Human neoplastic B cells express more than two isotypes of immunoglobulins without deletion of heavy-chain constant-region genes

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Flow cytometric analyses of surface immunoglobulins of human neoplastic B cells from four patients indicated that more than two isotypes were expressed on the surface of each patient's neoplastic B cells. Southern blot analysis of DNAs of these cells showed that each patient's neoplastic cells had the monoclonal rearrangement profile of the *J* segment of the immunoglobulin heavy-chain gene. Heavy-chain constant-region genes of these cells had no deletion associated with S-S recombination. Northern blot analysis of RNA from two neoplastic cells revealed that each RNA contained comparable amounts of mRNAs for two different isotypes which were identified by surface staining. These results support the hypothesis that the simultaneous expression of two different isotypes in a certain stage of B-cell differentiation is mediated by alternative RNA splicing without DNA deletion.

[**Key Words:** Immunoglobulin class switch; alternative splicing; Northern blot analysis; Southern blot analysis; flow cytometry]

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During differentiation of B lymphocytes (B cells), IgM is the first antibody expressed on the cell surface. IgG- and IgA-secreting cells are the descendants of cells that initially expressed IgM. Since the assembled variable (*V*) gene segments do not change in a lineage of the B cells, the switch of immunoglobulin class does not alter the antigen specificity of the antibody. Molecular mechanisms to replace the expressed constant region (*C*) of the immunoglobulin heavy-chain (*H*) without affecting the *V* region have been suggested to involve deletion of *C_H* genes 5' to the expressed *C_H* gene either by intrachromatid or sister chromatid recombination (Honjo and Kataoka 1978; Cory and Adams 1980; Rabbitts et al. 1980; Yaoita and Honjo 1980 a,b). Since deletion of *C_H* genes is mediated by recombination between the switch (*S*) regions located 5' to each *C_H* gene, except for the *C_δ* gene, this rearrangement is referred to as S-S recombination (Kataoka et al. 1980). The switch regions are composed of tandem arrays of short repetitive sequences (Kataoka et al. 1981; Nikaido et al. 1981).

B cells expressing two isotypes such as IgM and IgG have often been reported during normal B-cell development. The simultaneous expression of two immunoglobulin classes is difficult to explain by a deletion model. Yaoita et al. (1982) reported that there were no *C_H* gene deletions in $\mu^+\epsilon^+$ B lymphocytes sorted from

spleens of hookworm (*Nippostrongylus brasiliensis*)-injected SJA/9 mice whereas *I_H* loci were rearranged. The results imply that the simultaneous expression of the *C_μ* and *C_ε* genes may be mediated by differential RNA splicing of an mRNA precursor containing a single *V_H* and multiple *C_H* region sequences. On the basis of this experiment we have proposed a two-step model for class switching (Yaoita et al. 1982). The first step involves synthesis of a long primary transcript including a *V_H* and multiple *C_H* genes, and its differential RNA splicing. In the second step the intervening DNA sequence is deleted by S-S recombination. Subsequently, several other groups reported experiments in support of (Perlmutter and Gilbert 1984; Chen et al. 1986) and against (Katona et al. 1985) this model. However, the recent report that B cells of SJA/9 mice lack *F_ε* receptors (Adachi et al. 1985) does not agree with the claim that $\mu^+\epsilon^+$ B cells of hookworm-injected SJA/9 mice are due to cytophilic association of IgE (Katona et al. 1985).

In this study we will describe the organization and expression of *I_H* and *C_H* genes of human neoplastic B cells from four patients that express more than two isotypes on their surface. Expression of mRNA of two isotypes and the absence of the *C_H* gene deletion in these leukemic cells support the model proposed previously (Yaoita et al. 1982).

Table 1. Profile of surface immunoglobulins of neoplastic cells

Neoplastic cells	Pheno-types	Percentages of cells expressing immunoglobulins						
		anti-Ig	μ	δ	γ	α	κ	λ
145/85	$\mu\delta\alpha/\lambda$	89	85	30	1	41	3	85
268/85	$\mu\delta\gamma/\lambda$	ND ^a	39	60	56	1	2	82
302/78	$\mu\delta\gamma/\kappa$	87	83	25	75	<1	78	2
L161	$\delta\alpha/\kappa$	92	<1	85	<1	60	95	<1

Cells were stained with class-specific and anti-human immunoglobulin (anti-Ig) antibodies and positive cells were counted by flow cytometry. Neoplastic cells are chronic leukemia cells, except in the case of 145/85 cells, which are malignant lymphoma cells. Numbers represent percentages of cells stained by FITC-labeled isotype-specific antisera (Dalsø, Copenhagen, Denmark).

^a (ND) Not determined.

Results and discussion

Characterization of surface immunoglobulins of leukemic cells by flow cytometry

Peripheral blood leukocytes were isolated from patients (three Norwegian and one British) with chronic lymphocytic leukemia or lymphoma of B-cell origin. We analyzed their surface immunoglobulins by flow cytometry. As shown in Table 1, about 90% of these cells were surface immunoglobulin-positive with a predominant light-chain isotype. They were stained with antibodies (monoclonal or affinity-purified) specific to each class. Interestingly enough, the additive ratios of positive cells stained with antibodies against different isotypes exceeded 1. This result indicates the presence of cells expressing more than two classes of immunoglobulins simultaneously. All the specimens expressed predominantly either κ or λ light chain. The 145/85 cells seem to express the δ - or α -chain in addition to the μ -chain. About half of the 268/85 cells expressed only one of the μ -, δ -, and γ -chains, indicating that a significant portion

of 268/85 cells carried more than two isotypes. The major populations of the 302/78 and L161 cells were $\mu^+\gamma^+$ and $\delta^+\alpha^+$, respectively. A significant portion of the 302/78 cells also expressed the δ -chain.

Monoclonal origins of the neoplastic cells

We investigated the profile of the J_H segment rearrangement and deletion of immunoglobulin C_H genes in the neoplastic cells of the four patients. High-molecular-weight DNAs extracted from the peripheral blood cells of the patients were digested with *EcoRI*, *BamHI*, and *HindIII*. DNA fragments were electrophoresed and transferred to nitrocellulose filters, which were hybridized with DNA probes to test the rearrangement of the immunoglobulin loci. Figure 1 shows the probes and restriction enzyme cleavage sites of the human immunoglobulin loci.

Any rearrangements in the J_H locus involving V_H -D- J_H joining would alter the size of *EcoRI*, *BamHI*, and *HindIII* fragments hybridized with the J_H probe (a in Fig. 1). Since the rearrangement profile of the J_H segment is unique to each lymphocyte, examination of the J_H rearrangement of each patient's cells provides a direct test for the clonality of neoplastic cells as well as extent of contamination by non-B cells. As shown in Figure 2 (lanes 1 and 4), all the DNA preparations contained one or two rearranged J_H segments, indicating that the majority of all the patients' cells were monoclonally expanded B cells. DNAs of the 145/85 (Fig. 2A), 268/85 (Fig. 2B), and L161 (Fig. 2D) cells had two rearranged J_H segments and a faint germ line J_H segment. The results show that both alleles of the J_H segments are rearranged. Furthermore, the contamination by non-B cells was negligible for the present analyses. DNA of the 302/78 cells contained one germ line and one rearranged J_H band. The intensity of the germ line J_H band was less than the rearranged band, probably because of the longer size of the fragment. The results also indicate that the contamination with other cells was not significant.

Figure 1. The restriction maps of the immunoglobulin locus. Horizontal arrows indicate the DNA fragments that were used as probes; (a) J_H probe (*EcoRI*–*HindIII* fragment); (b) 5' C_μ probe (*SacI*–*EcoRI* fragment); (c) C_μ probe (*EcoRI* fragment); (d) $C_{\gamma 1}$ probe (*SacII* fragment); (e) $C_{\epsilon 1}$ (*BamHI* fragment); (f) $C_{\alpha 1}$ probe (*XhoI*–*HindIII* fragment). H24 (Takahashi et al. 1980), Igγ1-10 (Takahashi et al. 1982), CH4A·H·Igε1 (Nishida et al. 1982), and CH4A·H·Igα25 (Hisajama et al. 1983) are original clones for probes a–c, d, e, and f, respectively. (Closed box) Structural sequences; (dotted box) S regions.

Figure 2. Southern blot analyses of neoplastic cells. Each lane is a pair of patient DNA (L) and human placental DNA (P). (A) 145/85 cell DNA was digested with *Eco*RI (lanes 1–3) or *Bam*HI (lanes 4–6). Probes used are: I_H probe for lanes 1 and 4, 5' C_μ probe for lanes 2 and 5, and $C_{\alpha 2}$ probe for lanes 3, and $C_{\gamma 1}$ probe for lane 6. (B) 268/85 cell DNA was digested with *Eco*RI (lanes 1–3) or digested DNA, *Bam*HI (lanes 4–6). Probe used are: I_H probe for lanes 1 and 4, 5' C_μ probe for lanes 2 and 5, $C_{\epsilon 1}$ probe for lane 3, and $C_{\gamma 1}$ probe for lane 6. (C) 302/78 cell DNA was digested with *Eco*RI (lanes 1–3) or *Bam*HI (lanes 4–7). Probes used are: I_H probe for lanes 1 and 4; 5' C_μ probe for lanes 2 and 5; $C_{\alpha 2}$ probe for lanes 3 and 6; and $C_{\gamma 1}$ probe for lane 7. (D) L161 cell DNA was digested with *Eco*RI (lanes 1 and 2), *Bam*HI (lanes 3–5), or *Hind*III (lane 6). Probes used are I_H probe for lanes 1 and 3; 5' C_μ probe for lanes 2 and 4; $C_{\alpha 2}$ probe for lane 5; and $C_{\gamma 1}$ probe for lane 6.

Absence of recombination in the S_μ region

To assess whether or not the neoplastic cells underwent S–S recombination involving rearrangements in the S_μ region located between the I_H and C_μ genes, we used the 5' C_μ probe (b in Fig. 1) which contains the immediate 5'-flanking region of the C_μ gene. Both the I_H and 5' C_μ probes should hybridize with the same *Eco*RI or *Bam*HI fragment as long as the I_H and C_μ sequences are physically linked. In the 145/85 cells, the 5' C_μ probe hybridized with two rearranged fragments (12.5- and 9.8-kb *Eco*RI fragments, and 23- and 20-kb *Bam*HI fragments) that are identical to those detected by the I_H probe, indicating that the I_H and C_μ genes were physically linked in the 145/85 cells (Fig. 2A, lanes 2 and 5). In the 268/85 cells I_H loci of both alleles rearranged to give rise to two new fragments, namely 13.2-kb and 12.5-kb *Eco*RI fragments and 15.5-kb and 13-kb *Bam*HI fragments. These rearranged I_H bands were physically linked with the C_μ

gene as the *Eco*RI and *Bam*HI fragments of the same sizes were hybridized with the 5' C_μ probe (Fig. 2B, lanes 2 and 5). The 302/78 cells contained one rearranged I_H fragment which was physically linked with the C_μ gene because the rearranged bands (12.6-kb *Eco*RI and 15.9-kb *Bam*HI fragments) hybridized with the C_μ and I_H probes were superimposable (Fig. 2C, lanes 2 and 5). In the L161 cells, the sizes of two rearranged I_H fragments were identical to those hybridized with the 5' C_μ probe (Fig. 2D, lanes 2 and 4), again indicating that the I_H and the C_μ genes are physically linked on both alleles of the L161 cells.

Absence of C_H gene deletion

We further investigated the genomic contexts of other C_H genes in the four neoplastic cells. The human C_H genes are clustered on chromosome 14 in the order of 5'- C_μ - C_δ - $C_{\gamma 3}$ - $C_{\gamma 1}$ - $C_{\epsilon 2}$ - $C_{\alpha 1}$ - ψC_γ - $C_{\gamma 2}$ - $C_{\gamma 4}$ - $C_{\epsilon 1}$ - $C_{\alpha 2}$ -3' (Flanagan and Rabbitt 1982; Bech-Hansen et al. 1983). The

$C_{\epsilon 1}$ probe (e in Fig. 1) cross-hybridizes with the $C_{\epsilon 2}$ and $C_{\epsilon 3}$ genes, and the $C_{\alpha 2}$ probe (f in Fig. 1) cross-hybridizes with the $C_{\alpha 1}$ gene. In addition, the $C_{\epsilon 1}$ probe detects a third fragment containing the $C_{\epsilon 3}$ gene which is located on chromosome 9 (Max et al. 1982). Since each pair of the C_{ϵ} and C_{α} genes ($C_{\epsilon 2}$ - $C_{\alpha 1}$ and $C_{\epsilon 1}$ - $C_{\alpha 2}$) is located on a single *EcoRI* fragment (30 kb and 28 kb, respectively) (Max et al. 1982; Nishida et al. 1982), both $C_{\epsilon 1}$ and $C_{\alpha 2}$ probes (e and f in Fig. 1, respectively) hybridize to the two identical *EcoRI* fragments (30 and 28 kb) in the human germ line DNA. All C_{ϵ} and C_{α} genes in the four neoplastic cells were in the germ line context (lane 3 in A and B, lanes 3 and 6 in C, and lane 5 in D of Fig. 2), excluding deletion of the C_{ϵ} and C_{α} genes.

In the 302/78 cells one additional band (14.5-kb *EcoRI* or 10-kb *BamHI* band) appeared when hybridized with the $C_{\alpha 2}$ probe. However, the possibility that this fragment was created by S_{μ} - S_{α} recombination and linked with the J_H segment was excluded because the sizes of the *EcoRI* and *BamHI* fragments hybridized with the $C_{\alpha 2}$ probe were different from those hybridized with the J_H and 5' C_{μ} probes. Since the 30-kb *EcoRI* (or 19.1-kb *BamHI*) band of 302/78 DNA was fainter than those of placental DNA, we considered that the 14.5-kb *EcoRI* (or 10-kb *BamHI*) fragment of the 302/78 cells originated by polymorphic deletion somewhere around the $C_{\alpha 1}$ gene on one allele of 302/78 cells.

The $C_{\gamma 1}$ probe (d in Fig. 1) which cross-hybridized with all the subclasses of the C_{γ} genes revealed that the C_{γ} genes of the neoplastic cells were also in the germ line context (lane 6 in A, B, and D, and lane 7 in Fig. 2C). Two polymorphic counterparts of the 15.5-kb ($C_{\gamma 2}$) and 9.4-kb ($C_{\gamma 4}$) bands in human placental DNA (Takahashi et al. 1982; Noma et al. 1984) were not seen in the 268/85 cells. The results indicate that the C_{γ} , C_{ϵ} , and C_{α} genes were not deleted from the four neoplastic cells.

Presence of mRNAs for two isotypes

We analyzed transcripts of immunoglobulin genes in the 145/85 and 268/85 cells because the transcripts are more abundant in these cells than in the other cells. Total RNA extracted from the 145/85 leukemic cells contained 2.4-kb and 2.1-kb μ -chain mRNA, which encodes the membrane-bound and secretory forms of the μ -chain, respectively, as shown in Figure 3A. The 145/85 RNA also contained 1.9-kb mRNA for the membrane-bound form of the α -chain. The amount of the C_{α} transcripts was about one-half of that of the C_{μ} transcripts, judging from intensities of the bands.

The 268/85 cells contained 2.4-kb and 2.1-kb μ -chain mRNAs, and 3.0-kb (membrane-bound form) and 1.7-kb (secretory form) γ -chain mRNAs (Fig. 3B). The γ -chain mRNA bands were rather broad because of partial degradation of RNA as identified by ethidium bromide staining. α -Chain mRNA was not detected in the 268/85 cells, even after long exposure (data not shown). The amount of the C_{γ} gene transcripts was estimated to be about one-half of the C_{μ} gene transcripts by densitometric tracing.

Figure 3. Presence of C_{μ} , C_{γ} , and C_{α} mRNAs in neoplastic cells. Total RNAs (10 μ g each) of 145/85 (A) and 268/85 (B) cells were electrophoresed in agarose gels, blotted onto filters, and hybridized with the C_{μ} (lanes 1 and 3), $C_{\alpha 2}$ (lane 2), and $C_{\gamma 1}$ (lane 4) probes. Filters were autoradiographed for 30 min (lanes 1 and 2), 1 hr (lane 3), and 2 hr (lane 4).

Differential splicing model

The results of DNA and mRNA analyses are summarized in Table 2. All four cases contained one or two rearranged J_H segments, but no evidence for S-S recombination was found in C_H region genes. In two of the four cases the class and the amount of the heavy-chain mRNA correlated with those of the surface immunoglobulin was determined by flow cytometry.

These results could be explained by a mixture of leukemic cells expressing IgM and contaminant non-neoplastic lymphocytes expressing other classes of immunoglobulins or by a mixture of two populations of leukemic cells, each expressing different immunoglobulin classes on their surface. These possibilities are, however, unlikely because most of the peripheral leukocytes of these patients were monoclonal cells on the basis of surface marker studies and the profiles of J_H rearrangement.

The most plausible explanation for these results is that the neoplastic cells express different classes of immunoglobulins simultaneously by alternative RNA splicing of a long primary transcript as proposed by Yaotita et al. (1982). The two-step model for class switching assumes that a long transcript including a V_H - D - J_H complex and various C_H gene sequences is synthesized and its alternative splicing gives rise to mRNAs with the same V_H - D - J_H and different C_H sequences. Since the long transcript may be 180 kb long in order to express IgM and IgA, it is impossible to analyze it by gel electrophoresis. Because the amounts of RNAs were limited, we could not demonstrate that different classes of immunoglobulin had the same V_H - D - J_H complex. We considered that these neoplastic cells derived

Table 2. Summary of organization of the immunoglobulin C_H genes and their expression in human leukemic cells

Neoplastic cells	Phenotype	I_H^a	Linkage of I_H-C_μ	C_H genes ^a			mRNA
				C_γ	C_α	C_ϵ	
145/85	$\mu\delta\alpha/\lambda$	R/R	+ / +	G/G	G/G	G/G	$\mu^+ \alpha^+$
268/85	$\mu\delta\gamma/\lambda$	R/R	+ / +	G/G	G/G	G/G	$\mu^+ \gamma^+$
302/78	$\mu\delta\gamma/\kappa$	R/G	+ / +	G/G	R*/G	G/G	ND ^b
L161	$\delta\alpha/\kappa$	R/R	+ / +	G/G	G/G	G/G	ND

Rearrangement at I_H loci and C_H genes, and I_H-C_μ linkage were investigated.

^a (R) Rearranged; (G) germ line; (R/G and R/R) rearrangement in one and both alleles, respectively; (R*) rearrangement is due to the deletion somewhere around the $C_{\alpha 1}$ gene on one allele (see the text).

^b (ND) Not determined because of scarce supply of the cells.

from transformation of double producers that existed at a certain stage during differentiation of B cells.

Perlmutter and Gilbert (1984) also demonstrated different C_H sequences in a single transcript by sandwich RNA hybridization. More recently, Chen et al. (1986) showed that a variant of a murine B-cell leukemic line BCL₁ 2.58 produced the μ - and γ -chain mRNA as well as the two isotypes. BCL₁ 2.58 contained a single rearranged I_H segment. Taken together, these observations suggest that double producers exist and that these cells produce different classes of the immunoglobulin by alternative splicing during differentiation, possibly before S–S recombination.

Materials and methods

FACS analysis

Mononuclear cells from the blood of patients with chronic lymphatic leukemia or from lymphoid tissues in the case of lymphoma were stained with antibodies against the λ -, κ -, μ -, γ -, and α -chains. Cells were analyzed further by flow cytometry (FACS). We have collected multi-isotype-bearing specimens (other than or in addition to μ and δ), in which the total number of cells stained with antibodies against heavy chains exceeded that of cells stained with anti- λ and anti- κ antibodies (see Table 1).

DNA isolation and Southern hybridization

High-molecular-weight DNA from peripheral mononuclear cells was extracted as described (Yaoita and Honjo 1984). Approximately 1–2 μ g of DNA was digested with appropriate restriction enzymes, electrophoresed in 0.5% agarose gels, and transferred to nitrocellulose filters by the method of Southern (1975). Hybridization to nick-translated ³²P-labeled probes was carried out for 18 hr in 1 M NaCl at 65°C. Filters were washed in 0.015 M NaCl, 1.5 mM sodium citrate, 0.1% SDS and exposed to Kodak XRP films at –80°C with an intensifying screen.

Northern blot hybridization

Total RNA was prepared by homogenization of cells in guanidium thiocyanate and subsequent centrifugation on a CsCl cushion (Ullrich et al. 1977). Approximately 10 μ g of total RNA was treated with glyoxal and dimethylsulfoxide, fractionated by electrophoresis in agarose gels containing 10 mM sodium phosphate buffer (pH 7.0), and transferred to nitrocellulose filters (Makmaster and Carmichael 1977; Thomas 1980). The filters were hybridized with appropriate probes as described by Thomas (1980).

Materials

Restriction enzymes were obtained from Takara Shuzo Co. (Kyoto). Radioactive nucleotides were purchased from Amersham.

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