk function, although other genetic or undefined factors might also modify it. XLA cases with significant levels of serum immunoglobulins and older age at diagnosis were generally considered as less severe phenotype [20,21]. However, at least in the present case, older age at diagnosis was associated with significant immunoglobulin concentration, but not with less severe phenotype. This case suggests that significant levels of serum immunoglobulins could be one of the factors associated with older age at diagnosis regardless of the severity of clinical phenotypes. Thus, significant levels of serum immunoglobulins in XLA patients do not necessarily mean less severe phenotype. More detailed criteria for classifying severe and less severe phenotype might be necessary to discuss genotype–phenotype correlations.

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