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Severe Neutropenia in Japanese Patients with X-Linked Agammaglobulinemia

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X-linked agammaglobulinemia (XLA) is clinically characterized by recurrent bacterial infections during early infancy. Although it is not a phagocytic disorder, XLA is sometimes associated with neutropenia. We conducted a nation-wide survey to determine the frequency of neutropenia among Japanese XLA patients. Responses were received from 87 (86%) of 101 patients in which *BTK* mutations were previously identified, and of these, 16 (18%) had neutropenia. All episodes of neutropenia occurred before initiation of intravenous immunoglobulin (IVIG) replacement therapy. Two XLA patients died of multiple organ failure caused by severe neutropenia and *Pseudomonas* sepsis before initiation of IVIG replacement therapy. These results suggest that, in some cases, severe bacterial infections in XLA patients might be caused not only by antibody deficiencies but also by neutropenia.

KEY WORDS: X-linked agammaglobulinemia (XLA); Bruton's tyrosine kinase (*BTK*); neutropenia; intravenous immunoglobulin (IVIG).

INTRODUCTION

X-linked agammaglobulinemia (XLA) is the most common form of antibody deficiency syndrome, accounting for approximately half of all primary immunodeficiency diseases (1). XLA patients experience recurrent bacterial infections from early childhood, and laboratory data show hypogammaglobulinemia and markedly reduced numbers of peripheral blood B cells (2). However, occasionally, XLA patients show atypical phenotypes including late onset or subnormal levels of serum IgG. It is often difficult to clinically diagnose these individuals if they have no family history of XLA (3–6).

In 1993, the gene responsible for XLA was identified as intracytoplasmic tyrosine kinase, Bruton's tyrosine kinase (*BTK*) (7, 8). BTK plays a crucial role in early B cell development; however, it is also expressed in myeloid cells, monocytes, and platelets as well as during all the stages of B cell development, except in plasma cells (9–11). The function of BTK in all cells, except B cells, has yet to be determined; however, phagocytosis and chemotaxis of monocytes previously appeared reduced in XLA patients (12). It has also been demonstrated that lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production is diminished in XLA patients (13). These data suggest that BTK protein in monocytes could play a functional role in the immune response.

XLA patients exhibit recurrent bacterial infections caused by hypogammaglobulinemia, and are sometimes associated with neutropenia (14–19). In the present study, we conducted a nation-wide survey of neutropenia among Japanese XLA patients.

PATIENTS AND METHODS

In Japan, 101 XLA patients have been identified through flow cytometric detection of the BTK protein and mutational analysis of the *BTK* gene (20, 21). Primary questionnaires regarding the association between severe neutropenia (neutrophil counts $<500/\mu$l) and XLA were sent to the physicians of these 101 XLA patients. Subsequently, to obtain detailed clinical and laboratory data of white blood cell (WBC) and neutrophil counts and immunoglobulin levels, a second series of questionnaires was sent to those respondents whose XLA patients were associated with neutropenia. Immunoglobulins levels were determined by nephelometry in each institute.

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For definite diagnosis of XLA, heparinized venous blood was obtained from each patient after obtaining informed consent from each individual or his parents. Samples were transferred to our laboratory where peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient centrifugation. The percentage of circulating B cells was evaluated using an immunofluorescence method with anti-CD19 and CD20 monoclonal antibodies (Dako Japan, Kyoto, Japan). Intracellular BTK staining of the monocytes was performed using an anti-BTK monoclonal antibody (48-2H) as described previously (10). Stained cells were analyzed by flow cytometry. Detection of *BTK* mutations was performed as described previously (20). RNA was extracted from the PBMC using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), then subjected to reverse transcriptase-polymerase chain reaction (RT-PCR). BTK cDNA was amplified from the first strand cDNA with seven overlapping PCR primers. Genomic DNA was extracted from the PBMC using a QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany), then subjected to PCR. The sequencing reaction was performed with a BigDye Terminator Cycle Sequencing Kit with an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA). Two patients with presumed XLA died of sepsis and, therefore, PBMC could not be obtained. Alternatively, genomic DNA was extracted from skin fibroblasts established from these deceased patients, then subjected to DNA-based PCR and subsequent direct sequencing reactions.

RESULTS

Primary questionnaires for a total of 87 patients (86%) were returned, and of these, 16 (18%) had associated neutropenia (Table I). Two patients (P6 and P7) experienced 3 episodes of neutropenia; therefore, 20 episodes were identified in total. The three neutropenic episodes experienced by P6 were associated with viral infections (exanthema subitum, rubella, and measles), whereas those observed by P7 were all associated with pneumonia. The mean age at diagnosis of XLA associated with neutropenia was 2.1 years, and five patients were diagnosed at less than 1 year of age. All episodes of neutropenia were recognized before diagnosis of XLA, indicating no episodes of neutropenia after initiation of intravenous immunoglobulin (IVIG) replacement therapy. WBC counts were highly variable with two and three episodes showing less than 1000 and more than 10,000 μl^{-1}, respectively. Eighteen episodes demonstrated an absolute neutrophil count (ANC) of less than 200 μl^{-1}, while five displayed an ANC of 0 μl^{-1}; thus, most cases were associated with profound neutropenia. Although 2 patients (P4 and P13) died within 24 h of disease onset, 6 of 11 episodes showed neutropenia for less than 1 week, after which ANCs returned to a normal level ($>1500\ \mu l^{-1}$) within 11 days.

All XLA patients were associated with certain infections during episodes of neutropenia. While viral infections were identified in P6 in association with three episodes of neutropenia, all other patients showed moderate to severe bacterial infections such as otitis media and pneumonia. P4 and P13 died of multiple organ failure caused by septic shock within 24 h of onset. The bacteria responsible for infection were identified in seven episodes as follows: *Pseudomonas aeruginosa* ($n = 4$), *Staphylococcus aureus* ($n = 1$), *Moraxella* (*Branhamella*) *catarrhalis* ($n = 1$), and *Hemophilus influenzae* ($n = 1$). The pseudomonas infections were associated with severe illnesses including the two fatal sepses in P4 and P14 and necrotizing otitis media in P1. Antibiotics were administered to all patients except P4, and IVIG was given during nine episodes before diagnosis of XLA. Six patients were also treated with granulocyte-colony stimulating factors (G-CSF).

Typical XLA patients show serum IgG levels of <200 mg/dl, and IgA and IgM are undetectable. In this study, immunoglobulin data was not available for four patients (P2, P11, P14, and P15) because of prior IVIG treatment (Table II). P6 and P16 displayed serum IgG levels of 427 and 611 mg/dl, respectively, indicating atypical XLA. In addition, P16 showed a serum IgM level of 135 mg/dl and was misdiagnosed with selective IgA deficiency before diagnosis of XLA.

All XLA patients presented here showed *BTK* gene mutations (Table. III). Various types were identified: four missense mutations, one nonsense mutation, four small deletions, two small insertions and five splicing errors. P6 and P14 showed the same mutations because they were siblings.

DISCUSSION

The present study disclosed that 18% of Japanese XLA patients have associated neutropenia. Lederman and Winkelstein (14) retrospectively analyzed 96 patients clinically diagnosed with XLA, and reported a 10% incidence of neutropenia. In addition, Farrar *et al*. (16) showed that 13 (26%) of 50 XLA patients in whom *BTK* gene mutations were identified had neutropenia. Plo Rodriguez *et al*. (17) similarly reported that 4 (11%) of 37 XLA patients had experienced episodes of neutropenia. These studies indicate that approximately 20% of XLA patients might be associated with neutropenia despite the fact that

Table I. Clinical Characteristics of the XLA Patients with Neutropenia

Patient No.	Age at diagnosis	Age at diagnosis neutropenia	WBC (μl^{-1})	Neutrophil (%)	ANC (μl^{-1})	Infection at time of neutropenia	Causal bacteria	Treatment	Duration of neutropenia
P1	4 months	3 months	2,400	0	0	Otitis media	*P. aeruginosa*	Antibiotics+IVIG+G-CSF	4 days
P2	6 months	6 months	1,600	1	16	Pneumonia, otitis media	*P. aeruginosa*	Antibiotics	2 days
P3	7 months	5 months	1,400	0	0	Impetigo	*S. aureus*	Antibiotics+IVIG+G-CSF	4 days
P4	7 months	7 months	400	0	0	Sepsis	*P. aeruginosa*	IVIG+G-CSF+steroid	Dead
P5	11 months	11 months	7,400	0	0	Pharyngitis, otitis media	Unknown	Antibiotics+IVIG	6 days
P6	1 year 3 months	5 months	5,780	2	116	Exanthema subitum		Antibiotics	Unknown
		8 months	3,480	2	70	Rubella		Antibiotics	Unknown
		10 months	6,140	7	430	Measles		Antibiotics+steroid	11 days
P7	1 year 7 months	8 months	13,000	1	130	Pneumonia, otitis media	Unknown	Antibiotics	Unknown
		1 year 2 months	10,100	1.5	152	Pneumonia, otitis media	*M. catarrhalis*	Antibiotics	4 days
		1 year 7 months	19,000	2	380	Pneumonia, atelectasis	Unknown	Antibiotics+IVIG+G-CSF	4 days
P8	1 year 8 months	1 year 8 months	200	0	0	Pneumonia	Unknown	Antibiotics+IVIG	3 days
P9	1 year 9 months	1 year 9 months	7,600	2	152	Suspected sepsis	Unknown	Antibiotics+IVIG	8 days
P10	2 years 5 months	2 years 5 months	6,850	1	68	Pharyngitis	Unknown	Antibiotics	Unknown
P11	3 years 7 months	2 years 4 months	5,100	2	102	Pharyngitis	Unknown	Antibiotics	6 days
P12	5 years 3 months	5 years	1,600	2	32	Pneumonia, otitis media	Unknown	Antibiotics	8 days
P13	5 years 5 months	5 years 5 months	1,500	6	90	Pneumonia, sepsis	*P. aeruginosa*	Antibiotics+IVIG+G-CSF+steroid	Dead
P14	5 years 7 months	3 years 5 months	3,850	5	193	Pharyngitis	Unknown	Antibiotics	Unknown
P15	5 years 9 months	5 years 9 months	4,400	1	44	Septic arthritis	*H. influenzae*	Antibiotics+IVIG+G-CSF	6 days
P16	11 years	10 years	2,500	3	75	Skin infection	Unknown	Antibiotics	9 days

Table II. Serum Immunoglobulin Levels of the XLA Patients with Neutropenia

Patient	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)
P1	167	2	17
P2	NA[a]	NA	NA
P3	141	<2	9
P4	9	<6	7
P5	8	<26	66
P6	427	<5	47
P7	1	3	18
P8	11	<6	7
P9	<100	<8	22
P10	187	18	39
P11	NA	NA	NA
P12	220	<10	28
P13	17	<8	22
P14	NA	NA	NA
P15	NA	NA	NA
P16	611	<8	135

[a]Not applicable.

XLA is principally an antibody deficiency syndrome. It is worth noting that all episodes of neutropenia observed here occurred before initiation of IVIG replacement therapy. In this study, two patients (P6 and P7) experienced recurrent episodes of neutropenia; however, no incidence was observed after diagnosis of XLA. It was previously reported that neutropenia was not found in XLA patient receiving IVIG replacement therapy (16, 17). IVIG replacement therapy might therefore reduce the incidence of infection, thus eliminating occurrence of neutropenia.

Other primary antibody deficiencies are also associated with neutropenia, but the pathogenesis of neutropenia in XLA patients remains unclear (22). Patients with common variable immunodeficiency often have neutropenia, usually concomitant with thrombocytopenia and/or hemolytic anemia, and it is therefore possible that neutropenia occurs as a result of autoimmune mechanisms. Neutropenia is also seen in about half of all patients with X-linked hyper IgM syndrome, which is caused by a mutation in the CD40 ligand gene. Neutropenia associated with X-linked hyper IgM syndrome might also be caused by autoimmunity, since patients sometimes manifest autoimmune diseases. Another possibility is that interaction between CD40 on stromal cells and CD40 ligand on T cells produces granulocyte colony-stimulating factor (G-CSF), and therefore, abnormalities of CD40 ligand might contribute to reduced G-CSF synthesis leading to neutropenia (23). Kozlowski and Evans (15) suggested that XLA-associated neutropenia might be caused by increased destruction of neutrophils by bacterial toxins. However, in this study, neutropenia was observed in patients with mild bacterial infections, and even in those with viral infections.

Table III. BTK Protein Expression and *BTK* Gene Mutations in the XLA Patients with Neutropenia

Patient No.	BTK protein expression (%)	Nucleotide substitution	Amino acid substitution
P1 (P52-2)[a]	4.6	787delG	V219delX228
P2 (*P9*)[b]	0.2	NE[c]	Exons16-18 skip
P3 (P17)	2.7	1816C>T	R562W
P4	NE	1925delAT	Y598X
P5 (P78)	0.5	2011del(5nt) /ins(13nt)	Exons16-18 skip /Exons17-18 skip
P6 (P57-2)	8.3	1089delGT	V319delX321
P7 (P77)	5.8	NE	Exons9-11 skip /Exons14-16 skip
P8 (P73-1)	7.1	612insA	K160ins193X
P9 (P19)	4.1	1877C>T	A582V
P10 (P58)	8.2	IVS12+1G>A	Exon12 skip
P11 (P33)	5.8	888G>A	W252X
P12 (P43)	8.0	IVS2-1G>T	R48delX51
P13	NE	861insCT	I243ins277X
P14 (P57-1)	6.2	1089delGT	V319delX321
P15 (P6)	3.5	424A>G	F98V
P16 (P18)	59.1	1833A>C	E567D

[a]Described previously (7).
[b]Reported previously (18).
[c]Not examined.

The *BTK* gene responsible for XLA is expressed in monocytes as well as B cells in peripheral blood (10). In XLA patients, BTK protein expression in monocytes was reduced, but the roles of monocytes in XLA remains unknown. Recently, it was reported that monocyte phagocytosis and chemotaxis are impaired in XLA patients (12). In addition, LPS-induced TNF-α production in monocytes of XLA patients previously appeared diminished (13), and it is possible that BTK might be involved in a toll-like receptor pathway of monocytes (24). These studies suggest that BTK might play a role in the immunological response of monocytes. Deficient cytokine or chemokine production in the monocytes might be involved with neutropenia in XLA patients.

In this study, approximately 20% of the Japanese XLA patients studied had associated profound neutropenia as demonstrated in previous studies (14, 16, 17). Some neutropenic patients had severe bacterial infections, and two suffered from fatal *Pseudomonas* sepsis. Severe bacterial infections among XLA patients might therefore be caused not only by antibody deficiencies but also by neutropenia. After the initiation of IVIG treatment, neutropenia was not observed. Therefore, in conclusion, early diagnosis and initiation of IVIG therapy is important for better prognosis of XLA patients.

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