

ORIGINAL ARTICLE

An Antibody-Deficiency Syndrome Due to Mutations in the *CD19* Gene

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ABSTRACT

BACKGROUND

The CD19 protein forms a complex with CD21, CD81, and CD225 in the membrane of mature B cells. Together with the B-cell antigen receptor, this complex signals the B cell to decrease its threshold for activation by the antigen.

METHODS

We evaluated four patients from two unrelated families who had increased susceptibility to infection, hypogammaglobulinemia, and normal numbers of mature B cells in blood. We found a mutation in the *CD19* gene in all four patients. The *CD19* gene in the patients and their first-degree relatives was sequenced, and flow-cytometric immunophenotyping of B cells, immunohistochemical staining of lymphoid tissues, and DNA and messenger RNA analysis were performed. B-cell responses on the triggering of the B-cell receptor were investigated by in vitro stimulation; the antibody response after vaccination with rabies vaccine was also studied.

RESULTS

All four patients had homozygous mutations in the *CD19* gene. Levels of CD19 were undetectable in one patient and substantially decreased in the other three. Levels of CD21 were decreased, whereas levels of CD81 and CD225 were normal, in all four patients. The composition of the precursor B-cell compartment in bone marrow and the total numbers of B cells in blood were normal. However, the numbers of CD27+ memory B cells and CD5+ B cells were decreased. Secondary follicles in lymphoid tissues were small to normal in size and had a normal cellular composition. The few B cells that showed molecular signs of switching from one immunoglobulin class to another contained V_H-C_α and V_H-C_γ transcripts with somatic mutations. The response of the patients' B cells to in vitro stimulation through the B-cell receptor was impaired, and in all four patients, the antibody response to rabies vaccination was poor.

CONCLUSIONS

Mutation of the *CD19* gene causes a type of hypogammaglobulinemia in which the response of mature B cells to antigenic stimulation is defective.

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PRIMARY ANTIBODY DEFICIENCIES, WHICH are associated mainly with susceptibility to bacterial infections, can be caused by mutations in genes involved in B-cell differentiation.¹ Such genetic defects block the differentiation of immature B cells in bone marrow, thereby causing both a deficiency of mature B cells in the peripheral circulation and agammaglobulinemia.²⁻⁸

In disorders involving defects in the maturation process of antigen-responsive B cells, by contrast, there are mature B cells in blood and a deficiency of some, but not all, immunoglobulin classes (dysgammaglobulinemia) or hypogammaglobulinemia. These disorders fall into two categories. The hyper-IgM syndromes are those in which a genetic defect impairs immunoglobulin-class switching (e.g., IgM to IgG) or impairs somatic mutation of immunoglobulin genes (a molecular sign of a response to an antigen), or both.⁹⁻¹² The second category is common variable immunodeficiency, a syndrome involving hypogammaglobulinemia, recurrent bacterial infections, and a lack of antibody production in response to vaccination. Genetic defects have been found in some patients with common variable immunodeficiency,^{13,14} but no genetic abnormality is evident in more than 80 percent of such patients.

During B-cell differentiation in the bone marrow, the surface molecule CD19 appears early and remains on the B cell until it differentiates into a plasma cell. Four proteins on the surface of mature B cells — CD19, CD21, CD81, and CD225 — form the CD19 complex, which signals in conjunction with the B-cell antigen receptor, thereby decreasing the threshold for receptor-dependent signaling.¹⁵

We describe four patients, from two unrelated families, who had hypogammaglobulinemia and defective CD19. These patients had normal levels of B cells in blood but had undetectable or very low levels of surface CD19, owing to a homozygous mutation in the *CD19* gene. The deficiency of antibody in these patients resulted from a subnormal response to antigen by mature B cells.

CASE REPORTS

Patient 1, a girl of Turkish descent and the second child of consanguineous parents (second cousins), was referred to the Selçuk University Hospital in

Konya, Turkey, at 10 years of age with a seven-month history of intermittent hematuria. She also had a history of recurrent bronchiolitis and bronchopneumonia starting at one year of age and meningitis starting at eight years of age. On physical examination, her heart rate was 118 beats per minute and auscultation of the lung revealed rhonchi and wheezing. The differential blood count was normal, but urinalysis showed microscopic hematuria without proteinuria. The complement levels were normal, tests for antinuclear antibodies were negative, the erythrocyte sedimentation rate was 41 mm per hour, and the level of C-reactive protein was 93 mg per deciliter. The dermal response to purified protein derivative after two vaccinations with bacille Calmette–Guérin was 12 mm. Renal ultrasonography showed no abnormalities; a renal biopsy revealed proliferation of mesangial cells, interstitial edema, and an apparently normal basement membrane of the glomerulus. She was given a diagnosis of postinfectious glomerulonephritis and was also found to have hypogammaglobulinemia (Table 1).

Patients 2, 3, and 4 were siblings, children of unrelated parents of Colombian descent, and were examined at the Primary Immunodeficiencies Clinic of the University of Antioquia in Medellín, Colombia, at 35, 33, and 49 years of age, respectively. All three had had otitis media, sinusitis, and pharyngitis during childhood. In addition, Patient 2 (the index patient) had had four bouts of pneumonia between the ages of 18 and 35 years; more recently, he had received diagnoses of bacterial conjunctivitis and chronic gastritis (*Helicobacter pylori* infection). He was given a diagnosis of hypogammaglobulinemia due to common variable immunodeficiency (Table 1). His two sisters also had hypogammaglobulinemia (Table 1) and histories of recurrent upper respiratory tract infections. Patient 3 first had pneumonia at 20 years of age, herpes zoster at 30 years of age, and more recently, recurrent bacterial conjunctivitis with dacryocystitis and diarrhea. Patient 4 had had pneumonia at eight years of age, recurrent skin abscesses and more bouts of pneumonia during childhood, and chronic diarrhea, bronchitis, and recurrent bacterial conjunctivitis during adulthood. These three patients had had surgery for chronic sinusitis.

Intravenous immune globulin therapy was initiated in all four patients soon after the diagnosis of hypogammaglobulinemia. All patients

Table 1. Baseline Characteristics and Laboratory Findings in Four Patients with CD19 Deficiency.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Sex	F	M	F	F
Age at onset — yr	1	7	6	5
Age at diagnosis — yr	10	35	33	49
Lymphocytes — cells/mm ³				
Total lymphocytes	4480	2182	2508	2059
CD3+ T cells	3270	1520	1855	1384
CD4+ T cells	1792	713	1070	620
CD8+ T cells	1478	720	692	696
CD20+ B cells	806	286	521	268
CD16+CD56+ natural killer cells	313	277	348	288
Serum immunoglobulin level — mg/dl (95% CI for age-matched controls)*				
IgG	325 (842–1943)	204 (804–2212)	198 (814–2047)	256 (814–2047)
IgA	292 (62–390)	18 (99–489)	7 (99–489)	19 (81–538)
IgM	25 (54–392)	47 (58–324)	30 (68–379)	63 (42–600)
Blood group	A, Rh+	O, Rh+	O, Rh+	O, Rh+
Isohemagglutinin levels	Not detectable	Low	Low	Low

* Serum levels at diagnosis are shown. Subnormal values are given in bold type. CI denotes confidence interval. Age-matched controls have been described previously.¹⁶

are now clinically well, with no signs of lymphoproliferation, cancer, or autoimmunity. The other siblings of both families have normal serum immunoglobulin levels and no symptoms of immunodeficiency. However, one sibling of Patients 2, 3, and 4 was given a diagnosis of systemic lupus erythematosus at 13 years of age, according to the criteria of the American Rheumatism Association.¹⁷

METHODS

Diagnostic workups of blood, biopsy of bone marrow, cervical lymph node, and tonsil, and rabies vaccination were carried out in patients and family members after they provided oral informed consent and according to the guidelines of the local medical ethics committees in Turkey, Colombia, and the Netherlands. Controls provided written consent.

AMPLIFICATION AND SEQUENCE ANALYSIS OF THE CD19 GENE

The *CD19* gene on chromosome 16 (16p11.2) spans 7.5 kb of genomic DNA and codes for a protein of

556 amino acids, including the signal peptide.¹⁸ DNA was isolated from granulocytes that had been isolated with the use of Ficoll gradient centrifugation. All 15 exons were amplified by the polymerase chain reaction (PCR) (the primer sequences are listed in Part A of the Supplementary Appendix, available with the full text of this article at www.nejm.org) and were sequenced on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

Total complementary DNA (cDNA) was prepared from messenger RNA (mRNA) that had been isolated from the patients' mononuclear cells, as described previously.¹⁹ Fragments of *CD19* cDNA were amplified and sequenced to verify the mutations found in the genomic DNA.

CD19 TRANSCRIPTS

Levels of the *CD19* transcript in blood B cells were quantified from reverse-transcribed mRNA from patients' blood mononuclear cells with the use of TaqMan real-time quantitative PCR with newly designed primers and a FAM-TAMRA-labeled fluorogenic probe (sequences are given in Part B of the Supplementary Appendix). The transcript lev-

els were normalized against those of a control *ABL* gene and corrected for the numbers of B cells as determined by means of flow-cytometric analysis.²⁰

ANALYSIS OF SOMATIC HYPERMUTATION

Hypermuation of immunoglobulin genes was studied in the V_H3-C_α , V_H4-C_α , V_H3-C_γ , and V_H4-C_γ fragments, which were amplified from the cDNA and cloned into a pGEM-T easy vector (Promega). Twenty-one clones of Patient 1, 44 clones of Patient 2, 39 clones of Patient 3, and 39 clones of Patient 4 were sequenced. The International ImmunoGeneTics information system (<http://imgt.cines.fr/>) was used to identify the V, D, and J segments and somatic mutations.²¹ The ratio of replacement and silent mutations was determined for the framework regions and the complementarity-determining regions, and the distribution of replacement mutations in the framework regions was analyzed according to the binomial distribution model of Chang and Casali.²²

FLOW CYTOMETRY

Flow-cytometric immunophenotyping of blood samples from four patients, nine relatives (including eight carriers), and eight controls was performed to evaluate markers and subgroups of B cells. We also analyzed bone marrow samples from Patient 1 and from five controls, as previously described.²³ Antibodies against CD21 (LB21), CD81 (JS-81), and CD225 (Leu13) were used to analyze the CD19 complex further.^{15,24}

WESTERN BLOTTING

B cells in blood were sorted from mononuclear cells with the use of the magnetic affinity cell-sorting B-cell purification kit (Miltenyi Biotec). Lysates of B cells (2×10^6) were separated by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and immunoblotted with the use of a polyclonal rabbit antiserum against the intracellular portion of human CD19.²⁵

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The biopsy specimen of a cervical lymph node from Patient 1 and the tonsil-biopsy specimen from Patient 2 were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Immunohistochemistry was performed according to standard procedures involving monoclonal antibodies against CD20 (L26), CD21 (1F8),

CD79a (JCB117), BCL-2 (124), BCL-6 (PG-B6p), and Ki67 (MIB-1), all obtained from DakoCytomation.

B-CELL ACTIVATION

Blood mononuclear cells were incubated with $6 \mu\text{g}$ of indo-1 per milliliter of cell suspension (Molecular Probes) to evaluate calcium fluxes after stimulation. Levels of free intracellular calcium were measured in CD20+ B cells with the use of flow cytometry in a FACSVantage station (BD Biosciences) before and after stimulation with *Staphylococcus aureus* Cowan I (Calbiochem) in a 1:500 dilution or with $20 \mu\text{g}$ of goat antihuman IgM F(ab')₂ per milliliter (Jackson ImmunoResearch Laboratories). Subsequently, $2 \mu\text{g}$ of ionomycin per milliliter (Molecular Probes) was added to control for intracellular loading of indo-1.

B-CELL PROLIFERATION

Enriched blood B cells (1×10^5 per well) were cultured in 96-well flat-bottomed plates for 48 or 72 hours at 37°C and stimulated with *S. aureus* Cowan I in a 1:10,000 dilution or with $20 \mu\text{g}$ of anti-IgM F(ab')₂ per milliliter. Proliferation of the B cells was assessed by incorporating $0.5 \mu\text{Ci}$ of [³H]thymidine in each well.

IN VIVO VACCINATION WITH RABIES VACCINE

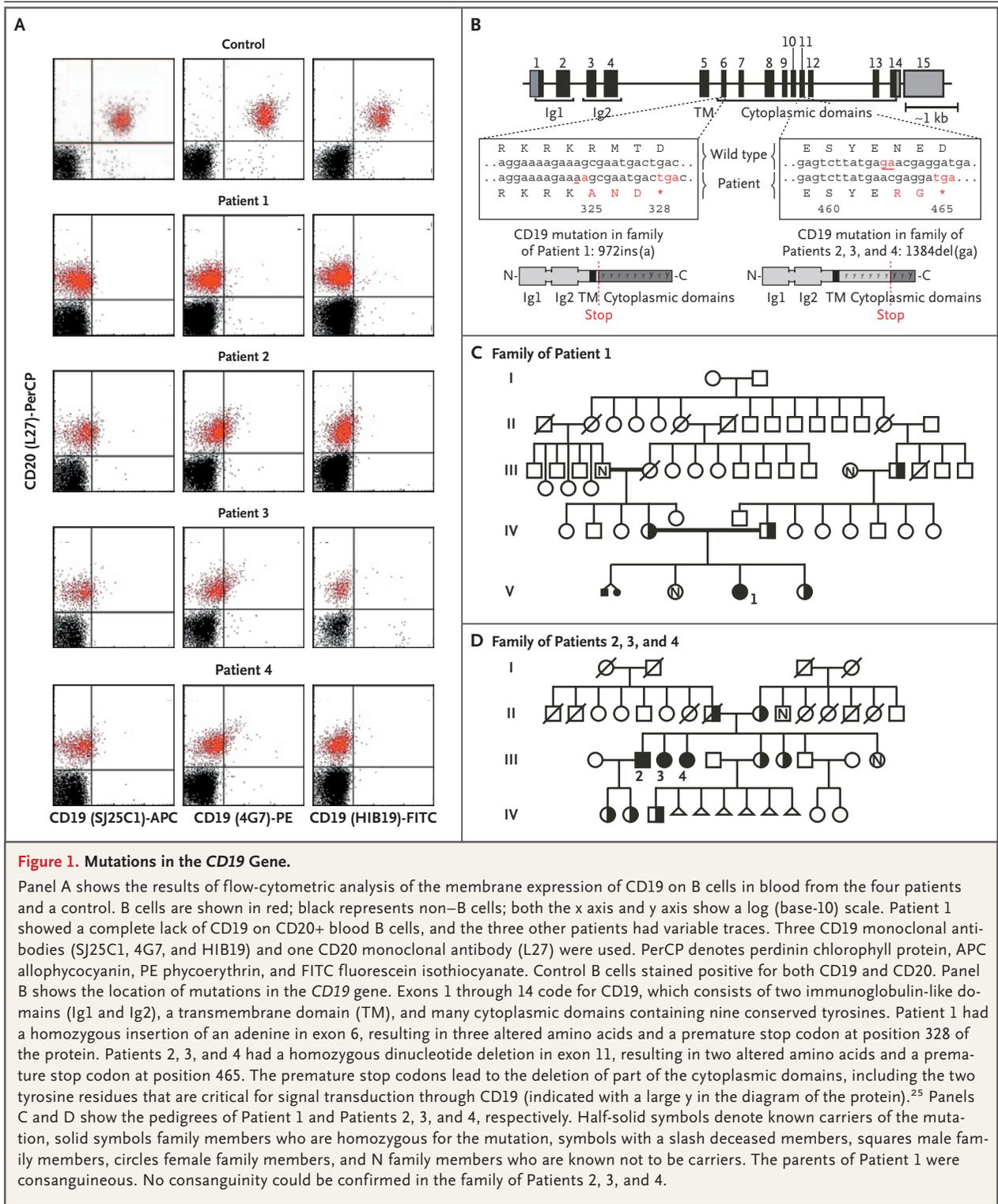
Patients, relatives, and controls were vaccinated twice with the human diploid-cell rabies vaccine (Institut Pasteur Merieux, Merck Sharp & Dohme) according to standard protocols. Antibody levels and avidity were measured as previously described.²⁶

RESULTS

IDENTIFICATION OF CD19 DEFECTS

Flow-cytometric immunophenotyping with antibodies against the cell-surface markers CD3 (on T cells), CD16 or CD56 or both (on natural killer cells), and CD19 or CD20 (on B cells) results in the identification of more than 95 percent of lymphocytes. All four patients had normal absolute numbers of CD20+ B cells. In contrast, membrane expression of CD19 on B cells was not detectable in Patient 1 and was barely detectable in Patients 2, 3, and 4 (Fig. 1A).

Sequencing of the *CD19* gene in Patient 1 revealed an insertion of an adenine in exon 6 of both alleles of *CD19*. This insertion causes a frame shift,



and thus there is a premature stop codon at the intracellular amino acid 328 (Fig. 1B). Patients 2, 3, and 4 were homozygous for a dinucleotide deletion of guanine and adenine in exon 11, resulting in a premature stop codon at the intracellular amino acid 465 (Fig. 1B). Living parents and several members of both families carried one mutated allele (Fig. 1C and 1D) but had no clinical features of immunodeficiency.

Normal levels of *CD19* transcripts were found in B cells from all patients (data not shown). Western blotting did not detect the CD19 protein in B cells from Patient 1, because the CD19 in this patient lacked most of the cytoplasmic domains. However, a reduction in intracellular levels of CD19 was observed in B cells from Patient 4 (data not shown), confirming the flow-cytometric results.

MEMBRANE EXPRESSION OF THE CD19 COMPLEX

The level of expression of CD19 in the carriers of the mutations was lower than that in controls (Fig. 2A). In addition, CD21 expression on the surface of patients' B cells was also reduced (Fig. 2B). CD81 and CD225 molecules were expressed at normal levels by the B cells of all patients and carriers (data not shown).

B-CELL DIFFERENTIATION

The precursor B-cell compartment in the bone marrow of Patient 1 showed a normal relative distribution of all differentiation stages (data not shown).²³ Ig- κ + B cells and Ig- λ + B cells were present in normal ratios in blood samples from all patients. Moreover, B cells from all four patients had normal expression of other membrane-bound molecules, including IgM, IgD, CD20, CD22, CD38, CD40, beta₂-microglobulin, and HLA-DR. However, all four patients had decreased levels of CD5+ B cells (8 to 12 percent) and CD27+ memory B cells (1 to 6 percent) in blood, as compared with levels in age-matched controls and carriers (19 to 41 percent for CD5+ and 17 to 28 percent for CD27+) (Fig. 2C and 2D). Memory B cells that switched immunoglobulin class were CD27+IgD-, lacking IgM and IgD, and were present in decreased numbers in blood samples from all patients. Sequencing of the V_H-C_α and V_H-C_γ transcripts showed somatic hypermutations in a pattern suggesting the potential to produce an antigen-selected B-cell-receptor repertoire, which is a molecular sign of a response to antigenic stimulation (data not shown).

Figure 2 (facing page). Immunophenotype of Mature B Cells in Patients with a CD19 Mutation.

All four patients had a reduced level of expression of CD19 or no expression on B cells in blood, as compared with levels in the controls and the mouse IgG1 isotype control (Panel A). In addition, the level of expression of CD19 on B cells in blood from carriers was lower than that in the controls. The level of expression of CD21 on B cells in blood from the patients was lower than that in carriers and controls (Panel B). The level of expression of CD5 on CD20+ B cells (shown in red; black represents non-B cells) was decreased in all patients as compared with carriers and controls, whereas it was normal on CD20- cells (Panel C; log scale). The level of expression of CD27+IgD+ and CD27+IgD- memory B cells was lower in the patients than in age-matched controls (Panel D; log scale). PerCP denotes peridinin chlorophyll protein, APC allophycocyanin, PE phycoerythrin, and FITC fluorescein isothiocyanate.

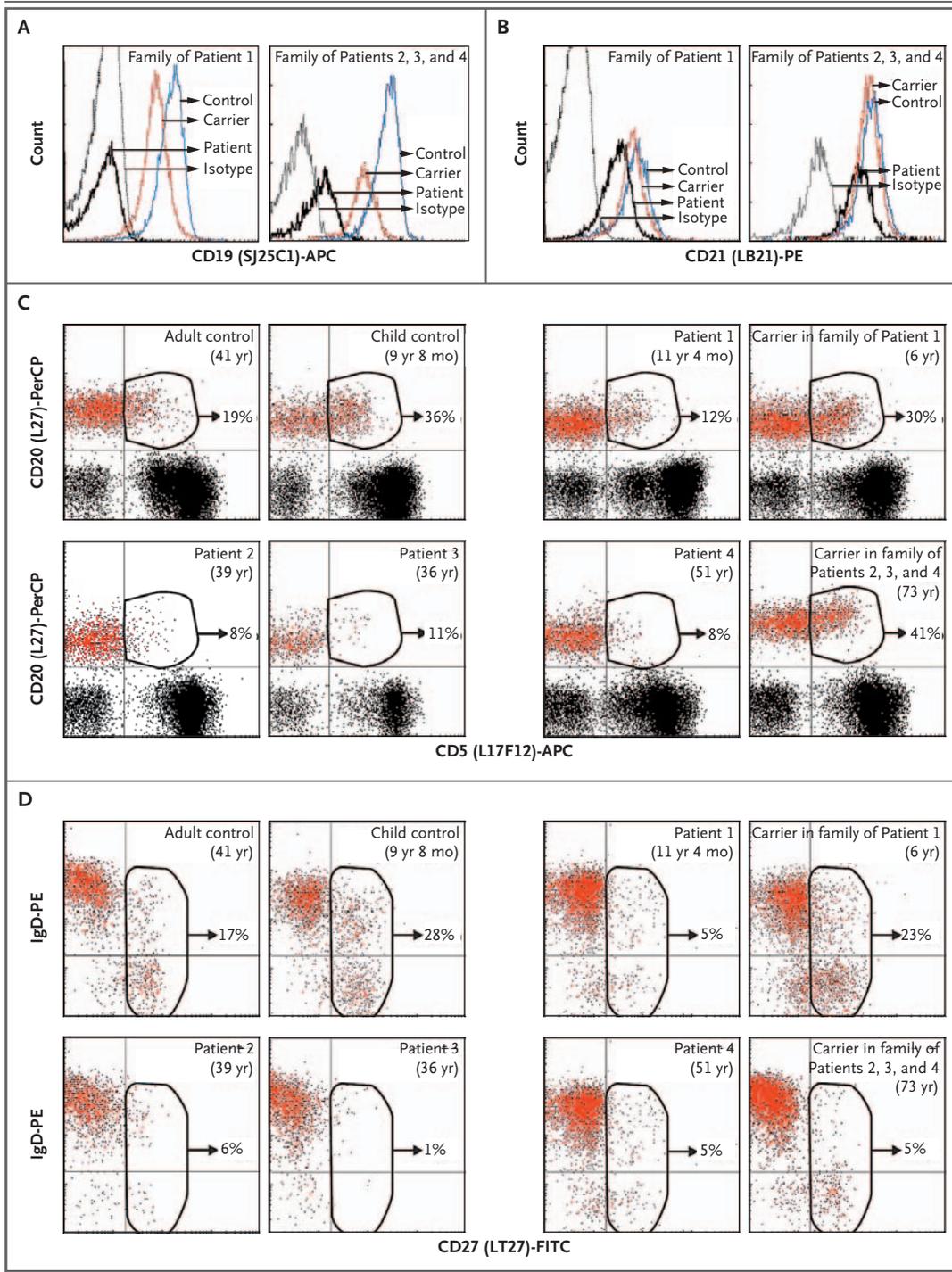
The numbers of CD27+ memory B cells in carriers in the family of Patient 1 were similar to those in controls, whereas carriers in the family of Patients 2, 3, and 4 had fewer memory B cells than controls (Fig. 2D). In general, however, in contrast to the patients, the carriers in the family of Patients 2, 3, and 4 had mainly memory B cells that had switched immunoglobulin class and normal serum immunoglobulin levels (data not shown).

GERMINAL CENTERS IN SECONDARY LYMPHOID ORGANS

Histologic analysis of a sample of cervical lymph node from Patient 1 showed multiple B-cell follicles of small-to-moderate size with proliferation centers on immunohistochemical staining for CD79a, BCL-2, BCL-6, CD21, and Ki67 (Fig. 3A). Follicles of normal size and architecture were also found in the tonsil specimen from Patient 2 (Fig. 3B).

CALCIUM MOBILIZATION AND PROLIFERATION ON STIMULATION OF THE B-CELL RECEPTOR

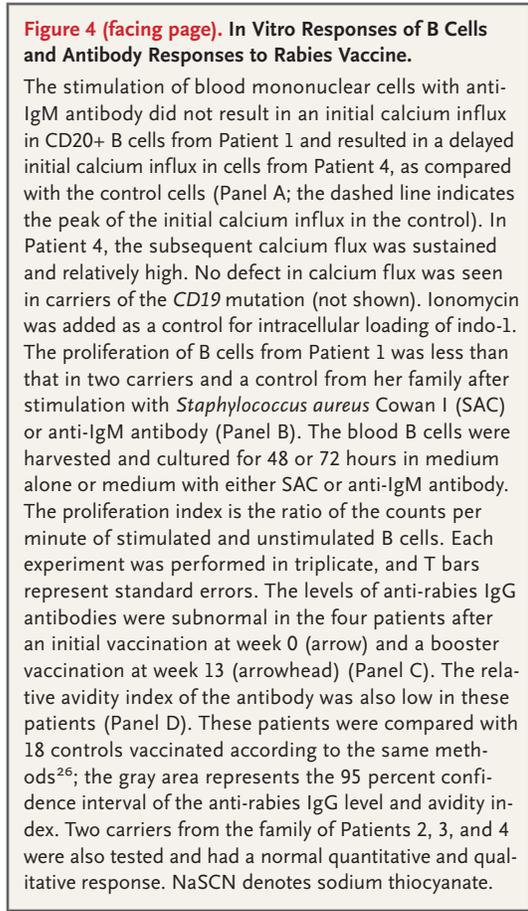
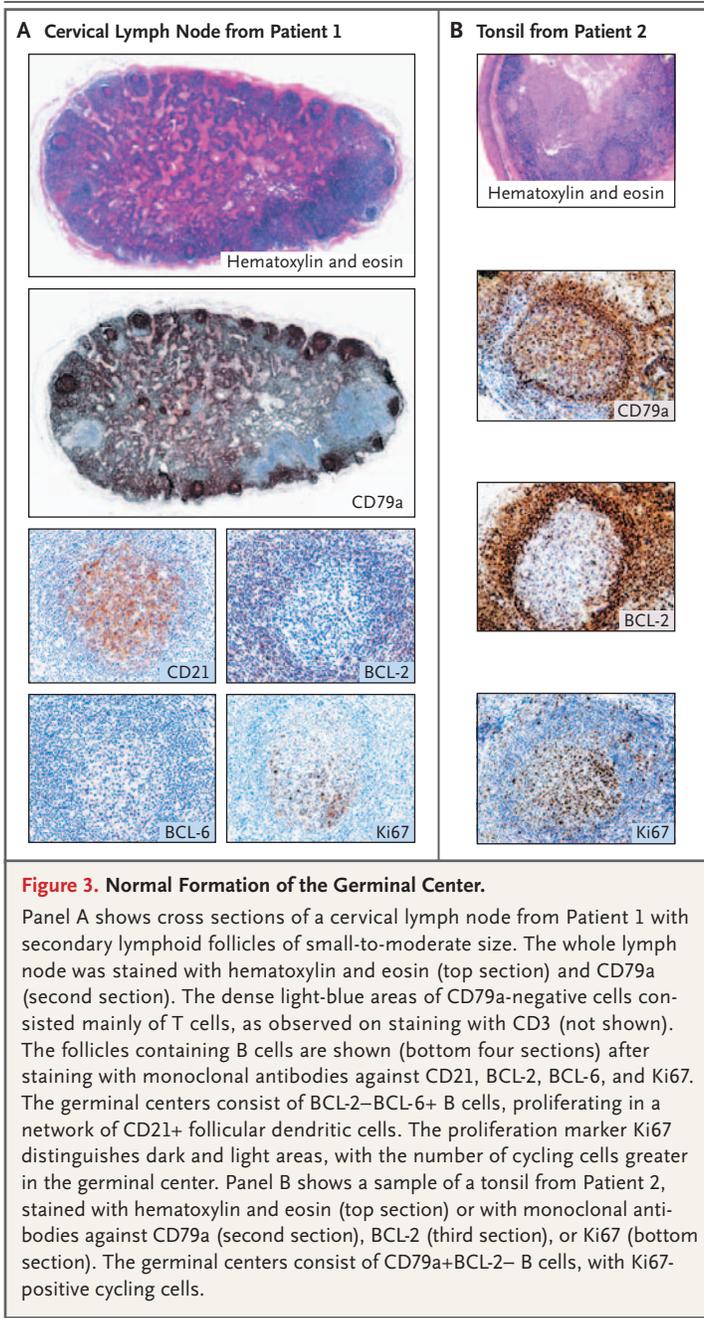
B-cell receptor-mediated activation causes an initial flux of calcium from the rough endoplasmic reticulum into the cytoplasm, followed by a sustained influx of extracellular calcium.²⁷ As compared with control B cells, the B cells from all four patients with CD19 deficiency exhibited defective calcium fluxes after stimulation with an anti-IgM antibody, which cross-links IgM molecules on the B-cell surface. No calcium influx or efflux was observed in the B cells from Patient 1,



whereas the initial calcium influx was delayed in B cells from Patients 2, 3, and 4. Furthermore, calcium efflux in all four patients was delayed and remained low for the duration of the experiment (Fig. 4A; data for Patients 2 and 3 not shown). B cells from heterozygous relatives had

normal calcium fluxes (data not shown). Normal calcium fluxes were found in Patients 2, 3, and 4 in response to *S. aureus* Cowan I, which activates B cells by means of innate immune receptors and the B-cell receptor (data not shown).

B cells from Patient 1 proliferated poorly after

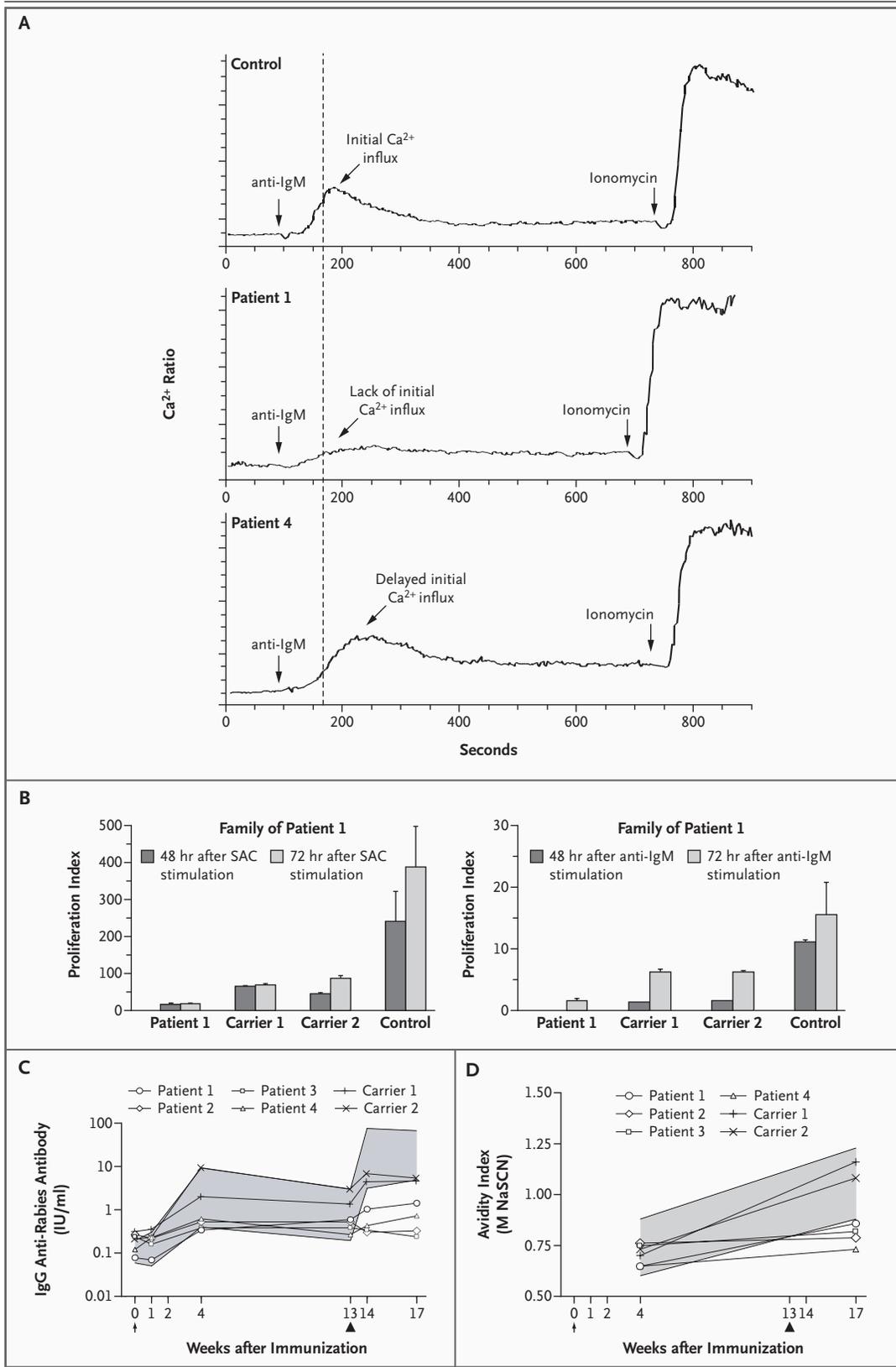


stimulation with *S. aureus* Cowan I or an anti-IgM antibody, as compared with B cells from the control (Fig. 4B). B cells from the parents of Patient 1, who are carriers of the mutation, showed intermediate proliferation in response to both *S. aureus* Cowan I and anti-IgM antibody (Fig. 4B). B cells from carriers in the family of Patients 2, 3, and 4 proliferated normally after stimulation

with *S. aureus* Cowan I or anti-IgM antibody (not shown).

ANTIBODY RESPONSE TO RABIES VACCINE

To test the primary and secondary antibody responses in vivo, all patients were vaccinated twice with rabies vaccine (at week 0 and week 13). The primary response of production of IgG anti-rabies antibody in Patient 1 was low but detectable, whereas Patients 2, 3, and 4 did produce IgG anti-rabies antibodies after the first vaccination. The secondary IgG response, after week 13, in all patients was below the 95 percent confidence interval of the normal response (Fig. 4C).²⁶ Furthermore, no increase in avidity was observed in Patient 2, 3, or 4, and Patient 1 had only a marginal increase in avidity during the secondary response (Fig. 4D). The second antibody response and the increase in avidity maturation were normal in the two carriers evaluated in the family of Patients 2, 3, and 4 (Figs. 4C and 4D).



DISCUSSION

We described four patients (from two different families) with homozygous mutations in *CD19* and showed that their profound hypogammaglobulinemia resulted from a poor response to antigen by mature B cells. Both *CD19* mutations were frameshift mutations, causing premature stop codons that result in the deletion of the total cytoplasmic domain (in the family of Patient 1) or a major part of the cytoplasmic domain (in family B). Both truncations result in the lack of the three C-terminal tyrosine residues, two of which have been shown to be critical for signal transduction through *CD19* (Fig. 1B).²⁵

These mutations are not likely to be polymorphisms; *CD19* is the most broadly used B-cell marker for flow-cytometric immunophenotyping of lymphocytes and has not been found to be lacking in large-scale studies of healthy children.^{16,28} Moreover, the B cells of heterozygous carriers of the mutation expressed *CD19*, albeit at slightly lower levels than noncarriers. Furthermore, *CD19* — together with *CD21*, *CD81*, and *CD225* — forms a signaling complex that decreases the threshold for the activation of B cells as mediated by the B-cell receptor.²⁹

B cells from *CD81*-knockout mice have decreased *CD19* expression.^{30,31} The mature B cells from our patients, however, had a normal level of expression of *CD81* and *CD225* but a reduced level of expression of *CD21*, suggesting a dependency of *CD21* on *CD19*. Normal levels of *CD19* transcripts in the B cells of these patients indicate that the reduction in the level of the protein does not result from nonsense-mediated decay of mRNA, but rather from the instability of the truncated *CD19* proteins. This interpretation is consistent with the observation that truncated (but otherwise stable) *CD19* molecules can associate with *CD81* and *CD225* on the membranes of B cells in which 95 percent of the cytoplasmic domains of *CD19* are deleted.³²

The *CD19* deficiency did not disturb the differentiation of precursor B cells in the bone marrow. Still, *CD19*-deficient B cells may undergo differentiation less efficiently than *CD19*⁺ B cells, as has been observed in *CD19*-knockout mice.^{33,34} Nevertheless, our patients had normal numbers of mature B cells in blood.

The numbers of *CD5*⁺ B cells in our patients were decreased, as they are in *CD19*-knockout

mice.^{35,36} *CD5* is a negative regulator of B-cell receptor signaling, and in mice, this marker defines the subgroup of autoreactive B-1a cells.^{37,38} It is likely that, in our patients, the inhibitory effect of *CD5* on B-cell–receptor signaling is superfluous in the absence of *CD19*.

The numbers of *CD27*⁺*IgD*⁺ and *CD27*⁺*IgD*[−] memory B cells in blood from all four patients were decreased. Together with the finding of decreased serum immunoglobulin levels, this finding suggests that terminal differentiation of B cells into memory B cells and plasma cells is affected. In two patients, secondary lymphoid tissues contained follicles of small-to-moderate size with germinal centers that appeared normal. *CD19*-knockout mice lack germinal centers, but centers appear when the animals are challenged with live virus.³⁹ *IgG* antibodies and circulating memory B cells are defective in these mice,³⁹ as they are in our four patients.

IgG and *IgA* transcripts with antigen-driven hypermutation were found in our patients at variable levels, indicating that *CD19*-deficient B cells are in principle able to undergo antigen-dependent differentiation, albeit with reduced efficiency. In mice, defects in *CD19* signaling due to mutations in critical intracellular tyrosines result in the absence of high-affinity antibodies owing to low B-cell proliferation and abnormal immunoglobulin-class switching.^{25,40,41}

The inefficient response of *CD19*-deficient B cells in these patients probably results from the loss of dual antigen recognition through the B-cell receptor and the *CD19* complex.^{15,42} The B cells from all four patients had abnormal calcium fluxes on stimulation, but the differences between Patient 1 and the other three patients may be due to the presence of different mutations. Patient 1 lacked all the intracellular domains responsible for signal transduction³²; in Patients 2, 3, and 4, the *CD19* molecule lacked only 92 amino acids at the intracellular C-terminal end, which contains two critical tyrosines that are required for most functions of *CD19*.²⁵ Most of the intracellular domains were present, potentially permitting some B-cell signaling.⁴³ Nevertheless, all four patients with *CD19* deficiency had substantially decreased antibody responses (levels and avidity) after vaccination with rabies vaccine. We conclude that the *CD19* signals that normally enable B-cell responses to antigenic stimuli are lacking in our patients.

This study shows that the disruption of *CD19*

signaling results in a primary antibody deficiency, mainly characterized by a poor antigen-specific response. On the basis of the crucial role of CD19 in signaling by the B-cell receptor on antigen recognition, it is likely that defects in other members of the CD19 complex (CD21, CD81, and CD225) also lead to antibody deficiency in humans.

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