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Persistent Hypogammaglobulinemia Following Mononucleosis in Boys Is Highly Suggestive of X-Linked Lymphoproliferative Disease—Report of Three Cases

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Hypogammaglobulinemia is a common symptom in different immunodeficiencies. It is, however, not usually associated with Epstein–Barr virus (EBV) infections. The X-linked lymphoproliferative disease (XLP) on the other hand shows immunological changes in response to the EBV. Here we report three previously healthy boys, all of which developed persistent hypogammaglobulinemia following severe acute infectious mononucleosis. All three patients revealed T-cell abnormalities including inverted CD4/CD8 and increased $CD8^+$ T-cell numbers. The number of IFN-γ-producing T cells were markedly increased in the two patients studied so far. In addition, patient 2 showed mainly T cells, instead of B cells, to be infected with the EBV. Apart from an uncle of patient 3, who died of malignant lymphoma, family history was unremarkable in all cases. All three patients exhibited mutations in the SH2D1A gene, establishing the diagnosis of XLP. Protein expression was found on immunoblot analysis in one patient with a missense mutation. Development of persistent hypogammaglobulinemia after severe primary EBV infection seems to be a specific diagnostic sign for XLP even in males with unremarkable family history.

KEY WORDS: X-linked lymphoproliferative disease; Epstein–Barr virus infection; hypogammaglobulinemia; SH2D1A gene mutations.

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INTRODUCTION

Infectious mononucleosis as manifestation of primary Epstein–Barr virus (EBV) infections in young children is rare. The course of the infection is usually asymptomatic or accompanied by mild, nonspecific manifestations The full clinical picture of acute infectious mononucleosis typically occurs in adolescents and adults (1, 2).

Here we report three unrelated boys who all suffered from unusually severe acute infectious mononucleosis at ages between 1 and 7 years followed by the development of persistent panhypogammaglobulinemia.

PATIENTS AND METHODS

Patients

Case 1

This boy suffered from severe acute infectious mononucleosis with hepatic involvement at the age of 2 years. During acute illness serum immunoglobulin levels were markedly increased (IgG 1833 mg/dL, normal range for the age: 200–1000; IgM 1214 mg/dL, normal range for the age: 40–170; IgA 349 mg/dL, normal range for the age: 15–120) (Table I). Serum IgM and IgG antibodies against EBV viral capsid antigen (VCA) were found only transiently. The patient was followed up 6 weeks after discharge due to persistent illness, and decreasing serum immunoglobulin levels were noted (IgG 551 mg/dL, IgM 52 mg/dL, IgA 52 mg/dL). In the course of the following months, the patient suffered from recurrent respiratory tract infections and developed panhypogammaglobulinemia 6 months after diagnosis of infectious mononucleosis (IgG 133 mg/dL, IgM 13 mg/dL, IgA 15 mg/dL) (Table I). CD4/CD8 ratio was decreased initially (0,4) and

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Table I. Immunological and Virological Findings in Three Boys Who Developed Persistent Hypogammaglobulinemia After Severe Acute Infectious Mononucleosis

	Patient 1	Patient 2	Patient 3
Acute infectious mononucleosis, at age	2 year	12 month	7 year 3 month
EBV status during EBV infection	anti-VCA-IgM+ anti-VCA-IgG+	anti-VCA-IgM+ anti-VCA-IgG+ anti-EBNA+ 12 d later	anti-VCA-IgM+ anti-VCA-IgG- anti-EA – anti-EBNA –
EBV status after EBV infection, at age	2 year 2 month EBV serology completely negative	1 year 2 month anti-VCA-IgG+[b] anti-EBNA+[b]	7 year 4 month EBV serology completely negative
Serum immunoglobulins during EBV infection, at age[a]	2 year	12 month	7 year 4 month
IgG (mg/dL)	1833 (200–1000)	875 (200–1000)	normal gamma-globulin peak (serum protein electrophoresis)
IgM (mg/dL)	1214 (40–170)	770 (40–170)	n.d.
IgA (mg/dL)	349 (15–120)	144 (15–120)	n.d.
Serum immunoglobulins after EBV infection, at age	2 year 6 month	12 month 2 week	8 year 11 month
IgG (mg/dL)	133	200	< 150
IgM (mg/dL)	13	5	< 20
IgA (mg/dL)	15	1	< 8
T-cell subpopulations, at age[a]	2 year	12 month	8 year 11 month
CD4/CD8 ratio	0.4 (1.0–1.6)	0.6 (1.5–2.9)	0.5 (1.1–1.4)
$CD3^+$ T cells (absolute)	14589/mL (1800–3000)	5472/mL (1460–5440)	5286/mL (1400–2000)
$CD3^+$ T cells (%)	91% (62–69)	96% (53–81)	80% (66–76)
$CD8^+$ T cells (absolute)	10 614/mL (630–1910)	2900/mL (570–2230)	3425/mL (600–900)
$CD8^+$ T cells (%)	66% (22–38)	53% (16–38)	52% (27–35)
$CD4^+$ T cells (absolute)	4462/mL (1000–1800)	1751 (1020–3600)	1544 (700–1100)
$CD4^+$ T cells (%)	28% (30–40)	32% (31–54)	23% (30–41)
HLA-DR^+ $CD3^+$ T cells (absolute)	7576/mL (92–316)	3228/mL (60–280)	1960/mL (160–320)
HLA-DR^+ $CD3^+$ T cells (%)	47% (6–16)	59% (4–9)	30% (9.5–17)
IFN-γ producing $CD3^+$ T cells (%)	n.d.	73,6% (25.5—38.5)[c]	70% (23–33)
SH2D1A gene mutations			
Nucleotide change	G378 → A	C462 → T	deletion of all exons
Effect on coding sequence	G27S (missense)	R55X (nonsense)	deletion

Note. EA, (Epstein–Barr virus) early antigen; VCA, EBV viral capsid antigen; EBNA, Epstein–Barr nuclear antigen; n.d., not determined.
[a] In parentheses: normal values for age.
[b] During IVIG therapy.
[c] At age 13.

was still low after 6 months (0,59), with highly elevated $CD8^+$-T-lymphocytes (66%, absolute count 10.614/μL) both at time of diagnosis and 6 months later (absolute count 4400/μL, 50%). $CD19^+$-B-lymphocytes were elevated during acute infectious mononucleosis (absolute count 5133/μL, 32%) and within normal range on follow-up. Further immunological investigations revealed decreased function of lymphocytes and granulocytes (lymphocyte proliferative response to mitogens such as phytohemagglutinin, Concavalin A, Interleukin-2 and OKT3, stimulation of granulocytes with NBT) and weak intracutaneous reaction to recall antigens. Furthermor, no specific serum antibodies against different protein and polysaccharide antigens (including EBV antigens) were found in this patient.

This patient has been treated with intravenous immunoglobulin every 3–4 weeks ever since and has not presented with severe infections again apart from an episode of chronic sinusitis.

Case 2

The first clinical and laboratory data of this patient have already been published (3). At the age of 12 months this boy was diagnosed with severe acute infectious mononucleosis and EBV-associated meningoencephalitis with seizures, impaired liver function and depletion of bone marrow. The patient was treated with acyclovir (50 mg/kg/day, for a period of 4 months), blood transfusions and prednisolone (1 mg/kg/day). While liver and bone marrow function tests returned to normal within 4 weeks after onset of therapy, his neurological status fully recovered 8 weeks after admission. Electroencephalography (EEG), abnormal at admission to the hospital, returned to normal. Cranial magnetic resonance imaging (MRI) showed multiple lesions in both hemispheres.

On admission (at 12 months of age), serum immunoglobulin levels were normal for IgG with 875 mg/dL (normal range for the age: 200–1000) and increased for IgM with 770 mg/dL (normal range for the age: 40–170)

and IgA with 144 mg/dL (normal range for the age 15–120), but decreased within 2 weeks to almost undetectable levels (IgG 200 mg/dL, IgM 5 mg/dL, and IgA 1 mg/dL) (Table I). Since then, secondary hypogammaglobulinemia has persisted and the patient has been treated with IVIG monthly.

During the acute phase of infectious mononucleosis, EBV genome and EBV latent membrane antigen (LMP) was shown to be present in 70% of the boy's peripheral $CD3^+$ T cells (3). The patient presented initially with positive serum IgM and IgG antibody titers for EBV VCA. Twelve days later, serum antibodies against Epstein–Barr nuclear antigen 1 (EBNA-1) were also detected. Anti-VCA and anti-EBNA-1 antibodies have persisted up to the present date.

At onset of EBV infection, peripheral $CD3^+$ T cells, $CD8^+$ T cells, and HLA-$DR^+/CD3^+$T cells were increased, whereas $CD4^+$ T cells were within normal range (Table I). CD4/CD8 ratio was decreased (0.6; normal for the age: 1.5–2.9). The lymphocyte proliferative response to mitogens (PWM, PHA, Con A) was markedly decreased. NK cell activity, as shown by ^{51}Cr-release assay, was increased. Altered lymphocyte subsets and impaired lymphocyte function returned to normal after 8 months. Recent analysis now showed elevated numbers of interferon γ (IFNγ) producing $CD3^+$ (73.6%, normal for the age: $32.0 \pm 6.5\%$) and $CD8^+$ T cells (73.4%, normal for the age: $46.2 \pm 22.9\%$) after stimulation with PMA and Ionomycin for 6 h in this patient (at age 13) (Table I).

Further development of the child was quite encouraging. The boy started walking at the age of 24 months and has been attending school since age 7. A single focal seizure occurred at age 11 due to residual lesions caused by the meningoencephalitis. An EEG study showed focal activity over the right occipital hemisphere. Since then, anticonvulsive treatment with carbamazepine has successfully prevented further seizures. EEG patterns have returned to normal on retesting, but cranial MRI findings remained unchanged.

Case 3

The pedigree of this family is shown in Fig. 1. Subject II:1, an uncle of patient 3, died from malignant lymphoma at age 35 years. The mother (II:2) is affected by neurofibromatosis type I (NF-I) and exhibits mild psychomotor retardation. The patient (III:1) and his otherwise healthy sister (III:2) show similar features of NF-I (cafe-au-lait spots, axillary freckling, hamartomas of cerebellum). At age 7 years 3 months the boy suffered from severe acute infectious mononucleosis but completely recovered within 5 weeks. Serum protein electrophoresis revealed a gammaglobulin fraction of 11.5% (normal range 10–19%). During the acute phase of disease, serum IgM antibodies against EBV VCA were detectable, whereas IgG antibodies against VCA, EA or EBNA were absent. In the course of the following year recurrent upper respiratory infections and several episodes of pneumonia occurred. At age 8 years 11 months a diagnosis of severe bronchiectasis of both lungs and panhypogammaglobulinemia (Serum IgG $<$ 150 mg/dL, IgM $<$ 20 mg/dL, IgA $<$ 8 mg/dL) was made. Specific serum antibodies against EBV, other viruses and recall antigens were negative. Absolute number of peripheral lymphocytes (5700/mL; normal for the age: 2400 (2000–2700)/mL) and $CD8^+$ T cells (3425/mL; normal for the age (600–900)/mL) were increased, while the CD4/CD8 ratio was decreased (0.5; normal for the age 1.1–1.4) (Table I). After stimulation with PMA/Ionomycin, 70% of $CD3^+$ T cells showed intracytoplasmic IFNγ (normal for the age $28.0 \pm 5.3\%$) (Table I).

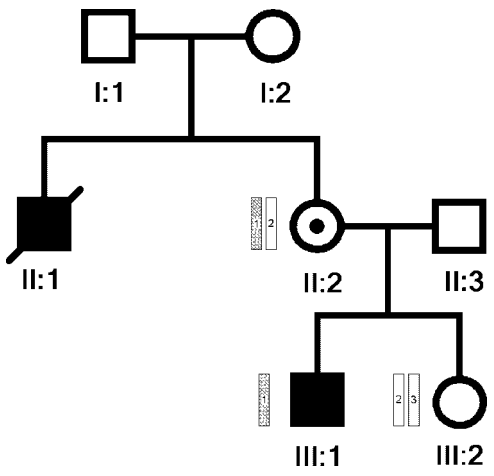

Fig. 1. Pedigree of family 3.XLP (black symbols) and the DXS424 genotype (allele 1, marked by a grey bar) were coinherited by the mother (I:2, healthy carrier) and passed on to the affected son (III:1) only. SH2D1A sequence analysis revealed a large deletion covering all four exons in the patient and his mother. The sister (III:2) exhibited the wild type only. Independently, neurofibromatosis type I was inherited from the mother (II:2) to both children (III:1 and III:2) (not shown in pedigree).

Under monthly IVIG substitution (480 mg/kg body weight) respiratory infections decreased and the patient's clinical condition is now in a sufficient state.

Molecular Genetic Studies

Patient Samples and Cell Preparation

Heparinized or EDTA-treated peripheral blood was obtained by venipuncture from patients with X-linked

lymphoproliferative disease (XLP), family members of the patients, and healthy volunteers. Informed consent was obtained in each instance prior to analysis from adult patients and healthy controls or for children from both their legal guardians.

Lymphocyte subpopulations were either analyzed in peripheral blood by flow cytometry or extracted by a Ficoll-Hypaque gradient separation and stored in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin solution, and 10% DMSO in liquid nitrogen for analysis at a later date.

Mutation Analysis of the SH2D1A Gene

Genomic DNA, extracted from peripheral whole blood by standard methods, was amplified by PCR with primers covering all four exons of the SH2D1A gene. Amplified DNA products were directly sequenced as described previously (4). Haplotype analysis with X-chromosomal marker *DXS424*, which is located proximally to the SH2D1A gene, was performed as previously described (5).

Southern Blot Analysis

Genomic DNA was digested with *Hin*dIII or *Pst*I according to the manufacturer's recommendations, electrophoresed in 0.8% agarose gels and blotted onto a nylon membrane. The filter was hybridized with ^{32}P-labeled SH2D1A cDNA as described previously (4).

Flowcytofluorometric Analysis

Cells were stained with fluorescein isothiocyanate or phycoerythrin (FITC or PE)-conjugated murine monoclonal antibodies (mAbs). The following mAbs were used for two-color analysis: anti-CD3-FITC, anti-CD4-PE, anti-CD8-PE, anti-CD14-PE, anti-CD16-PE, anti-CD19-PE, anti-CD45-FITC, anti-CD56-PE, anti-HLA-DR-PE (BD Biosciences), and anti-IgG1-FITC/anti-IgG2a-PE (BD Biosciences) as isotype control. The method for intracellular staining was adapted from that of Pala *et al.* (6). Cells were incubated with phorbol myristate acetate 10 μg/mL, ionomycin 1 μg/mL, and monensin 2 mmol/L (each Sigma-Aldrich, Taufkirchen, Germany) for 24 h. Cells were washed twice in PBS/1% FCS, then fixed with 0.5 mL ice-cold 4% paraformaldehyde/PBS for 10 min. Following that, cells were resuspended in PBS containing 0.3% saponin (Sigma). The cells were incubated for simultaneous surface and intracellular staining with anti-CD16/CD56-FITC (BD Biosciences) and anti-IFN-gamma-PE (Coulter/Immunotech, Krefeld, Germany) for 30 min at 4°C. After washing, the cells were analyzed by flow cytometry on a FACScan® (BD Biosciences) instrument.

Antibody Generation and Immunoblot Analysis

SH2D1A monoclonal antibodies were induced in Lou/C rats with full length HIS-tagged XLP protein according to standard procedures. XLP 1D12 of rat IgG2a subclass was tested positive in western blotting and used for this study.

PBMCs resuspended in RPMI 1640 with added 10% FCS and 1% Penicillin/Streptomycin were stimulated in 24-well flat-bottomed culture plates with 10 μg/mL phytohaemagglutinin (PHA) for 96 h. Cells were then lysed in Bellido lysis buffer (10% Brij 97 in 40 mM Tris-HCl pH 7.4, 300 mM NaCl, 2 mM EDTA, 20 mM NaF, 2 μg/mL Pepstatin, 5 μg/mL Leupeptin, and 0.14 U/mL Aprotinin) and protein concentration was measured by reaction with Bradford reagent (Bio-Rad Laboratories, Munich, Germany). μwenty microgram of protein lysate was mixed with SDS sample buffer and boiled for 5 min. Samples were size fractionated on a 15% acrylamide gel and transferred to a PVDF membrane (Amersham, Freiburg, Germany). The membrane was then split in two parts (carrying the 42 kDa β-Actin band and the 16 kDa SH2D1A band, respectively), incubated in 0.1 M phosphate buffer (pH 2.0) and subsequently blocked in 5% skimmed milk. β-Actin was detected using mouse anti-β-actin mAb (Sigma, St.Louis, USA) and peroxidase-labeled rabbit anti-mouse antibody (Daco A/S, Glostrup, Denmark). SH2D1A detection was performed using supernatant from 1D12-producing hybridoma cells at 1:10 dilution and peroxidase-labeled goat anti-rat antibody (Zymed, San Francisco, USA).

RESULTS

All three patients suffered from severe acute infectious mononucleosis at the age of 12 months, 2 years, and 7 years 3 months. Within a period of 2 weeks (patient 2), 6 months (patient 1), and 19 months (patient 3) all subjects developed persistent hypogammaglobulinemia. During acute EBV infection, serum IgM and IgA were temporarily increased in patients 1 and 2 (not tested in patient 3). The CD4/CD8 ratio was decreased and the number of $CD3^+$ and $CD8^+$ T cells were increased in patients 1 and 2 (measured during acute infectious mononucleosis) and in patient 3 (measured 19 months after acute infectious mononucleosis). Most of $CD3^+$ T cells were in an activated state (HLA DR+). The frequency of IFN-gamma producing $CD3^+$ T cells, so far tested for patients 2 and 3 only, was shown to be markedly increased (Table I). EBV serological response was normal only in patient 2 and deficient in patient 1 and 3.

In all three patients, the SH2D1A gene was mutated: patient 1 exhibited a missense mutation G27S, patient

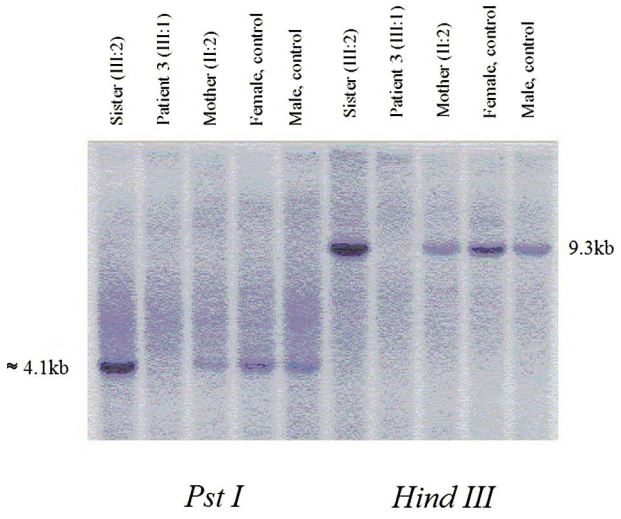

Fig. 2. Southern blot hybridization with ^{32}P-labeled SH2D1A cDNA (*Pst*I and *Hin*dIII digest): Patient 3 and other healthy family members. For details see text.

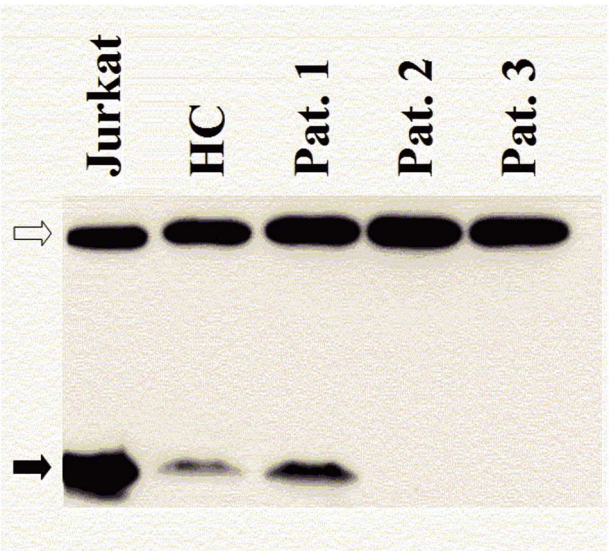

Fig. 3. Immunoblot analysis of three patients with XLP. Lysates from Jurkat cells and PBMC from a healthy control (HC) and the three patients were electrophoresed, transferred on PVDF membrane and cut in two. The upper half of the membrane (42 kDa, marked by white arrow) was blotted with antibody against β-actin as loading control. The lower half was blotted with 1D12 antibody against SH2D1A (16 kDa, marked by black arrow). Patient numbers correspond with numbering in the text.

2 a nonsense mutation R88X (Table I). In patient 3 the SH2D1A gene was completely deleted (Fig. 2). Indirect genotype analysis confirmed the maternal inheritance of the gene (Fig. 1). SH2D1A protein expression was tested in all patients and was found to be completely absent compared to normal controls in patients 2 and 3 (Fig. 3). However, patient 1 exhibited a SH2D1A band of normal size, indicating the production of a missense protein.

DISCUSSION

Hypogammaglobulinemia in early childhood is a common manifestation of different immunodeficiencies and is in clinical practice mostly treated as common variable immunodeficiency. In many cases, no discernible reason for the lack of immunoglobulin production can be found, although the "triggering" effect of infections, including EBV, has been described in several instances (7, 8). XLP (McKusick's OMIM number 308 240) is a rare immunodeficiency presenting in the majority of cases as fatal infectious mononucleosis at an unusually low age (9). Hypogammaglobulinemia occurs in approximately one third of patients and can manifest with or, more rarely, without prior exposure to EBV (10–12). The gene causing XLP when defective was identified and described in 1998 and termed SH2D1A or SAP (for signalling lymphocyte activation molecule (SLAM) associated protein) (13). This gene encodes a protein which interacts with the SLAM (or CD150), a T-cell surface marker and self-ligand, and several related receptors (14). It has been shown in animal models that the absence of SH2D1A leads to a shift towards a T_H1 cytotoxic immune response poorly controlled by the immune system (15). Apart from the "classical" XLP manifestations, SH2D1A mutations have been found in different immunodeficiencies with widely varying clinical pictures (16–18).

Persistent hypogammaglobulinemia following acute infectious mononucleosis should be considered highly suspicious of XLP. Considering the facts that 1) all cases reported so far, including this report, are male and 2) all patients presented with manifest mononucleosis at a very young age, we can assume that most—if not all—of these cases were caused by SH2D1A mutations. In addition, all patients studied so far exhibited further immunologic T-cell abnormalities (e.g., increased numbers of activated $CD8^+$ T cells, inverted CD4/CD8 ratio) (7). However, the two patients of Zuccaro *et al*. (7) and Lanning *et al*. (8) were not examined for mutations in the SH2D1A gene.

In patient 2 we identified the SH2D1A gene nonsense mutation R58X, which so far has been shown to be the most common mutation in XLP patients (19–23). Patients with the R58X mutation exhibit a wide phenotypic variability (21, 24) suggesting additional genetic or environmental influencing factors. In patient 3, a large deletion was found encompassing all four exons of the SH2D1A gene. Both patients 2 and 3 showed complete lack of SH2D1A production on immunoblot (Fig. 3). Patient 1

presented with the missense mutation G27S, previously reported in an XLP patient of Japanese origin suffering from fulminant acute infectious mononucleosis at age 1 year, who died 1 month after onset of disease (25). Despite the mutation and the demonstrated lack of SH2D1A function, immunoblotting blotting of protein from patient 1 showed a distinct band of appropriate size (Fig. 3). Considering experiences with protein assays for other immunodeficiencies, the production of a presumably nonfunctional SH2D1A protein, while not yet described, is not completely unexpected (26). Morra *et al*. have already described XLP missense mutations producing SH2D1A with normal half-life in human cell lines (27).

Of note is the fact that patient 2 is the first patient with XLP reported so far in whom EBV genome and antigen could be detected in approximately 70% of his peripheral T cells (3). In general, B cells are the primary target of EBV viral infection in both mononucleosis and other EBV-associated disorders (28). EBV-infected T cells have been found in only one case of fatal acute mononucleosis (29) and in patients with severe chronic active EBV-infection (30, 31), the latter group having an increased risk for the development of malignant T-cell lymphoma (32, 33). Only 6% of XLP-associated lymphoma are of T-cell origin, thus, a general association of XLP with an increased rate of T-cell infection by EBV seems unlikely (24, 34).

The development of bronchiectasis in XLP patients seems to be a rare event. Only five cases have been reported so far, including patient 3 of this report (16, 18, 35). Somewhat surprisingly, in two of these patients this complication developed despite normal serum immunoglobulin levels (35). Patient 3 also shows a coincidental occurrence of both XLP and NF-I, independently inherited from the mother. A similar concomitant occurrence has been reported only once for common variable immunodeficiency (CVI) and NF-I (36) (Pub Med Search 1966–2003).

Hypogammaglobulinemia in XLP mice has been shown to be the result of failure to develop long-lived memory B cells. SH2D1A seems to have a crucial role in late B cell maturation while leaving the early humoral response intact (37, 38). The development of persistent hypogammaglobulinemia in XLP has also been attributed to a sustained regulatory "suppressor" T-cell activity, which would presumably influence late B cell maturation: In XLP patients with hypogammaglobulinemia, the *in vitro* synthesis of IgG, IgM, and IgA by lymphoblastoid cell lines (B-LCL) is markedly decreased only in the presence of autologous $CD8^+$T cells (39, 40). The degree of this $CD8^+$ T cell-mediated suppression of immunoglobulin (Ig) synthesis correlated with the serum levels of IgM and IgG in these patients (39). The pathogenesis and the characteristics of these regulatory T cells is still unknown. The T-cell abnormalities found in our three XLP patients might possibly reflect this sustained "suppressor" activity.

As mentioned above, *in vitro* and *in vivo* findings suggest that mutations in the SH2D1A gene lead to a block of the final stages of T helper 2 cell development (Th2) resulting in a skewing towards an overwhelming Th1 response (20). SH2D1A-knockout mice ($SH2D1A^{null}$ mice) show increased numbers of $CD8^+$ and $CD4^+$ IFNγ-producing T cells after infection with lymphocytic choriomeningitis virus (LCMV), a virus that elicits strong and well-defined immune responses. Moreover, T cells of these animals exhibit an inherited deficiency in IL-4 gene activation and increased IFNγ production following TCR triggering (41). In accordance with this SH2D1A-knockout mouse model, we were able to detect an increased frequency of IFNγ-producing $CD3^+$ T cells in XLP patients (patients 2 and 3). This finding was recently corroborated in a third patient with XLP (42). A shift towards Th1 memory T cells was also recently described in an XLP patient (38).

In conclusion, severe acute infectious mononucleosis followed by persistent hypogammaglobulinemia in boys is strongly indicative of XLP. Diagnostic screening for SH2D1A, preferably by mutation screening, might help identifying other family members at risk. The role of the SH2D1A protein in the pathogenesis of hypogammaglobulinemia including potential regulatory "suppressor" T-cells mechanisms remains to be elucidated.

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