

Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM

(isotype switching/immunodeficiency/switch recombination)

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ABSTRACT B lymphocytes from patients with X chromosome-linked immunoglobulin deficiency with normal or elevated serum IgM are unable to switch from the synthesis of IgM/IgD to that of other immunoglobulin isotypes. Isotype switch recombination was evaluated in three affected males by examining interleukin 4-driven IgE synthesis. T-cell-dependent IgE synthesis was completely absent in the B lymphocytes of the patients. In contrast, CD40 mAb plus interleukin 4 induced the patients' B cells to synthesize IgE and to undergo deletional switch recombination. Because interaction between CD40 and its ligand on activated T cells is critical for T-cell-driven isotype switching, we examined CD40 ligand expression. In contrast to normal T cells, lymphocytes from the patients expressed no detectable CD40 ligand on their surface after stimulation with phorbol 12-myristate 13-acetate and ionomycin, although the mRNA of the ligand was expressed normally. These results suggest that defective expression of the CD40 ligand underlies the failure of isotype switching in this disease.

Immature B cells express IgM and IgD on their surface. During an immune response, a B lymphocyte can express different heavy chain isotypes sharing the same variable, variable(diversity)joining [V(D)J], region (1). Heavy chain class (C_H) switching allows a single B-cell clone to produce antibodies that retain variable region specificity in association with different C_H region genes that possess different effector functions. Class switching is thought to result from a deletional recombination event that juxtaposes a downstream C_H gene to the rearranged V(D)J genes. Recombination involves switch (S) regions located 5' of the C_μ gene and corresponding S regions located immediately 5' of each C_H gene except C_δ.

Deletional switch recombination in human B cells is best understood in the case of isotype switching to IgE. Interleukin 4 (IL-4), the switch factor for IgE, induces the transcription of a 1.8-kb ϵ germ-line mRNA, which initiates 5' of the S _{ϵ} region (2). This transcript is sterile as it is not translated into a functional protein. A second signal, delivered by T cells, is required for deletional switch recombination. This T-cell signal can be replaced by Epstein-Barr virus, hydrocortisone, monoclonal antibody (mAb) to the B-cell antigen CD40 (3), or a soluble form of the CD40 ligand (4). The CD40 ligand is expressed on T cells upon activation (5). T-cell-dependent isotype switching to IgE appears to depend on interaction between CD40 and its ligand because it is inhibited by soluble (s) CD40 (6).

X chromosome-linked immunoglobulin deficiency with normal or elevated serum IgM (HIGMX-1) is characterized

by undetectable serum levels of IgG, IgA, and IgE with normal or elevated levels of IgM (7). Circulating B cells are normal in number but are exclusively surface IgM/IgD positive with no detectable cells expressing surface IgG or IgA. Affected males are unduly susceptible to recurrent pyogenic infections, autoimmune diseases, and lymphoproliferative disease. The underlying defect in these patients appears to be an inability to switch from IgM/IgD secretion to the production of other immunoglobulin isotypes, IgG, IgA, or IgE (8, 9). We demonstrate that B cells from three males with this syndrome synthesize IgE in response to CD40 mAb plus IL-4 and undergo deletional switch recombination. In contrast, T cells from these patients fail to induce IgE synthesis in IL-4-treated B cells and are unable to express the ligand for CD40 on their surface.

METHODS

Patients. Three unrelated male patients, a 2-year-old Chinese (patient 1), a 10-year-old Lebanese (patient 2), and a 14-year-old of Italian descent (patient 3) fitted the World Health Organization criteria for the diagnosis of HIGMX-1 (10). They suffered from recurrent pulmonary infections beginning in the first year of life and had affected lateral male relatives. Their serum immunoglobulin levels and B-cell phenotype are summarized in Table 1.

Cell Preparation, Culture, and IgE Assay. PBMC and B cells were isolated and cultured as described (11). Recombinant IL-4 (Amgen Biologicals) was used at 100 units/ml; CD40 mAb 626.1 (12) was used at 5 μ g/ml. Day 10 culture supernatants were assayed for IgE as described (13); the average of two replicate samples is given in the results. sCD40 and sCD44 were products of fusion of cDNA segments encoding the extracellular domain of CD40 (14) and CD44 (15) to genomic DNA segments encoding human IgG1 (5, 16) and were purified on a protein A column.

Nested Polymerase Chain Reaction (PCR) Runs for the S _{μ} /S _{ϵ} "Switch Fragments." This was performed on high molecular weight DNA isolated from cultured B cells using two sets of nested primers located 5' of S _{μ} and 3' of S _{ϵ} as described (17).

Cell Surface Expression of the CD40 Ligand. PBMC were left untreated or stimulated with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 μ M; Calbiochem). PBMC (10⁷ per ml) were suspended in staining buffer (18) and incubated successively for 30 min on ice with sCD40 or sCD44 (20 μ g/ml), biotinylated protein G (10 μ g/ml;

Table 1. Serum immunoglobulin levels and B-cell phenotype

Subject	Serum immunoglobulin, mg/dl			% CD19 ⁺ cells	% CD19 ⁺ cells with surface (s) immunoglobulin staining			
	IgM	IgG	IgA		sIgM	sIgD	sIgG	sIgA
Patient								
1	82	20	<7	27	99	99	<1	<1
2	672	20	<7	8	99	99	<1	<1
3	89	50	<7	13	99	99	<1	<1
Control (n = 3)								
	50–200	>600	50–200	12–20	60–80	60–70	10–20	10–20

Serum immunoglobulin levels were determined using a radial immunodiffusion technique. Peripheral blood mononuclear cells (PBMC) were stained for B-cell number using a phycoerythrin-labeled anti-CD19 mAb (Beckton Dickinson), and surface immunoglobulin staining was assessed by fluorescein-labeled F(ab')₂ fragments of goat anti-human immunoglobulin (Boehringer Mannheim). CD19⁺, CD19-positive.

Calbiochem), and phycoerythrin-labeled streptavidin (R & D Systems, Minneapolis). CD25 expression was determined using a fluorescein-labeled mAb (Beckton Dickinson).

Northern Blot Analysis. Total RNA was extracted from PBMC stimulated with PMA plus ionomycin for 3 hr (19). Ten micrograms of RNA was fractionated on a 1.5% formaldehyde/agarose gel, blotted onto nitrocellulose, and hybridized with random primer labeled (Pharmacia) human CD40 ligand probe (20).

RESULTS

We examined the capacity of PBMC from the patients to undergo IL-4-driven isotype switching to IgE. Table 2 shows that, in contrast to PBMC from three age-matched controls, PBMC from all three patients completely failed to secrete IgE in the presence of IL-4. Induction of IgE synthesis in PBMC by IL-4 is strictly dependent on T cells; thus the failure of the patients' PBMC to respond to IL-4 could be due to a defect in the T cells or the B cells. We therefore examined the response of the patients' lymphocytes to stimulation with CD40 mAb plus IL-4. Table 2 shows that PBMC from the patients synthesized IgE in response to CD40 mAb plus IL-4. The effect of CD40 mAb was independent of T cells because purified B cells from the patients synthesized IgE in response to CD40 mAb plus IL-4.

The ability of the patients' B cells to undergo deletional switch recombination was evaluated directly using a nested PCR method that detects S_μ/S_ε switch fragments. The distance from S_μ to S_ε is ≈250 kb. After productive switch recombination, S_μ and S_ε are juxtaposed, thereby enabling detection of DNA fragments of 0.6–1.5 kb by nested PCR. Variable-size DNA fragments are amplified because switch recombination occurs at variable sites within the S_μ and S_ε regions. If switch recombination has not occurred, no DNA fragments will be detected. Fig. 1 shows that S_μ/S_ε switch fragments were amplified from DNA isolated from patient B

cells treated with CD40 mAb plus IL-4, and it can be concluded that deletional switch recombination has occurred. On the other hand, no switch fragments were detected in normal or patient B cells treated with IL-4 alone or CD40 mAb alone (data not shown).

The above results indicated that B cells from the patients were capable of undergoing isotype switching to IgE and that the defect lies at the level of the T cells. Interaction between CD40 on B cells and its ligand on T cells is critical for T-cell-directed isotype switching of IL-4-treated B cells (4, 6). We therefore examined the expression of the ligand for CD40 by the patients' cells. Fig. 2 shows that the CD40 ligand is not detectable on resting T cells. Activation of normal T cells by PMA and ionomycin induced CD40 ligand expression. Expression peaked at 6 hr and was lost by 24 hr. In contrast to normal T cells, T cells from the patients completely failed to express the CD40 ligand. The failure to detect the CD40 ligand on the patients' cells was not simply due to a lag in CD40 ligand expression because the ligand remained undetectable up to 24 hr after stimulation. Furthermore, stimulation with anti-CD3 mAb induced CD40 ligand expression on normal T cells but not on patient T cells (data not shown). The failure to express the CD40 ligand was specific because activated T cells from the patients normally expressed CD25 (interleukin 2 receptor α). Defective expression of the CD40 ligand was not simply a consequence of agammaglobulinemia because T cells from two patients with common variable immunodeficiency (acquired agammaglobulinemia) expressed the CD40 ligand upon activation (Fig. 2).

We next examined whether the defect in CD40 ligand expression resided at the mRNA level. CD40 ligand mRNA was detected in normal cells by Northern blotting 1 hr after stimulation with PMA plus ionomycin and remained detect-

Table 2. IgE synthesis by PBMC and by B cells

Cells	Stimulus	Patient			Control		
		1	2	3	1	2	3
PBMC	—	<150	<150	<150	200	<150	<150
	IL-4	<150	<150	<150	3200	4700	5800
B cells	CD40 mAb						
	+ IL-4	7100	2400	3700	7700	7600	9400
B cells	—	<150	<150	<150	<150	<150	<150
	IL-4	<150	<150	<150	<150	300	200
B cells	CD40 mAb						
	+ IL-4	6600	5200	5400	10700	5320	7400

Net IgE synthesis (pg/ml) above background. Background IgE levels were detected in the presence of cycloheximide (100 μg/ml) and were always <500 pg/ml.

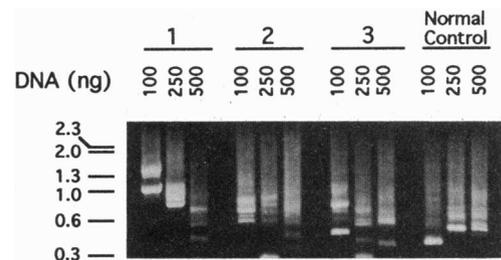


FIG. 1. Nested primer PCR amplification of S_μ/S_ε switch fragments. Aliquots of total cellular DNA from patient (1, 2, and 3) and from control B cells stimulated with CD40 mAb plus IL-4 were amplified by nested primer PCR. DNA amounts utilized in the first round of PCR are noted above the gel. The second round of PCR was performed on a 1/10th aliquot of the first round PCR mixture. Final PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. Positions of molecular weight markers (kb) are shown on the left.

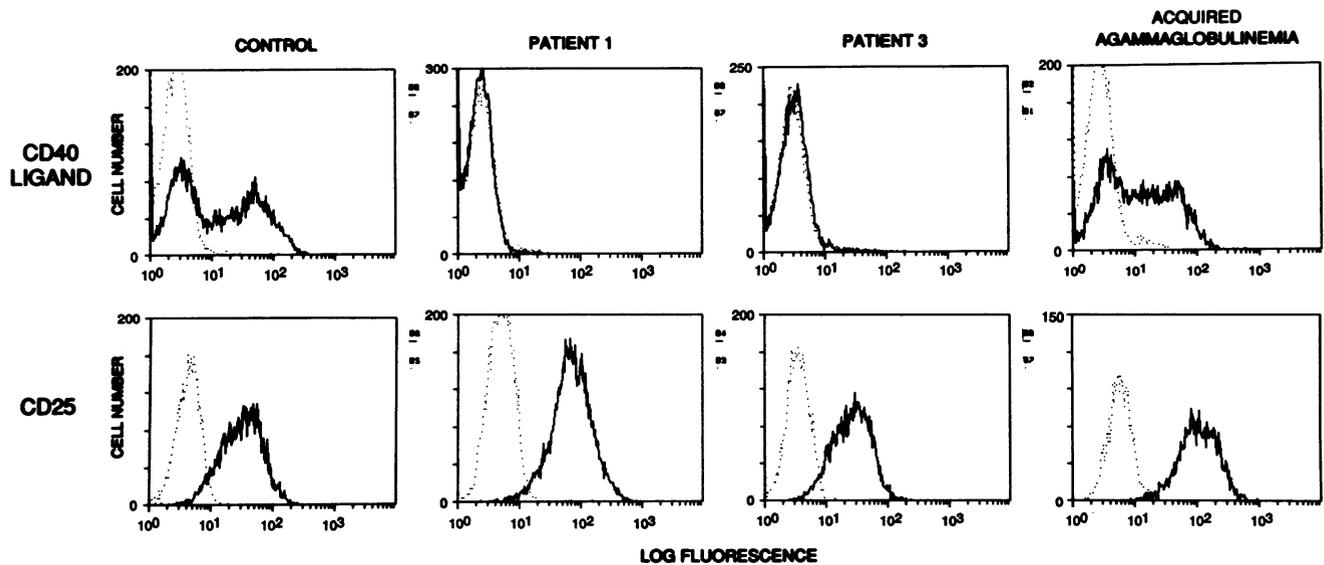


FIG. 2. Cell surface expression of the CD40 ligand. PBMC were left untreated (dotted line) or stimulated with PMA plus ionomycin (continuous line). CD40 ligand expression was assessed at 6 hr and CD25 expression was assessed at 24 hr. There was no detectable binding of sCD44 with or without stimulation in patients or controls (data not shown). Patient 2 was also studied and similar results were obtained. Four healthy subjects and two patients with acquired agammaglobulinemia were used as control. Similar results were obtained, and one representative result from each group is shown.

able at 3 and 6 hr (data not shown). Fig. 3 shows that normal cells stimulated for 3 hr expressed a major ≈ 2.2 -kb band and a minor ≈ 1.8 -kb band that hybridized with the human CD40 ligand cDNA probe. The 1.8-kb band was visible on the original autoradiograph in patient 1. Hybridizing bands of identical size were detected in RNA from patient cells stimulated in parallel.

DISCUSSION

Our results demonstrate that peripheral blood lymphocytes of patients with HIGMX-1 fail to express the ligand for CD40 on their cell surface. Given the role of the CD40 ligand in T-cell-directed isotype switching, these findings suggest that

failure of CD40 ligand expression underlies the isotype switch defect in these patients.

Our results clearly place the defect in isotype switching at the T-cell level. This is consistent with previous findings by Mayer *et al.* (21), who reported that a Sézary T-cell line could induce isotype switching in B cells from patients with HIGMX-1 and concluded that the defect was in switch T cells. The observation that B cells from these patients could undergo deletional switch recombination is consistent with the findings of Hendriks *et al.* (22), who reported nonrandom X chromosome inactivation in IgG- and IgA-secreting Epstein-Barr virus-transformed B-cell lines derived from two female carriers and concluded that the defect did not involve the immunoglobulin C_H switch mechanism.

T-cell number, subset distribution, and proliferation to mitogens and antigens were normal in all three patients studied. This suggests that expression of the ligand for CD40 does not play a critical role in T-cell maturation and proliferative responses to antigen and nonspecific mitogens. Furthermore, T cells from patients with HIGMX-1 can help normal B cells to synthesize IgG in response to pokeweed mitogen (8, 9). Because pokeweed mitogen-driven IgG synthesis does not involve isotype switching (23–25), these results do not contradict our present findings. Rather, they suggest that the CD40 ligand is not required for all T-cell helper activities but is selectively involved in T-cell-driven isotype switching. Interestingly, PBMC from these patients failed to synthesize IgE in response to IL-4 and hydrocortisone, an agent shown to induce isotype switch recombination (ref. 26; data not shown). This suggests that interactions that involve the CD40 ligand may be necessary for B cells to respond to hydrocortisone.

The molecular basis of defective CD40 ligand expression has yet to be defined. Genetic linkage analysis has localized the defective gene in HIGMX-1 to the q24-27 region of the X chromosome (27). In view of the presence of normal-sized mRNA for the CD40 ligand, the defective gene on the X chromosome may encode for the CD40 ligand itself or for a gene product that is essential for cell surface expression of the CD40 ligand. Chromosomal mapping of the gene for the CD40 ligand and characterization of the CD40 ligand mRNA in the patients are necessary. Ultimately, it will be important

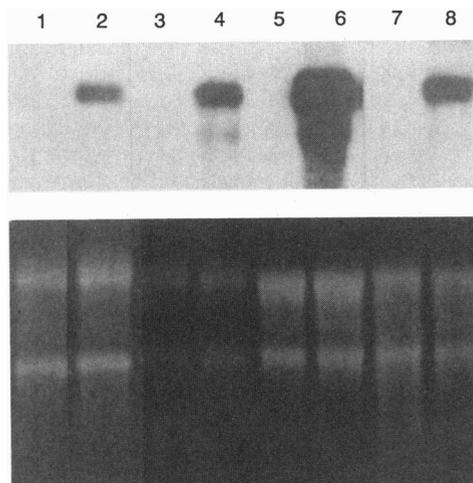


FIG. 3. Northern blot analysis of CD40 ligand mRNA. (Upper) Autoradiograph of RNA hybridized with the human CD40 ligand probe. Odd-numbered lanes, unstimulated cells; even-numbered lanes, cells stimulated for 3 hr with PMA plus ionomycin. Lanes 1 and 2, patient 1; lanes 3 and 4, control donor 1; lanes 5 and 6, patient 3; lanes 7 and 8, control donor 2. (Lower) Ethidium bromide staining of 28S and 18S RNA illustrating the relative RNA concentrations. Patient 2 was also studied and similar results were obtained.

to demonstrate, by transfection studies, that expression of the CD40 ligand in the patients' T cells corrects the defect in T-cell-driven isotype switching.

Note Added in Proof. To date, we have examined surface expression of CD40 ligand in nine patients from eight unrelated families. Cells obtained from all nine patients failed to express CD40 ligand upon stimulation. Mutational analysis of CD40 ligand cDNA from patient 1 revealed a 58-base-pair deletion and of cDNA from patient 3 showed a C → A transversion at position 590 and the deletion of an adjacent C residue (unpublished results) indicating that a defect in the CD40 ligand gene is the underlying cause of HIGM1.

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