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Research Article

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Absence of IgD$^-$CD27$^+$ Memory B Cell Population in X-linked Hyper-IgM Syndrome

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Abstract

The present study analyzed peripheral blood B cell populations separated by IgD and CD27 expression in six males with X-linked hyper-IgM syndrome (XHIM). Costimulation of mononuclear cells from most of the patients induced no to low levels of class switching from IgM to IgG and IgA with *Staphylococcus aureus* Cowan strain (SAC) plus IL-2 or anti-CD40 mAb (anti-CD40) plus IL-10. Measurable levels of IgE were secreted in some of the patients after stimulation with anti-CD40 plus IL-4. Costimulation with SAC plus IL-2 plus anti-CD40 plus IL-10 yielded secretion of significant levels of IgG in addition to IgM, but not IgA. The most striking finding was that peripheral blood B cells from all of the six patients were composed of only IgD$^+$ CD27$^-$ and IgD$^+$ CD27$^+$ B cells; IgD$^-$ CD27$^+$ memory B cells were greatly decreased. IgD$^+$ CD27$^+$ B cells from an XHIM patient produced IgM predominantly. Our data indicate that the low response of IgG production in XHIM patients is due to reduced numbers of IgD$^-$ CD27$^+$ memory B cells. However, the IgG production can be induced by stimulation of immunoglobulin receptors and CD40 in cooperation with such cytokines as IL-2 and IL-10 in vitro. (*J. Clin. Invest.* 1998. 102:853–860.) Key words: hyper-IgM syndrome • memory B cells • CD40 • CD27 • CD70

Introduction

Hyper-IgM syndrome is characterized by an increased susceptibility to infections and markedly decreased levels of serum IgG, IgA, and IgE, but normal or elevated levels of IgM (1, 2). An X-linked form of hyper-IgM syndrome (XHIM)[1] has been shown to result from mutations in the CD40 ligand (CD154) gene (3–5). CD154 is a TNF-like type II membrane protein expressed on the surface of activated T cells (6). CD40 is expressed ubiquitously throughout the B cell lineage (7). Triggering via CD40 on B cells by CD154 stimulates B cell proliferation (8) and germinal center formation (1, 9–12), and promotes the expansion of the memory B cell pool (13, 14). The CD40-mediated signal in combination with IL-2 or IL-10 stimulates B cells to secrete IgG, IgM, and IgA (15, 16) and in the presence of IL-4 to secrete IgE (17). The vital role of CD40 molecule for immunoglobulin production is underlined by low levels of serum IgG, IgA, and IgE in XHIM patients. There is, however, little information on the reason for the inability of various B cell activation systems, including stimulation via CD40, to produce immunoglobulins, even though CD40 in the patient B cells is intact and the CD40-mediated signal transduction in B cells is not impaired (3, 4).

Recently, we have demonstrated that the interaction between CD27, which belongs to the NGFR/TNFR family (18), and CD27 ligand (CD70), a member of the TNF family (19) that is expressed not only on activated B cells but also on T cells, especially activated CD4$^+$ CD45RO T cells (20), can enhance immunoglobulin production by B cells (21, 22). Furthermore, adult peripheral blood B cells can be divided into three discrete subtypes: IgD$^-$ CD27$^+$, IgD$^+$ CD27$^+$, and IgD$^+$CD27$^-$ B cells. IgD$^-$ CD27$^+$ B cells produce IgG, IgM, and IgA, whereas IgD$^+$ CD27$^+$ B cells predominantly produce IgM (23). The striking function of CD27 in B cells is that CD27/CD70 interaction promotes the differentiation of CD27$^+$ memory B cells toward plasma cells (24, 25). To date, the information available from XHIM patients is not conclusive concerning the generation of memory B cells in the absence of CD40 ligation. Little is known as to whether a lack of memory B cell response represents the impaired IgG and IgA production in the disorder.

In the present study, we have analyzed peripheral blood cells in XHIM patients with the surface phenotype of memory B cells using CD27 as a memory marker, and discuss the role in CD40/CD154 interaction in the generation of memory B cells.

Methods

Patients. The patient population ranged from 6 to 24 yr in age. The clinical diagnosis of XHIM was made by the following criteria: *a*) males; *b*) onset of recurrent infections before 2 yr of age; *c*) low serum IgG and IgA levels, and normal to elevated serum IgM concentrations; and *d*) normal numbers of peripheral blood B cells. Ages of six patients are given in Table I, and the ages and serum immunoglobulin levels at the time of diagnosis before γ-globulin replacement therapy are shown in parentheses. CD40 expression on CD19$^+$ B cells analyzed by the flow cytometry was normal in all patients (data not shown). Routine clinical laboratory studies and treatment including immunoglobulin replacement therapy were performed at the referring hospitals.

Sequence analysis of genes coding for CD154. Isolation and char-

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1. *Abbreviations used in this paper:* MNC, mononuclear cell; RT, reverse transcriptase; SAC, *Staphylococcus aureus* Cowan strain; XHIM, X-linked hyper-IgM syndrome.

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acterization of CD154 cDNA were described previously (3, 26). Briefly, total RNA was extracted from activated lymphocytes of patients and reverse transcription of CD154 mRNA to cDNA was done. The cDNA was amplified by PCR using primers at the 5′ and 3′ ends of the coding region of the CD154 gene. The PCR products were sequenced using the dideoxy chain termination methods with an automated DNA sequencer (Perkin Elmer, Urayasu, Japan).

Antibodies and reagents. Anti-CD27 mAb (8H5; IgG1), which does not block the ligation of CD27/CD70 and anti-CD70 mAb (HNE51; IgG1), were previously described (21, 27). Polyclonal anti-IgD Ab was purchased from DAKO Japan (Tokyo, Japan). FITC-conjugated CD40 mAb, PerCP-conjugated CD20 mAb, and APC-conjugated CD19 mAb were from Becton Dickinson (Mountain View, CA), and purified anti-CD40 mAb (MAB89, IgG1) from Immunotech (Westbrook, ME). Anti-CD154 mAbs (1.7 and 106) and CD40-Fc fusion protein were gifts from T. Siadak and A. Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) and 5c8 was a gift from Biogen (Cambridge, MA). Conjugation of biotin to anti-CD27 mAb was performed by the standard technique using *N*-hydroxysuccinimido-biotin (Sigma Chemical Co., St. Louis, MO) in our laboratory. *Staphylococcus aureus* Cowan strain (SAC) was obtained from Sigma Chemical Co., IL-4 and IL-10 from Genzyme (Boston, MA), and IL-2 from Takeda Seiyaku Co. (Osaka, Japan).

Cell preparation. Heparinized cord blood was collected after obtaining informed consent. Peripheral blood samples of children at various ages were obtained at the time of clinical examination and were finally found to have no immunological abnormalities. Peripheral blood samples were also collected from healthy volunteers and XHIM patients. Mononuclear cells (MNCs) were isolated from the cord blood and peripheral blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The MNCs from healthy volunteers were separated with 5% sheep erythrocytes into erythrocyte rosette-positive (E^+) and -negative (E^-) populations (28). After depleting monocytes by silica (IBL, Fujioka, Japan) or adherence to a plastic surface, E^- cells were further enriched for B cells by positive selection with anti-CD19 mAb-coated immunomagnetic beads (Dynal, Oslo, Norway). Anti-CD19 mAb was subsequently removed by use of Detach a bead (Dynal). 97% of the cells in the B cell population thus obtained were reactive with anti-CD20 mAbs. $CD19^+$ IgD^+ $CD27^-$ and $CD19^+$ IgD^+ $CD27^+$ B cells of healthy adults or Patient IN were isolated from the monocyte-depleted E^- cells by sorting using a FACStar™ Plus (Becton Dickinson) under sterile conditions. Both populations thus obtained were > 98% pure.

Flow cytometric analysis. Peripheral blood MNCs or E^- cells were stained with a combination of IgD–FITC, biotin-CD27 followed by streptavidin-PE, and CD20-PerCP. Three color analysis was then performed by FACScan™ (Becton Dickinson). E^+ cells were stimulated with PMA (Life Technology, Grand Island, NY) plus ionomycin (Sigma Chemical Co.) for 18 h. After activated E^+ cells were stained with anti-CD154 mAbs (5c8, 1.7, 106) or CD40-Fc fusion protein followed by goat anti–Ig-FITC (Southern Biotechnology, Birmingham, AL), single color analysis was carried out.

Preparation of CD70 transfectants and fixation of the transfectants. CD70 transfectants were prepared as previously described (23). The CD70/300-19 and mock/300-19 transfectants were incubated with 1% paraformaldehyde in PBS for 5 min. After washing with PBS three times, the cells were cultured in RPMI 1640 + 10% FCS for 30 min, and used for the analysis.

Immunoglobulin assay by ELISA. MNCs (0.5×10^6/ml for IgG, IgM, and IgA, and 10^6/ml for IgE) or purified peripheral blood B cells were cultured with various stimuli in a volume of 200 μl/well for 8 d for IgG, IgM, and IgA assay and 12 to 14 d for IgE assay at 37°C in a humidified atmosphere with 5% CO_2. In some experiments, MNCs were treated with 2.5 μg/ml of anti-CD70 blocking mAb (HNE51) before the stimulation. The cultured supernatants were harvested and added to goat anti–human Ig (Southern Biotechnology) or anti-IgE (a kind gift from A. Saxon, Division of Clinical Immunology/Allergy, UCLA School of Medicine, Los Angeles, CA), coated 96-well flat ELISA plates with control human IgG, IgM, IgA purchased from Sigma Chemical Co., and IgE from Chemicon International Inc. (Temecula, CA). After an overnight incubation, supernatants were discarded and the wells were washed with 0.05% Tween-20 in PBS. Bound human Ig was detected with alkaline phosphatase-labeled goat anti–human IgG, IgM, IgA, or IgE (Sigma Chemical Co.) at a dilution of 1/2,500, and color detection was performed. In this ELISA system, no cross-reaction between IgG, IgM, IgA, and IgE occurred. Background readings obtained for wells exposed to PBS in the absence of culture supernatants or control Ig were subtracted.

Reverse transcription-PCR (RT-PCR) of germline Cε. Highly purified $CD19^+$ IgD^+ $CD27^+$, $CD19^+$ IgD^+CD27^- B cells or whole B cells at the cell numbers of 10^6 cells were stimulated with or without 100 ng/ml IL-4 for 16 h. Total RNA was extracted by the single step method (29). First-strand cDNA copies were synthesized using mu-

Table I. Patient Clinical Data, CD154 Expression, and Mutations

Cases	Age/sex	Serum immunoglobulin levels				CD154 expression				Mutation of CD154		Current γ-globulin therapy
		IgG (mg/dl)	IgM (mg/dl)	IgA (mg/dl)	IgE (IU/ml)	CD40-Fc	5c8	1.7	106	Nucleotide change	Consequence	
Pt. IN	24 yr/M	40	1100	< 5	< 10	2.6	0.4	0.9	3.3	C782→T	Thr 254→Met	−
Pt. YF	23 yr/M	647	293	215	< 10	1.3	ND	ND	1.9	178–309 deletion	59–96 deletion	+
	(6 yr)*	(13)	(424)	(74)	(< 10)					(Exon-2 skipping)		
										19-bp insertion	Stop at 101	
Pt. TF	16 yr/M	654	1390	106	< 10	1.7	ND	ND	1.8	178–309 deletion	59–96 deletion	+
	(1 yr 6m)	(47)	(330)	(23)	(< 10)					(Exon-2 skipping)		
										19-bp insertion	Stop at 101	
Pt. TY	12 yr/M	365	358	44	< 10	1.0	ND	ND	0.9	T713→C	Leu 231→Ser	+
	(8 yr)	(26)	(153)	(< 5)	(< 10)							
Pt. AY	6 yr/M	433	80	5	< 10	0.9	ND	ND	0.9	T713→C	Leu 231→Ser	+
	(3 mon)	(234)	(31)	(< 5)	(ND)							
Pt. RM	13 yr/M	490	410	< 5	< 10	1.8	ND	ND	0.9	T128→G	Met 36→Arg	+
	(3 yr)	(290)	(120)	(< 5)	(ND)							
Cont.	30 yr/M					68.5	72.4	75.6	71			

*Parentheses show ages and serum immunoglobulin levels at the time of diagnosis before γ-globulin replacement therapy.

rine Moloney leukemia virus reverse transcriptase (GIBCO BRL, Grand Island, NY) with oligo (dT) (GIBCO BRL) as a primer in a total volume of 20 μl. PCR was then performed with Cε exon sense primer 5′-CATGCGGTCCACGACCAAGAC-3′ and antisense primer 5′-CCACTGCACAGCTGGATGGAG-3′. 2 μl of cDNA were amplified in PCR using each primer and Taq DNA polymerase (GIBCO BRL). The amplified products were analyzed on a 1.2% agarose gel containing ethidium bromide and visualized by ultraviolet light illumination. β_2-microglobulin sense primer 5′-GCTATGTGTCTGGGTTTCAT-3′ and antisense primer 5′-CCCACTTAACTATCTTGGGC-3′ were used as controls (30).

Results

CD154 expression and mutations in XHIM patients. Upon stimulation by PMA plus ionomycin, T cells obtained from all of the six patients with XHIM demonstrated no CD154 expression as determined by flow cytometric analysis using several mAbs specific for CD154 and CD40-Fc fusion protein (Table I). In contrast, T cell activation was associated with marked increases in the CD69 surface expression (data not shown). Approximately 70% of activated T cells obtained from healthy controls expressed CD154 on the surface after stimulation by PMA plus ionomycin (Table I).

Detailed sequence analysis of cDNA obtained from the six patients was previously described (26). Briefly, Patients TY and AY, who are siblings, revealed the same point mutation at nucleotide T 713 to C, resulting in a change of leucine 231 to serine. Patient IN had a point mutation at nucleotide C 782 to T, changing from threonine 254 to methionine, and Patient RM at nucleotide T 128 to G, resulting in amino acid change in transmembrane domain from methionine 36 to arginine. Two of the siblings (Patients YF and TF) had the same unique mutations within introns, resulting in alternative splicing. They had a single-point mutation at a splice site of intron 2 and subsequently had two different sizes of cDNA, demonstrating skipping of the entire exon 2 or the insertion of 19 nucleotides (Table I).

Immunoglobulin secretion from patients' MNCs. The ability of XHIM B cells to secrete immunoglobulins was assessed by stimulation with SAC plus IL-2, anti-CD40 plus IL-10, or IL-10 plus IL-2. Upon stimulation with three types of the combination, MNCs obtained from healthy volunteers produced IgG, IgM, and IgA normally (Table II). In other experiments with normal MNCs or purified B cells, anti-CD40 or IL-2 alone did not induce immunoglobulin secretion, whereas SAC or IL-10 alone induced low levels of immunoglobulin secretion (data not shown)(23). In contrast to the results obtained from normal MNCs, in all but two of the XHIM patients, IgA and IgG secretion was below detection to marginal levels. Adequate IgG production was found in Patients YF and TF when MNCs of the patients were stimulated with SAC plus IL-2, anti-CD40 plus IL-10, or IL-10 plus IL-2. On the other hand, IgM production in all of the six patients was moderate to the same as that of healthy controls (Table II). When MNCs were treated with anti–CD70-blocking mAb and cultured in the presence of SAC plus IL-2, the IgG production was reduced by 32±7% (mean±SD, $n = 5$) in controls and only 3% in Patient IN and 4% in Patient TY.

The stimulation with SAC plus anti-CD40 plus IL-10 was reported to induce maximum immunoglobulin secretion in XHIM (31). In disagreement with the data, Saiki et al. (32) reported that there was no induction of IgG and IgA secretion in four XHIM patients. In four of our patients (Patient IN, TY, AY, and RM), marginal levels of IgG secretion, but no IgA, were observed after the stimulation. Interestingly, costimulation of patients' MNCs with SAC plus IL-2 plus anti-CD40 plus IL-10 induced secretion of large amounts of IgG and IgM, but not IgA. These data demonstrate that, although there is a variation in the immunoglobulin production, B cells of XHIM patients produce no to marginal levels of IgG and IgA production, but adequate levels of IgM, and the cells have ability to induce large amounts of IgG and IgM secretion by triggering via immunoglobulin receptors and CD40 in cooperation with IL-2 and IL-10.

*Table II. Immunoglobulin Production by XHIM Patient MNC after Various Stimuli**

Donor Ig class	SAC‡+IL-2‡	Anti-CD40‡ +IL-10‡	IL-10+IL-2	SAC+IL-2+IL-10 +anti-CD40
Pt. IN IgA§	< 64	< 64	< 64	< 64
IgM§	768±27	544±21	450±42	2510±738
IgG§	670±41	546±39	234±25	2888±280
Cont. IgA	3283±973	890±78	811±113	1168±119
IgM	1241±837	249±11	241±17	257±28
IgG	8499±1371	4424±1397	3997±746	6977±1358
Pt. TF IgA	< 64	< 64	< 64	119
IgM	2129±434	1900±318	1677±578	2210±798
IgG	2378±222	1659±213	1053±130	3746±1135
Pt. YF IgA	< 64	89±2	< 64	< 64
IgM	1609±247	235±70	94±8	2119±473
IgG	3012±1454	794±214	612±79	2890±659
Cont. IgA	3054±1024	683±42	345±19	3832±824
IgM	3509±279	511±133	117±25	3009±245
IgG	6732±1825	3017±593	2403±771	5997±1432
Pt. TY IgA	< 64	< 64	< 64	< 64
IgM	1182±298	476±112	477±105	556±186
IgG	785±137	< 125	< 125	1800±246
Pt. AY IgA	< 64	< 64	< 64	< 64
IgM	1001±60	907±157	1087±72	1033±241
IgG	< 125	< 125	< 125	2013±119
Cont. IgA	7402±1957	5715±1239	582±1652	> 10000
IgM	7185±1427	2756±279	2832±110	8470±2438
IgG	9821±2153	6370±1413	5240±1257	> 10000
Pt. RM IgA	< 64	< 64	< 64	< 64
IgM	789±168	675±98	578±43	2908±463
IgG	< 125	< 125	< 125	1751±367
Cont. IgA	6341±987	3177±652	1009±610	3177±834
IgM	6579±1043	2356±590	820±52	6357±1436
IgG	> 10000	6549±1786	7390±1595	> 10000

*MNCs (10^5/well) were used in this study. ‡Final concentrations of SAC, IL-2, anti-CD40, and IL-10 were 0.01%, 50 U/ml, l μg/ml, and 50 ng/ml, respectively. §All immunoglobulin concentrations are given in ng/ml.

The defect of IgD^- $CD27^+$ B cells in patients with XHIM. The blockage of CD40/CD154 interaction leads to impairment of germinal center formation and development of immunological B cell memory (33, 34). The observation prompted us to investigate the function of peripheral blood B cells in XHIM patients. Experiments were therefore conducted in which phenotypic analysis of their peripheral blood B cells was performed. As previously reported (23), adult peripheral blood B cells could be subdivided into three discrete subtypes by the

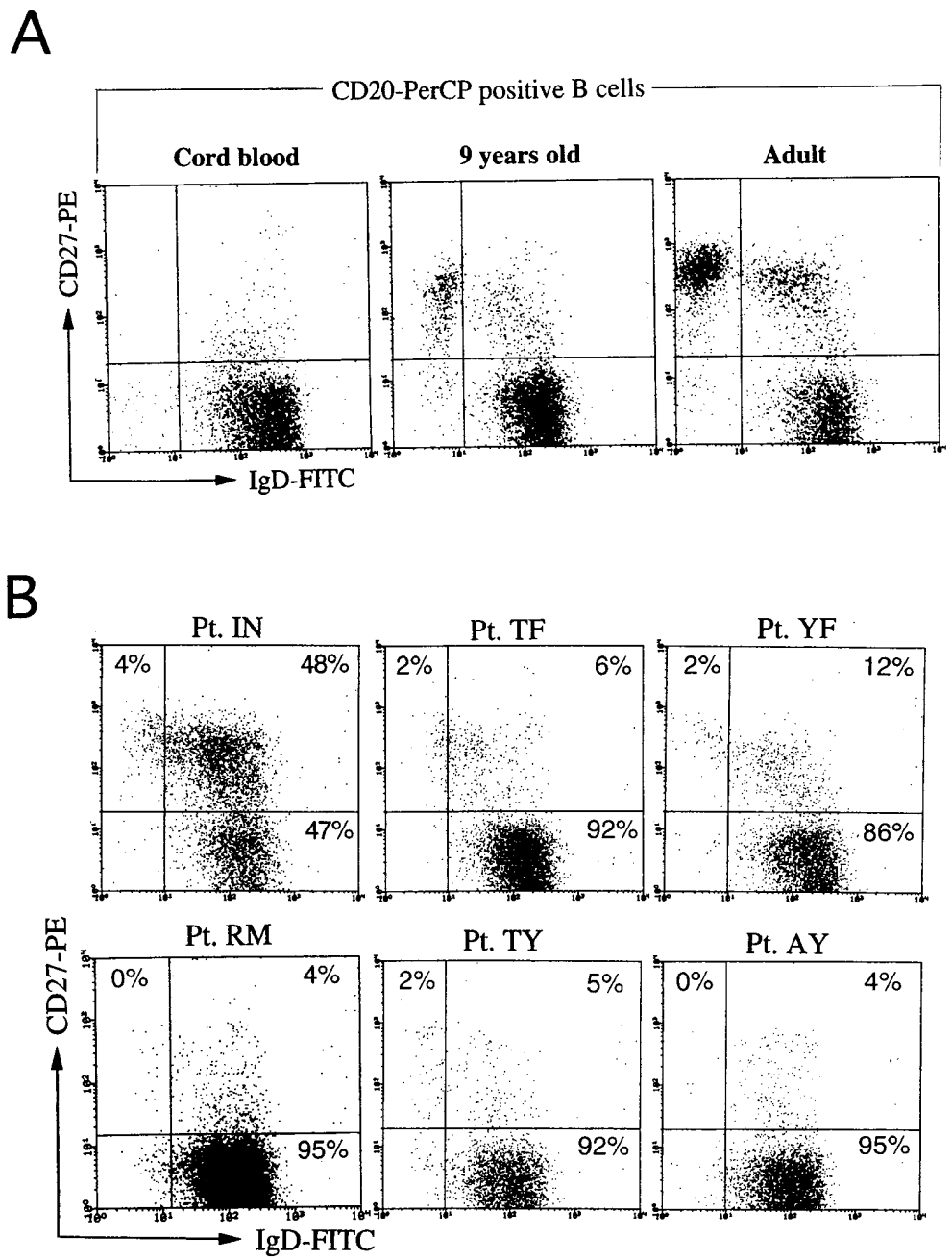

Figure 1. B cell subpopulations and defect of IgD$^-$ CD27$^+$ memory B cells in XHIM patients. MNCs of cord blood and peripheral blood of children aged 9 yr and of adult (*A*) or MNCs obtained from six XHIM patients (*B*) were stained with anti–IgD-FITC, anti–CD27-biotin followed by avidin-PE, and anti–CD20-PerCP. Three color analysis was carried out by gating CD20-PerCP positive B cells. Data were displayed as density plots with green (*FITC*) fluorescence, IgD, on the x-axis, and orange (*PE*) fluorescence, CD27, on the y-axis by the log scale. These data were representative of 15 cord blood samples, and peripheral blood samples of five 9-yr-old children and 20 adults. The percentage of positive cells is indicated.

IgD and CD27 expression, whereas cord blood B cells chiefly consisted of CD27$^-$ naive B cells and the CD27 expression on B cells increased with age (Fig. 1 *A*).

Three color flow cytometric analysis of peripheral blood cells in the XHIM patients revealed that the number of IgD$^-$ CD27$^+$ B cells was greatly decreased in all the patients as compared to that of normal volunteers (Fig. 1 *B*), implying that a generation of cells with the surface phenotype of memory B cells was diminished in the XHIM patients. Percentages of IgD$^+$ CD27$^+$ B cells were within normal levels, whereas percentages of IgD$^+$ CD27$^+$ B cells in Patient IN were increased, compared with those of normal adults (Fig. 1 *B*) (23). These data illustrate that peripheral blood B cell populations separated by the expressions of IgD and CD27 in XHIM patients are composed differently, supporting a view that an impaired germinal center formation due to defective CD154 expression results in impaired development of IgD$^-$ CD27$^+$ memory B cells.

Production of IgM from IgD$^+$ CD27$^+$ B cells in XHIM patient. A previous study has demonstrated that IgD$^-$ CD27$^+$ B cells produce IgG, IgM, and IgA, and that IgD$^+$ CD27$^+$ B cells predominantly produce IgM (23). Therefore, one may postulate that the IgD$^+$ CD27$^+$ B cell population in XHIM patients could produce IgM but little amounts of IgG and IgA. To explore this possibility, further experiments were carried out to examine whether the patient B cell populations separated by the expression of IgD and CD27 have different functions. We separated IgD$^+$ CD27$^+$ B cells of Patient IN (Fig. 2 *A*) and in-

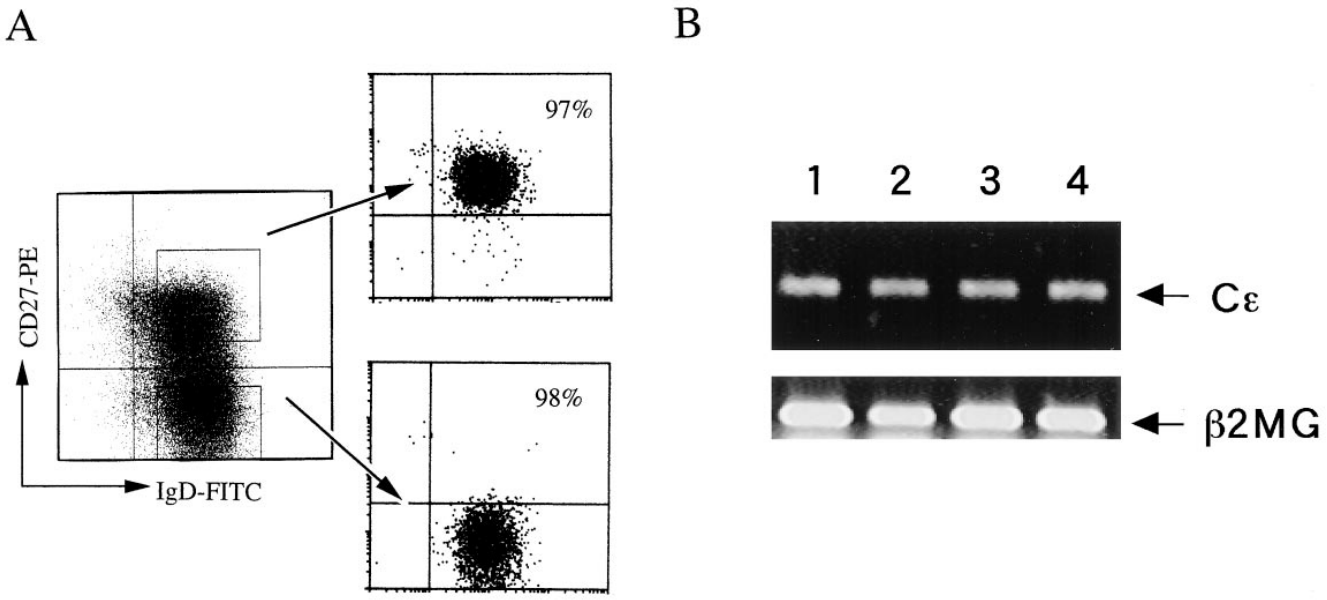

Figure 2. Purification of IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ B cell populations from the patient and germline ϵ transcripts. (*A*) MNCs obtained from Patient IN were stained with anti–IgD-FITC, biotin-labeled anti-CD27 followed by streptavidin-PE and anti–CD19-APC. IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ B cells were then purified by FACStar™ Plus. Purity is indicated as the percentage. (*B*) The highly purified IgD$^+$ CD27$^+$ B cells (lanes *1* and *3*) and IgD$^+$CD27$^-$ B cells (lanes *2* and *4*) were stimulated with 100 ng/ml IL-4 (lanes *1* and *2*) or IL-4 plus 1 μg/ml anti-CD40 (lanes *3* and *4*) for 16 h. After extraction of total RNA, RT-PCR was performed with Cϵ exon sense and antisense primers. β_2-microglobulin ($\beta_{2\,MG}$) was used as a positive control.

vestigated the immunoglobulin production in various B cell activation systems. IgD$^+$ CD27$^+$ B cells obtained from normal adults by sorting produced significant levels of IgM and moderate levels of IgG in the presence of SAC plus IL-2 or anti-CD40 plus IL-10, but no detectable levels of lgA. In contrast, IgD$^+$ CD27$^+$ B cells obtained from Patient IN produced moderate levels of IgM, mild IgG, and no IgA in the presence of SAC plus IL-2 or anti-CD40 plus IL-10 (Fig. 3). As for CD27$^-$ naive B cells, the cells of Patient IN, as well as healthy controls, could not produce IgG, IgM, and IgA in the presence of SAC plus IL-2 or anti-CD40 plus IL-10 (Fig. 3). Addition of CD70 transfectants to IgD$^+$ CD27$^+$ B cells in the presence of SAC plus IL-2 or anti-CD40 plus IL-10 augmented the IgG and IgM production by about twofold as compared to that of mock transfectants (data not shown). Very interestingly, the stimulation with SAC plus IL-2 plus anti-CD40 plus IL-10 promoted the production of IgG and IgM from IgD$^+$ CD27$^+$ B cells of both Patient IN and healthy controls. IgG and IgM production were also found in IgD$^+$CD27$^-$ B cells by the stimulation. However, no IgA production was recognized from two of the B cell populations by the stimuli.

Having shown the difference in IgA, IgM, and IgG synthesis between IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ B cells, we also examined the germline ϵ transcripts in two of the populations by RT-PCR. Germline Cϵ gene transcription was not found in unstimulated whole B cells obtained from healthy volunteers (data not shown). In the presence of IL-4 or IL-4 plus anti-CD40, both IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ B cell populations obtained from Patient IN equally expressed germline ϵ mRNA (Fig. 2 *B*). Similar results of germline ϵ gene transcripts were obtained when purified IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ B cells from healthy controls were stimulated with IL-4 or IL-4 plus anti-CD40 (data not shown).

Immunoglobulin production from cord blood B cells. A majority of cord blood B cells were composed of IgD$^+$CD27$^-$ B cells; IgD$^+$ CD27$^+$ B cells constituted a small population of B cells (Fig. 1 *A*). Highly purified cord blood B cells by positive selection with CD19 beads showed defective IgG and IgA secretion by the stimulation with SAC plus IL-2, anti-CD40 plus IL-10, or IL-10 plus IL-2. On the other hand, only low levels of IgM were observed after the addition of SAC plus IL-2 or anti-CD40 plus IL-10. Significant induction of IgG and IgM, but not IgA, was observed after the stimulation with SAC plus IL-2 plus anti-CD40 plus IL-10 (Table III). These data indicate that the composition of cord blood B cells separated by IgD and CD27 is in accordance with that of XHIM patient B cells, and that the pattern of IgG and IgA secretion from highly purified cord blood B cells is similar to that of XHIM patient B cells and that of IgD$^+$CD27$^-$ B cells obtained from normal adults.

IgE production. Anti-CD40 plus IL-4 was reported to induce class switching from IgM to IgE in XHIM patients (3, 4, 31, 35, 36). IgE production was examined by ELISA. In healthy controls, the stimulated MNCs with IL-4 plus anti-CD40, but not IL-4 alone, resulted in IgE secretion. The levels of IgE measured in the cultures varied among the patients. Three of the patients (Patients YF, TF, and RM) could produce IgE at the same levels as did healthy controls in the presence of IL-4 plus anti-CD40. Adequate IgE secretion was not detected in the other three patients. However, it is interesting to note that, in accordance with the results by Saiki et al. (32), the addition of IL-10 to anti-CD40 plus IL-4 resulted in measurable levels of IgE synthesis in Patient IN (Table IV). These data suggest that, although B cells of XHIM patients are composed of only IgD$^+$ CD27$^+$ and IgD$^+$CD27$^-$ cells, the B cells have ability to produce IgE via CD40 signaling in cooperation with IL-4 and IL-10.

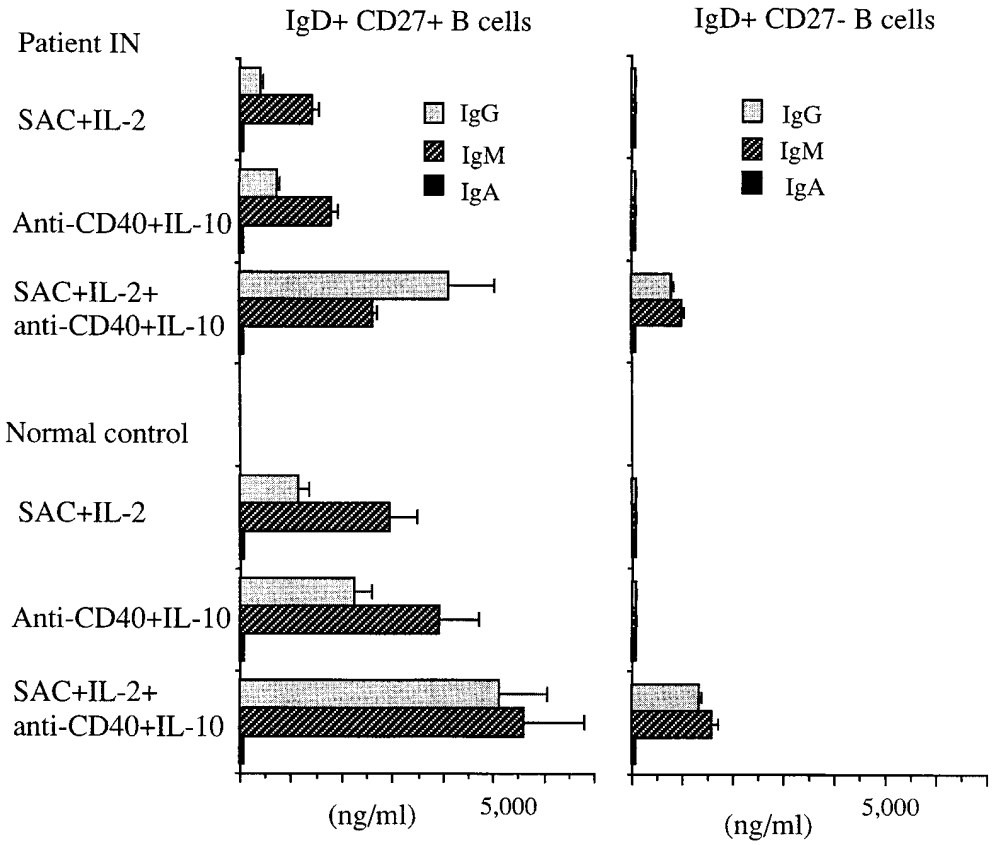

Figure 3. Immunoglobulin production by IgD^+ $CD27^+$ and IgD^+CD27^- B cells. Purified B cells (2.5×10^4/well) of Patient IN (Fig. 2 *A*) and normal adults were cultured in indicated B cell stimulation systems. After 8 d of culture, immunoglobulin levels in the supernatants were assayed by ELISA, as described in Methods.

Discussion

Germinal centers in the lymph node and spleen are sites of somatic mutation and affinity maturation of B cells. Though the molecular events required to drive proliferation, differentiation, and memory B cell formation in the germinal center are not completely understood, evidence from CD40/CD154 interaction and XHIM patients suggests that CD40 plays a major role in these events (1, 13, 33). For example, mice treated with an antibody to CD154 are incapable of germinal center formation, and are unable to produce memory B cells (37). CD154-deficient mice generated by gene-targeted disruption do not develop germinal centers in response to antigens, suggesting inability to develop memory B cell responses (9). These findings prompted us to examine whether memory B cells defined by CD27 molecule in the peripheral blood of XHIM patients were present, which may provide evidence for the lack of memory B cells as a cause of the impaired immunoglobulin production in vivo and in vitro in XHIM.

Based on the published reports, hyper-IgM syndrome can be classified into two types due to CD40 abnormalities and mutations in CD154 (37, 38). To clarify B cell functions in XHIM patients due to CD154 gene defects but not CD40 signal abnormality in CD40 pathway, we enrolled the patients with mutations in CD154. These mutations were confirmed by flow cytometric analysis using anti-CD40 mAbs and CD40-Fc

*Table III. Immunoglobulin Production by Purified Cord Blood B Cells**

	IgG (ng/ml)	IgM (ng/ml)	IgA (ng/ml)
SAC‡+IL-2‡	< 125	596±168	< 64
CD40‡+IL-10‡	< 125	378±96	< 64
IL-2+IL-10	< 125	< 96	< 64
SAC+IL-2+CD40+IL-10	6860±1370	6452±987	< 64

*Highly purified B cells (2.5×10^4/well) were used in this study. ‡Final concentrations of SAC, IL-2, anti-CD40, and IL-10 were 0.01%, 50 U/ml, 1 μg/ml, and 50 ng/ml, respectively.

*Table IV. IgE Secretion in XHIM Patients**

	IL-4‡	IL-4+anti-CD40‡	IL-4+anti-CD40+IL-10‡
Pt. IN	< 5§	< 5	11.3±2.1
Control	< 5	26.2±11.8	71.3±21.8
Pt. TF	< 5	19.3±5.0	ND
Pt. YF	< 5	36.1±13.2	ND
Control	< 5	42.9±17.2	ND
Pt. AY	< 5	7.8±4.3	ND
Pt. TY	< 5	6.9±3.5	ND
Control	< 5	21.0±6.8	ND
Pt. RM	< 5	15.1±6.1	96.0±18.4
Control	< 5	20.4±9.7	129.4±34.5

*MNCs (10^5/well) were used in this study. ‡Final concentrations of IL-4, anti-CD40, and IL-10 were 50 ng/ml, 1 μg/ml, and 100 ng/ml, respectively. §Immunoglobulin E concentration is given in ng/ml.

fusion protein as well as sequencing of CD154 gene. In addition, because CD27 expression on B cells increases with age (23), we selected older patients with XHIM in Japan.

Several reports indicate that the patient B cells have ability to produce IgG, IgA, or IgE in the presence of anti-CD40 plus cytokines (3, 31, 32, 36, 39). On the other hand, it is reported that apparent IgG and IgA production is not observed with anti-CD40 plus IL-10 (32). In our cases, B cells from some but not all of our XHIM patients did not secrete IgG in response to anti-CD40 plus IL-10 or SAC plus IL-2, which is similar to the reported findings of several XHIM patients (32, 40). However, in disagreement with the XHIM patients reported (35), IgA secretion was absent in all our patients regardless of the B cell stimulation systems (Table II). The reasons for the discrepancies of IgA, IgM, and IgG production among published data and the present study are not clear. One possibility is that there exists variations in the patient population due to different mutations in CD154 gene and that ability to produce immunoglobulins gradually changes with age. Alternatively, the sensitivity of ELISAs for detecting immunoglobulins may vary in laboratories. Very interestingly, IgG and IgM, but not IgA, was dramatically increased in cultures of XHIM B cells upon addition of anti-CD40 plus IL-10 plus IL-2 plus SAC. These results are similar to the findings of IgG, IgM, and IgA secretion by cord blood B cells and adult IgD^+CD27^- B cells. IgD^+CD27^- naive B cells have ability to produce, at least, IgG and IgM, in response to signaling via immunoglobulin receptors and CD40 in cooperation of cytokines such as IL-10 and IL-2 in vitro.

Activation with anti-CD40 or CD154 molecule is shown to induce proliferation in XHIM B cells (3, 4), indicating that the B cell function in XHIM patients may be normal. However, one important issue that has not been clearly addressed in studies of XHIM is the mechanism underlying the inability to produce adequate levels of IgG and IgA in response to various stimuli including stimulation via CD40 in vitro. Gray et al. (13) demonstrated that the development of memory B cell populations measured by adoptive transfer was grossly impaired by administration of soluble CD40 fusion protein, so as to block the CD40/CD154 interaction. They also indicated that by the blockage antigen-specific IgG responses were grossly inhibited, whereas the IgM response was augmented greatly, suggesting that there is a CD40 ligand-independent pathway of B cell activation that leads to IgM response.

It is generally admitted that cord blood and neonate B cells produce very low levels of immunoglobulin, presumably because of T cell immaturity and poor helper activity, including the undetectable CD154 expression on T cells (41, 42, 43). On the other hand, functional expression on neonatal T cells can be induced by their activation with anti-CD3 (44). Recently, we have demonstrated that cord blood B cells are mainly composed of IgD^+ $C27^-$ cells, but not IgD^- $CD27^+$ B cells with capacity to produce IgG, IgM, and IgA, implying that the impaired immunoglobulin secretion from cord blood cells is due to the composition of unprimed naive B cells (23). In this respect, the impairment of CD40/CD154 interaction in XHIM patients grossly inhibits the generation of $CD27^+$ memory B cells. The condition is very similar to that of cord blood B cells, which may not yet be primed in the germinal center through immunoglobulin receptors and CD40.

IgE synthesis is preceded by transcription of the germline ϵ RNA, and the relationship between this expression and subsequent switching to $C\epsilon$ has been proposed. IgD^+ $CD27^+$ and IgD^+CD27^- B cells of the patient studied here, as well as healthy adults, as expected, did not differ in their ability to produce germline ϵ transcripts by exogenous IL-4. Also, anti-CD40 stimulation or anti-CD40 plus CD70 transfectants did not upregulate IL-4–induced ϵ transcripts in B cell subpopulations from healthy adults even in IgD^- $CD27^+$ memory B cells (data not shown), suggesting that CD27/CD70 interactions do not participate in IL-4–induced ϵ transcripts. We have found that cross-linking through CD27 on B cells enhanced IgE synthesis by promoting the differentiation into plasma cells in the presence of IL-4 plus anti-CD40 mAb (Nagumo, H., K. Agematsu, S. Hokibara, K. Yasui, and A. Komiyama, manuscript in preparation). Therefore, the absence of IgD^- $CD27^+$ memory B cells in XHIM patients may contribute to the possibly impaired IgE synthesis.

CD40 is expressed on most B cells including naive and memory B cells. On the other hand, CD27 is expressed on memory B cells, but not on naive B cells (23). CD154 is expressed on T cells soon after activation, and CD70 was expressed on T cells later after activation (24). On the basis of these findings, mature B cell differentiation and immunoglobulin production are regulated by at least two of the major cell to cell signaling pathways. CD40/CD154 interactions act in an early phase of B cell activation and induce the expansion of a memory B cell pool. Most recently, we demonstrated that memory B cells differentiated into plasma cells by the activated helper T cells via CD27/CD70 in the presence of several cytokines such as IL-10 and IL-2 (24). These findings strongly suggest that IgD^- $CD27^+$ memory B cells are necessary to produce large amounts of immunoglobulins by differentiating into plasma cells through the contact with CD70.

In conclusion, the present data indicate that the absence of IgD^- $CD27^+$ memory B cells largely accounts for impaired immunoglobulin production despite functional CD40 signaling in XHIM patients.

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