A Novel Anti-Vpre-B Antibody Identifies Immunoglobulin-Surrogate Receptors on the Surface of Human Pro-B Cells

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Summary

Vpre-B and λ5 genes, respectively, encode V-like and C-like domains of a surrogate immunoglobulin light chain (ΨL). Such ΨL complex is expressed in early progenitor B (pro-B) cells, before conventional immunoglobulin heavy (μH) and light (L) chains are produced. We raised a wide panel of monoclonal antibodies (mAbs) against soluble recombinant Vpre-B proteins to study early events in human B cell development. One of these antibodies, B-MAD688, labeled surrogate Ig-complexes on the surface of μH- pro-B cell lines and normal bone marrow cells in immunofluorescence assays. Immunoprecipitations using surface-labeled pro-B cells and B-MAD688 mAb indicated that human ΨL is associated with high molecular weight components homologous to the surrogate heavy (ΨH) chains described in mouse. Using B-MAD688 and SLC2 mAbs, we were able to distinguish between ΨHΨL and μHΨL complexes on the surface of human pro-B and later precursor, pre-B, cells. The finding of ΨHΨL complexes in mouse and man lead us to hypothesize a role for ΨH-containing receptors in B cell development.

Materials and Methods

Production of Human Soluble Vpre-B Proteins. Plasmids pCEH-Vpre-B/mCx and pCEH-Vpre-B/try, were constructed by insertion of a human full-length Vpre-B DNA into two distinct expression vectors containing either mouse Cx or the hinge-CH2-CH3 domains of human IgG heavy chain, respectively, kindly provided by Dr. Karjalainen (10). The published sequence for the hVpre-B gene ends prematurely at a Psil site upstream to the termination codon due to the sequencing strategy. To allow for a full-length cloning, we have obtained the last 18 residues sequence from a genomic Vpre-B clone, pHVPB-6 created by...
The selected anti-soluble Vpre-B mAb do not bind in parallel assays to Western blots with either the B-MAD688 mAb (lanes proteins employed.

hinge-CH2-CH3 constant domains (h~/1, left) or the constant domain of vectors used (10) splice the genes of interest into either the human Ig'y~

Figure 1. B-MAD688 mAb specifically recognizes soluble Vpre-B recombinant proteins. A palette of single-chain chimeric proteins was created using expression vectors that allow the generation of fusion proteins through the splicing of their coding RNA messages (4). The expression vectors used (10) splice the genes of interest into either the human Ig'y, hinge-CH2-CH3 constant domains (h~/1, left) or the constant domain of mouse κ light chain (mCk, right). They contain the adequate sequences for the expression of the chimeric proteins in myeloma cells (κ-P, κ-promoter; HCE, heavy chain core enhancer, κ-E, κ-enhancer; polyadenylation) and selection (amp, ampicillin; gpt, mycophenolic acid resistance). In B, purified recombinant proteins were probed in several independent Western blots with either the B-MAD688 mAb (lanes 1–4), or antisera specific for the fusion domains (lanes 5 and 6, anti-mCk; lane 7, anti-hVpre-B/mCk, lanes 1 and 5; mCD2/ mCk, lanes 2 and 6; hVpre-B/hVpre-B/h~/1, lanes 3 and 7; and hCD4/h~/1, lane 4). Similar results were obtained for the other B-MAD mAbs reported here. The selected anti-soluble Vpre-B mAb do not bind in parallel assays to several other natural and recombinant proteins that lack Vpre-B but include the same fusion domains (hIL-2/mCk and hIL-2/h~/1, chimeras; and, hlgG1 and mlgG1 monoclonals, data not shown). The apparent molecular weights matched the predicted chimeric nature of the purified proteins employed.

Identification of B Cell Hybridomas that Produce Antibodies Specific against Soluble Human Vpre-B. BALB/c female mice were immunized four times with purified hVpre-B/h~/1 protein (5 μg/mouse/d) in the rear left footpad. The antigen administered at day 0 was emulsified in complete Freund adjuvant, and in PBS for days 3, 7, and 14. Samples from tail blood were monitored for anti-hVpre-B/mCk activity. The left popliteal lymph node from the best responder was obtained 24 h after the final boost, a singlecell suspension was prepared, and the lymphocytes fused to the X63 myeloma using the Köhler and Milstein's protocol. Supernatants from the hybridomas clones arising from the fusion were tested for anti-Vpre-B activity using ELISA and Western assays, including hVpre-B/mCk and hVpre-B/h~/1 proteins. The mAb specificity was established using in parallel a broad palette of recombinant and natural proteins containing the fusion domains. The palette included hIL-2/mCk, mCD2/mCk, hIL-2/h~/1, hCD4/h~/1, pure human IgG1 from a myeloma patient and mouse Ck or Ck-containing monoclonals. mAbs recognizing the Vpre-B domain in the two chimeric soluble proteins, that did not cross-react to mouse Ck or the human IgG1 domain were selected for further immunofluorescence screening of binding to surface Vpre-B on several human cells. Four mAbs which reacted specifically and in a dose-dependent fashion on ELISA plates coated with the recombinant Vpre-B proteins are characterized here.

Human Cells. We used cell lines that represented different stages in the development of the B cell lineage. They included REH and 207 pro-B cells, 697 and Nam-6 pre-B cells, and Daaudi (lgM, κ), Ramos (lgM, λ), and JY (lgG, κ) mature B cells (6). Other hematopoietic and non-hematopoietic cell lines included were K562, Jurkat, U937, and HeLa. The origins and the state of Ig gene loci of the cell lines used are listed elsewhere (6, see Table 1). All cells were maintained in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS (BioWhittaker, Verviers, B) and 5 × 10−5 M 2-ME (Fluka, Buchs, CH). Samples from bone marrow were obtained after informed consent from healthy donors involved in allogeneic transplants.

Antibodies and Immunofluorescence Assays. Mouse mAb were DA4.4 (lgG1, κ) specific for human µ chain, SLCL (lgG1, κ), SLC2 (lgM, κ), and SLC3 (lgM, κ) specific for human `H chain components (6). These were a kind gift of Dr. Lassoued. CD19 and CD10 mAb directly conjugated with FITC were purchased from Becton-Dickinson (MountainView, CA). B-MAD mAbs reported herein (lgM, κ) were purified by size-exclusion chromatography, and DA4.4 was purified by ProtA columns. They were biotin-labeled using standard protocols. In three-color studies with biotinylated-DA4.4 we used streptavidin-TC (CALTAG Labs., South San Francisco, CA). For immunofluorescence staining, cells (1.5 × 105/test) were incubated with 1 μg of purified mAb or 50 μl of culture supernatant in 96-well conical bottom plates at 4°C for 20 min. They were then washed three times with PBS-gelatin. Where necessary, they were incubated with the ap-
Production of Human Soluble Vpre-B Proteins and Isolation of Hybridoma Clones that Produce Specific Antibodies to Vpre-B.

To raise anti-human Vpre-B specific antibodies we produced two distinct soluble Vpre-B proteins. Vpre-B was fused to Ig-constant domains to create hVpre-B/λγ1 or hVpre-B/mCκ recombinant single-chain molecules (Fig. 1 A). It is similar to the strategy we have used to map the subunit specificity of mAbs against the human CD3 transduction subunits of the T cell receptor for antigen (12).

Using purified hVpre-B/λγ1 protein as immunogen, we produced a panel of mAbs that indeed recognizes Vpre-B, as they specifically bind to the hVpre-B/λγ1 and hVpre-B/mCκ protein chimeras, as demonstrated in ELISA and Western assays (see Materials and Methods and Fig. 1 B).

We next screened the panel of anti-soluble Vpre-B mAbs for binding to Vpre-B on the surface of B cell precursors. Four mAbs were selected and further characterized in immunofluorescence and quantitative flow-cytometry assays. These anti-Vpre-B mAbs, the B-MAD 176, 688, 792, and 1112 mAbs, label precursors of B lymphocytes but neither mature B cell lines nor cells from other lineages (Table 1 and Fig. 2 A).

Interestingly, the analysis of the staining patterns of two pro-B (REH and 207 IgH-L-) cells and two pre-B (Nalm6 and 697 IgH-L- cells) human lines revealed that the surface expression of Vpre-B emerges already in μH- pre-B cells, as shown using the B-MAD688 mAb (Fig. 2 A). The SLC1 and SLC2 mAbs rendered a distinct pattern (Fig. 2 A), which is the reported pre-B cell-confined staining (6).

In this regard, the ability of anti-soluble Vpre-B mAbs to bind their ligand on the surface of both pro-B and pre-B cells was rare, whereas the pre-B cell-restricted cluster (i.e., SLCs (6), B-MAD176 or 792) is large and appears immunodominant. Other specificities however occurred, like the B-MAD1112 mAb that binds to the pre-B cells and the REH pro-B cell but not to the 207 pro-B cell (Fig. 2 A).

Vpre-B is expressed on the surface of B cell progenitors from normal human bone marrow. We examined whether B-MAD688 mAb might be a tool for fluorescence-activated cell-sorting of equivalent Vpre-B+ precursor populations from bone marrow.

Two-color flow cytometry analyses showed that B-MAD688 mAb labels well discrete populations of bone marrow cells which are either CD10+/CD19+ (Fig. 2 B). As B cell precursors mature they lose CD10 and gain CD19 (9). In this regard, the Vpre-B+ subsets were predominantly CD10[bright] and CD19[null] cells. Triple-color studies showed that Vpre-B expression emerges in cells that...
lack surface $\mu H$ and are CD34+. These cells are the major Vpre-B$^+$ bone marrow subset detected (i.e., 3/4 of the 688$^+$ cells express CD34). No CD3+$^+$, CD14+$^+$, CD16+$^+$, CD33+$^+$, or CD56+$^+$ cells expressed Vpre-B (not shown). Thus, in normal cells, surface expression of Vpre-B emerges early in B cell development.

**Surface Vpre-B Is Associated with Surrogate-Heavy Chains ($\Psi H$) in Human Pro-B Cells.** The existence of human $\Psi H$-$\Psi L$ complexes has remained elusive due to the lack of antibodies able to immunoprecipitate surface $\Psi L$ before $\mu H$ chain expression (6, 9). We sought to reassess this issue using the B-MAD688 mAb. Immunoprecipitations were carried out after surface biotin-labeling of lines arrested at distinct stages in B cell development. The precipitates were resolved by SDS-PAGE, Western blot and chemiluminescence.

Interestingly, the anti-Vpre-B B-MAD688 mAb does not co-precipitate $\mu H$ among $\Psi L$-associated proteins, but other components of higher molecular weight, from pre-B and pro-B cell lysates (Fig. 3). A major band of 125-kD substitutes for the absent $\mu H$ in the pro-B line REH, and was consistently detected in all nine experiments performed. Other weaker bands (roughly p200, p100, and p70-40) were evident upon longer exposure in some experiments. In contrast, analyses using the SLC2 mAb revealed the known subset of surface $\Psi L$ that coprecipitates with the surface $\mu H$ but does not associate with $\Psi H$. The identity of conventional $\mu H$ was readily determined using the DA4.4 anti-$\mu$ mAb in parallel tracks. Both anti-$\Psi L$ mAbs did not recognize mature B cells (Fig. 3). The proteins co-precipitated by B-MAD688 mAb resemble the $\Psi H$ chains found by Karasuyma, Rolink, and Melchers associated to

![Figure 2.](image)

**Figure 2.** FACS analyses of Vpre-B/A5 surrogate L ($\Psi L$) chains expressed on the surface of human pro- and pre-B cell lines, and normal bone marrow cells as detected by specific mAbs. In A, pro-B (207 and REH), pre-B (697 and Nalm6) and mature B (Ramos and JY) cell lines were incubated first with either control mAb, soluble Vpre-B specific mAbs B-MAD688 and 1112, $\Psi L$-specific mAb SLC1 or $\mu H$-specific mAb SLC2 (6), and then with FITC-conjugated anti-mouse Ig antisera. Immunofluorescence was quantitated on an EPICS XL™ analyzer. Histograms depict the fluorescence distribution curves in a four decade logarithmic scale. In the case of control staining more than 99% of cells were below the C (right) statistic analysis bar. SLC3 rendered similar results to SLC1 and 2 mAb (not depicted in the sake of clarity). In B, Vpre-B-bearing cells in normal bone marrow were phenotyped by two-color flowcytometry in a FACScan™ analyzer. The lymphoid population was identified by its light scatter features and gated for these analyses. Cells were stained with control mAbs or anti-soluble Vpre-B mAb B-MAD688 (using an orange-red PE-label) and either CD10 or CD19 mAb (green FITC-labeled). In control stainings more than 99% of the cells were in the lower-left quadrant defined by the crossed statistics bars. In the dot plots depicted, 36% of the CD10$^+$ cells (which are 3.5% of total) and 29% of the CD19$^+$ cells (which are 8% of total) bound the B-MAD688 mAb.

![Figure 3.](image)

**Figure 3.** Vpre-B/A5 surrogate L ($\Psi L$) chains are associated to both conventional ($\mu H$) and surrogate ($\Psi H$) heavy chains on the surface of B-cell precursors in immunoprecipitation assays. Cells representative of distinct stages of B-lymphocyte development, pro-B (697), pre-B (REH) and mature B (Ramos) cell lines, were surface labeled with biotin and lysed in 1% NP40 lysis buffer. Detergent-soluble lysates were reacted with either anti-$\mu H$ mAb DA4.4 (lanes 1), anti-VpreB mAb B-MAD688 (lanes 2) or anti-A5 mAb SLC2 (lanes 3), immunoprecipitates were resolved by 10% SDS-PAGE under reducing conditions, Western blotted, and revealed by chemiluminescence. Open arrowheads point to conventional heavy ($\mu H$) and light ($L$) chains, filled arrowheads point to surrogate heavy chains ($\Psi H$). Apparent molecular mass, in kD, were estimated using a set of biotin-conjugated protein standards.
The vectorial labeling of the cell surface proteins using either p125 as the major $\Psi H$ component parallels an independent report also using mouse pro-B tumor lines (14).

Human Vpre-B is not readily available during selective, vectorial labeling of the cell surface proteins using either $^{125}$I (6) or biotin. Aliquots of pro-B and pre-B cell lysates were precipitated with B-MAD688 mAb, submitted to electrophoresis and revealed by Western blot using B-MAD688 mAb. The anti-Vpre-B antibody reacted with a 18-kD band (i.e., native Vpre-B size), but not with the 125-kD protein or other higher molecular weight bands (not shown).

We studied whether anti-Vpre-B mAbs B-MAD688 and 1112, and SLC mAbs recognize different epitopes on surface $\Psi L$ chains from pre-B cells, as suggested by the immunofluorescence clusters and immunoprecipitation results. The SLC1, SLC2, and SLC3 mAb epitopes are overlapping (6). Our results of cross-competition competition analyses evidenced that SLC1 showed a clear but partial (30%) competition with B-MAD1112 mAb, again indicative of overlapping or neighbor, but not identical, epitopes. This competition pattern was distinct to the observed for the B-MAD688 mAb and for the anti-\mu DA4.4 mAb, that did not show any cross-blocking of their binding by the mAb tested (data not shown).

Since B-MAD688 mAb recognizes VpreB and SLC2 mAb binds to $\lambda 5$ (6), it is not surprising that they could define distinct epitopes in human $\Psi L$. Our results however underscore that these two mAbs discriminate among $\Psi H\Psi L$ and $\mu H\Psi L$ due to a differential display of $\Psi L$ epitopes in those complexes. As B-MAD688 mAb recognizes only the former receptor, it defines a novel anti-$\Psi L$ specificity (6, 9, 13).

Two other reactivities were established in the mouse (5, 13). First, mAbs that selectively bind to $\Psi L$ when assembled in $\mu H\Psi L$ complexes (i.e., do not detect $\Psi H\Psi L$ complex although may recognize free Vpre-B or $\lambda 5$, as shown before for SLC mAbs in man (6)). Second, mAbs that recognize $\Psi L$ subunits but do not discriminate whether Vpre-B and $\lambda 5$ are associated to $\Psi H$ or $\mu H$ chains; perhaps similar to some anti-$\Psi L$ mAbs in man (9). The staining pattern of B-MAD 1112 mAb is yet difficult to interpret because the mAb does not render immunoprecipitates from pro-B or pre-B cell lysates.

*Light and Enigmas on the Use of Ig-Surrogate Complexes and on the Definition of Human B Cell Developmental Pathways.*

Our results indicate that Vpre-B is a subunit shared by two Ig-like surface complexes, homologous in mouse and man. First, it emerges in $\Psi H\Psi L$ receptors on pre-B cells. Second, it is component of $\mu H\Psi L$ complexes on pre-B cells. The existence of $\Psi H$ has been controversial in man (6, 9).

The fact that SLC2 mAb binds to surface $\Psi L$ chains only when they are associated with $\mu H$ chains might explain why $\Psi H\Psi L$ complexes escaped detection by other authors (6).

The emphasis in the analyses of Ig-surrogate chains has been placed on human pre-B cells that are $H^+L^-$, on which $\mu H\Psi L$ pre-B receptors would play a developmental role (6, 9). Less attention was payed to a reciprocal $H^-L^+$ human pre-B cell pathway, defined by Kubagawa et al. (1). The finding of surface $\Psi H$ chains in man opens the possibility that $\Psi H$ may assemble with $L$ into putative surface $\Psi H\Psi L$ receptors on $H^+L^-$ pre-B cells in the Kubagawa's pathway. By analogy to the major pathway (15, 16), $\Psi H\Psi L$ pre-B receptors could serve to sustain the rare cycling pre-B cells that bear only productive V-J-C$L$ rearrangements. The $\Psi H\Psi L$ receptors might allotypically exclude the $L$ loci in pre-B cells at the minority pathway, while $\mu H$ loci attempts recombination and surface $\mu H$ can replace $\Psi H$ in the successful B cell progeny. Two B cell differentiation pathways also occur in mouse but only the $\Psi L$-containing pre-B receptors were considered in the schemes (2, 3). Other authors pointed that a $\Psi L$-independent pathway predominates early in ontogeny when B-1a/CD5$^+$ cells preferentially develop (17). To test whether the $\Psi L$-independent pathway uses $\Psi H$ pre-B receptors requires the availability of probes for $\Psi H$ chain components, especially considering that $H^-L^+$ cells are infrequent (2, 18).

Another intriguing observation, divergent from previous models (2-4, 6, 9), concerns the coexpression of $\Psi H\Psi L$ and $\mu H\Psi L$ surrogate receptors on pre-B cell tumors. We could not readily detect a similar B-MAD688$^{bright}$, SLC$^+$ bone marrow subpopulation (not shown). Similarly a "transition" $\mu H\Psi L^+$, $\mu HkL^+$ step was shown in human tumors, but its in vivo counterpart was undetectable to the same mAb (6). It is worthy of note that, using similar methods in man (6) or mouse (7, 13), faint levels of surface $\mu H$ were reported in normal $\mu H\Psi L^+$ pre-B cells. Also, $\Psi H\Psi L^+$ cells are readily shown ex vivo, whereas a majority of cytoplasmic $\mu H^+$ cells lack surface $\Psi H\Psi L$ complexes (4, 6, 7). A sensitive analysis of surrogate chains expression and immunoglobulin loci status in single cells will be required to order the B cell development steps.

In summary, we raised mAbs against soluble human Vpre-B. We show that Vpre-B is expressed associated to $\Psi H$ chains on the surface of pro-B cell tumors, and that the Vpre-B$^+/\Psi H\Psi L$ complex detected by B-MAD688 mAb is a good marker to identify early B cell progenitors in human bone marrow. We propose that $\Psi H$-containing receptors might also participate in a second class of $\Psi H\Psi L$ pre-B receptors devoted to drive the development of rare precursors that rearrange L-chains first.
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