Brief Definitive Report

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.

B-lymphocyte lineages are best defined by the expression of their clonal receptors for antigen, the immunoglobulins (Ig). Hence, stages in B cell development pathways are drawn in terms of the rearrangement state of Ig heavy (H) and light (L) chain genes. Rearrangement of H and L chain loci is markedly asynchronous, creating H+L- or H-L+ intermediate receptors that might be implicated in further B cell differentiation (1-4).

Whereas H chains are not transported to the cell surface of mature B cells in the absence of L chains, this is not the case for B cell progenitors (3-6). Best characterized is the association of the proteins encoded by the Vpre-B and λ5 genes that constitutes a surrogate L (ΨL) complex, which replaces the requirement for L in the transport of Ig-like complexes to the cell surface (5-7). Mouse mutants deficient in this ΨL complex, created by targeted disruption of λ5 gene, show a B cell deficiency phenotype (8). Analyses of ΨH and ΨL expression patterns and function are therefore of interest to know how B cell progenitors differentiate, and to study key molecular events in B cell development.

Mouse models suggest that the ΨL proteins couple first with ΨH on the surface of pro-B cells and later with conventional μH chains on pre-B cells (3-5). Similar steps were proposed in man (9), although none of the monoclonal antibodies (mAbs) reported yielded any immunoprecipitate. Contrary to this hypothesis, other anti-human ΨL mAb recognized μHΨL receptors in pre-B cells but failed to detect surface ΨHΨL complexes in human pro-B cells, suggesting that ΨL associates only with μH chains (6).

We have produced human recombinant soluble Vpre-B molecules to characterize novel mAbs against ΨL-containing receptors. One of these mAbs, B-MAD688, labels Vpre-B on the surface of H-L- pro-B tumors 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 above findings suggest a B cell development scheme. B-MAD688 mAb may facilitate the analyses of Ig-surrogate receptors, and their relationship with the growth and differentiation requirements of B cell progenitors in man.

Materials and Methods

Production of Human Soluble Vpre-B Proteins. Plasmids pCEH-Vpre-B/mCk and pCEH-Vpre-B/Ihy, were constructed by insertion of a human full-length Vpre-B DNA into two distinct expression vectors containing either mouse Ck or the hinge-CH2-CH3 domains of human IgG1, heavy chain, respectively, kindly provided by Dr. Karjalainen (10). The published sequence for the hVpre-B gene ends prematurely at a PstI 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. (roCK, lanes 2 and 6; hVpre-B/h3q, lanes 3 and 7; and hCD4/h3q, lane 4. hinge-CH2-CH3 constant domains (h~/1, left) or the constant domain of through the splicing of their coding RNA messages (A). The expression B-MAD mAbs reported here. Similar results were obtained for the other B-MAD mAbs for the expression of the chimeric proteins in myeloma cells (K-P, K-pro-mericators; HCE, heavy chain core enhancer, k-E, k-enhancer; polyadenyla-tion) 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-hyA). The chimeric proteins were hVpre-B/mCk, lanes 1 and 5; mCD2/ mCk, lanes 2 and 6; hVpre-B/hy1, lanes 3 and 7; and hCD4/hy1, 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 of which Vpre-B but in-clude the same fusion domains (hIL-2/mCk and hIL-2/hy1, chimeras; and, hlgG1 and mlgG K monoclonals, data not shown). The apparent molecular weights matched the predicted chimeric nature of the purified proteins employed.

**Figure 1.** B-MAD688 mAb specifically recognizes soluble Vpre-B recombinant proteins. A palette of single-chain chimeric proteins was cre-ated using expression vectors that allow the generation of fusion proteins through the splicing of their coding RNA messages (A). The expression ve-crors used (10) splice the genes of interest into either the human hyA, hinge-CH2-CH3 constant domains (hy1, left) or the constant domain of mouse k light chain (mCk, right). They contain the adequate sequences for the expression of the chimeric proteins in myeloma cells (k-P, k-pro-mentor; HCE, heavy chain core enhancer, k-E, k-enhancer; polyadeny-la-tion) 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-hyA). The chimeric proteins were hVpre-B/mCk, lanes 1 and 5; mCD2/ mCk, lanes 2 and 6; hVpre-B/hy1, lanes 3 and 7; and hCD4/hy1, 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 of which Vpre-B but in-clude the same fusion domains (hIL-2/mCk and hIL-2/hy1, chimeras; and, hlgG1 and mlgG K monoclonals, data not shown). The apparent molecular weights matched the predicted chimeric nature of the purified proteins employed.
Table 1. Reactivity of the B-MAD mAbs with a Panel of Human Cell Lines

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Line</th>
<th>Ig gene Rearrangement</th>
<th>mAb Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro B (H:[H]=L)</td>
<td>REH</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Pre B (H:[H]=L)</td>
<td>697</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Nalm6</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Mature B (HL)</td>
<td>Ramos ([m]=L)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Dauidi ([m]=L)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>JY ([\gamma])</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>T</td>
<td>Jurkat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Erythroleukemia</td>
<td>K562</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Promyelocytic</td>
<td>U937</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Epithelial</td>
<td>HeLa</td>
<td>N</td>
<td>N</td>
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Reactivity was determined by cell surface immunofluorescence as described in Materials and Methods. Y, functional rearrangement/protein available; N, no functional rearrangement/no protein.

propriate second layer and washed three additional times. In the epitope competition assays, the cells were incubated in criss-cross combinations with 10 μg/test of the competing mAb (unlabeled and purified) for 20 min. This was followed by 1 μg/test of the relevant mAb (which was biotinylated or displayed a distinct isotype) for 20 min. The unbound mAbs were removed after three washes with PBS and the bound relevant mAbs were revealed using the chemiluminescence ECL kit (Amersham, UK) after 1–5-s exposures to X-Omat films (Kodak).

Results and Discussion

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/\[h\]=\[\gamma\] and hVpre-B/m\[\alpha\]=\[\gamma\] 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/\[h\]=\[\gamma\] protein as immunogen, we produced a panel of mAbs that indeed recognizes Vpre-B, as they specifically bind to the hVpre-B/\[h\]=\[\gamma\] and hVpre-B/ m\[\alpha\]=\[\gamma\] 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 IgH− pro-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+ or 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\[dim\] cells. Triple-color studies showed that Vpre-B expression emerges in cells that...
A Pro B (H- L-) Pre B (H+ L-) Mature B (H+ L+)

18 Z 0 W

Z m,

log RED FLUORESCENCE

LOG GREEN FLUORESCENCE

Figure 2. FACS™ analyses of Vpre-B/A5 surrogate L (ΨL) 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, ΨL-specific mAb SLC1 or ΨH-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-labeled) 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.

lack surface ΨH 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 (ΨH) in Human Pro-B Cells. The existence of human ΨH-ΨL complexes has remained elusive due to the lack of antibodies able to immunoprecipitate surface ΨL before ΨH 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 μH among ΨL-associated proteins, but other components of higher molecular weight, from pro-B and pro-B cell lysates (Fig. 3). A major band of 125-kD substitutes for the absent μH 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 ΨL that coprecipitates with the surface μH but does not associate with ΨH. The identity of conventional μH was readily determined using the DA4.4 anti-μ mAb in parallel tracks. Both anti-ΨL mAbs did not recognize mature B cells (Fig. 3). The proteins co-precipitated by B-MAD688 mAb resemble the ΨH chains found by Karasuayama, Rolink, and Melchers associated to ΨL.
ψL in mouse μH− pro-B cells (5, 7, 13). The finding of p125 as the major ψH 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 125I (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 ψL 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-criss 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-μ 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 λ5 (6), it is not surprising that they could define distinct epitopes in human ψL. Our results however underscore that these two mAbs discriminate among ψHψL and μHψL due to a differential display of ψL epitopes in those complexes. As B-MAD688 mAb recognizes only the former receptor, it defines a novel anti-ψL specificity (6, 9, 13).

Two other reactivities were established in the mouse (5, 13). First, mAbs that selectively bind to ψL when assembled in μHψL complexes (i.e., do not detect ψHψL complex although may recognize free Vpre-B or λ5, as shown before for SLC mAbs in man [6]). Second, mAbs that recognize ψL subunits but do not discriminate whether Vpre-B and λ5 are associated to ψH or μH chains; perhaps similar to some anti-ψL 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 ψHψL receptors on pro-B cells. Second, it is component of μHψL complexes on pre-B cells. The existence of ψH has been controversial in man (6, 9).

The fact that SLC2 mAb binds to surface ψL chains only when they are associated with μH chains might explain why ψHψL 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 μHψL 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 ψH chains in man opens the possibility that ψH may assemble with L into putative surface ψHψL receptors on H−L+ pre-B cells in the Kubagawa’s pathway. By analogy to the major pathway (15, 16), ψHψL pre-B receptors could serve to sustain the rare cycling pre-B cells that bear only productive V-J-C3 rearrangements. The ψHψL receptors might allelically exclude the L loci in pre-B cells at the minority pathway, while μH loci attempts recombination and surface μH can replace ψH in the successful B cell progeny. Two B cell differentiation pathways also occur in mouse but only the ψL-containing pre-B receptors were considered in the schemes (2, 3). Other authors pointed that a ψL-independent pathway predominates early in ontogeny when B-1a/CD5+ cells preferentially develop (17). To test whether the ψL-independent pathway uses ψH pre-B receptors requires the availability of probes for ψH 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 ψHψL and μHψL surrogate receptors on pre-B cell tumors. We could not readily detect a similar B-MAD688bright, SLC1bright bone marrow subpopulation (not shown). Similarly a “transition” μHψL+, μHκL+ 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 μH were reported in normal μHψL+ pre-B cells. Also, ψHψL+ cells are readily shown ex vivo, whereas a majority of cytoplasmic μH+ cells lack surface μHψL 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 ψH chains on the surface of pre-B cell tumors, and that the Vpre-B+/ψHψL complex detected by B-MAD688 mAb is a good marker to identify early B cell progenitors in human bone marrow. We propose that ψH-containing receptors might also participate in a second class of ψH pre-B receptors devoted to drive the development of rare precursors that rearrange L-chains first.

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2697  Sanz and de la Hera  Brief Definitive Report
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