Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency

Pernille Andersen, Henrik Permin, Vagn Andersen, Lone Schejbel, Peter Garred, Arne Svejgaard, and Torben Barington

Reduced levels of somatic hypermutation (SHM) have recently been described in IgG-switched immunoglobulin genes in a minority of patients with common variable immunodeficiency (CVID), demonstrating a disruption of the normal linkage between isotype switch and SHM. To see if, irrespective of isotype, there is a tendency to use unmutated immunoglobulin genes in CVID, we studied SHM in κ light-chain transcripts using a $V_{\kappa}A27$-specific restriction enzyme-based hot-spot mutation assay (IgκREHMA). Hot-spot mutations were found in 48% (median; reference interval, 28%-62%) of transcripts from 53 healthy controls. Values were significantly lower in 31 patients (median, 7.5%; range, 0%-73%; $P < .0000001$) of whom 24 (77%) had levels below the reference interval. Low levels of SHM correlated with increased frequency of severe respiratory tract infection (SRTI; $P < .005$), but not with diarrhea ($P = .8$). Mannose-binding lectin (MBL) deficiency also correlated with SRTI score ($P = .009$). However, the correlation of SHM and SRTI was also seen when only patients with normal MBL genotypes were analyzed (n = 18, $P = .006$). A slight decline of mutated fractions over years was noted ($P = .01$). This suggests that most patients with CVID fail to recruit affinity-maturated B cells, adding a qualitative deficiency to the quantitative deficiency characterizing these patients. (Blood. 2005;105:511-517)

Introduction

Common variable immunodeficiency (CVID) is a heterogeneous primary immunodeficiency characterized by acquired quantitative IgA and IgG deficiency and recurrent infections.[1-4] IgM levels are normal or reduced. The most common infections involve the upper and lower respiratory tract followed by gastrointestinal infections, but susceptibility, severity, and location of infections vary considerably among patients.[5-8] The most important features of the disease are recurrent bacterial pneumonias often leading to bronchiectasis, fibrosis, and eventually to respiratory insufficiency, the leading cause of death among patients with CVID. Autoimmune and allergic manifestations are common.[5,8] Cancer occurs frequently in CVID.[5,9] In 176 patients with CVID, Mellemkjaer et al[10] found a 10-fold increased incidence of malignant lymphoma and stomach cancer.

Several attempts have been made to subgroup patients with CVID based on clinical or immunologic parameters,[11,12] but no generally accepted criteria exist. Laboratory evidence for isolated B- and T-cell malfunction has been presented, and pathogenetic mechanisms may vary between patients.[13-24] Circulating B cells are normal or reduced in numbers, but rarely absent. Hypogammaglobulinemia results from failure to produce sufficient numbers of functional plasma cells. Recent data suggest that circulating memory B cells may be an important source of plasma cells.[25] It is therefore interesting that the generation of memory phenotype ($CD27^+$) B cells is reduced in most patients.[26,11,14,27] The plasma level of IgG is a poor predictor of disease severity because patients with slightly decreased levels of IgG may be highly susceptible to infections and benefit from immunoglobulin substitution therapy.[28] Thus, reliable prognostic criteria are currently lacking and qualitative aspects of the produced antibodies should be considered.

Impaired somatic hypermutation (SHM) of IgG was described in a minor subpopulation (23%) of CVID patients using hot-spot–specific restriction enzymes after polymerase chain reaction (PCR) amplification of *V3-23*-IgG heavy-chain transcripts.[29,30] Thus, the normal coupling of isotype switching and SHM may fail in some patients. SHM is essential for the generation of normal long-lived plasma cells and memory B cells secreting and encoding high-affinity antibodies, respectively. SHM normally occurs in the germinal centers, where high-affinity variants are selected in a process involving antigen-coated follicular dendritic cells and T cells. By this process affinity may increase 10- to 1000-fold.[31-36] The finding that IgG-secreting cells in some patients lack somatic mutations indicates that the IgG produced in these patients is of reduced quality with low affinity for the eliciting antigens. However, affinity maturation by SHM does not depend on isotype switching and may occur in B cells producing IgM.[37-39] The failure to use affinity-maturated antibody within all isotypes might be a better predictor of a qualitative immunodeficiency than the mutations of isotype-switched genes.

In this study, SHM of the κ light chain was investigated in patients with CVID as a measure of affinity maturation irrespective of isotype and correlated to clinical features of the disease. We developed a quantitative restriction enzyme-based hot-spot mutation assay (IgκREHMA). Using this technique, impaired SHM was detected in 77% of CVID patients and shown to correlate with

From the Departments of Clinical Immunology and Infectious Diseases, University Hospital, Copenhagen, Denmark; Department of Internal Medicine, Bispebjerg Hospital, Copenhagen, Denmark; Department of Medicine TA and Institute for Inflammation Research, University Hospital, Copenhagen, Denmark; and Department of Clinical Immunology, Odense University Hospital, Odense, Denmark.

Submitted December 22, 2003; accepted August 30, 2004. Prepublished online as *Blood* First Edition Paper, September 14, 2004; DOI 10.1182/blood-2003-12-4359.

Supported by Danish Medical Research Council grants 22-00-0151 and 22-01-0156.

Reprints: Pernille Andersen, Department of Clinical Immunology, Blood Bank, University Hospital, Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; e-mail: pernille.andersen@dadlnet.dk.

frequency of severe respiratory tract infection (SRTI). Quantification of the level of SHM may turn out to be an important parameter in the clinical management of patients with CVID and improve our understanding of the cellular mechanisms underlying the disease.

Patients, materials, and methods

Patients and controls

Thirty-one patients (13 men and 18 women) with CVID were included. All were referred to the Department of Clinical Immunology for evaluation of their immunodeficiency either during the study period (n = 26) or previously (n = 5) if frozen mononuclear cells were available. The inclusion criteria were primary hypogammaglobulinemia with a decrease of IgG to less than 5 g/L with IgA less than 0.7 g/L or IgM less than 0.5 g/L and fulfilling the following criteria: (1) onset of immunodeficiency later than 2 years of age and (2) exclusion of defined causes of hypogammaglobulinemia. Heparinized blood samples were collected and mononuclear cells isolated by density gradient centrifugation, frozen, and stored in liquid nitrogen until use. Samples were available from the time of diagnosis and prior to IgG substitution therapy for 17 patients; 14 patients were diagnosed several years earlier (median, 11 years; range, 3-26 years) and all were in substitution therapy at the time of the sampling. Frozen cell samples available from other time points were included to study the course of SHM.

The median age of the patients at the time of diagnosis was 35 years (range, 8-73 years) with histories of increased frequency of infections for 5 years (range, 0-48 years) prior to diagnosis. At the time of the first sample tested for SHM the median age was 41 years (range, 8-75 years). As controls, blood was obtained from 53 healthy adults after informed consent was given (9 men and 44 women; median age, 41 years; range, 23-65 years). Three patients were younger (ages 8, 14, and 22 years) and 3 older (ages 66, 74, and 75 years) than the controls.

Twenty cord blood samples were included as negative controls, donated after informed consent was obtained from the delivering women. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg Counties.

IgκREHMA

cDNA synthesis. Mononuclear cells were thawed, and mRNA was isolated from 3 to 6×10^6 cells using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway) as instructed by the manufacturer. cDNA synthesis was performed by the Perkin Elmer reverse transcription PCR kit using an oligo-dT reverse primer (DNA Technology, Aarhus, Denmark) as instructed by the manufacturer.

PCR. PCR was performed using fluorochrome-coupled primers specific for the signal peptide region and the third framework region (FR3), respectively, of the κ light-chain gene $V_\kappa A27$ (*IGKV3-20* in ImMunoGenetics (IMGT) nomenclature). The primers were: TET-coupled (tetrachloro-6-carboxyfluorescein) 5′-CAGAGGGAACCATGGAAA-3′ for the signal peptide region and FAM-coupled (6-carboxyfluorescein) 5′-CCACTGCCACTGAACCTGT-3′ for the FR3 region (Oswel DNA Services, Hampshire, United Kingdom; Figure 1). The PCR conditions were: primers 0.1 μM, $MgCl_2$ 1.5 μM, × 1 PCR buffer (50 mM KCL, 10 mM Tris (tris(hydroxymethyl)aminomethane)–HCl), platinum *Taq*-polymerase 1.25 U (Invitrogen, Life Technology, Taastrup, Denmark) in a reaction volume of 50 μL. Amplification conditions were 94°C for 2 minutes, 25 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute with a final elongation at 72°C for 10 minutes.

Cleavage reaction. The 271–base pair (bp) PCR product was digested by the restriction enzyme *Fnu*4HI (Medinova Scientific, Hellerup, Denmark) recognizing 3 sites (GCNGC) in the unmutated gene product, one in the signal peptide/first framework region and 2 adjacent (AGCAGCAGC) in the CDR1 hot-spot region at codons 29-31 (classification by Kabat; 30-32 in IMGT nomenclature) of the variable region CDR1 (Figure 1). Prevention of cleavage in the CDR1 hot spot would demand either at least one mutation in both GCs of codons 29 and 31, or at least one mutation in the G or C of codon 30. The reaction conditions were as follows: 16 μL PCR product, 3.75 μL × 1 PCR buffer, and 2.5 U *Fnu*4HI incubated at 37°C for 2 hours. A parallel control digestion using 5.0 U *Dde*I (Medinova Scientific) instead of *Fnu*4HI was included as control for gDNA (genomic DNA) contaminating the cDNA, which would reveal such contamination by the presence of a FAM-coupled fluorescent 131-bp fragment. Throughout the study no DNA contamination was seen.

Fragment analysis by capillary electrophoresis. A preparation of 1 μL of an internal size standard (Prism Genescan 350 TAMRA, Applied Biosystems, Naerum, Denmark), 13 μL formamide, and 2 μL of the cleavage reaction product was made prior to fragment analysis by capillary electrophoresis (Applied Biosystems prism 310 genetic analyzer). A FAM-coupled fluorescent fragment of 106 or 109 bp indicated cleavage in the hot spot, whereas a length of 244 bp indicated cleavage in the signal peptide site but not in any of the sites in the CDR1 hot spot due to one or more mutations in the latter (Figure 1). The fraction of PCR products with mutations preventing cleavage was calculated as the proportion between the 244-bp fragment peak area and the sum of the peak areas of 244-, 106-, and 109-bp fragments (Figure 2).

Absence of a TET-coupled fluorescent 163/166-bp fragment was used as an internal control demonstrating complete cutting by the *Fnu*4HI nuclease. Throughout the study failure of the restriction enzyme was not seen.

Cloning and sequencing

PCR was performed as described using primers without fluorochromes. The PCR products were cloned into a PCR II vector (TOPO cloning kit; Invitrogen, Life Technologies) as instructed by the manufacturer and transformed into competent cells (One Shot Competent cells; Invitrogen, Life Technologies). Colonies from transformation vials grew on Luria-Bertani (LB) agar plates with ampicillin/ITPG/X-Gal at 37°C for 18 hours. Plasmids were extracted and purified (GFX Micro Plasmid Prep Kit; Amersham Pharmacia Biotech, Uppsala, Sweden), and $V_\kappa A27$ inserts sequenced by the automatic 310 genetic analyzer using Dye Terminator Kit, Ampli*Taq* polymerase and primers supplied by the kit.

Classification of the CVID patients by clinical and paraclinical scores

Case records from the 31 CVID patients were studied by 2 medical specialists unaware of the mutational status of the patients. Four clinical scoring systems (I-IV) were used to evaluate the severity of disease and complications, I to III describing lower respiratory tract involvement and IV describing diarrhea. Score system I divided the patients based on the number of SRTIs recorded within the last 2 years prior to initiation of IgG

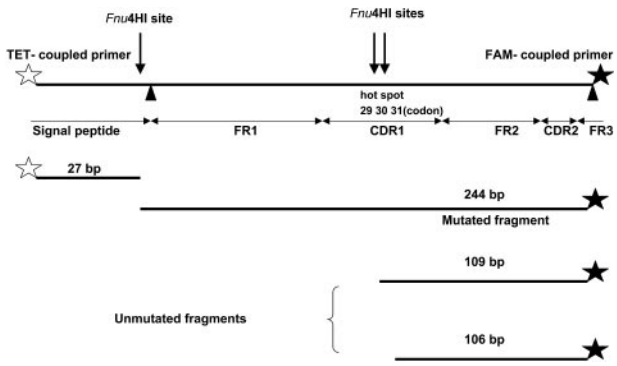

Figure 1. REHMA visualized. The top line illustrates the 271-bp sequence of the $V_\kappa A27$ light-chain V gene representing the signal peptide region, framework regions (FR1, FR2, FR3), and the complementarity determining regions (CDR1, CDR2). *Fnu*4HI sites, hot-spot, and TET- and FAM-fluorochrome-coupled primers (marked by an open and a black star, respectively) are indicated. Vertical black arrows indicate the sequenced cloned PCR product, codon 1-66, which is illustrated in Figure 4. The three bottom lines represent the 244-bp fragment of noncleaved hot-spot mutated $V_\kappa A27$ and the 106/109-bp fragments of cleaved hot-spot unmutated $V_\kappa A27$, respectively.

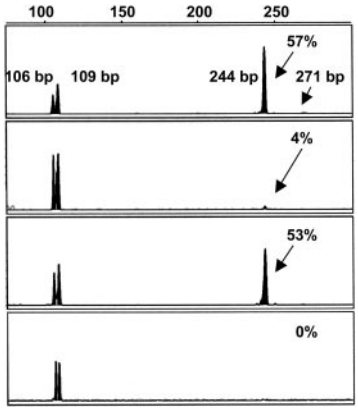

Figure 2. Fragment length analysis by capillary electrophoresis of cut $V_\kappa A27$ PCR products ($V_\kappa A27$ REHMA). Visualization of both TET- and FAM-coupled fragments was allowed, but only FAM fragments appeared in the shown length interval and are indicated in black. Size markers are not visualized for clarity reasons. Diagrams from top: patient no. 11 with a high-mutated fraction, followed by patient no. 4 with a low-mutated fraction, control no. 2 with a mutated fraction of 53%, and an unmutated cord blood sample. The peaks of 106 and 109 bp represent the quantity of $V_\kappa A27$ fragments cleaved in one of the 2 hot-spot *Fnu*4HI restriction sites, respectively. The peaks of 244 bp represent the quantity of $V_\kappa A27$ fragments cleaved in the signal peptide *Fnu*4HI site, but not cleaved in the hot spot as a consequence of one or 2 mutations eliminating the *Fnu*4HI sites. The peak of 271 bp (in general a fraction $< 1\%$, as illustrated here) represents $V_\kappa A27$ sequences in which cleavage is prevented by mutation in both the hot spot and the signal peptide restriction sites, as well.

substitution or diagnosis in case substitution was not initiated immediately (1, no SRTI; 2, ≤ 2 SRTIs per year, 3, > 2 SRTIs per year). SRTI was defined as symptoms of lower respiratory tract infection, elevated numbers of leukocytes and C-reactive protein, body temperature higher than 38.5°C, or antibiotic therapy, and severity of symptoms requiring hospitalization. Score system II described the presence and severity of sequelae to the disease: bronchiectasis, functional dyspnea, reduced forced expiratory volume at 1 second (FEV_1) at time of diagnosis (none, 1; light, 2; severe, 3). Score system III described the same sequelae but over the entire period of observation. Score system IV classified the patients in 2 groups without or with intermittent or daily diarrhea at the time of diagnosis. Basic data included age at onset of symptoms, age at diagnosis of CVID, age at investigation by IgκREHMA analysis, age at registration of the data from case records, and plasma concentrations of IgG, IgA, IgM, and IgG subclasses.

Cell separation and flow cytometry

To analyze the expression of CD27 on B cells (memory B cells), fresh peripheral blood mononuclear cells (PBMCs) were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-CD27, phycoerythrin (PE)–conjugated anti-CD19, and peridinin chlorophyll protein (PerCP)–conjugated anti-CD3 (Becton Dickinson, Heidelberg, Germany) and analyzed by fluorescence-activated cell sorting (FACS) on a FACScan (Becton Dickinson). For analysis of the mutation fractions of $CD19^+/CD27^+$ B cells, frozen PBMCs were thawed and enriched for B cells using CD3 depletion with Dynabeads M-450 (Dynal) as instructed by the manufacturer. The cells were incubated with allophycocyanin (APC)–conjugated anti–human CD19 and R-phycoerythrin RPE-conjugated anti–human CD27 (Dako, Glostrup, Denmark) and analyzed on a FACSCalibur (Becton Dickinson). $CD19^+/CD27^+$ cells from 2 patients (women, aged 34 and 49 years) and 2 healthy adults (women, aged 34 and 48 years) were sorted into carrier cells (monocyte cell line U937). The purity of the sorted cells was tested with FACS (CVID patients 60% and 78%, healthy adults 95% and 86%, respectively). The sorted cells were used as a template in the REHMA analysis as described (see "IgκREHMA").

Mannose-binding lectin (MBL) analysis

Plasma concentrations of mannose-binding lectin (MBL) and MBL (*mbl2*) genotypes (wild-type A allele, mutant D; codon 52, mutant B; codon 54 and mutant C; codon 57) were assessed by conventional enzyme-linked immunosorbent assay (ELISA) and PCR restriction fragment length analyses.[40,41]

Calculations and statistics

Confidence limits for the median were calculated based on the binomial distribution. Reference interval for IgκREHMA results was defined by the values of the second smallest and the second largest value from 53 healthy adults (equal to 96% confidence limits). The Mann-Whitney *U* test and Kruskal-Wallis test were used to test for different medians of 2 or 3 samples, respectively. *P* less than .05 was considered statistically significant.

Results

IgκREHMA

To investigate the status of κ light-chain transcripts in patients with CVID, compared to controls, we developed a quantitative method, $V_\kappa A27$-specific restriction enzyme based hot-spot mutation assay (IgκREHMA). The method, illustrated in Figure 1, takes advantage of 2 *Fnu*4HI restriction sites, both included in the CDR1 hot spot. Mutations in both of these restriction sites, or at least one in the central GC in codon 30 shared by both sites, are needed to abrogate cutting by *Fnu*4HI. Figure 2 shows representative fragment length analyses for 2 CVID patients (a high- and a low-mutating individual), one adult control, and a cord blood sample. Calculated values are given for fraction of $V_\kappa A27$ transcripts with at least one mutation in the CDR1 hot spot, preventing cutting by *Fnu*4HI restriction nuclease.

Hot-spot mutations in healthy individuals

IgκREHMA values in blood samples from 53 healthy adults ranged from 27% to 67%, yielding a calculated 96%-based reference interval for the assay of 28% to 62%. No effect of age was noted within this reference material (Spearman rank correlation coefficient $\rho = 0.1$, $P = .5$). The IgκREHMA values of 20 cord blood samples were all 0%.

Most CVID patients had reduced levels or absence of hot spot mutations

IgκREHMA values in the 31 CVID patients ranged from 0% to 73% in the first blood sample taken after diagnosis. The median was 7.5%, which was significantly lower than control values ($P < .0000001$). This difference also held if patients younger ($n = 3$) or older ($n = 3$) than the age span of the controls (23-65 years) were omitted (medians, 8.5% and 7%, respectively; $P < .0001$). Mutations were undetectable (0%) in 4 (13%) of the patients and below the reference interval in 24 (77%; Figure 3).

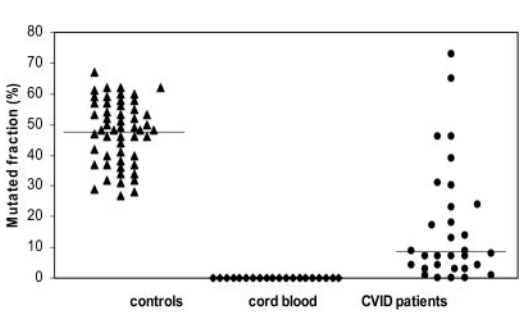

Figure 3. Mutated fraction in adult controls, cord blood samples, and CVID patients. Medians are indicated by horizontal lines. Values of mutated fractions in CVID patients were found to be significantly lower compared to adult controls.

Table 1. Mutated fraction of $V_\kappa A27$ in CVID patients over time

Patient no.	Year of diagnosis	Mutated fractions measured by $V_\kappa A27$ REHMA, %															
														2000		2001	
		1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	1	2	1	2
1	1979	—	—	—	—	—	—	—	—	3	—	—	0	2	7	0	0
2	1998	—	—	—	—	—	—	—	—	—	—	—	39	31	24	19	12
5	1997	—	—	—	—	—	—	—	—	—	3	—	4	—	4	—	0
7	1982	—	—	—	—	—	—	—	—	7	—	—	—	5	0	0	2
9	1997	—	—	—	—	—	—	—	—	—	—	9	—	6	1	8	19
10	1987	—	—	—	—	—	—	—	—	9	—	—	—	6	7	—	—
12	1996	—	—	—	—	—	—	—	—	4	2	—	—	—	—	0	—
13	1988	17	—	—	—	—	6	14	—	4	8	—	—	—	—	—	—
17	1983	—	—	—	30	33	—	—	—	—	—	—	—	42	—	39	16
19	1994	—	—	—	—	—	—	—	—	—	—	46	—	41	31	27	33
21	1992	—	—	—	—	—	—	—	—	4	—	—	—	5	5	4	5
22	1995	—	—	—	—	—	—	13	—	17	—	—	—	8	11	—	7
23	1992	—	—	—	—	7	—	—	—	6	—	—	—	8	8	7	0

Mutated fractions of $V_\kappa A27$ in 13 CVID patients followed for 2 to 10 years. In 2000 and 2001, mutated fractions were measured both in the first and second part of the year. — indicates no blood sample available.

Two patients had values above the reference interval. If only values from patients tested within the first year of diagnosis and before institution of substitution therapy (n = 17) were considered, the IgκREHMA values ranged from 0% to 65% with a median of 9%. These values did not differ significantly from the remaining patients tested at later time points ($P > .1$).

Mutation levels over time

An important question is whether the levels of hot-spot mutations are stable in CVID patients or change with time. Table 1 lists values for 13 patients from whom several frozen cell samples were available, covering at least 2 years of observation after diagnosis. Patients who had low values in the first sample continued with low values for the entire period (patient nos. 1, 5, 7, 9, 10, 12, 13, 21, 22, and 23 with 3-9 years of observation). Three patients (nos. 2, 17, and 19) who initially had a normal value continued so for 1, 10, and 3 years of observation, respectively, but 2 of them eventually acquired subnormal values. Overall, a slight declining trend was noted by comparing initial values with last values ($P = .01$, Wilcoxon matched-pairs test), but the decline was very slow. For the 2 patients with supranormal values, the samples were taken 6 years after diagnosis (no. 14) and at the time of diagnosis (no. 11), respectively.

Mutations in cloned sequences of $V_\kappa A27$

From 2 patients (a low and a highly mutated) and 2 controls, 62 cloned sequences were obtained. Sixty had maximal homology with the published germline gene $V_\kappa A27$ confirming the gene specificity of the IgκREHMA. Two sequences had maximal homology with $V_\kappa L6$, which in turn has 98% homology with $V_\kappa A27$ in the investigated regions (signal peptide, FR1, CDR1, and FR2).[42] Figure 4 illustrates the frequency and distribution of mutations in $V_\kappa A27$ sequences from these 4 individuals. For comparison, the same number (11) of randomly selected sequences was used from each individual. Among all 60 $V_\kappa A27$ sequences, the average prevalence of mutations per nucleotide (codons 1-66, 198 nucleotides) was 3% and 2% for the 2 controls, 0.2% and 4% for the 2 CVID patients, and 0% for the cord blood sample, respectively. The 3 GC pairs critical for cutting by *Fnu*4HI on average had mutation prevalences per nucleotide of 17% and 14% (controls), 2.8% and 20% (patients), and 0% (cord blood). Also in the poorly mutating CVID patient, mutations were preferentially affecting the CDR1 hot spot (Figure 4). The mutation prevalences of the sequences correlated well with results of the IgκREHMA analysis (Figure 5).

The fraction of hot spot-mutated transcripts correlated with incidence of SRTI at time of diagnosis

Based on the case reports, the 31 CVID patients were classified clinically by medical specialists unaware of the mutational data. The patients were scored with respect to SRTI within the last 2 years prior to diagnosis (score system I), complications of SRTI (II, III), and presence of diarrhea (IV). Figure 6 shows that patients suffering from SRTI (groups 2 and 3) had significantly lower levels of SHM than patients without reported SRTI (group 1, $P < .005$). A similar correlation was found when only patients tested for hot-spot mutations within 1 year of the time of diagnosis were analyzed (n = 17, $P = .015$). Less than half the patients had complications of SRTI (II and III) at diagnosis (n = 13) or during follow up (n = 14) and the levels of SHMs showed no correlation to the scores ($P > .38$). Thirteen patients suffered from diarrhea, but the presence of diarrhea was neither correlated to light- chain mutations ($P = .8$), nor to IgG levels at diagnosis, nor to MBL

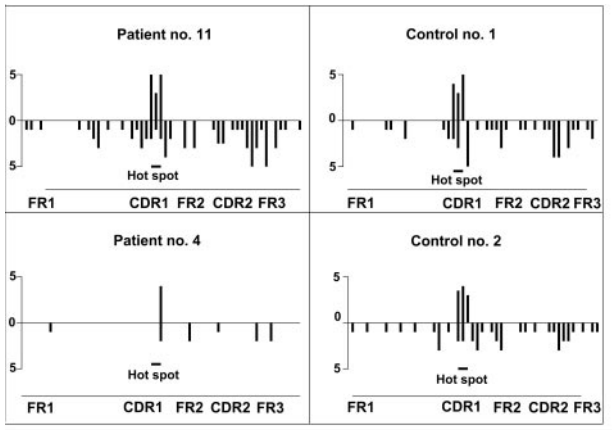

Figure 4. Mutations of cloned $V_\kappa A27$ sequences from 2 CVID patients (high and low mutated) and from 2 adult controls. Mutations in $V_\kappa A27$ are represented by 11 sequences randomly selected from each patient and control. Hot spot indicates the two *Fnu*4HI restriction sites in the CDR1.The columns illustrate the number of mutations in each codon; above the axis, preventing cleavage in the hot spot; below the axis, not affecting cleavage.

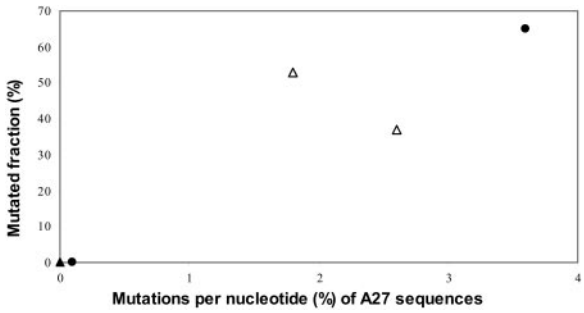

Figure 5. Correlation of hot-spot mutations to overall mutations per nucleotide. The correlation of mutated fraction (%) established by *V*κ*A27* REHMA to mutations per nucleotide in sequences of A27 immunoglobulin is illustrated by the 2 adult controls (△), the 2 patients (●) shown in Figure 4, and a cord blood sample (▲).

status (data not shown). A correlation of IgκREHMA results with the plasma concentration of IgG at diagnosis was noted (Spearman $\rho = 0.5$, $P = .003$), as well for IgA ($\rho = 0.47$, $P = .008$) and IgM plasma levels ($\rho = 0.39$, $P = .03$). IgG level, however, was a poor predictor of SRTI score ($P = .07$, Kruskal-Wallis). A correlation of IgA and IgM level with SRTI score was, however, noted ($P = .02$ and $P = .015$, Kruskal-Wallis). Neither IgκREHMA results nor SRTI score correlated with the concentrations of B or T lymphocytes in the blood, the B-cell fraction, or age at onset of symptoms (data not shown).

Correlation of hot spot-mutated transcripts with SRTI in CVID patients with or without MBL deficiency

Thirteen of the patients (42%) were heterozygotic for one of the 3 known structural mutations of the m*bl2* gene (genotype AB, AC, or AD) and had reduced levels of plasma MBL (median, 148 μg/L; range, 0-1648 μg/L) compared with the remaining patients (genotype AA, 2832 μg/L; range, 384-12 096 μg/L; $P < .0001$). Heterozygosity (open circles in Figure 6) was associated with high SRTI score (I; $P = .009$, Mann-Whitney *U* test). However, the correlation between the prevalence of SHM and SRTI score was also seen when only patients with normal MBL genotypes were analyzed ($n = 18$, $P = .006$; filled circles in Figure 6). It was noted that among those with SRTIs, the level of SHM was similar, whereas the patients with MBL deficiency almost exclusively clustered in the most severely affected group ($P = .049$, Fisher exact test).

Expression of the memory marker CD27 and SHM

Flow cytometric analysis of CD27 expression on B cells was performed in 18 CVID patients and compared with the results of the IgκREHMA analysis. With a few exceptions, the fraction of $CD27^+$ B cells in the circulation correlated well with the fraction of mutated κ transcripts (Figure 7; Spearman $\rho = 0.6$, $P = .01$). REHMA analysis was performed on FACS-sorted (Fluorescence-Activated Cell Sorter) $CD19^+/CD27^+$ B cells from 2 patients with 6% and 3% $CD19^+/CD27^+$ cells, respectively, and 2 healthy adults. The fractions of mutated κ transcripts of the $CD19^+/CD27^+$ B cells were reduced in the CVID patients compared with healthy adults (Figure 7B).

Discussion

The clinical importance of affinity maturation of antibody responses is currently unknown. Patients with reduced levels of SHMs due to mutations of the activation-induced cytidine deaminase gene or CD40 ligand gene are highly susceptible to infections including recurrent bacterial infections of the respiratory tract.[43,44] In both conditions, however, isotype switching is also inhibited and contributes to the increased susceptibility to infections.

SHMs may increase the affinity of the antibody 10- to 1000-fold.[31-36] Sufficient binding of antibody to a given pathogen should therefore be possible with much less affinity-maturated antibody than with the unmutated version. Because the total levels of circulating immunoglobulins are restricted, SHM allows for more specificities in the immune antibody repertoire or for longer duration of antibody-mediated memory or both. In humans, who are dependent on antibody-mediated protection for decades after immunization, affinity maturation may prove necessary to combine long-lasting immunity with a sufficiently broad antibody repertoire.

Immunoglobulin mRNA obtained from the blood is predominated by transcripts from circulating plasmablasts and plasma cells.[45] In healthy adults, about 1000 immunoglobulin-secreting cells are found per milliliter of blood (approximately 1% of B cells).[46] Almost all (> 90%) of the Igκ transcripts carry SHMs, which tend to concentrate in certain hot spots located in CDR1 in particular. The V_κ gene *A27* is the most commonly used light-chain gene and normally accounts for about 10% to 15% of V_κ transcripts in the circulation.[47-49] In this study, *V*κ*A27* transcripts from healthy adults demonstrated SHMs with an average of 4 mutations per transcript equivalent to 2% of the nucleotides (codon 1-66). The *V*κ*A27* CDR1 contains a strong hot spot consisting of 3 overlapping RGYW motifs; this motif is known to be preferentially targeted by SHM.[50] On the average, 16% of the GC nucleotides of the target sequence AGCAGCAGC were mutated, which is 7 times the average level for nucleotides of the V region. The sequence contains 2 overlapping *Fnu*4HI restriction sites both of which were modified by mutations in 27% and 31% of the sequences of the 2 healthy adults, respectively (Figure 3). Therefore, these restriction sites were exploited in this study to quantify mutation.

We demonstrate reduced levels of SHM of *V*κ*A27* transcripts in 77% of CVID patients. In comparison, Levy et al[29] and Bonhomme et al[30] found reduced levels of SHM in IgG *VH3-23* transcripts in 23% of CVID patients. The reason for this discrepancy is most likely the fact that the IgG-based method measures mutations only in isotype-switched cells. Even if T/B interactions are malfunctioning, it is possible that the (few) B cells, which obtain sufficient T-cell help for isotype switching, also mutate to a normal extent. In fact, Piqueras et al[27] found normal levels of mutation in IgG transcripts from switched memory B cells from patients with CVID. This would yield a normal IgG mutation rate despite a possible skewing of the overall antibody response from mutated IgG to unmutated IgM. In light chain–based SHM analysis,

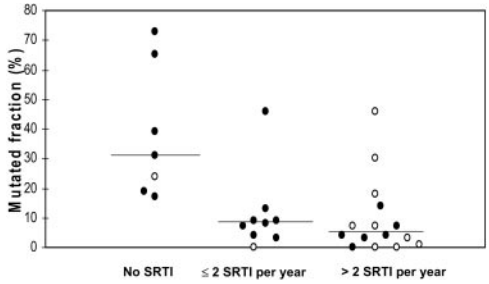

Figure 6. Correlation of mutated fractions to frequency of SRTI. Thirty-one CVID patients categorized in 3 groups by frequency of SRTI during the 2 years up to the diagnosis or initiation of IgG substitution therapy. Median fractions are indicated by horizontal lines; ○ indicates CVID patients with MBL deficiency.

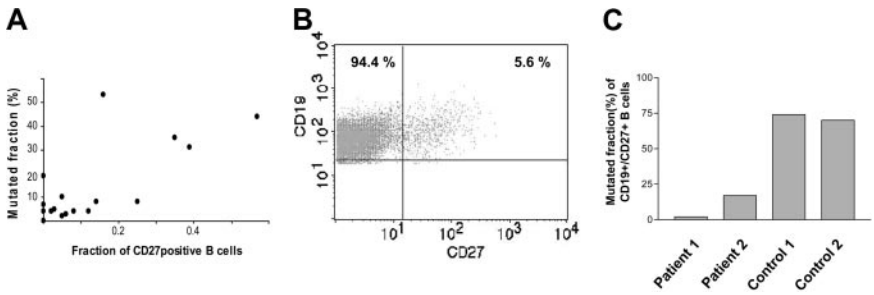

Figure 7. Relationship between mutated fractions of *V*κ*A27* transcripts and the presence of CD19$^+$/CD27$^+$ memory B cells. (A) Samples from 18 CVID patients were analyzed by *V*κ*A27* REHMA and flow cytometry. The abscissa shows the fractions of CD27$^+$ cells among B cells. In 2 patients, the values of mutated fraction and CD27$^+$ fraction are both 0%. (B) CD27 positivity of CD19- gated B cells from one of the 2 CVID patients from whom CD19$^+$/CD27$^+$ memory B cells were sorted. Percentage of B cells (CD19$^+$) negative or positive for CD27 are given in the upper quadrants. (C) Mutated fractions of purified CD19$^+$/CD27$^+$ B cells from 2 CVID patients and 2 healthy adults illustrating reduced light-chain mutation in the patients.

however, the cells donating the mRNA are not necessarily isotype-switched, and low levels of mutations may reflect a reduction of SHM within the individual isotypes as well as a shift from highly mutated IgG- or IgA-secreting cells to less mutated (or unmutated) IgM-secreting cells. Thus, the IgκREHMA result reflects the overall tendency to use mutated (affinity-maturated) versus unmutated antibody in ongoing antibody responses.

If affinity maturation is important for protection against infections, it should be revealed by a propensity for infections in individuals with low levels of light-chain mutations. Indeed, we found a good correlation between low levels of light-chain mutation and a high frequency of SRTIs in this study. Mutation rates appeared more important than IgG levels at the time of diagnosis because the latter did not correlate significantly with the susceptibility to SRTI though a trend for more infections with lower IgG levels was noted ($P = .07$). Structural mutations in MBL, however, did predict more SRTI episodes. Nevertheless, low IgκREHMA levels remained well correlated with the frequency of SRTI even when patients with MBL deficiency were excluded. This was also the case when patients with lower than median (1.7 g/L) IgG levels were excluded from analysis ($P = .04$). These results indicate an independent impact of somatic mutation on the susceptibility to SRTI.

It was somewhat surprising not to find a correlation of Igκ mutation to sequelae of lung disease. This could, however, be explained by the extreme variation in time from the first symptoms of immunodeficiency to measurement of SHM (0-51 years; median, 14 years) providing very different conditions for complications to occur. Indeed, the 4 patients with sequelae despite Igκ mutation values above the lower limit of the reference interval had had symptoms for a median of 40 years (range, 9-51 years). The lack of correlation between SHM and prevalence of diarrhea could be related to the poor correspondence between the function of the systemic humoral immune system and that of the small intestine as reported by Herbst et al.[51]

Important questions relate to whether reduced somatic light-chain hypermutation is a fixed feature characterizing a subgroup of patients or rather a dynamic feature changing with the course of the disease. The relative stability of the mutation levels over several years demonstrated in Table 1 may support the first possibility and makes it meaningful to evaluate SHM and probably to use it prognostically at the time of diagnosis. Normal values were in one case seen over a period of 10 years, and in others 6, 7, and 18 years after diagnosis and, in several cases, more than 40 years after onset of symptoms (reported increased susceptibility to upper respiratory tract infections). Likewise, patients with low values remained so for up to 9 years of observation without any patient demonstrating recovery of SHM. Nevertheless, 3 of 3 patients who initially presented with normal mutation levels and who were followed for more than 2 years did eventually acquire reduced levels of mutation suggesting that an inherent but slow progression with time of this qualitative immunodeficiency may be the rule in CVID. Bonhomme et al found that hypomutation of isotype-switched immunoglobulin genes is correlated to late onset of disease.[30] In our study, we did not find this correlation for light-chain mutation.

The correlation of light-chain mutation to the fraction of B cells that carried the memory marker CD27 (Figure 7), however, could indicate that the light-chain mutation levels (unlike mutation of switched heavy chains) might relate to the CVID subgroups proposed by Warnatz et al[11] or Piqueras et al[27] based on the absence of different memory subpopulations. However, the finding of reduced levels of light-chain mutations among CD27$^+$ cells sorted from CVID patients (Figure 7) indicates that a low number of memory cells per se does not explain the lgκREHMA results and points to defects in the SHM machinery itself as at least a part of the explanation.

The influence of MBL deficiency on the predisposition to SRTIs in CVID patients has not previously been reported and should be confirmed in larger patient populations. This finding suggests that MBL may be an important protective factor in CVID patients. Indeed, the 60% of patients with more than 2 episodes of SRTI per year carried structural mutations in the MBL gene while approximately 34% of the background population are heterozygotes.[40,41] This fits well with the demonstration that MBL deficiency increases the susceptibility to respiratory infections in age groups with poorly developed humoral immunity as demonstrated in Greenland infants.[52] It may explain the finding of Mullighan and colleagues that MBL deficiency correlated with early onset of CVID.[53] We cannot, however, confirm this relation in the present study because neither age at onset of symptoms, nor age at clinical diagnosis correlated significantly with MBL status. This discrepancy could be related to different sampling of patients in the 2 studies.

This study shows that failure of SHM is more common in CVID patients than previously recognized and indicates that poor affinity maturation may predispose to lower respiratory tract infections, which are of paramount importance for the prognosis of these patients. Furthermore, it suggests that deficiency of MBL may also contribute independently to this predisposition. Measurement of light-chain SHM and MBL could prove useful in the management of this disease. Inquiries into mechanisms necessary for SHM might shed light on the pathogenesis of CVID.

References

1. Primary immunodeficiency disease. Report of an IUIS scientific committee. Clin Exp Immunol.1999;118(suppl 1):1-28.
2. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Clin Immunol. 1999;93:190-197.
3. Sneller MC. Common variable immunodeficiency. Am J Med Sci. 2001;321:42-48.
4. Cunningham-Rundles C. Common variable immunodeficiency. Curr Allergy Asthma Rep. 2001; 1:421-429.
5. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol. 1999;92:34-48.
6. Van der Hilst JC, Smits BW, van der Meer JW. Hypogammaglobulinaemia: cumulative experience in 49 patients in a tertiary care institution. Neth J Med. 2002;60:140-147.
7. Martinez Garcia MA, Hernandez F de Rojas MD, Nauffal Manzur MD, et al. Respiratory disorders in common variable immunodeficiency. Respir Med. 2001;95:191-195.
8. Ballow M. Primary immunodeficiency disorders: antibody deficiency. J Allergy Clin Immunol. 2002; 109:581-591.
9. Cunningham-Rundles C, Cooper DL, Duffy TP, Strauchen J. Lymphomas of mucosal-associated lymphoid tissue in common variable immunodeficiency. Am J Hematol. 2002;69:171-178.
10. Mellemkjaer L , Hammarström L, Andersen V, et al. Cancer risk among patients with IgA deficiency or common variable immunodeficiency and their relatives: a combined Danish and Swedish study. Clin Exp Immunol. 2002;130:495-500.
11. Warnatz K, Denz A, Dräger R, et al. Severe deficiency of switched memory B cells ($CD27^+IgM^-IgD^-$) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood. 2002;99: 1544-1551.
12. Bryant A, Calver NC, Toubi E, Webster AD, Farrant J. Classification of patients with common variable immunodeficiency by B cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. J Clin Immunol Immunopathol. 1990;56: 239-248.
13. Schwartz R, Porat YB, Handzel Z, et al. Identification of a subset of common variable immunodeficiency patients with impaired B-cell protein tyrosine phosphorylation. Clin Diagn Lab Immunol. 1999;6:856-860.
14. Sawabe T, Horiuchi T, Nakamura M, et al. Defect of Ick in patient with common variable immunodeficiency. Int J Mol Med. 2001;7:609-614.
15. Brouet J-C, Chedeville A, Fermand J-P, Royer B. Study of the B cell memory compartment in common variable immunodeficiency. Eur J Immunol. 2000;30:2516-2520.
16. Groth C, Dräger R, Warnatz K, et al. Impaired up-regulation of CD70 and CD86 in naive ($CD27^-$) B cells from patients with common variable immunodeficiency (CVID) Clin Exp Immunol. 2002;129:133-139.
17. Jacquot S, Macon-Lamaître L, Paris E, et al. B cell co-receptors regulating T cell-dependent antibody production in common variable immunodeficiency: CD27 pathway defects identify subsets of severely immuno-compromised patients. Int Immunol. 2001; 13:871-876.
18. Guazzi V, Aiuti F, Mezzaroma I, et al. Assessment of thymic output in common variable immunodeficiency patients by evaluation of T cell receptor excision circles. Clin Exp Immunol. 2002;129: 346-353.
19. Rump JA, Jahreis A, Schlesier M, Drager R, Melchers I, Peter HH. Possible role of IL-2 deficiency for hypogammaglobulinaemia in patients with common variable immunodeficiency. Clin Exp Immunol. 1992;89:204-210.
20. Eisenstein EM, Jaffe JS, Strober W. Reduced interleukin-2 (IL-2) production in common variable immunodeficiency is due to a primary abnormality of $CD4^+$ T cell differentiation. J Clin Immunol. 1993;13:247-258.
21. Zielen S, Dengler TJ, Bauscher P, Meuer SC. Defective CD2 T cell pathway activation in common variable immunodeficiency (CVID). Clin Exp Immunol. 1994;96:253-259.
22. Kondratenko I, Amlot PL, Webster AD, Farrant J. Lack of specific antibody response in common variable immunodeficiency (CVID) associated with failure in production of antigen-specific memory T cells. Clin Exp Immunol. 1997;108:9-13.
23. Boncristiano M, Majolini MB, D'Elios MM, et al. Defective recruitment and activation of ZAP-70 in common variable immunodeficiency patients with T cell defects. Eur J Immunol. 2000;30:2632-2638.
24. Pozzi N, Gaetaniello L, Martire B, et al. Defective surface expression of attractin on T cells in patients with common variable immunodeficiency (CVID). Clin Exp Immunol. 2001;123:99-104.
25. Bernasconi, NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298:2199-2202.
26. Agematsu K, Futatani T, Hokibara S, et al. Absence of memory B cells in patients with common variable immunodeficiency. Clin Immunol. 2002; 103:34-42.
27. Piqueras B, Lavenu-Bombled C, Galicier L, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol. 2003;23:385-400.
28. Busse PJ, Razvi S, Cunningham-Rundles C. Efficacy of intravenous immunoglobulin in the prevention of pneumonia in patients with common variable immunodeficiency. J Allergy Clin Immunol. 2002;109:1001-1004.
29. Levy Y, Gupta N, Le Deist F, et al. Defect in IgV gene somatic hypermutation in common variable immuno-deficiency syndrome. Proc Natl Acad Sci U S A. 1998; 95:13135-13140.
30. Bonhomme D, Hammarström L, Webster D, et al. Impaired antibody affinity maturation process characterizes a subset of patients with common variable immunodeficiency. J Immunol. 2000;165: 4725-4730.
31. Neuberger MS, Ehrenstein MR, Rada C, et al. Memory in the B-cell compartment: antibody affinity maturation. Philos Trans R Soc Lond B Biol Sci. 2000;355:357-360.
32. Foster SJ, Dorner T, Lipsky PE. Targeting and subsequent selection of somatic hypermutations in the human V_κ repertoire. Eur J Immunol. 1999; 29:3122-3132.
33. Berek C, Milstein C. The dynamic nature of the antibody repertoire. Immunol Rev. 1988;105:5-26.
34. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell. 1991; 67:1121-1229.
35. Storb U, Shen HM, Michael N, Kim N. Somatic hypermutation of immunoglobulin and non-immunoglobulin genes. Philos Trans R Soc Lond B Biol Sci. 2001;356:13-19.
36. Sale JE, Bemark M, Williams GT, et al. In vivo and in vitro studies of immunoglobulin gene somatic hypermutation. Philos Trans R Soc Lond B Biol Sci. 2001;356:21-28.
37. Muramatsu M, Sankaranand VS, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem. 1999; 274:18470-18476.
38. Spencer J, Dunn M, Dunn-Walters DK. Characteristics of sequences around individual nucleotide substitutions in IgVH genes suggest different GC and AT mutators. J Immunol. 1999;162:6596-6601.
39. Harris RS, Kong Q, Maizels N. Somatic hypermutation and the three R's: repair, replication and recombination. Mutat Res. 1999;436:157-178.
40. Garred P, Madsen HO, Kurtzhals JAL, et al. Diallelic polymorphism may explain variations of blood concentration of mannan-binding protein in Eskimos, but not in black Africans. Eur J Immunogenet. 1992;19:403-412.
41. Madsen HO, Garred P, Kurtzhals JAL, et al. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics. 1994;40:37-44.
42. Schäble KF, Zachau H-G. The variable genes of the human immunoglobulin kappa locus. Biol. Chem Hoppe Seyler. 1993;374;1001-1022.
43. Allen RC, Armitage RJ, Conley ME, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science. 1993;259:990-993.
44. Revy P, Muto T, Levy Y, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the hyper-IgM syndrome (HIGM2). Cell. 2000;102:565-575.
45. Manz RA, Acre S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. Curr Opin Immunol. 2002;14:517-521.
46. Barington T, Heilmann C, Andersen V. Quantitation of antibody-secreting cells in the blood after vaccination with *Haemophilus influenzae* type b conjugate vaccine. Scand J Immunol. 1990;31: 515-522.
47. Foster SJ, Brezinschek HP, Brezinschek RI, Lipsky PE. Molecular mechanisms and selective influences that shape the kappa gene repertoire of IgM^+ B cells. J Clin Invest. 1997;99:1614-1627.
48. Tomlinson IM, Cox JP, Gherardi E, Lesk AM, Chothia C. The structural repertoire of the human V kappa domain. EMBO J. 1995;14:4628-4638.
49. Weber J-C, Blaison G, Martin T, Knapp A-M, Pasquali J-L. Evidence that the V kappa III gene usage is nonstochastic in both adult and newborn peripheral B cells and that peripheral $CD5^+$ adult B cells are oligoclonal. J Clin Invest. 1994;93: 2093-2105.
50. Jacobs H, Bross L. Towards an understanding of somatic hypermutation. Curr Opin Immunol. 2001;13:208-218.
51. Herbst EW, Armbruster M, Rump JA, Buscher HP, Peter HH. Intestinal B cell defects in common variable immunodeficiency. Clin Exp Immunol. 1994;95:215-221.
52. Koch A, Melbye M, Sorensen P, et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA. 2001; 285:1316-1321.
53. Mullighan CG, Marshall SE, Welsh KI. Mannose binding lectin polymorphisms are associated with early age of disease onset and autoimmunity in common variable immunodeficiency. Scand J Immunol. 2000;51:111-122.