

Summary

Tyrosine kinase 2 (Tyk2) is a nonreceptor tyrosine kinase that belongs to the Janus kinase (Jak) family. Here we identified a homozygous Tyk2 mutation in a patient who had been clinically diagnosed with hyper-IgE syndrome. This patient showed unusual susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. The patient's cells displayed defects in multiple cytokine signaling pathways including those for type I interferon (IFN), interleukin (IL)-6, IL-10, IL-12, and IL-23. The cytokine signals were successfully restored by transducing the intact Tyk2 gene. Thus, the Tyk2 deficiency is likely to account for the patient's complex clinical manifestations, including the phenotype of impaired T helper 1 (Th1) differentiation and accelerated Th2 differentiation. This study identifies human Tyk2 deficiency and demonstrates that Tyk2 plays obligatory roles in multiple cytokine signals involved in innate and acquired immunity of humans, which differs substantially from Tyk2 function in mice.

Introduction

The majority of helical bundled cytokines transduce their signals via the Janus kinase (Jak) family kinases that are constitutively associated with cytokine receptors. Four mammalian Jak proteins have been described to date: Jak1, Jak2, Jak3, and Tyk2 (Ihle, 1995; Liu et al., 1998; O'Shea et al., 2002). When cytokines bind to their corresponding receptors expressed on the cell surface, receptor-associated Jaks are activated, and they in turn phosphorylate both cytokine receptors and neighboring Jaks. Phosphorylation of specific tyrosine motifs in the cytokine receptors provides docking sites for signal transducers and activators of transcription (STAT), and STATs recruited to the receptor are phosphorylated by the Jaks. Upon phosphorylation, STATs dimerize via their Src homology domain-2 (SH2) domains and translocate to the nucleus, where they activate different genes. Recent studies have demonstrated that several inhibitory mechanisms are involved in the regulation of the Jak-STAT pathway to prevent it from overactivation, including SOCS (suppressor of cytokine signaling) proteins that are induced through the Jak-STAT pathway to make a negative feedback loop (Alexander and Hilton, 2004).

The functional importance of the Jaks was first investigated in studies that used cell lines defective in cytokine signaling and was subsequently confirmed by the establishment of Jak-deficient mice (Velazquez et al., 1992; Muller et al., 1993; Neubauer et al., 1998; Nosaka et al., 1995; Parganas et al., 1998; Park et al., 1995; Rodig et al., 1998). *Jak1*^{-/-} mice die perinatally and exhibit profound defects in lymphoid development (Rodig et al., 1998). Analyses of cells from *Jak1*^{-/-} mice demonstrated that Jak1 is essential for signaling by type I and II IFN, in agreement with prior studies that used human mutant cell lines. *Jak2*^{-/-} mice show embryonic lethality due to failure of erythropoiesis, while *Jak3*^{-/-} mice suffer from severe combined immunodeficiency in accord

with observations for human Jak3 deficiency (Macchi et al., 1995; Russell et al., 1995). These findings clearly demonstrated the nonredundant roles of Jak1, Jak2, and Jak3 in signaling through the corresponding cytokine receptors.

Tyk2 is the first member of the Jak kinase family to be isolated and was originally described as essential for type I IFN signaling in a human fibroblast cell line (Firmbach-Kraft et al., 1990; Velazquez et al., 1992). However, the establishment of Tyk2-deficient mice revealed that Tyk2 was not absolutely essential for type I IFN signaling in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*^{-/-} mice displayed a lack of responsiveness to a small amount of IFN α , but a high concentration of IFN α could fully transduce its signals even in the absence of Tyk2. Although Tyk2 is a component of several other cytokine signaling pathways, including those of IL-6 and IL-12 (Bacon et al., 1995; Ihle, 1995; Stahl et al., 1994), in *Tyk2*^{-/-} mice the IL-6-mediated signals are essentially normal and IL-12 signaling is partially impaired. Thus, the function of Tyk2 in mice appears to be compensated for in vivo, partly by other kinase(s), most likely other Jak(s). Whether the discrepancy in the stringency of Tyk2 requirement in cytokine signaling between the human cell lines and the mutant mice can be attributed to the difference between established cell lines and primary cells or the species difference as observed in several primary immunodeficiencies such as the Btk, BLNK, or λ 5 deficiency has been unclear (Conley et al., 2000; Minegishi et al., 1998, 1999b).

In the present study, we investigated immunological abnormalities in a patient who had been clinically diagnosed with hyper-IgE syndrome (HIES). HIES is a unique primary immunodeficiency, characterized by recurrent skin abscesses, pneumonia, and highly elevated serum IgE (Grimbacher et al., 2005). Its etiology is totally unknown even though the autosomal dominant or recessive inheritance has been reported (Grimbacher et al., 1999a; Renner et al., 2004). The patient investigated in this study showed susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. Peripheral blood cells from the patient showed almost complete defects in both IL-12 and IFN α/β signaling, prompting us to identify a homozygous mutation of Tyk2 in this patient, a signaling component shared by both cytokines. The patient's cells also displayed severe defects in IL-6 and IL-10 signaling, in contrast to previous observations in Tyk2-deficient mice. Transduction of the intact Tyk2 gene rescued the patient's cells from the cytokine signaling defects. Thus, the present study identified human Tyk2 deficiency as a type of primary immunodeficiency displaying the phenotype of the autosomal recessive HIES accompanied by susceptibility to intracellular bacterial infection and highlighted a unique and indispensable role played by Tyk2 in the innate and acquired immune responses in humans.

Results

Both IL-12 and IFN α Signaling Pathways Are Defective in T Cells from a Patient with HIES

We investigated, from an immunological point of view, a 22-year-old Japanese male who had been clinically

diagnosed as HIES. According to the HIES scoring system developed by the NIH group (Grimbacher et al., 1999b), the patient scored 48 points, similar to those with typical HIES (see Experimental Procedures). He showed complex clinical manifestations, including susceptibility to various types of microorganisms (virus, intracellular and extracellular bacteria, and fungi), atopic dermatitis-like skin inflammation starting from 1 month of age, and a high amount of serum IgE (2100 IU/ml). He has suffered recurrently from otitis media, sinusitis, pneumonias, skin abscesses, oral candidiasis, molluscum contagiosum, and herpes simplex infection in skin and mucosa. He also experienced an episode of Bacille Calmette-Guerin (BCG) infection at 22 months and nontyphi salmonella gastroenteritis at 15 years of age. The numbers of T, B, and NK cells in the peripheral blood were all within the normal range, and neutrophils showed normal functions in laboratory tests (data not shown). The patient's parents are consanguineous, indicating a possible recessive hereditary disorder. Because the patient experienced an infection with BCG, we first analyzed the genes encoding IL-12 p40, IL-12R β 1, IFNGR1, IFNGR2, and STAT1, which have been identified as responsible genes for primary immunodeficiency with susceptibility to mycobacterial infection (Casanova and Abel, 2004). No mutation was detected in any of the corresponding cDNAs isolated from the patient (data not shown).

We next examined whether the patient had any defect in IL-12 signaling. When peripheral blood mononuclear cells (PBMCs) from the patient were stimulated with mitogens, IL-12 production was normal (data not shown). On the other hand, CD4⁺ T cells isolated from the patient's PBMCs failed to produce detectable amounts of IFN γ when stimulated with IL-12 plus IL-18, a potent IFN γ inducer for T cells (Figure 1A). The failure could not be attributed to a total defect in the machinery for IFN γ production, because the patient's T cells produced considerable amounts of IFN γ in response to a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin, although the amount was a bit lower than normal (Figure 1A). Furthermore, IL-18 signaling appeared to be intact in the patient's T cells, as assessed by I κ B α degradation in response to IL-18 stimulation (Figure 1B), implying a defect in the IL-12 signal. Indeed, the patient's T cells showed almost no response to IL-12 in contrast to those from a normal subject, as judged by the tyrosine phosphorylation of STAT4, a component of the IL-12 signaling pathway (Figure 1C). Interestingly, the patient's T cells also failed to respond to IFN α (Figure 1C). This finding was in sharp contrast to human IL-12R β 1 deficiency, the only known human defect in IL-12 signaling, in which STAT4 phosphorylation is defective in response to IL-12, but its phosphorylation is intact in response to IFN α (Fieschi et al., 2004).

A Homozygous Mutation in the Patient's Tyk2 Gene

The defect in both IL-12 and IFN α signaling strongly suggested an abnormality in one or more molecules involved in a signaling pathway shared by both cytokines. STAT4 was a good candidate; however, STAT4 was expressed at a normal amount in the patient's T cells (Figure 1C), and no sequence alteration was found in the coding region of the STAT4 gene (data not shown).

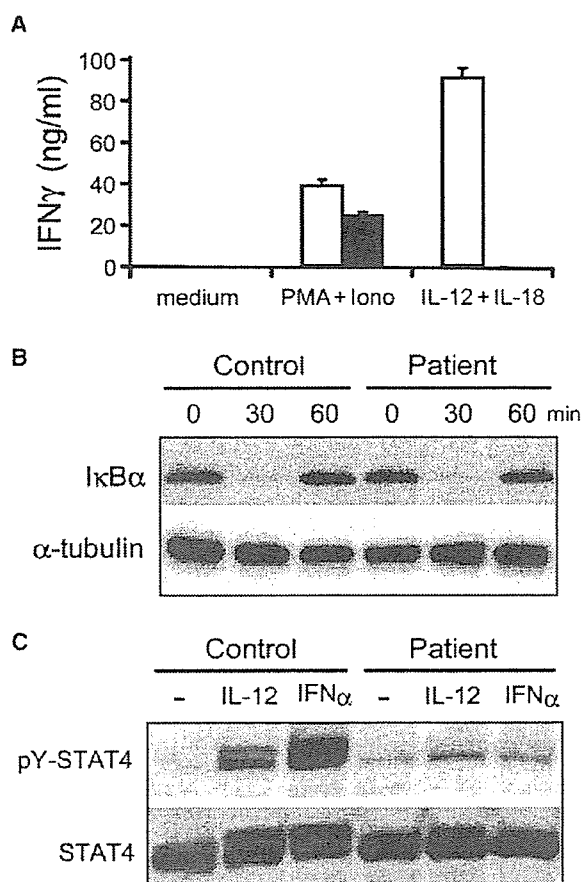


Figure 1. Impaired Responses to IL-12 and IFN α in a Patient with HIES

(A) The patient's T cells were defective in IFN γ production in response to IL-12 and IL-18. The concentration of IFN γ in culture supernatants was measured after CD4⁺ T cells from a healthy control and the patient were cultured for 24 hr with medium alone, a combination of PMA and ionomycin, or a combination of IL-12 and IL-18. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(B) The patient's T cells had intact IL-18 signaling. I κ B α and α -tubulin proteins were detected by immunoblotting at the indicated time points after the T cells from a control subject and the patient were stimulated with IL-18.

(C) The patient's T cells had defects in both IL-12 and IFN α signaling. Tyrosine 694-phosphorylated and total STAT4 proteins were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with IL-12 or IFN α for 15 min. Results are representative of at least three independent experiments.

The second candidate we investigated was Tyk2, because a mutant human cell line unresponsive to IFN α turned out to be deficient in Tyk2 (Velazquez et al., 1992), even though Tyk2 appeared to be not absolutely essential for IL-12 and IFN α signals in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Sequencing of the patient's cDNA and genomic DNA coding for Tyk2 revealed a homozygous deletion of four nucleotides, GCTT, at nt 550–553, that was not found in 50 unrelated healthy individuals (Figures 2A and 2B). This deletion resulted in a frame-shift mutation from codon 70 to 89 and generated a premature stop codon at aa 90 in the 1187 amino acid-long Tyk2 protein (Figure 2B). The predicted truncation of Tyk2 deletes most of the protein, including

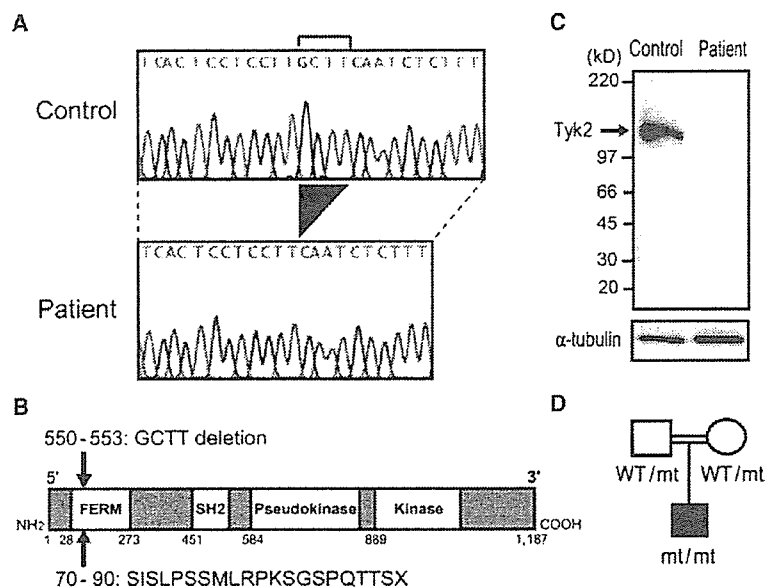


Figure 2. A Homozygous Mutation of the Tyk2 Gene in the Patient

(A) Electropherograms show the part of the Tyk2 cDNA sequences from a control subject and the patient, from which a deletion of four nucleotides, GCTT, was found in the patient. (B) The structure of Tyk2 is shown schematically, and the mutation is indicated: the 4-nucleotide deletion (above) and the frame-shift and truncation of the protein (below). (C) The Tyk2 protein was undetectable in the patient's T cells. Tyk2 and α -tubulin proteins in the T cells from a healthy control subject and the patient were detected by immunoblotting. (D) The patient was homozygous for the mutation, and both parents were heterozygous for it. WT, wild-type Tyk2; mt, mutated Tyk2.

the FERM domain, the SH2 domain, and the pseudo-kinase and kinase domains. Indeed, no Tyk2 protein was detected by immunoblotting with two specific antibodies that recognize different regions of Tyk2 (Figure 2C). Both parents of the patient were apparently healthy and showed normal amounts of serum IgE, but the analysis of genomic DNAs from the parents revealed that both were heterozygous for the same deletion (Figure 2D), establishing that the patient had an autosomal recessive Tyk2 deficiency.

Tyk2 Is Indispensable for Type I IFN Signaling in Humans

Stimulation of the patient's T cells with various concentrations of IFN α or IFN β up to 50 ng/ml (equivalent to 10,000 IU/ml) failed to induce tyrosine phosphorylation of not only STAT4 (Figure 1C) but also Jak1, STAT1, STAT2, and STAT3 (Figure 3A and see Figure S1 in the Supplemental Data available online), in contrast to the observation in *Tyk2*^{-/-} mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Furthermore, no tyrosine phosphorylation of STAT1 and STAT2 was detected at any time points (5, 15, 30, and 60 min) after the treatment with IFNs (Figure S2). Interestingly, the amount of STAT1 protein was reduced in the patient's T cells in accord with the observations in *Tyk2*-deficient mice (Karaghiosoff et al., 2000), whereas those of STAT2, STAT3, and STAT4 proteins appeared unaffected (Figures 1C and 3A, and Figure S3). The patient's T cells displayed a reduced amount of surface IFNAR1 expression (Figure S4). This is consistent with previous observations in a *Tyk2*-deficient human cell line but in contrast with that in the *Tyk2*-deficient mice (Gauzzi et al., 1997; Karaghiosoff et al., 2000; Shimoda et al., 2000). Tyk2 has been shown to stabilize IFNAR1 at the cell surface in human cells (Gauzzi et al., 1997; Ragimbeau et al., 2003). Thus, the defect in the type I IFN signaling in the patient's cells could be explained by the absence of Tyk2 accompanied with reduced amounts of STAT1 and IFNAR1.

Consistent with the signaling defects, the transcriptional upregulation of a panel of IFN-inducible genes

including NMI and MxA, in response to IFN α or IFN β , was completely abrogated in PBMCs from the patient (Figure 3B and Figure S5). We further investigated biological consequences of the compromised IFN signaling. The upregulation of HLA class I expression in response to IFN α was not observed in patient PBMCs (Figure 3C). Unexpectedly, the patient's cells displayed a higher basal amount of HLA class I expression than the control cells, suggesting a possible compensatory mechanism to regulate expression in a type I IFN-independent manner under conditions of the *Tyk2* deficiency. Even at high concentrations, neither IFN α nor IFN β produced any antiviral protection against herpes simplex virus (HSV) within the patient B cells (Figures 3D and 3E). Thus, human *Tyk2* deficiency showed a complete defect in type I IFN responses under our experimental conditions, in contrast to observations in *Tyk2*^{-/-} mice (Karaghiosoff et al., 2000; Shimoda et al., 2000).

We next examined the response to IFN γ in the patient (Figure 3F). Stimulation with IFN γ upregulated the transcription of two IFN γ -responsive genes, IRF1 and RING4, in PBMCs from the patient and from a healthy control. However, the extent of the induction was much smaller in the patient's PBMC sample, in accord with the impairment of IFN γ signaling reported in *Tyk2*^{-/-} mice (Karaghiosoff et al., 2000).

Tyk2 Is Critical for IL-23, IL-6, and IL-10 Signaling in Humans

We next examined the response to IL-23 in the patient's cells, because IL-23 shares signaling pathways with IL-12 (Watford et al., 2004). The patient's T cells showed no detectable tyrosine phosphorylation of STAT4 in response to IL-23 even at high concentrations (500 ng/ml) in contrast to T cells from a control subject (Figure 4A). Control T cells secreted IFN γ in response to IL-23 in a dose-dependent manner (Figure 4B) as reported previously (Oppmann et al., 2000). In contrast, the patient's T cells showed no response to IL-23 in IFN γ secretion even though the background amount of

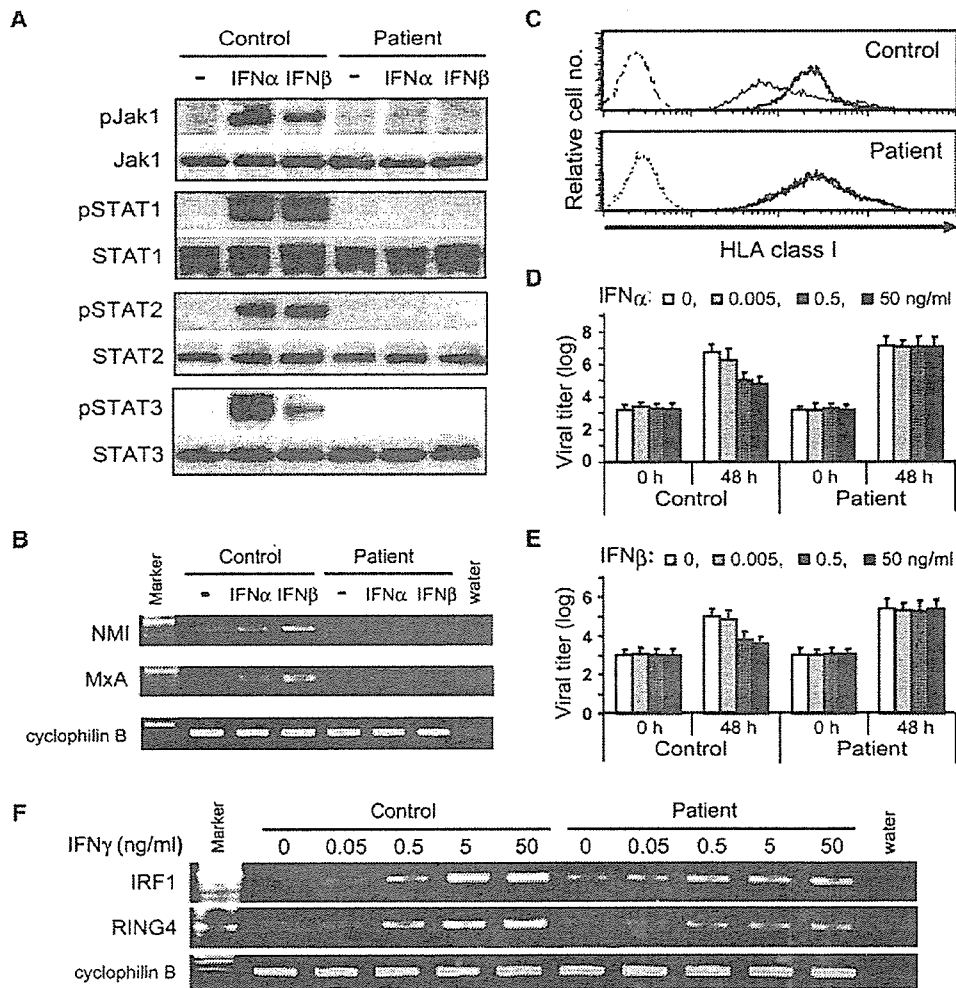


Figure 3. Defects in the Type I and II IFN Signals in the Patient's T Cells

(A) No detectable phosphorylation of the Jak and STAT molecules in the patient's T cells was seen upon stimulation with type I IFN. The indicated proteins (p, phosphorylated form) were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with or without 50 ng/ml (equivalent to 10,000 IU/ml) of IFN α or IFN β for 15 min.

(B) There was no detectable upregulation of the IFN-inducible gene expression in the patient's cells upon stimulation with type I IFN. The amounts of NMI, MxA, and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control subject and the patient were stimulated or not with IFN α or IFN β for 2 hr.

(C) No detectable upregulation of HLA class I in the patient's cells upon stimulation with type I IFN. The level of HLA class I on PBMCs from a control subject and the patient was determined with flow cytometry after the cells were cultured with (histograms with thick gray lines) or without (histograms with thin black lines) IFN α for 48 hr. Histograms with dotted lines show the control staining with an isotype-matched mAb.

(D and E) No antiviral activity was detected in the patient's B cells upon stimulation with type I IFN. EBV-transformed B cells from a control subject and the patient were treated for 24 hr with indicated concentrations of IFN α (D) or IFN β (E) prior to the infection with HSV. The viral titers in culture supernatant of 0 and 48 hr culture were measured by plaque assay with Vero cells. Data are representative of two independent experiments. Error bars are standard deviations.

(F) Impaired response to IFN γ in the patient's cells. The amounts of IRF1, RING4, and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR, after PBMCs from a control subject and the patient were stimulated with the indicated concentration of IFN γ for 2 hr.

IFN γ was slightly higher in the patient's T cells than in the control T cells (Figure 4B). These results indicated that human Tyk2 played an essential role in IL-23 signaling, in accord with observations in the *Tyk2*-deficient mice (Shaw et al., 2003).

It has been demonstrated that Tyk2 is also activated upon stimulation with IL-6 or IL-10 (Ihle, 1995; Stahl et al., 1994). PBMCs from the patient failed to upregulate SOCS3 transcripts in response to IL-6 stimulation (Figure 4C). They secreted IgM when stimulated by infection with Epstein-Barr Virus (EBV), as did those

from a healthy control subject. However, further stimulation with IL-6 induced only a little increase in IgM secretion from the patient's PBMCs, although IgM secretion did increase 3 times in the control PBMCs (Figure 4D). The patient's PBMCs had a very poor response to IL-10 stimulation in their induction of SOCS3 (Figure 4E). Moreover, the inhibition of LPS-induced TNF α production by IL-10 was severely impaired in macrophages from the patient (Figure 4F). These results indicated that Tyk2 plays an obligatory role in signaling of IL-6 and IL-10 in humans, in contrast to *Tyk2*^{-/-} mice, which

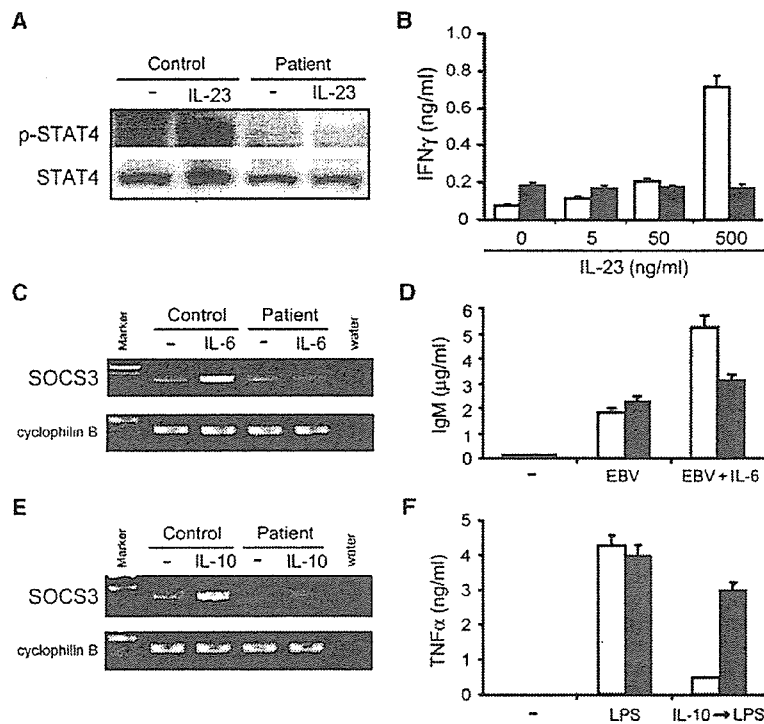


Figure 4. Defects in IL-23, IL-6, and IL-10 Signals in the Patient's Cells

(A) The patient's T cells showed impaired IL-23 signaling. Tyrosine phosphorylated and total STAT4 proteins were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with IL-23 (500 ng/ml) for 15 min.

(B) The patient's T cells showed no substantial response to IL-23 in the IFN γ production. The concentration of IFN γ in culture supernatants was determined by ELISA after the T cells from a control subject and the patient were cultured with the indicated concentrations of IL-23 for 48 hr. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(C) No detectable upregulation of the SOCS3 gene expression in the patient's cells upon stimulation with IL-6. The amounts of SOCS3 and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control subject and from the patient were stimulated with or without IL-6 for 4 hr. (D) Little enhancement of IgM secretion in the patient's B cells upon stimulation with IL-6. The concentration of IgM in the culture supernatants was determined by ELISA after PBMCs from a control subject and the patient were cultured for 10 days with or without Epstein-Barr virus (EBV), alone or in combination with IL-6. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(E) Poor upregulation of the SOCS3 gene expression in the patient's cells upon stimulation with IL-10. The amounts of SOCS3 and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control subject and the patient were cultured with or without IL-10 for 4 hr. (F) Poor inhibition of LPS-induced TNF α production by IL-10 in the patient's macrophages. The concentration of TNF α in the culture supernatants was determined by ELISA after macrophages derived from a control subject's and the patient's PBMCs were cultured with or without IL-10 for 24 hr, and then stimulated or not with LPS for 48 hr. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations. Results are representative of at least three independent experiments.

respond normally to IL-6 (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*^{-/-} mice were originally reported to respond normally to IL-10 (Karaghiosoff et al., 2000; Shimoda et al., 2000), while a recent study demonstrates partial impairment in IL-10 signaling (Shaw et al., 2006).

Imbalance in Th1 and Th2 Differentiation in the Patient

We next examined in vitro the Th1 and Th2 differentiation of T cells from the patient (Figure 5), because the patient displayed Th2-dominant phenotypes such as atopic dermatitis and highly elevated serum IgE. Even under Th1-biased culture conditions, the frequency of IFN γ -producing Th1 cells remained low in the patient's T cells (Figure 5A, middle). On the other hand, their differentiation into IL-4-producing Th2 cells under Th2-biased culture conditions appeared to be accelerated (Figure 5A, right). When cultured for 14 days in the presence of anti-CD3 and IL-2, the patient's T cells showed helper T cell differentiation that was extremely skewed toward Th2, compared with the control T cells (Figure 5B). Naive CD4⁺ T cells isolated from the patient's PBMCs showed reduced secretion of IFN γ and enhanced secretion of IL-5 and IL-13 as compared to the control T cells when stimulated with anti-CD3 and anti-CD28 (Figure 5C). These results clearly indicated that *Tyk2* is critical for the IL-12-induced Th1 differentiation and represses the development of Th2 cells, in

accord with the observations in mice (Seto et al., 2003; Shaw et al., 2003).

Expression of the Intact *Tyk2* Gene Rescues the Patient's T Cells from Defects in IL-12 and Type I IFN Signaling

The wild-type *Tyk2* gene in a retroviral vector was introduced into activated primary T cells from the patient. The infected T cells acquired the expression of *Tyk2* proteins at approximately 70% of the normal amount (Figure 6A). Upon combined stimulation with the IL-12 and IL-18, the infected *Tyk2*-expressing cells produced IFN γ , as did T cells from a normal control subject (Figure 6B). In contrast, the patient's T cells infected with virus carrying the empty vector did not produce IFN γ in response to this stimulation, nor did uninfected cells. Furthermore, *Tyk2*-infected T cells from the patient acquired the ability to upregulate the transcription of *MxA* in response to IFN α (Figure 6C). A complementary experiment was performed in which human HeLa cells were subjected to the knockdown of *Tyk2* gene expression with siRNAs. In cells treated with *Tyk2*-specific siRNAs, the tyrosine phosphorylation of STAT1 and STAT2 in response to IFN α was almost completely abrogated, concomitantly with the suppression of *Tyk2* expression, as observed in the patient's cells (Figure S6). These results indicated that the defects in the IL-12 and type I IFN signaling observed in the patient's T cells were caused primarily by the absence of functional *Tyk2*.

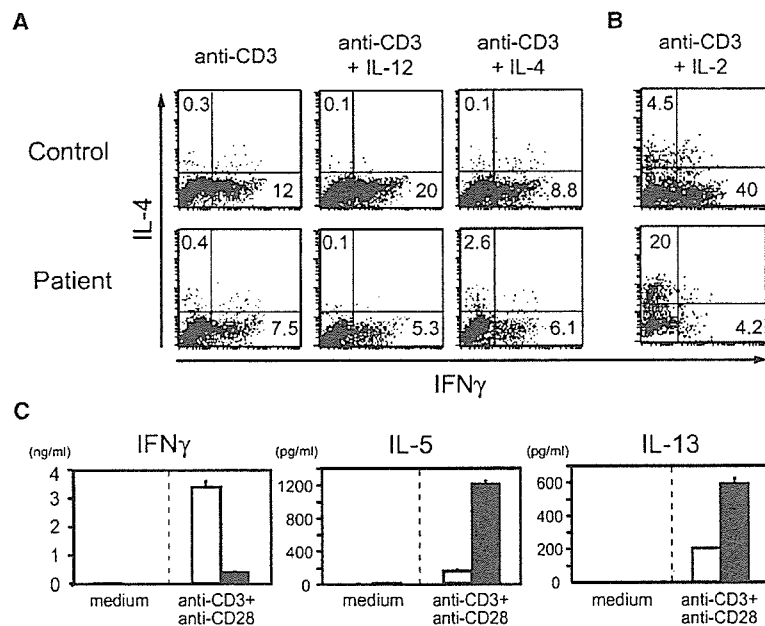


Figure 5. Impaired Th1 and Accelerated Th2 Differentiation in the Patient's T Cells

(A) Intracellular staining with anti-IFN γ and anti-IL-4 was performed after PBMCs from a control subject and the patient were stimulated with immobilized anti-CD3 alone, in combination with IL-12 and anti-IL-4 (Th1-promoting condition) or in combination with IL-4 (Th2-promoting condition) for 5 days. The percentage of IFN γ - or IL-4-producing cells is shown in each panel.

(B) PBMCs from a control subject and the patient were stimulated with immobilized anti-CD3 and IL-2 for 14 days, and intracellular staining was performed as in (A).

(C) Naive CD4⁺ T cells from a control subject and the patient were stimulated with anti-CD3 and anti-CD28 for 3 days, and the concentration of IFN γ , IL-5, and IL-13 in culture supernatants was measured by ELISA. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations. Results are representative of at least three independent experiments.

Discussion

We have identified a human deficiency in Tyk2. Among the four members of the Jak family, Jak3 deficiency was previously reported in human, demonstrating the crucial role of Jak3 in lymphocyte development, in accord with findings in Jak3-deficient mice (Macchi et al., 1995; Nosaka et al., 1995; Park et al., 1995; Russell et al., 1995). The identification of a human Tyk2 deficiency highlighted the obligatory roles of human Tyk2 in multiple cytokine signals involved in innate and acquired immune responses, including type I IFN, IL-6, IL-10, IL-12, and IL-23 signals. Although Tyk2 is ubiquitously expressed (unlike Jak3), the Tyk2 deficiency displayed apparently normal embryogenesis, postnatal growth, and hematopoiesis, while it resulted in a primary immunodeficiency with clinical manifestations characteristic to HIES, indicating a unique and indispensable role played by Tyk2 in the immune system.

The present study contrasted differences in the stringency of Tyk2 requirement between human and mice. The nonredundant roles of human Tyk2 in multiple cytokine signals *in vivo* could not be assumed from the studies on mouse Tyk2. The Tyk2^{-/-} mice responded normally to IL-6, even though IL-6 activated Tyk2 *in vitro*. The mice also showed a leaky phenotype in type I IFN responses: a high concentration of IFN α showed antiviral effects and upregulated MHC class I expression in cells from the Tyk2^{-/-} mice to an extent comparable to that observed in wild-type mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). This was a sharp contrast to the observation in human Tyk2-deficient fibroblast cell lines. Our results indicated that the discrepancy between these two observations was due to a species difference rather than the difference between primary cells and particular cultured cell lines. We observed no detectable tyrosine phosphorylation of Jak1 in response to type I IFN in the human Tyk2 deficiency, supporting the idea that Tyk2 and Jak1 transphosphorylate each

other, and hence both are independently required for the initial activation of IFN signaling. In contrast, in the mouse Tyk2 deficiency, the Jak1 phosphorylation was observed when stimulated with type I IFN, albeit to a lesser extent than in wild-type mice. Thus, Tyk2 is the obligatory requirement for the initiation of type I IFN signaling in human whereas it is redundant in the initiation and might function as an amplifier of signaling in mice. This also appears to be the case in the IL-12 signaling. Tyk2^{-/-} mice showed compromised signaling and biological effects in response to IL-12 (Karaghiosoff et al., 2000; Seto et al., 2003; Shaw et al., 2003; Shimoda et al., 2000), but the impairment was less drastic than that observed in IL-12 receptor-deficient mice (Wu et al., 1997).

The *in vitro* findings of defects in multiple cytokine signals due to the Tyk2 deficiency appeared to account for the complex clinical manifestations of the patient investigated in the present study. First, the susceptibility to viral infection could be attributed to the defect in IFN signaling. Indeed, even a high titer of type I IFNs did not show any protective effect against herpes simplex virus in the patient's cells, unlike in Tyk2^{-/-} mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Interestingly, the response to type II IFN (IFN γ) stimulation was also impaired in the patient's T cells, albeit to a lesser extent, even though Tyk2 has not been assigned to the type II IFN signaling pathway. The partial impairment of IFN γ signaling could be explained, at least in part, by the reduced amounts of STAT1 protein or by the observation that IFN γ signaling depends on constitutive subthreshold type I IFN signaling (Takaoka et al., 2000). Thus, the type II IFN response is reduced but not absent in the Tyk2 deficiency in contrast to its complete lack in the STAT1 deficiency (Dupuis et al., 2003), even though the type I IFN response is absent in both deficiencies. This could account for the apparently milder phenotype of the Tyk2 deficiency in viral infections, compared to the lethal phenotype of the STAT1 deficiency. It remains

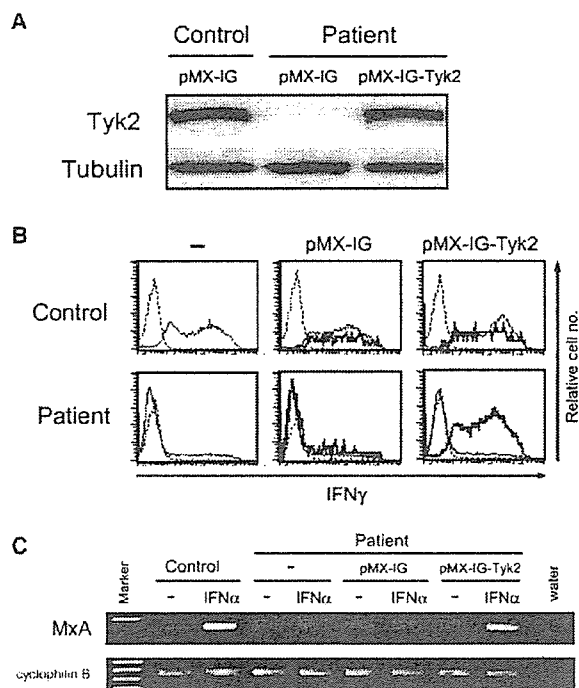


Figure 6. Transduction of the Intact Tyk2 Gene Rescued the Patient's T Cells from Defects in the IL-12 and Type I IFN Signals

(A) Reconstitution of Tyk2 in the patient's T cells. T cells from the patient and a control subject were infected with either the empty retroviral vector (pMX-IG) or the Tyk2 cDNA-containing vector (pMX-IG-Tyk2). The protein expression of Tyk2 and tubulin in GFP⁺-infected cells were detected by immunoblotting.

(B) Retrovirus-mediated Tyk2 transfer restored the IFN γ production in response to IL-12 and IL-18 in the patient's T cells. The production of IFN γ was detected by intracellular staining with an IFN γ mAb in uninfected and infected T cells after stimulation with IL-12 and IL-18 for 48 hr. Histograms with thick gray lines show the staining profile of GFP⁺-infected cells, and those with thin black lines show the staining profile of GFP⁻ uninfected cells. Histograms with dotted lines show the control staining with an isotype-matched mAb.

(C) Retrovirus-mediated Tyk2 transfer restored the upregulation of IFN-inducible gene expression in response to IFN α in the patient's T cells. The amounts of MxA and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after stimulation of the indicated T cells with IFN α for 2 hr. Results are representative of at least three independent experiments.

to be determined whether the type III IFN (IL-28 and IL-29) signaling is defective in the Tyk2 deficiency.

Second, the susceptibility of the patient to infection with intracellular bacteria, such as mycobacteria and salmonella, could be explained by the defect in IL-12 signaling, as seen in IL-12R β 1 deficiency (Altare et al., 1998; de Jong et al., 1998). The differentiation into Th1 cells, which are important for the elimination of these bacteria, was severely impaired in the patient's T cells, even under culture conditions that strongly promote the differentiation of Th1 cells. Given that type I IFNs promote Th1 differentiation in humans and not in mice, the defect in the IFN signal might also account in part for the failure of Th1 differentiation in this patient. The susceptibility of Tyk2 knockout mice to infection of intracellular bacterial has not been reported, but a natural mutant strain B10.Q/J, which is susceptible to toxoplasma infection and resistant to induction of autoimmune arthritis, was reported to carry Tyk2 mutation

(Shaw et al., 2003). Mice deficient for IL-23 showed the susceptibility to Klebsiella pneumonia owing to their impairment in IL-17 production by T cells (Happel et al., 2005). Therefore, the patient's susceptibility to extracellular bacteria might be explained at least in part by the defect in IL-23 signaling.

Third, the high amount of serum IgE and the atopic dermatitis-like skin inflammation in the patient could be due to the accelerated Th2 differentiation. Indeed, the Tyk2 deficiency was shown to accelerate Th2-mediated allergic airway inflammation in mice (Seto et al., 2003). In a previous report, enhanced Th2 differentiation was not observed in an IL-12R β 1 deficiency, although the Th1 differentiation was severely impaired (Wood et al., 2005). Thus, the defect in IL-12 signaling does not account for the abnormally accelerated Th2 differentiation in the Tyk2-deficient patient. PBMCs and macrophages from the patient showed a severe signaling defect in the IL-10 pathway. IL-10 has been reported to suppress the differentiation of both Th1 and Th2 cells, so the defect in the IL-10 pathway might explain, at least in part, the accelerated Th2 differentiation under conditions in which Th1 differentiation was defective as observed in mice deficient for both IL-10 and IL-12 (Hoffmann et al., 2000).

The patient investigated in the present study had been clinically diagnosed as HIES, fulfilling the criteria for the diagnosis according to the NIH scoring system (Grimbacher et al., 1999b). HIES is a primary immunodeficiency with unknown etiology, characterized typically by high serum IgE amounts, recurrent staphylococcal skin abscesses, and pneumonia with pneumatocele formation (Grimbacher et al., 2005). While most cases of HIES are sporadic, families displaying autosomal dominant (AD) or autosomal recessive (AR) inheritance have been reported (Grimbacher et al., 1999a; Renner et al., 2004). In most sporadic and AD cases, these clinical manifestations are part of a multisystem disorder including abnormalities of the soft tissue, skeletal, and dental systems (Grimbacher et al., 1999a). In contrast, patients with AR-HIES lack skeletal and dental involvement and lung cyst formation and suffer from fungal and viral infections such as herpes simplex and molluscum contagiosum (Renner et al., 2004). According to this classification, the present case could be categorized into the AR-HIES. It is notable that the infection with intracellular bacteria experienced by the present case has not been frequently observed in HIES patients, even though three cases with mycobacterial infection were reported in the literatures (Grimbacher et al., 1999a; Metin et al., 2004; Pasic et al., 1998). Therefore, we would like to propose that the present case represents a subset of the AR-HIES. The whole picture of HIES including its etiology has not yet drawn clearly, so further genetic analyses of HIES is needed to evaluate this proposal and define the Tyk2 deficiency as either a variant of HIES or a distinct disease entity. In either case, the present study highlighted a requisite and nonredundant role played by Tyk2 in the innate and acquired immune responses in human.

Experimental Procedures

Patient

This study was approved by the institutional review board at the Tokyo Medical and Dental University. Written informed consent

was obtained from all the subjects studied. The patient investigated in this study is a 22-year-old Japanese male with a history of infantile atopic dermatitis starting from 1 month of age and recurrent infections, such as otitis media, sinusitis, pneumonias, and skin abscesses, starting from 12 months of age. At 22 months, he developed BCG lymphadenitis. Histological examination revealed paucibacillary, well-circumscribed granulomas with epithelioid and giant multinucleated cells, as observed in the human IL-12R β 1 deficiency (Altare et al., 1998; de Jong et al., 1998). The immunological work-up performed at this occasion revealed mild eosinophilia (700–800/mm³) and high serum IgE (2100 IU/ml) with normal amounts of other Ig classes and subclasses. All other laboratory data examined were within the normal range, including complement components, the oxidative burst of granulocytes, the number and the size of platelets, lymphocyte subpopulations, and their proliferative responses to mitogens. The patient's illness was tentatively diagnosed as HIES though he did not show any apparent skeletal abnormality. Thereafter, in addition to infections with bacteria including *Staphylococcus aureus*, he recurrently suffered from viral and fungal infections, such as molluscum contagiosum, herpes simplex infection of skin and mucosa, and oral candidiasis. At 15 years of age, the patient developed severe non-typhi salmonella gastroenteritis leading to sepsis. According to the HIES scoring system (Grimbacher et al., 1999b), the patient gained 48 points; 10 in IgE titers, 8 in skin abscess, 8 in pneumonia, 3 in eosinophil count, 4 in newborn rash, 4 in eczema, 2 in upper respiratory infection, 1 in oral candidiasis, 4 in other serious infections, and 4 in fatal infection.

Antibodies and Cytokines

The mAbs for the N-terminal region (aa 46–258) of human Tyk2, IL-12R β 1, IL-12R β 2, I κ B α , IL-4, and IFN γ were purchased from BD Pharmingen, and the CD3 mAb (OKT3) was from Janssen Pharmaceutical. The Ab for the C-terminal region of Tyk2 (C-8) was from Santa Cruz. Rabbit antibodies for Jak1, STAT1, STAT2, STAT3, and STAT4 and their phosphorylated forms were purchased from Cell Signaling. Anti-IFNAR1 was from R&D Systems. HRP-conjugated rabbit anti-mouse and goat anti-rabbit antibodies were from Calbiochem, and mouse anti- α -tubulin was from Sigma-Aldrich. Recombinant human IL-4, IL-12, IFN α , IFN β , IFN γ , and GM-CSF were purchased from Peprotech, recombinant human IL-2 was from Shionogi, and IL-18 was from MBL.

Isolation and Culture of T Cells and Macrophages from PBMCs

Heparinized blood samples were obtained from the patient and from healthy age-matched control subjects. PBMCs were isolated from blood samples through density gradient centrifugation via Histopaque-1077 (Sigma-Aldrich). For T cell activation, PBMCs were cultured with 5 μ g/ml plate-bound CD3 mAb and 300 U/ml IL-2 in RPMI 1640 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 50 μ M 2-mercaptoethanol (Life Technologies), 2% L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1% penicillin-streptomycin. CD4⁺ T cells were enriched with immunomagnetic beads (BD Pharmingen) with more than 98% purity. After 14 days of culture, more than 98% of the cells from both the patient and control subjects were effector memory T cells (CD45RO⁺CGR7⁻). Naive CD4⁺ T cells were purified by removing CD45RO⁺ cells from CD4⁺ T cells by immunomagnetic beads (BD Pharmingen) with more than 90% purity. To obtain macrophages, PBMCs were cultured for 2 hr, and the adherent cells were further cultured with 10 ng/ml GM-CSF for 7 days as described (Hart et al., 1995). All the experiments were performed at least three times with three different controls.

Construction of Retroviral Vectors and Infection of Cells

The retroviral vector pMX-IRES-GFP-Tyk2 was constructed by inserting the Tyk2 cDNA into the BamHI and NotI sites of the pMX-IRES-GFP vector (a gift from T. Kitamura) (Nosaka et al., 1999). Infectious retroviral particles were generated with the Plat-GP cell line (a gift from T. Kitamura and H. Miyoshi). Retroviral supernatant stocks were produced by culturing the producer cells at 37°C for 72 hr. For the infection, PBMCs were stimulated in culture with 5 μ g/ml plate-bound CD3 mAb and 300 IU/ml IL-2. Cells (1 \times 10⁶/ml) were then spin-infected with the retroviral supernatant at 32°C for 30 min. The transduction procedure was repeated on days 2 and 3. The

efficiency of infection was approximately 3% as judged by the frequency of GFP⁺ cells.

Tyk2 Knockdown

RNA interference for Tyk2 was designed with the aid of the online software from Invitrogen. The sequences of siRNA sense strands (5' \rightarrow 3') were as follows: Tyk2 siRNA1, CCCAGAGAUGCAAGCCU GAUGCUAU, and Tyk2 siRNA2, CCAUUCUGAAGACAGUCCAUGA GAA. HeLa cells were grown in DMEM supplemented with 10% FBS. Transfection of the cells with siRNAs was performed with Lipofectamine-RNAiMAX reagent (Invitrogen). 48 hr after transfection, the cells were treated with IFN α (50 ng/ml) for 15 min before lysis. Lysates were analyzed by immunoblotting with the antibodies indicated.

RT-PCR and Direct Sequence Analysis

Extraction of total RNA, cDNA synthesis, PCR, and semiquantitative RT-PCR were performed as previously described (Minegishi et al., 1999a). PCR primers were designed as follows: Tyk2, sense, 5'-TTGCTTGAGTTGACACAGGGAGCT-3', antisense, 5'-TCTCTAGAC AGGAGTAAGGCACAC-3'; STAT4, sense, 5'-CTGGGACCTGTGCTG AGAGAGC-3', antisense, 5'-ACTTTTTTCATTTGCTTCCTT-3'; NMI, sense, 5'-TTAAGGAGCATTCGCCAGAT-3', antisense, 5'-TTCGAGC TCACTTGAAACGA-3'; MxA, sense, 5'-GAGGTGCAGGAGAACAG CTC-3', antisense 5'-CTCCTCATACTGGCTGCACA-3'; cyclophilin B, sense, 5'-GCCCAAAGTCACCGTCAAGG-3', antisense, 5'-GGAA GCGCTCACCGTAGATG-3'; IRF1, sense, 5'-GCTGGGACATCAACA AGGAT-3', antisense, 5'-GTGGAAGCATCCGGTACACT-3'; RING4, sense, 5'-AGGGCTGGCTGGCTGCTTGA-3', antisense, 5'-ACGTG GCCCATGGTGTGTTAT-3'; and SOCS3, sense, 5'-TTCAGCTCCA AGAGCGAGTA-3', antisense, 5'-CGGAGTAGATGTAATATGGCTC-3'. Sequencing was performed with an ABI Prism dRhodamine Terminator kit and analyzed with an ABI Prism 310 DNA Sequencer (Perkin-Elmer Applied Biosystems).

Stimulation of Cells with Cytokines and the Measurement of Cytokine and Ig Production

Cells were stimulated for the indicated time in culture with PMA (40 nM), ionomycin (0.5 μ g/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), IFN α (50 ng/ml), IFN β (50 ng/ml), IL-6 (10 ng/ml), or IL-10 (100 ng/ml). The concentration of IFN γ in the culture supernatants was determined by ELISA (BD-PharMingen) according to the manufacturer's instructions, after the cells (1 \times 10⁶/ml) were cultured in 96-well plates at 37°C for 24 hr with or without stimulation. The concentration of TNF α in the culture supernatants was determined by ELISA (BD-PharMingen), after the cells (1 \times 10⁵/well) were cultured in 96-well plates at 37°C for 48 hr with or without stimulation. The measurement of IgM secretion from Epstein-Barr virus-infected B cells was determined by ELISA as previously described (Minegishi and Conley, 2001). To evaluate helper T cell differentiation in vitro, PBMCs were cultured for 5 days with immobilized anti-CD3 alone or in combination with IL-12 (10 ng/ml) and anti-IL-4 (10 μ g/ml) to promote Th1 differentiation, or in combination with IL-4 (20 ng/ml) to promote Th2 differentiation, or the PBMCs were cultured for 14 days with immobilized anti-CD3 and IL-2 (300 IU/ml). Alternatively, naive CD4⁺ T cells were cultured with immobilized anti-CD3 and soluble anti-CD28 for 3 days. The concentration of IFN γ , IL-5, and IL-13 in the culture supernatants was determined by ELISA (BD Pharmingen).

Flow Cytometric Analysis

The surface immunophenotype was analyzed as described (Minegishi et al., 1999a). For cytokine intracellular staining, cells were restimulated with PMA and ionomycin in the presence of 2 μ M of monensin for 5 hr, followed by fixation and cytoplasmic staining with FITC- or PE-conjugated mAbs specific for the indicated cytokines. An irrelevant FITC- or PE-conjugated IgG1 mAb was used as the negative control.

Immunoblotting and Immunoprecipitation

Cells were washed and cultured for 18 hr in starvation medium consisting of RPMI 1640 and 1% FBS. Cells were then stimulated at 37°C for 15 min with medium alone, 10 ng/ml IL-12, 50 ng/ml IL-18, 50 ng/ml IFN α , or 50 ng/ml IFN β . After the stimulation, the cells

were lysed on ice for 30 min in lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 2 μ g/ml aprotinin, 100 μ g/ml PMSF, 1 mM sodium orthovanadate, and 1 mM NaF. The cell lysates were subjected to SDS-PAGE, followed by electrotransfer to PVDF membranes and immunoblotting with the indicated antibodies.

Antiviral Activity Assay

EBV-transformed B cells were pretreated with or without 0.005, 0.5, 50 ng/ml IFN α or IFN β for 24 hr, and then infected with HSV (10^3 plaque-forming units/ml). The viral titers in culture supernatants were determined at the indicated time points by plaque assay with Vero cells.

Supplemental Data

Six Supplemental Figures can be found with this article online at <http://www.immunity.com/cgi/content/full/25/5/745/DC1/>.

Acknowledgments

We appreciate the willingness of the patient and the parents to participate in this research study. This work is supported by Grants-in-Aid 16616004, 17047013, and 18659299 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology and Grants-in-Aid 2212131 and 2211932 from the Japanese Ministry of Health, Labor, and Welfare.

Received: May 3, 2006

Revised: June 27, 2006

Accepted: September 19, 2006

Published online: November 9, 2006

References

- Alexander, W.S., and Hilton, D.J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu. Rev. Immunol.* **22**, 503–529.
- Altare, F., Durandy, A., Lammas, D., Emile, J.F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Doffinger, R., Bernaudin, F., et al. (1998). Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* **280**, 1432–1435.
- Bacon, C.M., McVicar, D.W., Ortaldo, J.R., Rees, R.C., O'Shea, J.J., and Johnston, J.A. (1995). Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J. Exp. Med.* **181**, 399–404.
- Casanova, J.L., and Abel, L. (2004). The human model: a genetic dissection of immunity to infection in natural conditions. *Nat. Rev. Immunol.* **4**, 55–66.
- Conley, M.E., Rohrer, J., Rapalus, L., Boylin, E.C., and Minegishi, Y. (2000). Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. *Immunol. Rev.* **178**, 75–90.
- de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., Boer, T., van Breda Vriesman, P.J., Kabel, P.J., Draaisma, J.M., van Dissel, J.T., Kroon, F.P., et al. (1998). Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* **280**, 1435–1438.
- Dupuis, S., Jouanguy, E., Al-Hajjar, S., Fieschi, C., Al-Mohsen, I.Z., Al-Jumaah, S., Yang, K., Chapgier, A., Eidenschenk, C., Eid, P., et al. (2003). Impaired response to interferon- α/β and lethal viral disease in human STAT1 deficiency. *Nat. Genet.* **33**, 388–391.
- Fieschi, C., Bosticardo, M., de Beaucoudrey, L., Boisson-Dupuis, S., Feinberg, J., Santos, O.F., Bustamante, J., Levy, J., Candotti, F., and Casanova, J.L. (2004). A novel form of complete IL-12/IL-23 receptor β 1 deficiency with cell surface-expressed nonfunctional receptors. *Blood* **104**, 2095–2101.
- Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J.J. (1990). tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* **5**, 1329–1336.
- Gauzzi, M.C., Barbieri, G., Richter, M.F., Uze, G., Ling, L., Fellous, M., and Pellegrini, S. (1997). The amino-terminal region of Tyk2 sustains

the level of interferon alpha receptor 1, a component of the interferon α/β receptor. *Proc. Natl. Acad. Sci. USA* **94**, 11839–11844.

Grimbacher, B., Holland, S.M., Gallin, J.I., Greenberg, F., Hill, S.C., Malech, H.L., Miller, J.A., O'Connell, A.C., and Puck, J.M. (1999a). Hyper-IgE syndrome with recurrent infections—an autosomal dominant multisystem disorder. *N. Engl. J. Med.* **340**, 692–702.

Grimbacher, B., Schaffer, A.A., Holland, S.M., Davis, J., Gallin, J.I., Malech, H.L., Atkinson, T.P., Belohradsky, B.H., Buckley, R.H., Cossu, F., et al. (1999b). Genetic linkage of hyper-IgE syndrome to chromosome 4. *Am. J. Hum. Genet.* **65**, 735–744.

Grimbacher, B., Holland, S.M., and Puck, J.M. (2005). Hyper-IgE syndromes. *Immunol. Rev.* **203**, 244–250.

Happel, K.I., Dubin, P.J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L.J., Odden, A.R., Shellito, J.E., Bagby, G.J., Nelson, S., and Kolls, J.K. (2005). Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* **202**, 761–769.

Hart, P.H., Jones, C.A., and Finlay-Jones, J.J. (1995). Monocytes cultured in cytokine-defined environments differ from freshly isolated monocytes in their responses to IL-4 and IL-10. *J. Leukoc. Biol.* **57**, 909–918.

Hoffmann, K.F., Cheever, A.W., and Wynn, T.A. (2000). IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J. Immunol.* **164**, 6406–6416.

Ihle, J.N. (1995). Cytokine receptor signalling. *Nature* **377**, 591–594.

Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., et al. (2000). Partial impairment of cytokine responses in Tyk2-deficient mice. *Immunity* **13**, 549–560.

Liu, K.D., Gaffen, S.L., and Goldsmith, M.A. (1998). JAK/STAT signaling by cytokine receptors. *Curr. Opin. Immunol.* **10**, 271–278.

Macchi, P., Villa, A., Giliani, S., Sacco, M.G., Frattini, A., Porta, F., Ugazio, A.G., Johnston, J.A., Candotti, F., O'Shea, J.J., et al. (1995). Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* **377**, 65–68.

Metin, A., Uysal, G., Guven, A., Unlu, A., and Ozturk, M.H. (2004). Tuberculous brain abscess in a patient with hyper IgE syndrome. *Pediatr. Int.* **46**, 97–100.

Minegishi, Y., and Conley, M.E. (2001). Negative selection at the pre-BCR checkpoint elicited by human μ heavy chains with unusual CDR3 regions. *Immunity* **14**, 631–641.

Minegishi, Y., Coustan-Smith, E., Wang, Y.H., Cooper, M.D., Campana, D., and Conley, M.E. (1998). Mutations in the human λ 5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J. Exp. Med.* **187**, 71–77.

Minegishi, Y., Coustan-Smith, E., Rapalus, L., Ersoy, F., Campana, D., and Conley, M.E. (1999a). Mutations in Ig α (CD79a) result in a complete block in B-cell development. *J. Clin. Invest.* **104**, 1115–1121.

Minegishi, Y., Rohrer, J., Coustan-Smith, E., Lederman, H.M., Pappu, R., Campana, D., Chan, A.C., and Conley, M.E. (1999b). An essential role for BLNK in human B cell development. *Science* **286**, 1954–1957.

Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvenoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., et al. (1993). The protein tyrosine kinase JAK1 complements defects in interferon- α/β and γ signal transduction. *Nature* **366**, 129–135.




Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998). Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* **93**, 397–409.

Nosaka, T., van Deursen, J.M., Tripp, R.A., Thierfelder, W.E., Witthuhn, B.A., McMickle, A.P., Doherty, P.C., Grosveld, G.C., and Ihle, J.N. (1995). Defective lymphoid development in mice lacking Jak3. *Science* **270**, 800–802.

Nosaka, T., Kawashima, T., Misawa, K., Ikuta, K., Mui, A.L., and Kitamura, T. (1999). STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. *EMBO J.* **18**, 4754–4765.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., et al. (2000). Novel p19 protein

- engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13, 715–725.
- O'Shea, J.J., Gadina, M., and Schreiber, R.D. (2002). Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 109 (Suppl), S121–S131.
- Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., et al. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* 93, 385–395.
- Park, S.Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T., and Saito, T. (1995). Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* 3, 771–782.
- Pasic, S., Lilib, D., Pejnovic, N., Vojvodic, D., Simic, R., and Abinun, M. (1998). Disseminated *Bacillus Calmette-Guerin* infection in a girl with hyperimmunoglobulin E syndrome. *Acta Paediatr.* 87, 702–704.
- Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G., and Pellegrini, S. (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. *EMBO J.* 22, 537–547.
- Renner, E.D., Puck, J.M., Holland, S.M., Schmitt, M., Weiss, M., Frosch, M., Bergmann, M., Davis, J., Belohradsky, B.H., and Grimbacher, B. (2004). Autosomal recessive hyperimmunoglobulin E syndrome: a distinct disease entity. *J. Pediatr.* 144, 93–99.
- Rodig, S.J., Meraz, M.A., White, J.M., Lampe, P.A., Riley, J.K., Arthur, C.D., King, K.L., Sheehan, K.C., Yin, L., Pennica, D., et al. (1998). Disruption of the *Jak1* gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell* 93, 373–383.
- Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.S., Noguchi, M., Markert, M.L., Buckley, R.H., et al. (1995). Mutation of *Jak3* in a patient with SCID: essential role of *Jak3* in lymphoid development. *Science* 270, 797–800.
- Seto, Y., Nakajima, H., Suto, A., Shimoda, K., Saito, Y., Nakayama, K.I., and Iwamoto, I. (2003). Enhanced Th2 cell-mediated allergic inflammation in Tyk2-deficient mice. *J. Immunol.* 170, 1077–1083.
- Shaw, M.H., Boyartchuk, V., Wong, S., Karaghiosoff, M., Ragimbeau, J., Pellegrini, S., Muller, M., Dietrich, W.F., and Yap, G.S. (2003). A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. *Proc. Natl. Acad. Sci. USA* 100, 11594–11599.
- Shaw, M.H., Freeman, G.J., Scott, M.F., Fox, B.A., Bzik, D.J., Belkaid, Y., and Yap, G.S. (2006). Tyk2 negatively regulates adaptive Th1 immunity by mediating IL-10 signaling and promoting IFN- γ -dependent IL-10 reactivation. *J. Immunol.* 176, 7263–7271.
- Shimoda, K., Kato, K., Aoki, K., Matsuda, T., Miyamoto, A., Shibamori, M., Yamashita, M., Numata, A., Takase, K., Kobayashi, S., et al. (2000). Tyk2 plays a restricted role in IFN α signaling, although it is required for IL-12-mediated T cell function. *Immunity* 13, 561–571.
- Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., Pellegrini, S., et al. (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* 263, 92–95.
- Takaoka, A., Mitani, Y., Suemori, H., Sato, M., Yokochi, T., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Cross talk between interferon- γ and α/β signaling components in caveolar membrane domains. *Science* 288, 2357–2360.
- Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992). A protein tyrosine kinase in the interferon α/β signaling pathway. *Cell* 70, 313–322.
- Watford, W.T., Hissong, B.D., Bream, J.H., Kanno, Y., Muul, L., and O'Shea, J.J. (2004). Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.* 202, 139–156.
- Wood, P.M., Fieschi, C., Picard, C., Ottenhoff, T.H., Casanova, J.L., and Kumararatne, D.S. (2005). Inherited defects in the interferon- γ receptor or interleukin-12 signalling pathways are not sufficient to cause allergic disease in children. *Eur. J. Pediatr.* 164, 741–747.
- Wu, C., Ferrante, J., Gately, M.K., and Magram, J. (1997). Characterization of IL-12 receptor β 1 chain (IL-12R β 1)-deficient mice: IL-12R β 1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol.* 159, 1658–1665.



American Journal of Medical Genetics Part A e-mail  print What is RSS?Early View (Articles online in advance of print)

Published Online: 7 Mar 2007

Copyright © 2007 Wiley-Liss, Inc., A Wiley Company



Go to the homepage for this journal to access trials, sample copies, editorial and author information, news, and more. >

SEARCH All Content
 Publication
Titles[Advanced Search](#)
[CrossRef / Google Search](#)
[Acronym Finder](#) [Save Article to My Profile](#) [Download Citation](#)< [Previous Article](#) | [Next Article](#) >[Abstract](#) | [References](#) | Full Text: HTML[View Full Width](#)

Clinical Report

Epstein-Barr virus-associated B-cell lymphoma in a patient with DNA ligase IV (LIG4) syndrome[†]

Nariaki Toita^{1*}, Norikazu Hatano¹, Satoru Ono¹, Masafumi Yamada¹, Ryoji Kobayashi¹, Ichiro Kobayashi^{1,7}, Nobuaki Kawamura¹, Motohiko Okano¹, Akira Satoh², Atsuko Nakagawa³, Koichi Ohshima⁴, Masanobu Shindoh⁵, Tsuyoshi Takami⁶, Kunihiro Kobayashi¹, Tadashi Ariga¹¹Department of Pediatrics, Graduate School of Medicine, Hokkaido University, Sapporo, Japan²Department of Oral Pathobiological Science, Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan³Second Department of Pathology, Aichi Medical University, Nagakute, Japan⁴Department of Pathology, School of Medicine, Fukuoka University, Fukuoka, Japan⁵Department of Oral Pathology, Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan⁶Department of Second Pathology, Gifu University School of Medicine, Gifu, Japan⁷Department of Pediatrics, Kitami Red Cross General Hospital, Kitami, Japanemail: Nariaki Toita (toita@med.hokudai.ac.jp)

* Correspondence to Nariaki Toita, Department of Pediatrics, Graduate School of Medicine, Hokkaido University, North-15 west-7, Kita-ku, Sapporo, Japan.

[†] How to cite this article: Toita N, Hatano N, Ono S, Yamada M, Kobayashi R, Kobayashi I, Kawamura N, Okano M, Satoh A, Nakagawa A, Ohshima K, Shindoh M, Takami T, Kobayashi K, Ariga T. 2007. Epstein-Barr virus-associated B-cell lymphoma in a patient with DNA ligase IV (LIG4) syndrome. *Am J Med Genet Part A*.

Funded by:

- Ministry of Education, Culture, Sports, Science and Technology; Grant Number: 14370237, 14570714
- Ministry of Health, Labor and Welfare; Grant Number: 25060018-15

KEYWORDS

LIG4 syndrome DNA ligase IV immunodeficiency EBV lymphoma

ABSTRACT

A 14-year-old Japanese girl with a progressing combined immunodeficiency had developed non-Hodgkin's diffuse large B cell lymphoma. Her molecular analysis showed a compound heterozygote of novel mutations in the *LIG4* gene, M249V substitution and a five nucleotides deletion from nucleotide

position 1,270-1,274. She had also a set of characteristic clinical features of LIG4 syndrome. Mutations in the *LIG4* gene, which plays a critical role in the repair of DNA double-strand breaks, imply a correlation with malignancies and several cases with leukemia or lymphoma have already been reported. We report here on a case of LIG4 syndrome complicated with distinct EBV-associated B-cell lymphoma. © 2007 Wiley-Liss, Inc.

Received: 7 September 2006; Accepted: 24 November 2006

DIGITAL OBJECT IDENTIFIER (DOI)

10.1002/ajmg.a.31644 [About DOI](#)

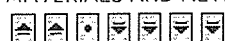
ARTICLE TEXT

INTRODUCTION



DNA ligase IV (LIG4) syndrome (OMIM #606593) is a rare autosomal recessive disorder arising from mutations in the DNA ligase IV gene, which plays a critical role in the repair of DNA double-strand breaks by non-homologous end-joining mechanism [Riballo et al., [1999]; O'Driscoll et al., [2001]]. It is characterized by chromosomal instability, immunodeficiency, and developmental delay. Out of 11 previously reported patients, two cases with T cell leukemia, one case with B-cell lymphoma and one case with myelodysplasia have been reported, suggesting an increased risk for lymphoid malignancies in this disorder [Riballo et al., [1999]; O'Driscoll et al., [2001]; Ben-Omram et al., [2005]; Buck et al., [2006]; Enders et al., [2006]; van der Burg et al., [2006]]. In addition, a possible correlation between polymorphisms in the *LIG4* gene and a risk for malignancies such as breast cancer and multiple myeloma has been suggested [Goode et al., [2002]; Roddam et al., [2002]]. We present here a case of LIG4 syndrome complicated with Epstein-Barr virus (EBV)-associated large B-cell lymphoma.

MATERIALS AND METHODS



Patient

A 14-year-old Japanese girl was admitted to Hokkaido University Hospital because of progressive gingival swelling and high fever. Her underlying clinical and laboratory features have already been precisely reported [Yamada et al., [2001]]. Briefly, she had polydactyly, proportional microcephaly, short stature, and progressively decreasing serum levels of IgG and IgM, and the numbers of both peripheral B and T cells. Her skin fibroblast showed both spontaneous chromosome aberration and ionizing irradiation hypersensitivity but not mitomycin C hypersensitivity. Her clinical features including microcephaly, immunodeficiency and radiosensitivity resembled that of Nijmegen breakage syndrome (NBS) other than Bloom syndrome, Fanconi anemia, ataxia telangiectasia, and radiosensitive SCID (Artemis deficiency). However, no mutation was found in the coding lesion of *NBS1*. As well, a normal level of NBS1 protein was expressed in her fibroblast. She had been protected from severe infections by monthly intravenous immunoglobulin replacement therapy.

On admission, she had a whitely coated tumor involving her left upper gingiva and hard palate without apparent lymphadenopathy or hepatosplenomegaly. Laboratory findings were as followings; white blood cell count $1.5 \bar{\tau} 10^9/L$ with 66% of neutrophils and 11% of lymphocytes, hemoglobin 97 g/L, platelet count $38 \bar{\tau} 10^9/L$, and C-reactive protein 26.3 mg/L. Flow-cytometry analysis of her peripheral blood mononuclear cells showed decreased numbers of both T (CD3⁺, 33.1%) and B cells (CD20⁺, 0.1%). T2-weighted magnetic resonance imaging showed a mixed intensity mass in the left maxillary sinus. The histopathological diagnosis was made as non-Hodgkin's diffuse large B cell lymphoma.

No abnormal cells were detected in either her peripheral blood or bone marrow. She was treated with the combination of reduced amounts of vincristine, cyclophosphamide, and prednisolone. Following this, erythematous desquamation of the skin, severe diarrhea, and severe persistent neutropenia developed. Finally, serious pulmonary aspergillosis developed despite anti-fungal therapy. She died of respiratory failure after four months of hospitalization. An autopsy was not performed.

Histopathology

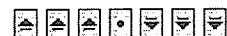
Tissue sample from the left maxillary sinus was fixed in 10% buffered formalin, embedded in paraffin,

and stained with H&E. immunostains for CD3, CD20/L26, CD79a (DAKO, Kyoto, Japan) were performed for phenotypic analysis of the proliferating lymphocytes. Detection of EBV was done by immunostains for latent membrane protein (LMP-1) and EBV-determined nuclear antigen 2 (EBNA2) (DAKO) and by in situ hybridization with an oligoprobe for EBV-encoded small non-polyadenylated RNAs (EBER) and with a sense probe (negative control) [Weiss et al., [1992]].

DNA Sequencing

Genomic DNA was extracted from both peripheral blood neutrophils and skin fibroblasts. Overlapping fragments of the coding lesions of *LIG4* were amplified by polymerase chain reaction (PCR; 35 cycles of 94-C for 30 sec, 60-C for 30 sec, and 72-C for 1 min) with primers according to the previous report [O'Driscoll et al., [2001]]. PCR products were supplied for direct sequencing reaction. Both PCR amplification and sequencing reaction were performed by GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA). Sequencing analyses were performed by ABI PRISM Gene Analyzer 310 (Perkin Elmer).

RESULTS



Histopathological Findings

Immunohistochemistry of the tumor cells demonstrated positive for both CD20 and CD79a but negative for CD3, and was histopathologically diagnosed as diffuse large B cell lymphoma (Fig. 1). Clonal immunoglobulin heavy chain gene rearrangement was noted by PCR analysis, suggesting monoclonal proliferation of the tumor cells. Although no EBV genome was demonstrated by semi-quantitative PCR analysis in her peripheral circulation, tumor cells were positive for EBV-encoded RNAs (EBERs), latent membrane protein-1, and EBV-determined nuclear antigen 2, indicating EBV-related type III latency [Okano and Gross, [2000]].

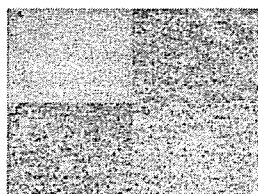


Figure 1. Histopathology of the tumor. Tumor section was stained with hematoxylin-eosin (A; original \times 100). Tumor cells are positive for CD79a (B; original \times 400) and latent membrane protein-1 (C; original \times 400) by immunohistochemistry. In situ hybridization study demonstrated EBV-encoded RNAs in the tumor cells (D; original \times 400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 88K | Magnified View 172K]

Sequencing Analysis and Immunoblot Analysis

We detected novel mutations, a five nucleotide deletion from 1,270 to 1,274 and an 745A > G substitution of the *LIG4* gene, which result in a frame shift at the amino acid position 424 and M249V amino acid substitution, respectively (Fig. 2). Compound heterozygosity was confirmed by sequencing analyses of both cloned DNA from the patient and DNA from her parents. The small deletion was inherited from her mother and the point mutation was identified in her father (data not shown). Western blot analysis revealed reduced LIG4 protein levels in her fibroblasts using monoclonal antibody for LIG4 (purchased from Santa Cruz Biotechnology, Santa Cruz, CA), compared with control cells (data not shown).

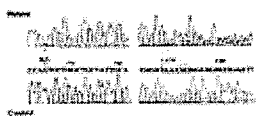


Figure 2. Direct sequencing analysis demonstrates mutations in *LIG4*. The sequence of DNA from the patient shows heterozygote of 745A and 745G (left upper panel). A five nucleotides deletion from nucleotide position 1,270-1,274 (AAAAG) results in frame shift (right upper panel). Each mutation was detected on a different allele by sequencing analyses of cloned DNAs (data not shown). Lower panels show the sequence of DNA from a normal individual. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 44K | Magnified View 69K]

DISCUSSION

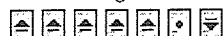


Here we describe a case of LIG4 syndrome complicated with distinct EBV-associated B-cell lymphoma, who had many phenotypic characteristics resembling NBS. O'Driscoll et al. [2001] screened NBS-like patients who had no mutations in *NBS1* and produced normal levels of nibrin, then identified several patients with mutations in *LIG4*. We also identified two novel mutations in the *LIG4* gene, a five-nucleotides deletion and M249V substitution. The small deletion result in a frame shift and its product lacking C-terminal XRCC4 binding site could be non-functional [Grawunder et al., 1998]. Methionine at amino acid 249 is located near an ATP-binding site and is conserved among other DNA ligases [Wel et al., 1995]. However, M249V mutant could be partially functional, because LIG4 knock-out mice show embryonic lethality [Frank et al., 2000]. LIG4 plays a critical role in both DNA replication and V(D)J recombination [Riballo et al., 1999; O'Driscoll et al., 2001]. Thus its deficit might cause the death of the cells with non-functional repair or recombination during meiosis or rearrangement of both T and B cell receptors, which promotes a progressive combined immunodeficiency in LIG4 syndrome. Such a slow progression of the disease as in our case might be one of the characteristic features of LIG4 syndrome.

We detected EBV-specific RNAs and latent proteins in the tumor cells in our case, although no EBV genome was detected in her peripheral blood by PCR. EBV infection to B cells causes infectious mononucleosis in immunocompetent individuals, but promotes lymphoproliferative disorders including malignant lymphoma in immunocompromised hosts particularly with profound T cell defect [Okano and Gross, 2000]. Thus, it is possible that her deficient immunosurveillance allowed the development of EBV-associated B cell lymphoma. Otherwise, the defects in damaged-DNA repair might be responsible for the development of her malignancy as seen in other chromosome breakage syndromes such as NBS and ataxia telangiectasia. There have been two cases with T cell leukemia, one case with B-cell lymphoma and one case with myelodysplasia complicated with LIG4 syndrome to date. Collectively, LIG4 syndrome patients are likely to be at increased risk for lymphoid malignancies.

The desquamative erythema, protracted diarrhea, and neutropenia during the reduced chemotherapy could be correlated with the defect in repair of DNA damage induced by the cytotoxic agents used for her treatment. Accordingly, haematopoietic stem cell transplantation that requires cytotoxic agents for myeloablation could be harmful for patients with this syndrome. Instead, anti-CD20 antibody (rituximab) or adoptive EBV-CTL therapy may be beneficial for EBV-associated lymphoma in such patients.

Acknowledgements



We thank Dr. H. Kikuta for his analyzing EBV-DNA by PCR, and to Dr. Y. Sakiyama, Dr. F. Kuroki, Dr. M. Kaneda and Dr. M. Yoshida, and to staff at the Pediatric Ward, Hokkaido University Hospital for their substantial contribution. We also thank Dr. Ochs HD, University of Washington, for his helpful suggestion. This work was supported in part by Grant-in-Aid (No.14370237: IK, NK, KK and No. 14570714: MO) from the Ministry of Education, Science, Sports and Culture, Japan, and Grant 25060018-15 from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES



- Ben-Omram TI, Cerosaletti K, Concannon P, Weitzman S, Nezarati MM. 2005. A patient with mutations in DNA ligase IV: Clinical features and overlap with Nijmegen breakage syndrome. *Am J Med Genet Part A* 137A: 283-287. [Links](#)
- Buck D, Moshous D, de Chasseval R, Ma Y, le Deist F, Cavazzana-Calvo M, Fischer A, Casanova JL, Lieber MR, de Villartay JP. 2006. Severe combined immunodeficiency and microcephaly in siblings with hypomorphic mutations in DNA ligase IV. *Eur J Immunol* 36: 224-235. [Links](#)
- Enders A, Fisch P, Schwarz K, Duffner U, Pannicke U, Nikolopoulos E, Peters A, Orłowska-Volk M, Schindler D, Friedrich W, Selle B, Niemeyer C, Ehl S. 2006. A severe form of human combined immunodeficiency due to mutations in DNA ligase IV. *J Immunol* 176: 5060-5068. [Links](#)
- Frank KM, Sharpless NE, Gao Y, Sekiguchi JM, Ferguson DO, Zhu C, Manis JP, Horner J, DePinho RA, Alt FW. 2000. DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via p53 pathway. *Mol Cell* 5: 993-1002. [Links](#)
- Goode EL, Dunning AM, Kuschel B, Healey CS, Day NE, Ponder BA, Easton DF, Pharoah PP. 2002. Effect of germ-line genetic variation on breast cancer survival in a population-based study. *Cancer Res* 62: 3052-3057. [Links](#)

- Grawunder U, Zimmer D, Leiber MR. 1998. DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. *Curr Biol* 8: 873-876. [Links](#)
- O'Driscoll M, Cerosaletti KM, Girard PM, Dai Y, Stumm M, Kysela B, Hirsch B, Gennery A, Palmer SE, Seidel J, Gatti RA, Varon R, Oettinger MA, Neitzel H, Jeggo PA, Concannon P. 2001. DNA ligase IV mutation identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell* 8: 1175-1185. [Links](#)
- Okano M, Gross TG. 2000. A review of Epstein-Barr virus infection in patients with immunodeficiency disorders. *Am J Med Sci* 319: 392-396. [Links](#)
- Riballo E, Critchlow SE, Teo S-H, Doherty AJ, Priestley A, Broughton B, Kysela B, Beamish H, Plowman N, Arlett CF, Lehmann AR, Jackson SP, Jeggo PA. 1999. Identification of a defect in DNA ligase IV in a radio sensitive leukemia patient. *Curr Biol* 9: 699-702. [Links](#)
- Roddam PL, Rollinson S, O'Driscoll M, Jeggo PA, Jack A, Morgan GJ. 2002. Genetic variants of NHEJ DNA ligase IV can affect the risk of developing multiple myeloma, a tumor characterised by aberrant class switch recombination. *J Med Genet* 39: 900-905. [Links](#)
- van der Burg M, van Veelen LR, Verkaik NS, Wiegant WW, Hartwig NG, Barendregt BH, Brugmans L, Raams A, Jaspers NG, Zdzienicka MZ, van Dongen JJ, van Gent DC. 2006. A new type of radiosensitive T-B-NK+ severe combined immunodeficiency caused by a LIG4 mutation. *J Clin Invest* 116: 137-145. [Links](#)
- Weiss LM, Jaffe ES, Liu XF, Chen YY, Shibata D, Medeiros LJ. 1992. Detection and localization of Epstein-Barr viral genomes in angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma. *Blood* 79: 1789-1795. [Links](#)
- Wel YF, Robins P, Carter K, Caldecott K, Pappin DJC, Yu GL, Wang RP, Shell BK, Nash RA, Schär P, Barnes DE, Haseltine WA, Lindahl T. 1995. Molecular cloning and expression of human cDNA encoding a novel DNA ligase IV and DNA ligase III, as enzyme active in DNA repair and recombination. *Mol Cell Biol* 15: 3206-3216. [Links](#)
- Yamada M, Matsuura S, Tsukahara M, Ebe K, Ohtsu M, Furuta H, Kobayashi I, Kawamura N, Okano M, Shouji R, Kobayashi K. 2001. Combined immunodeficiency, chromosomal instability, and postnatal growth deficiency in a Japanese girl: A new syndrome? *Am J Med Genet* 100: 9-12. [Links](#)

Successful Transduction of Mammalian Astrocytes and Oligodendrocytes by "Pseudotyped" Baculovirus Vector *in Vitro* and *in Vivo*

Hiroshi KOBAYASHI^{1,2}, Kazuhiko WATABE³, Sayoko IZUKA¹, Hideki TANI⁴,
Yoshiharu MATSUURA⁴, James BARSOUM⁵, Campbell KAYNOR⁵,
Toya OHASHI^{1,2}, and Yoshikatsu ETOH^{1,2}

¹Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine

²Department of Pediatrics, The Jikei University School of Medicine

³Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience

⁴Research Center for Emerging Infectious Diseases,

Research Institute for Microbial Diseases, Osaka University

⁵Biogen, Inc.

ABSTRACT

Baculovirus vectors can efficiently transduce human hepatoma cells and primary hepatocytes in culture. We report the potential use of baculovirus as a vector for gene delivery into cells of the mammalian central nervous system. We generated a "pseudotyped" baculovirus encoding the bacterial β -galactosidase (β -Gal) gene (LacZ), under the control of the cytomegalovirus promoter, and the vesicular stomatitis virus G protein gene, under the control of the polyhedrin promoter. This virus was used to infect primary cultures of rat glial cells. Three days after infection, these cells were immunostained for β -Gal, glial fibrillary acidic protein (for astrocytes), or galactocerebroside (for oligodendrocytes) to identify the infected cell types. Positive β -Gal immunofluorescence was observed in 10.4% of glial fibrillary acidic protein-positive cells and 35.6% of galactocerebroside-positive cells at a multiplicity of infection of 50. When the virus was injected into adult mouse striatum, β -Gal-positive cells were demonstrated, and no cytological or histological evidence of cell damage, inflammation, or cell infiltration was observed after infection. These findings suggest that baculovirus-mediated gene transfer can be used for gene therapy against nervous system diseases, especially demyelinating disorders, affecting mainly oligodendrocytes.

(Jikeikai Med J 2006 ; 53 : 55-62)

Key words : baculovirus, gene therapy, pseudotype, astrocytes, oligodendrocytes

INTRODUCTION

The baculovirus vector has been widely used to obtain high levels of expression of foreign genes under the control of the strong baculoviral promoter (polyhedrin promoter) in insect cells¹. Although its host specificity had been thought to be restricted to insect

cells, the recombinant baculovirus was recently shown to be capable of transferring and expressing foreign genes in mammalian cells, such as hepatocytes^{2,3} and nonhepatic cell lines⁴. For the original baculovirus vector the foreign gene is cloned next to the viral polyhedrin promoter, whereas for the recombinant baculovirus the gene is fused to a mammalian pro-

Received for publication, January 6, 2006

小林 博司, 渡部 和彦, 飯塚佐代子, 谷 英樹, 松浦 善治, James Barsoum, Campbell Kaynor, 大橋 十也, 衛藤 義勝

Mailing address : Hiroshi KOBAYASHI, Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, 3-5-28, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.

E-mail address : hr-kb@wd6.so-net.ne.jp

moter, such as cytomegalovirus immediate early promoter and inserted. Efficient gene transfer has been reported, and no viral DNA replication has been observed in mammalian cells. These results suggest that this virus system would be safe for humans.

Sarkis et al. have reported that the baculovirus vector can be used to transfer a marker gene to neurons and astrocytes *in vitro* and *in vivo*⁵. They have reported efficient gene transfer into astrocytes and neurons, but not into oligodendrocytes. Oligodendrocytes are the cells mainly affected in several neurological diseases, such as demyelinating disorders; therefore, for gene therapy in these diseases, investigating ways to transfer genes into oligodendrocytes is important. Barsoum et al. have reported that pseudotype baculovirus, expressing vesiculostomatitis virus G (VSVG) protein in its envelope, transduces mammalian cells much more efficiently than does nonpseudotype baculovirus⁶. In this study, we examined the ability of a pseudotype baculovirus vector to transfer a marker gene in central nervous system (CNS) cells, including oligodendrocytes.

MATERIAL AND METHODS

1. Construction of pseudotype transfer plasmids

The pseudotype baculovirus transfer vector, pCZPG (Fig. 1), was generated by inserting expression cassettes encoding the VSVG protein and bacterial β -galactosidase (β -Gal) gene (LacZ) into the standard baculovirus transfer vector BacPak9 (Clontech Laboratories, Inc., Palo Alto, CA, USA)⁶. First, the VSVG gene complementary DNA was ex-

cised from the plasmid pLGRNL⁷. This fragment was inserted into the *Bam*HI site of BacPak9, in a direct orientation with respect to the polyhedrin promoter, to create VSVG/BP9. Next, the LacZ gene preceded by the cytomegalovirus promoter and followed by the SV40 poly(A) signal was inserted into VSVG/BP9 such that the LacZ cassette was downstream from the VSVG gene and the direction of transcription was convergent⁶.

2. Pseudotype baculovirus production

Cells of the insect cell line Sf9 were grown in TC-100 medium (GIBCO/Invitrogen, Carlsbad, CA, USA) with 0.26% Bacto tryptose phosphate broth (Difco, Detroit, MI, USA), 100 μ g/ml kanamycin, and 10% fetal bovine serum (GIBCO/Invitrogen). Recombinant pseudotype baculovirus, CZPG, was generated by homologous recombination, as described previously^{4,9}. Briefly, pCZPG and the *Bsu* 36I-digested baculovirus genomic DNA were cotransfected into Sf9 cells by lipofectin (GIBCO/Invitrogen). Two days later, the culture medium was harvested and used to infect Sf9 cells in a standard plaque assay. The plaques were isolated and purified by a second round of plaque isolation. After the presence of the predicted recombinant DNA restriction digestion pattern was determined, selected plaques were expanded twice in tissue culture flasks and then on a large scale in 100-ml spinner cultures to obtain a significant volume of virus preparation. The viral titer was determined with a plaque assay¹⁰. To purify the virus, conditioned media of Sf9 cells infected with the virus were harvested 3 days after infection, and cell debris was removed by centrifugation at 6,000 g for 15

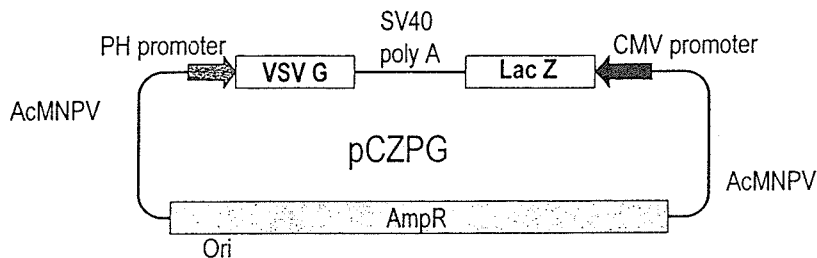


Fig. 1. Structure of pseudotype baculovirus transfer plasmid (pCZPG). AcMNPV, Baculovirus sequences; PH, polyhedrin promoter; AmpR, ampicillin-resistance gene; Ori, replication origin; VSVG, Vesicular stomatitis virus G protein gene.

minutes at 4°C. The virus was pelleted by ultracentrifugation at 80,000 g (RPS27-2 rotor, Hitachi, Tokyo) for 90 minutes and resuspended in 1 ml of phosphate buffered saline (PBS), loaded on 10% to 60% (w/v) sucrose gradients, and ultracentrifuged at 77,600 g (P-40ST rotor, Hitachi) for 90 minutes. The virus band was collected and resuspended in PBS and ultracentrifuged at 77,600 for 90 minutes. The virus pellet was resuspended in PBS, and infectious titers were determined with a plaque assay⁴.

3. Mixed glial cell culture

Cultures of rat astrocytes and oligodendrocytes were established with the enzyme digestion-Percoll (Pharmacia, Uppsala, Sweden) density gradient method¹¹⁻¹³. The brains of 10-week-old male Wistar rats were minced and incubated in 0.25% trypsin and 20 µg/ml DNase in calcium- and magnesium-free Hanks balanced salt solution (HBSS) for 40 minutes at 37°C. Dissociated cells were passed through a 100-µm nylon mesh. Isolated cells suspended in HBSS were mixed with Percoll, and a gradient was formed by centrifugation for 25 minutes at 15,000 g in a high-speed refrigerated centrifuge with a fixed-angle rotor (Hitachi). The final concentration of Percoll was 30% in HBSS. An astrocyte- and oligodendrocyte-enriched fraction, bound by an upper myelin layer and a lower erythrocyte layer, was collected and diluted in three volumes of HBSS, before being harvesting by low-speed centrifugation for 10 minutes. The cells were washed twice in HBSS, suspended in feeding medium (1×10^5 cells/ml), and seeded on polysine-coated 9-mm-diameter round coverslips (Aclar, Honeywell/AlliedSignal, Pottsville, PA, USA). The feeding medium consisted of 5% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin in Isocove's modified Dulbecco's minimum essential medium. The cultures were maintained in 5% CO₂ at 37°C for 2 weeks, with the culture medium being changed every 4 days.

4. Infection of glial cell cultures with CZPG

Living cells of mixed rat glial cultures on coverslips after 2 weeks in vitro were infected with a 10-µl solution of purified CZPG at a multiplicity of infection

of 50 for 1 hour and were incubated in feeding medium for 3 days in 5% CO₂ at 37°C. To detect β-Gal expression in infected cells, immunofluorescence was performed with a rabbit antibody to β-Gal. Cell types were identified with immunofluorescence using antibodies to glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), specific markers for astrocytes and oligodendrocytes, respectively. For double immunofluorescence staining for β-Gal and GFAP, cells on coverslips were fixed in 1% paraformaldehyde in PBS for 10 minutes at 4°C and in methanol for 10 minutes at -20°C and incubated at room temperature for 1 hour with a mixture of a rabbit antibody to β-Gal (Eppendorf-5 Prime, Boulder, CO, USA) and rat monoclonal antibody (hybridoma soup) to GFAP¹⁴ (kindly provided by Dr. Seung U. Kim, University of British Columbia) at final dilutions of 1:50 and 1:2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel, MP Biomedicals, Aurora, OH, USA) and rhodamine-conjugated goat anti-rat IgG (Cappel) at a final dilution of 1:50. For double immunofluorescence staining for β-Gal and GC, living cells on coverslips were incubated with mouse monoclonal antibody (hybridoma soup) to GC¹⁵ (kindly provided by Dr. Seung U. Kim) at a dilution of 1:2 for 30 minutes at room temperature. After being washed with Isocove's modified Dulbecco's minimum essential medium, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG (Cappel) at a final dilution of 1:50 for 30 minutes at room temperature. The cells were fixed with 1% paraformaldehyde in PBS at 4°C for 10 minutes and cold methanol at -20°C for 10 minutes. After being washed in PBS, the cells were incubated with rabbit antibody to β-Gal at a dilution of 1:50 for 1 hour at room temperature. After being washed, the cells were incubated with FITC-conjugated goat anti-rabbit IgG at a final dilution of 1:50 for 45 minutes at room temperature. After being washed, coverslips were mounted on glass slides with 20% glycerol/10% polyvinylalcohol in 0.1 M Tris-HCl buffer, pH 8.0. Cells were then examined under a universal microscope (Olympus Opti-

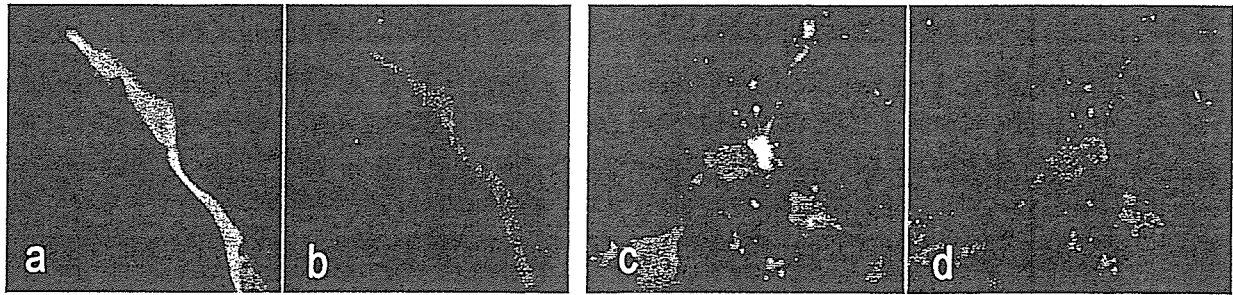


Fig. 2. β -Gal expression in rat primary culture astrocytes and oligodendrocytes *in vitro*. a, b: Astrocytes infected with CZPG *in vitro* for 3 days. β -Gal-FITC (a) and GFAP-rhodamine (b) double immunofluorescence. c, d: Oligodendrocytes infected with CZPG *in vitro* for 3 days. β -Gal-FITC (c) and GC-rhodamine (d) double immunofluorescence.

cal Co., Tokyo) equipped with fluorescein and rhodamine optics.

5. Infection of mouse brains with CZPG

Intracerebral injection of purified CZPG was carried out as follows: 6- to 8-week-old normal female C57BL/6 mice ($n=12$) were anesthetized with intraperitoneal injection of 40 mg/kg of pentobarbital sodium. Five microliters of purified CZPG (1×10^9 pfu/ml) was injected slowly into the left striatum (AP: to 1 mm; ML: 2 mm; DV: 3.6 mm to the bregma) or the ventricle (AP: to 1 mm; ML: 2 mm; DV: 2.6 mm to the bregma) through a syringe (Hamilton Co., Reno, NV, USA). Three days after the operation, the mice were deeply anesthetized through inhalation of diethyl ether and intracardially perfused with 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 24 hours, and cryoprotected in 30% sucrose in PBS, after which 20- μ m-thick coronal sections were cut with a cryostat. Gene transfer into the CNS was identified with β -Gal staining. These sections were washed four times in PBS and stained in a solution of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS¹⁶. Cell types were identified with immunofluorescence using antibodies to GFAP and 3',5'-cyclic nucleotide phosphodiesterase (CNP) for astrocytes and oligodendrocytes, respectively. The sections were washed in PBS and incubated in 0.1% Triton X-100 in PBS (PBST) for 30 minutes and in 3% normal goat serum in PBST for 1 hour at room temperature. For double immunofluorescence stain-

ing for β -Gal and GFAP, the sections were incubated at 4°C overnight with a mixture of rabbit antibody to β -Gal and rat monoclonal antibody to GFAP at final dilutions of 1:50 and 1:2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-rat IgG at a final dilution of 1:50. For double immunofluorescence staining for β -Gal and CNP, the sections were incubated at 4°C for 12 hours with a mixture of rabbit antibody to β -Gal and mouse monoclonal antibody to CNP at final dilutions of 1:50 and 1:100, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG at a final dilution of 1:50.

RESULTS

1. Transduction of cultured glial cells by CZPG

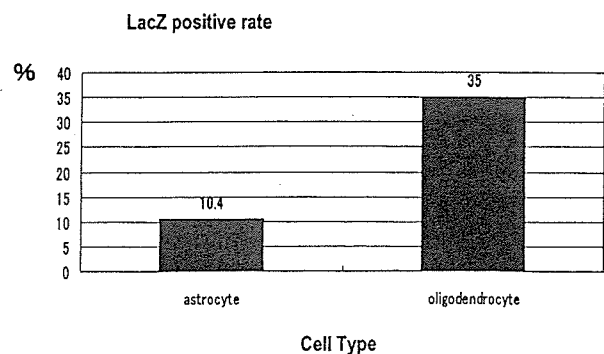


Fig. 3. Efficiency of LacZ gene transfer into rat primary culture *in vitro*.

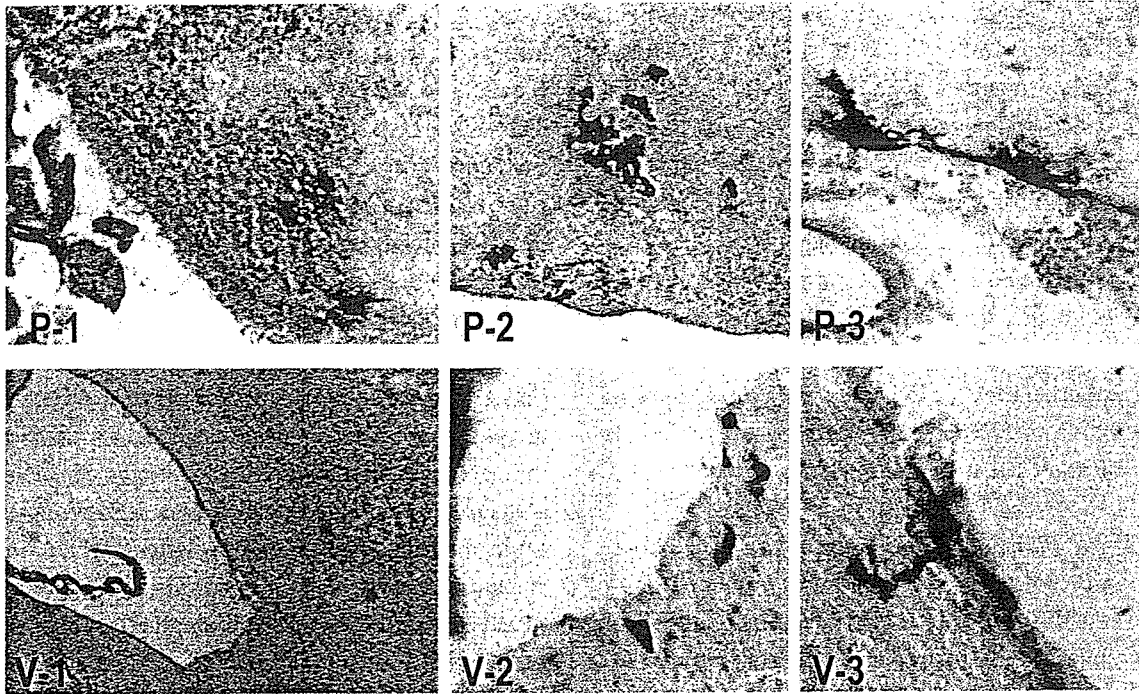


Fig. 4. β -Gal expression in normal mouse brain *in vivo*. 3 days after injection of CZPG into normal mouse (C57BL/6) striatum, gene transfer into CNS *in vivo* was identified by β -Gal staining. P1-P3; the slides of the mouse brain injected in the parenchyma. Lac Z positive cells were seen in near the choroid plexus (P-1), striatum (P-2), and corpus callosum (P-3). V1-V3; the slides of the mouse brain injected in the ventricle. Lac Z positive cells were seen in the endodermal cell (V-1, 2) and partially migrated into the parenchyma (V-3).

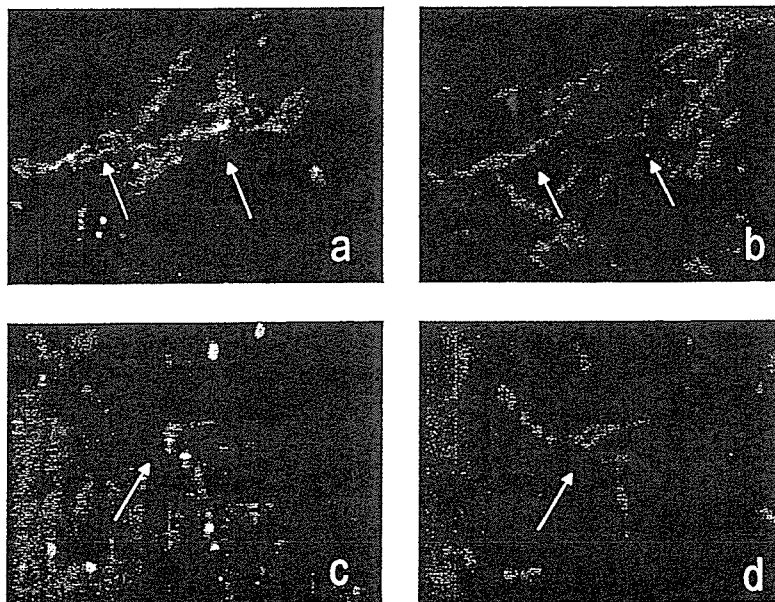


Fig. 5. The slides of the Immunohistochemistry of the mouse brain injected in the parenchyma, double-positive cells, i.e. β -Gal+/GFAP+ (an infected astrocytes; a, b) and β -Gal+/CNP+ (infected oligodendrocyte: c, d) cells were demonstrated by double immunofluorescence microscopy, indicating both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG.