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Immunology

Light-chain gene expression before heavy-chain gene rearrangement in pre-B cells transformed by Epstein–Barr virus

(immunoglobulin gene/transformation/trans-acting factors)

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ABSTRACT **Epstein–Barr virus transformation of B-cell-depleted bone marrow cells from human fetuses allowed us to identify novel cell types characterized by the expression of immunoglobulin κ or λ light chain without heavy chains. Four κ-only clones with normal karyotype were obtained and examined for their immunoglobulin gene configurations and expression. All four clones had κ-chain gene rearrangements at either one or both alleles, but the heavy-chain gene loci in these clones either were in germ-line context or had undergone only *D*–J_H rearrangements (*D* and J_H represent diversity and joining gene segments). All clones contained κ mRNA of normal size at levels consistent with the protein level, except for one clone that no longer produced κ protein. No μ mRNA or immunoglobulin heavy-chain molecules were detected in any of the κ^+ clones. The results suggest that the μ heavy-chain protein is not an obligatory prerequisite for light-chain gene rearrangements.**

Variable (*V*) regions of immunoglobulin molecules are encoded by separate clusters of germ-line gene segments: V_H, diversity (*D*), and joining (J_H) segments for immunoglobulin heavy (H) chains and V_L and J_L segments for immunoglobulin light (L) chains. Stem-cell progeny entering the B-cell pathway must undergo rearrangement of these gene segments in order to express H and L chains (1). These rearrangements have been thought to occur in an ordered fashion so that μ H-chain expression precedes L-chain expression during the conversion of pre-B cells to B cells (2–6). A model for the regulation of this ordered sequence of gene rearrangements has been proposed based primarily on studies of pre-B-cell lines transformed by the Abelson murine leukemia virus (Ab-MuLV) (7). According to this model, the μ polypeptide that is produced following a functional *V*–*D*–*J* rearrangement would play a dual role: (*i*) termination of further H-chain gene assembly to guarantee allelic exclusion and (*ii*) induction of L-chain gene assembly (7, 8).

We have used Epstein–Barr virus (EBV) transformation to analyze human B-cell precursors at different developmental stages. EBV has long been known to immortalize B cells, but its ability to transform B-cell precursors, which requires prior depletion of surface IgM-positive ($sIgM^+$) B cells, has been appreciated only more recently (9–11). Unlike many Ab-MuLV-transformed cell lines, which can continue to rearrange immunoglobulin genes in culture, EBV transformants stably maintain their immunoglobulin genes during *in vitro* propagation (10–13). In addition, EBV can transform a broad spectrum of B-lineage cells, including Ig^- "null" cells with germ-line H- and L-chain genes (11, 14, 15). Among the B-cell progenitors rescued by EBV transformation we have frequently observed novel cell types that produce κ or λ L chains in the absence of H chains. Cloned lines of cells producing κ L chain but no H chain had either maintained their H-chain loci in germ-line context or undergone only *D*–J_H rearrangements. The results suggest the existence of unanticipated pathways of B-cell development.

MATERIALS AND METHODS

EBV Tranformation of B-Cell Precursors. Preparation of marrow mononuclear cells from normal fetuses, depletion of sIg^+ B cells by fluorescence-activated cell sorting (FACS), *in vitro* infection with EBV, and culture conditions and subcloning procedures for transformed cells have been described (11).

Immunofluorescence Analysis of Cells. Paired combinations of mouse monoclonal antibodies (mAbs) specific for each isotype (μ, δ, γ, α, ε, κ, and λ) or for joining (J) chain and goat antibodies specific for human immunoglobulins, μ chain, or H chains (μ + δ + γ + α) were used for two-color immunofluorescence analysis in a manner previously described (6, 11, 16).

Biosynthetic Analysis of Immunoglobulins. Cells (10^7) were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (100 μCi each; 1 μCi = 37 kBq) for 4 hr at 37°C. Solid-phase immunoisolation from the cells solubilized in 1% Nonidet P-40 and from the culture supernatant was performed in microtitier wells precoated with rat mAb to mouse κ chains (187.1) and then with mouse mAbs to human isotypes as described (17). The isolated, radiolabeled proteins were analyzed by $NaDodSO_4$/PAGE under both reducing and nonreducing conditions.

DNA and RNA Blot Analyses. Isolation of genomic DNA and total cellular RNA, restriction enzyme digestions, agarose gel electrophoresis, DNA and RNA blotting procedures, and preparation of nick-translated, ^{32}P-labeled DNA probes were performed as described (11). The following DNA probes were used: a 2.0-kilobase (kb) *Sac* I J_κ probe (18), an ≈ 3.6-kb *Bgl* II J_H probe (19, 20), a 1.8-kb *Bam*HI D_2 probe (21), a 1.3-kb *Eco*RI μ constant-region (C_μ) probe (19), and a 2.5-kb *Eco*RI κ constant-region (C_κ) probe (22).

RESULTS

Transformation of Pre-B Cells. EBV was used to immortalize B-cell precursors in normal fetal bone marrow samples that had been depleted of B cells by FACS. Two to three months after EBV infection, transformed cells in 64 independent cultures derived from three fetuses were analyzed for immunoglobulin expression by two-color immunofluorescence using both monoclonal and polyclonal antibodies. Cells with a conventional μ^+ pre-B phenotype were found in ≈80%

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Abbreviations: *V*, *D*, *J*, and *C*, variable, diversity, joining, and constant gene segments; H, heavy; L, light; Ab-MuLV, Abelson murine leukemia virus; ALL, acute lymphocytic leukemia; EBV, Epstein–Barr virus; FACS, fluorescence-activated cell sorting; J chain, joining chain; mAb, monoclonal antibody; sIg, surface immunoglobulin.

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of the cultures, and Ig^- null cells that expressed J chain (11) were present in almost every well (Fig. 1). Surprisingly, two-thirds of the cultures contained variable proportions (1–35%) of cells that expressed κ L chains (κ^+) but no H chains (HC^-) in their cytoplasm. Cells with a λ^+HC^- phenotype were also observed, but at a much lower incidence (5 of 64 cultures) and at much lower frequencies (1–7%) than the κ^+HC^- cells. Such κ or λ "L-chain-only" cells were not found in EBV-transformed cultures from other tissues including neonatal and adult blood and palatine tonsils.

Four κ^+HC^- cell lines were cloned by limiting dilution and subjected to further phenotypic and genotypic analyses. All of the clones displayed similar morphology with the spectrum of lymphoblastoid to plasmacytoid cell types that has been described previously (11, 15). The frequency of cytoplasmic κ^+ cells among the clones varied from 30% to 70% with the exception of clone I. This clone initially contained $\approx$5% κ^+ cells but had become κ^-. Since the clone still contained abundant κ mRNA (see below), it was included in this analysis. Unexpectedly, one of the clones (III) expressed κ L chains (without H chains) on the cell surface. Chromosomal analysis revealed that all of the clones had a normal diploid karyotype.

Immunoglobulin Biosynthesis. A single polypeptide chain with a mobility corresponding to 20–25 kDa was immunoprecipitated with a monoclonal anti-κ antibody from the cell lysates of three κ^+ clones, whereas the same antibody immunoprecipitated both H and L chains from an EBV-transformed, $\mu^+\kappa^+$ clone (C1) used as a control (Fig. 2). In agreement with the immunofluorescence analysis, no specific bands were immunoprecipitated from the κ^+ clones II–IV with monoclonal anti-λ or anti-μ antibodies, nor were any immunoglobulin chains identified in clone I. Although by immunofluorescence clone III expressed κ L chains on its cell surface membrane, no proteins associated with κ L chains were detectable in this biosynthetic assay. Clone III was also found to secrete both monomeric and dimeric forms of κ L chains, a finding unique to this clone (data not shown).

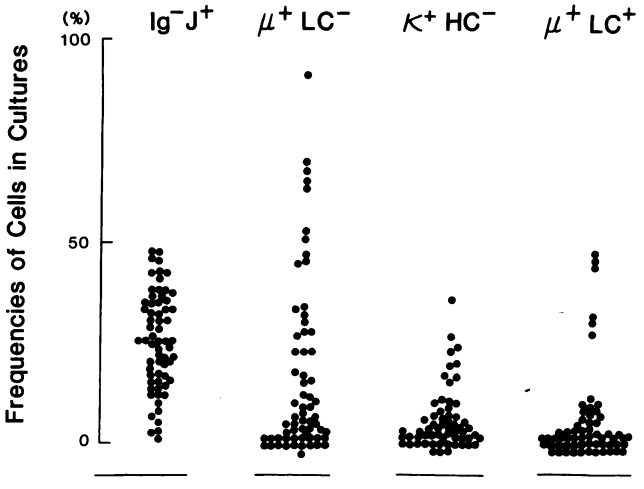

FIG. 1. Four major phenotypes of cells present in bulk cultures of EBV-transformed fetal bone marrow. B-cell-depleted bone marrow mononuclear cells from three normal fetuses (13–16 weeks of gestation) were infected with EBV and cultured in microtiter wells at a cell concentration of 10^5 per ml. Two to three months after infection, transformed cells (seen in 60–90% of the wells) were sedimented onto glass slides, fixed, and doubly stained for cytoplasmic immunoglobulin components. The combination of monoclonal anti-J antibody and goat anti-immunoglobulin antibodies was used to enumerate Ig^-J^+ cells, and the combination of a mixture of monoclonal anti-κ and anti-λ antibodies and goat anti-μ was used to identify μ^+LC^- cells and μ^+LC^+ cells. The κ^+HC^- cells were detected by staining with monoclonal anti-κ and goat anti-H chain antibodies. Single staining with isotype-specific mAbs was also conducted to confirm the results with polyclonal reagents. Four κ^+HC^- clones (clone I, from a 15-week fetus, and clones II–IV, from a 16-week fetus) were derived by limiting dilution.

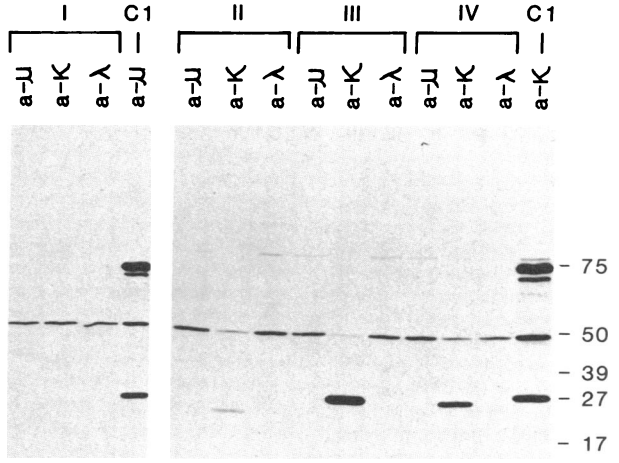

FIG. 2. Immunoglobulin biosynthetic analysis. The radiolabeled proteins immunoprecipitated with the indicated mAbs (a-, anti-) from the cell lysate of the κ^+ pre-B clones (I–IV) and a control $\mu^+\kappa^+$ B-cell clone (Cl) were analyzed by $NaDodSO_4$/10% PAGE under both nonreducing (data not shown) and reducing conditions. Mobilities and sizes (kDa) of prestained protein standards are indicated at right.

Immunoglobulin Gene Configurations. All four κ^+ clones had J_κ gene rearrangements at one (clone IV) or both (clones I–III) alleles as determined by *Bam*HI (Fig. 3*a*) or *Hin*dIII digestion and DNA blot analysis. Hybridization with a probe derived from upstream of $J_{\kappa 1}$ (a gift from R. P. Perry and G. G. Lennon, Fox Chase Cancer Center, Philadelphia) revealed conventional V_κ–J_κ rearrangements that resulted in deletion of intervening DNA rather than, e.g., inversions. There were no rearrangements of the λ-chain gene loci in any of the clones (data not shown).

Clones III and IV had two rearranged, J_H-containing fragments in *Hin*dIII digests, whereas the other two κ^+ clones (I and II) had no J_H gene rearrangements that could be visualized following *Hin*dIII, *Bam*HI, or *Bgl* II digestion (Fig. 3*b*). In all four clones, both J_H and C_μ probes gave the same hybridization results after *Bam*HI digestion, indicating that the C_μ gene was not deleted (data not shown).

To determine whether the J_H gene rearrangements in the two κ^+ clones (III and IV) were due to *D–J* or *V–D–J* recombination, we used the D_2 gene probe (21), which recognizes four *D* segments located $\approx$22 kb downstream of the most 3′ V_H gene (V_{H6}) in the order 5′-V_{H6}–D_4–D_1–D_2–D_3–J_H-3′ (refs. 21 and 23–25) and a fifth segment (D_5) located $\approx$1300 kb upstream of the V_{H6} gene (23). As shown in Fig. 3*c*, all five germ-line *D* segments were identified in three κ^+ clones (I–III). The other clone (IV) exhibited a complete deletion of the D_3 segment accompanied by two rearranged fragments that also hybridized to the J_H probe. When the DNAs of tonsillar B and T cells from a single normal individual were analyzed as controls, marked, but incomplete, deletions of four *D* gene segments (D_1–D_4) were observed in the B-cell population. Thus, these findings suggest that the J_H gene rearrangements seen in clones III and IV are due to *D–J* recombination rather than *V–D–J* joining.

Since the most 3′ *D* segment (D_{Q52}) in humans is located within the J_H gene cluster (19, 21), $\approx$90 base pairs upstream of the first functional *J* gene segment (J_{H1}), a D_{Q52}–J_{H1} recombination might not be distinguishable from the germ-line J_H fragment in *Hin*dIII, *Bam*HI or *Bgl* II digests. Double digestion with *Bgl* II and *Sma* I, which cuts between D_{Q52} and J_{H1}, was therefore performed to determine whether the κ^+ clones with apparent germ-line J_H regions (I and II) had

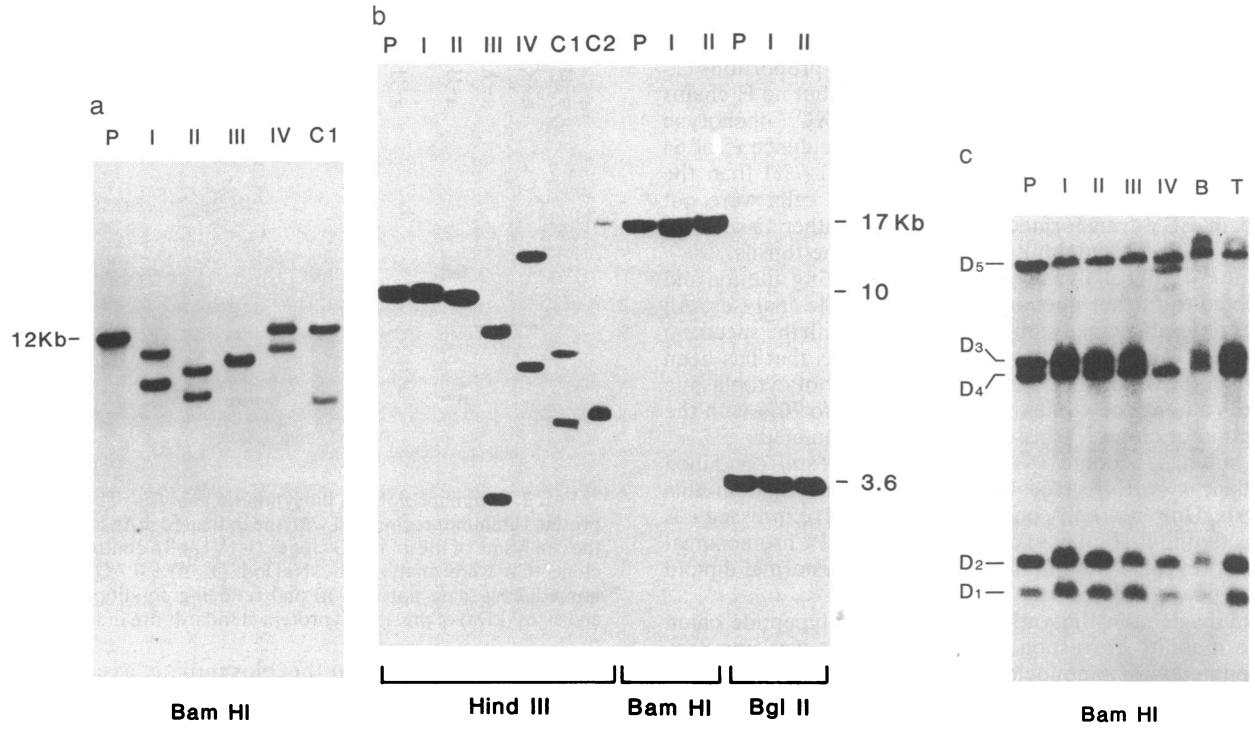

FIG. 3. DNA blot analysis. Samples (8 μg) of high molecular weight DNA from the κ^+ clones (I–IV), control $\mu^+\kappa^+$ B-cell clones (C1 and C2), placenta (P), and tonsillar B and T cells (B and T) were digested with the indicated restriction endonucleases. Hybridization probes corresponded to human J_κ (*a*), J_H (*b*), and D_2 (*c*) gene segments. The sizes of germ-line J_κ, J_H, and *D* fragments were estimated on the basis of the fragment sizes of *Hin*dIII-digested phage λ DNA.

instead a D_{Q52}–J_{H1} rearrangement. Three germ-line J_H-containing fragments of 1.4, 0.7, and 0.4 kb were demonstrated in both clones and in placenta; specifically, a fragment of $\approx$1 kb that would have been generated by D_{Q52}–J_{H1} recombination was not detected. These results strongly suggest that the immunoglobulin H-chain alleles in clones I and II were retained in their germ-line context.

The karyotypic and DNA blot analyses thus indicate that the κ^+ clones contain two copies of chromosome 14, and that the immunoglobulin H-chain loci on these chromosomes either are in germ-line context (clones I and II) or have undergone only *D*–J_H rearrangements (clones III and IV). However, in the former case it might be argued that the cells originally contained a productive *V*–*D*–*J* rearrangement on one allele. Loss of this chromosome 14 with subsequent duplication of the other (with a germ-line H-chain allele) would then yield the results we have obtained. This possibility can be excluded for clone I, since in humans a polymorphic region containing variable numbers of tandem repeats exists 5′ of the J_H gene segments (26). This is illustrated in Fig. 3*b*, where two bands of nearly identical mobility are found with a heterozygous placental DNA sample after *Hin*dIII digestion and hybridization with a J_H probe. The same two germ-line bands are found in *Hin*dIII-digested DNA from clone I, confirming that two nonidentical but germ-line H-chain alleles exist in this cell line.

Analysis of mRNA. All four clones contained κ mRNA of normal size at levels consistent with the protein levels except for clone I, which no longer produced κ protein but had abundant κ message (Fig. 4). No μ mRNA was detectable in any of the κ^+ clones, including those with *D*–J_H rearrangements, after a 16-hr autoradiographic exposure. However, a 5-fold longer exposure revealed faint, heterogeneous C_μ-hybridizing bands, suggesting limited μ gene transcription or mRNA stability in these clones or perhaps normal transcription levels in only a subpopulation of cells. The extremely low level of C_μ transcripts in the κ^+ pre-B clones was in striking contrast to our previous finding that sterile C_μ transcripts were easily detectable in Ig^- null cell clones where the H-chain locus had *D*–*J* rearrangements and the L-chain loci were retained in their germ-line configuration (11).

DISCUSSION

The concept that μ H-chain expression precedes L-chain expression during B-cell development is based on a number of observations, including our own. Hybridomas derived from fetal liver pre-B cells synthesized μ H chains but no L chains (2). Immunofluorescence studies as well as biosynthetic analysis of normal fetal liver and marrow pre-B cells also supported this asynchronous expression hypothesis (2, 4–6), as did an extensive series of studies using Ab-MuLV-transformed B-cell precursors (3, 7, 27). Molecular analyses of murine fetal liver-derived pre-B hybridomas (28, 29), Ab-MuLV transformants (7, 27, 30), FACS-enriched normal pre-B

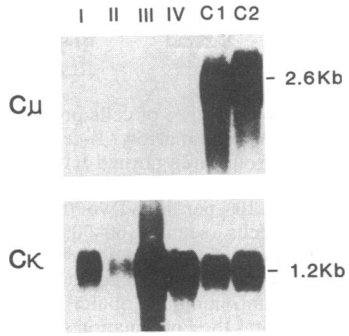

FIG. 4. RNA blot analysis. Samples (10 μg) of total cellular RNA from the indicated clones were electrophoresed in a 1.2% agarose/formaldehyde gel, blotted onto nylon membranes, and hybridized to nick-translated, ^{32}P-labeled C_μ or C_κ probe. The sizes of C_μ and C_κ transcripts were estimated on the basis of the sizes of an RNA "ladder" (BRL).

cell populations (31) as well as human acute lymphocytic leukemias (ALLs) (32, 33), and EBV-transformed pre-B-cell lines (15, 34) have demonstrated that many μ^+ pre-B cells have not yet rearranged a L-chain gene, consistent with the concept that H-chain expression precedes L-chain expression. Based on a series of elegant experiments using primarily Ab-MuLV-transformed cell lines, Alt *et al.* (7) proposed a model for regulation of the immunoglobulin gene rearrangement hierarchy. Immunoglobulin gene rearrangements would begin at the H-chain locus resulting in *D–J* rearrangements on both alleles (35, 36). The membrane form of μ polypeptide, which would be synthesized once a productive *V–D–J* rearrangement had occurred, would then serve two functions: (*i*) termination of any further J_H rearrangements and (*ii*) induction of κ gene rearrangement (7, 8).

Several apparent exceptions to the above model have been reported. The 18-4 Ab-MuLV cell line, which has deleted one J_H locus and has only a *D–J* rearrangement on the other, synthesizes κ L chains and can continue κ gene rearrangement *in vitro* (36, 37). A rare Ab-MuLV transformant derived from a *scid* (severe combined immunodeficiency) mouse was observed to undergo aberrant V–J_κ rearrangement without functional V_H–D–J_H assembly (48). Approximately 20% of Ig^+ hybridomas derived by fusion of neonatal mouse spleen cells synthesized L chains but no H chains, and a distorted κ/λ ratio (1:1) suggested that the L-chain-only phenotype was not due to random loss of H-chain production (38). A number of human ALLs have rearrangements of both H- and L-chain genes but produce no H- or L-chain protein (32, 33). Most of these exceptions can be accommodated by current theory if one proposes that the cells immortalized by fusion or transformation originally produced a μ chain. Clearly, however, this cannot be the case for the L-chain-only cells described here. A number of possible explanations for the unique phenotype and genotype of the L-chain-only cells will be discussed but, to summarize, we conclude that these cells are a normal component of human, and perhaps mouse, B-cell development. They have gone undetected in analyses of normal and transformed lymphopoietic cells due to an inhibitor of L-chain gene transcription present in pre-B cells. The ability of EBV to transform a broad (perhaps the complete) spectrum of B-cell precursors and induce their plasmacellular differentiation accounts for the frequent detection of L-chain-only cells in this transformation system and their rarity in others (Fig. 5).

Several possibilities concerning the origin of κ^+ pre-B cells have to be considered. The first is that they are an unusual artifact of EBV transformation. We consider this possibility unlikely, since we have observed cells with this phenotype only in transformed bone marrow cultures; they are not seen in cultures of blood or tonsil, even when IgM^+ B cells have been depleted prior to transformation. Since among these cell sources only bone marrow is a lymphopoietic site, the most straightforward interpretation is that the L-chain-only cells are transformed analogues of a previously unidentified member of the B-cell lineage. Another possibility is that EBV can induce a B-cell precursor having either *D–J* rearrangements or germline J_H loci to mature directly to a stage in differentiation where L-chain gene rearrangements take place, bypassing the normal intermediate stages where H-chain gene rearrangements are initiated or completed. This possibility is difficult to exclude formally, but several observations suggest that this is not the case. The immunoglobulin gene status of EBV-transformed cell lines is remarkably stable. Spontaneous *de novo* expression of H or L chains has not been found in Ig^- null cell clones or μ-only pre-B-cell clones (refs. 10 and 11; unpublished observations), nor has isotype switching been observed in IgM-producing B cells (12, 13). If EBV has the capacity to induce L-chain gene rearrangements, it must be an inefficient process, since Ig^- clones and μ^+ pre-B clones, neither of

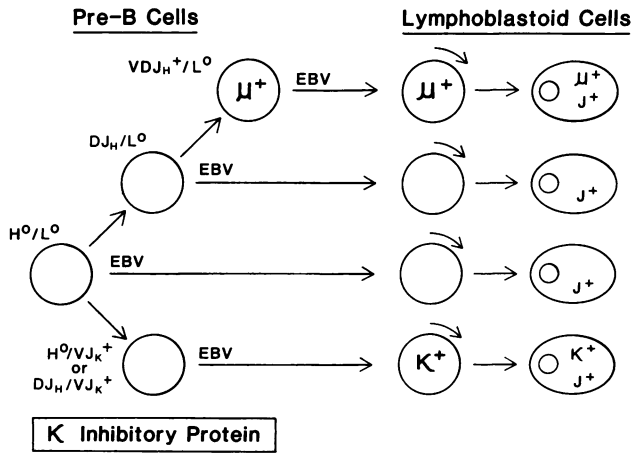

FIG. 5. EBV can transform the entire spectrum of B-cell precursors and induces them to differentiate into plasma cells. According to this model, subpopulations of stem cells with germ-line configuration of both H- and L-chain gene loci (H^0/L^0) can undergo immunoglobulin gene rearrangements at either the H-chain (DJ_H/L^0) or L-chain (H^0/VJ_κ in the case of κ L-chain genes) gene loci. In addition, cells that have undergone D–J_H rearrangements can rearrange their L-chain genes (DJ_H/VJ_κ). *In vivo*, the only cells that would produce immunoglobulin would be those with productive *V–D–J*$_H$ rearrangements (VDJ_H^+). Cells with productive κ gene rearrangement (VJ_κ^+) would not be detectable due to an inhibitor of κ gene transcription (κ inhibitory protein) present at this stage of differentiation (see text). EBV can transform any of these B-cell precursors, regardless of their immunoglobulin gene status, and induces them to differentiate into lymphoblastoid and J-chain-positive plasmacytoid cells in which the κ inhibitory protein would no longer be expressed. The ability of EBV to induce this cellular differentiation permits detection of κ-chain-only cells and explains why such cells are seen only rarely in other *in vitro* or *in vivo* transformation systems.

which express a L chain or have undergone L-chain gene rearrangements, were so frequently obtained. If κ gene rearrangements can indeed be induced by EBV, it would be of considerable interest since it might suggest that EBV receptor-mediated transmembrane signaling can replace the physiologic signal that normally induces V–J_κ recombination.

Since most of the data concerning the regulation of *V–(D)–J* rearrangements have come from studies in mice, it may be that the L-chain-only pre-B phenotype is unique to humans. There is nothing unusual about the general features of the recombinatorial signal sequences in humans that would suggest different mechanisms for *V* gene assembly in the two species. However, in mice most B-lineage cells have rearrangements on both alleles, often a *D–J* rearrangement on the non-productive one (36), whereas in humans this is not always the case (10, 11, 15, 32, 33). This raises the possibility of species differences in the regulation of the rearrangement process. In this regard, our previous analyses of two aspects of human immunoglobulin gene expression, the regulation of IgD during B-cell differentiation (39) and the specificity of isotype switch recombination (13, 20), have revealed potentially significant differences between mice and humans. Given these reservations, we nevertheless consider that the infrequent finding of L-chain-only cells in Ab-MuLV transformants is primarily due to differences in the transformable target-cell population and/or in the extent of maturation of transformants. EBV apparently has a broader spectrum of target cells than Ab-MuLV, including progenitors of pre-B cells that have not yet begun immunoglobulin gene rearrangements (11, 14, 15). In contrast, all Ab-MuLV transformants have, at minimum, *D–J* rearrangements on both alleles (36, 40). Moreover, EBV can induce plasmacellular differentiation accompanied by the expression of J chain and plasma-

cell differentitation antigens (11), whereas Ab-MuLV does not. If the expression of L-chain genes is regulated by non-immunoglobulin factors that are expressed at restricted stages of B-cell differentiation (see below), then differences in the phenotypes of Ab-MuLV and EBV transformants would be expected.

There could be several explanations for the fact that L-chain-only pre-B cells have not been detected by immunofluorescence in normal bone marrow (6) and ALL samples (16, 32, 33). It may simply be that the level of L chain is below the limits of detection by this technique. A more interesting possibility is suggested by studies of the murine pre-B-cell line 70Z/3 (41). Cells in this line constitutively express μ chains but no L chains, even though they contain a productive κ rearrangement. Transcription of this κ-chain gene can be induced by a variety of agents including bacterial lipopolysaccharide (42). Brief treatment with inhibitors of protein synthesis can also induce κ expression, suggesting the presence of a labile inhibitor of κ transcription in pre-B cells (41). Baeuerle and Baltimore (43) recently identified an inhibitor protein in 70Z/3 cells that associates with a κ enhancer-binding protein in the cytoplasm, preventing its translocation into the nucleus and thus inhibiting its activity for κ gene transcription. If such an inhibitor exists *in vivo*, pre-B cells with $H^0/VJ_\kappa{}^+$ or $DJ_H/VJ_\kappa{}^+$ genotype would not be detectable by immunofluorescence or other techniques requiring the production of κ mRNA or protein. The expression of the κ inhibitory protein would be down-regulated as pre-B cells differentiate to B lymphocytes and would remain so in their plasma-cell progeny. Pre-B cells normally do not differentiate into plasma cells but can do so following EBV transformation (11, 15, 34) (see Fig. 5). A few examples of leukemias/lymphomas having κ-chain gene rearrangements but germline J_H gene loci have been reported (44, 45), and $\approx$40% of Ig^- null ALLs have rearrangements of both H- and κ-chain gene loci (32, 33, 44, 46). Some of the H-chain gene rearrangements in these ALLs may be *DJ/DJ* and would thus correspond to clones III and IV described here. The finding that phorbol ester can induce the expression of both μ and κ chains in some of these leukemias (47) supports the possibility that κ-chain gene expression is regulated *in vivo* by an inhibitor protein, and might even suggest the existence of an analogous inhibitor of H-chain gene expression. Thus the L-chain-only pre-B cells described here could represent a significant but ordinarily undetectable component of the normal developmental pathway of B-lineage cells whose existence has been revealed by EBV transformation.

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