Resolution and Characterization of Pro-B and Pre-Pro-B Cell Stages in Normal Mouse Bone Marrow

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Summary

We have resolved $B220^+IgM^-$ B-lineage cells in mouse bone marrow into four fractions based on differential cell surface expression of determinants recognized by S7 (leukosialin, CD43), BP-1, and 30F1 (heat stable antigen). Functional differences among these fractions can be correlated with Ig gene rearrangement status. The largest fraction, lacking S7, consists of pre-B cells whereas the others, expressing S7, include B lineage cells before pre-B. These $S7^+$ fractions, provisionally termed Fr. *A*, Fr. *B*, and Fr. *C*, can differentiate in a stromal layer culture system. Phenotypic alteration during such culture suggests an ordering of these stages from Fr. *A* to Fr. *B* to Fr. *C* and thence to $S7^-$ pre-B cells. Using polymerase chain reaction amplification with pairs of oligonucleotide primers for regions 5′ of J_H1, DFL16.1, and J_k1, we find that the Ig genes of Fr. *A* are in germline configuration, whereas Fr. *B* and *C* are pro-B cell stages with increasing D-J rearrangement, but no V-D-J. Finally, functional analysis demonstrates that the proliferative response to IL-7, an early B lineage growth factor, is restricted to $S7^+$ stages and, furthermore, that an additional, cell contact-mediated signal is essential for survival of Fr. *A*.

The molecular events of Ig gene rearrangement that occur early in B lineage differentiation have been extensively characterized using A-MuLV-transformed cell lines (1). These studies provided evidence for an ordered rearrangement of the three Ig heavy chain gene elements (V, D, J) (reviewed in reference 2). Data from these lines also provided evidence for the sequential rearrangement of light chain following heavy chain; rearrangement of two additional gene segments (V and J) at either of the two light chain loci (κ or λ) consequently yields an intact Ig heterodimer (H_2L_2).

While the order of Ig gene rearrangement during B cell differentiation has been defined primarily through the use of in vitro transformed cell lines, it has never been clearly established for the normal cells present in sites of primary B cell differentiation, e.g., the bone marrow (BM)[1] of adult mice. According to the results with Abelson cell lines, one of the clearest changes expected to take place during early B cell differentiation involves the Ig heavy chain locus: D-J joining should precede complete V-D-J rearrangement (1). However, whether this can be observed in resolvable sets of cells in BM is not clear. An equally likely alternative is that Abelson virus transformation simply freezes as immortal cell lines in rapid transition. Furthermore, analysis of Ig rearrangements in such lines is complicated by potential oncogene rearrangements which would obscure the normal process.

These issues could be clarified if the early stages of B-lineage differentiation were defined more precisely through detailed investigation of the expression of cell surface molecules in normal BM cell populations coupled with determination of the status of Ig gene rearrangement. Recently developed culture techniques facilitate the growth and limited differentiation of B lineage cells using cloned stromal lines (3, 4). Therefore, analysis of changes in expression of surface molecules during short term culture of isolated BM populations should permit a provisional ordering of the differentiation stages. This ordering could then be confirmed by comparison with Ig gene rearrangement data. The definition of resolvable intermediates in normal populations would contribute greatly in defining the growth properties and requirements of cells at critical stages in the generation of functional B cells, work clearly not possible with transformed lines. Further, this determination should facilitate the study of gene regulation early in the B cell lineage.

At present, no single surface marker has been found that

[1] *Abbreviations used in this paper:* APC, allophycocyanin; APN, aminopeptidase; BI, biotin; BM, bone marrow; FL, fluorescein; HSA, heat stable antigen; TR, Texas Red.

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distinguishes the earliest B-committed cells before the pre-B stage from latter populations. We have approached this problem by using multicolor flow cytometry which enables us to determine the expression of several cell surface molecules simultaneously. We reasoned that the earliest B-committed cells could be found by investigating the correlated expression of determinants consistently present on B-lineage cells (such as B220; reference 5) together with those not found on surface IgM^+ B cells, but possibly expressed on B progenitor stages. Simultaneously, we have determined levels of several other cell surface molecules whose expression is known to change during differentiation and which might facilitate resolution of heterogeneity within the B lineage cells in BM.

We have found that the earliest stages of B lineage differentiation can be resolved by determining the level of expression of leukosialin (CD43) in the $B220^+$ BM fraction. The rat mAb S7 recognizes a determinant (which we term S7) present on a single chain polypeptide of Mr 85–95 kD (6) which has recently been shown to be the murine homologue of leukosialin (7, 8), previously characterized in the rat and human (C. M. Baecher-Allen, J. D. Kemp, and J. G. Frelinger, manuscript submitted for publication). S7 is expressed on granulocytes and all T cells (thymocytes and peripheral T cells), but is not found on most peripheral B (except plasma cells; reference 6). However, the regulation of S7 expression during early lymphoid cell development from hematopoietic stem cells was not known. As demonstrated in this paper, S7 is expressed on early precursors for B cells and is rapidly lost as these cells progress to pre-B and B cell stages during in vitro short term culture. Furthermore, multicolor immunofluorescence and sorting studies reveal that three subpopulations are clearly distinguishable within this early B fraction on the basis of differential expression of BP-1 and the heat stable antigen (HSA; detected by antibody 30F1; reference 9), surface molecules previously shown to be found at different levels during lymphoid differentiation (10–12).

We have determined the developmental relationships of cells in these three $S7^+$ subpopulations together with their rearrangement status, resolving cell fractions which show D-J rearrangement alone. Data to support the ordered differentiation pathway which we propose is obtained by culturing fractionated cells on a preestablished stromal line (FLST2; reference 13) where we observe progression of cell surface phenotype. The status of the Ig gene loci in these normal subpopulations, obtained by use of polymerase chain reaction (PCR) (14), reveals the existence of previously unresolved intermediates. Since the rearrangement of each Ig heavy chain gene element results in the deletion of known intervening DNA sequences, we used PCR to amplify fragments of these sequences along with a sequence that remains unchanged (from the actin gene). Using this approach we detect reduction in the fragment between D and J before any decrease in the fragment between V and D. Consequently, these data allow us to demonstrate phenotypic and physiological alterations in early B lineage cells according to their differentiation stage (pre–pro-B, early pro-B, late pro-B), progressing from absolute dependence on cell contact mediated signals to dependence only on a factor present in the stromal supernatant, identifiable as IL-7.

Materials and Methods

Animals and Cell Preparation. 2–4-mo-old female BALB/cAnN mice bred in the Institute for Cancer Research (ICR) animal facility were used in all experiments. A single cell suspension of BM (femur and tibia) was prepared by injecting medium (staining medium; deficient RPMI [Irvine Scientific, Santa Ana, CA], containing 10 mM HEPES, 3% FCS and 0.1% NaN_3) into the bone to flush out cells, followed by gentle mixing with a 1 ml syringe. Cells were treated with 0.165 M NH_4Cl to eliminate erythrocytes. Cells were recovered from cultures by gentle pipetting and then washing once with staining medium.

Immunofluorescence Staining, Analysis and Cell Sorting. Fractions A-C were prepared by incubating BM cells with a combination of fluorescein(FL)-S7, phycoerythrin (PE)BP-1, biotin(BI)-30F1 and allophycocyanin(APC)-6B2 in staining medium on ice for 15 min, washed three times with staining medium, then incubating a further 15 min with Texas Red(TR)-avidin to reveal the biotin reagent and finally washing twice with staining medium. Fractions D-F were prepared by staining with fluorescein (FL)-S7, PE–anti-IgM and APC-6B2. Stained cells ($2–6 \times 10^7$) were then applied to a dual laser/dye laser flow cytometer (FACStar$^{PLUS®}$, Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with appropriate filters for four color immunofluorescence. Samples were held on ice during sorting. Reanalysis of sorted fractions consistently showed purities in excess of 95%. Selected populations were sorted directly into 24- or 96-well plates (for functional analysis) or into microcentrifuge tubes (for DNA preparation). Analysis of cultured cell samples always included propidium iodide (1 μg/ml) for dead cell exclusion. Preparation of labeled reagents has been described previously (15).

Cell Cycle Analysis. Sorted cells ($1–2 \times 10^5$) were washed once in ice cold Tris-Saline (10 mM Tris, pH 7.0, 150 mM NaCl), then resuspended in 100-μl of this buffer to which was added 900 μl of ethanol chilled to −20°C. Cells were held for 10–20 min at 20°C, pelleted and then resuspended in 300 μl staining solution (1 mg/ml RNAase A, 20 μg/ml propidium iodide in PBS containing 0.01% NP40) and incubated for 10 min at 37°C. Cells were then analyzed by flow cytometry with pulse processing used to discriminate doublets.

Culture Conditions. The FLST2 stomal line (previously referred to simply as ST2) was established from C3H day 16 fetal liver and shown previously to support B lineage differentiation (13). The line was maintained in standard medium (RPMI-1640 supplemented with 5×10^{-5} M 2-ME, 5% FCS) in a humidified 5% CO_2 incubator chamber at 37°C and passaged weekly by treatment with trypsin-EDTA. Experimental cultures on FLST2 were carried out in 24-well plates (Nunc, Roskilde, Denmark) using 1 ml standard medium. Cultures were also performed by inserting diffusion chambers (Millicell; Millipore Corp., Bedford, MA) into wells containing preestablished FLST2 layers. Typically $1–5 \times 10^4$ cells were sorted per well. Cultures with rIL-7 (generously provided by Dr. S. Gillis; Immunex Corp., Seattle, WA) were done using 200 μl medium in 96-well plates with $0.5–2 \times 10^4$ cells per well. In some experiments, rabbit antibody to IL-7 (from Dr. S. Gillis; Immunex Corp.) or control rabbit serum was added immediately before sorting cells onto the layer.

DNA Preparation, PCR, and Data Analysis. Quantitative three pair PCR using sorted samples has been described previously for

two pair PCR (16). Briefly, 1–2 × 10^5 lymphocytes are sorted via flow cytometry according to phenotype directly into a microcentrifuge tube. Cells are washed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 8.0) at 4°C and then digested with 0.5 mg/ml proteinase K for 2 h at 50°C in buffer A (0.5% sodium lauroyl sarkosinate, 10 mM EDTA, 50 mM tris, pH 8.0) containing 1% low gelling temperature agarose. After digestion samples are allowed to gel on ice for 5 min, dialyzed against TE (three changes in 36 h), and then stored at 4°C. Before use, DNA samples are melted at 65°C, treated with RNAase (10 ng) and 10 U EcoRI restriction enzyme (37°C for 4–16 h), then gelled on ice and redialyzed three times against ddH_2O. Agarose containing the digested DNA is melted at 65°C, diluted 1:5 with 65°C ddH_2O (final volume 150 μl) and stored at 4°C. One fifth of the sample is analyzed by PCR using three sets of oligos (Table 1). These three sets are designated 5′DFL16.1, actin, and 5′J_H1; amplified regions shown in Fig. 6. For light chain (κ) rearrangement, a region 5′ of J_k1 was amplified (see Table 2) together with the actin fragment.

Conditions for PCR were: denaturation at 95°C for 1 min, annealing at 63°C for 30 s, and polymerization at 72°C for 1.5 min. Aliquots are withdrawn at 18, 20, and 22 cycles for separate analysis to ensure that amplification is within the linear range and care is taken to use relatively comparable levels (within a three-fold range) of DNA. 10 μl of the PCR samples are then separated overnight by 1.5% agarose gel electrophoresis, stained with ethidium bromide, photographed, and blotted onto Hybond N membrane (Amersham Corp., Arlington Heights, IL). Filters are UV crossed-linked, pre-hybridized for 1–3 h and then hybridized overnight (at 42°C) with riboprobes prepared from the PCR products (see below). Membranes are washed (twice for 30 min in 2× SSC and twice in 0.2× SSC at 65°C) and imaged on x-ray film (1–4 h exposure) and quantitated using a two dimensional proportional scintillation detector (Ambis Radioanalytic Imaging Systems, San Diego, CA). Radioactivity in individual bands representing each PCR product is measured and calculated as a percentage of germline (unrearranged) DNA. Each sample was separately amplified two to four times and several independently sorted samples of each cell phenotype were analyzed.

Generation of Probes from PCR Products. PCR products were cloned and riboprobes made as this gave a very high signal with low background when hybridized. Clones were made in the following manner. Individual PCR products were amplified using BALB/c liver DNA for 30 cycles and the appropriate size ethidium bromide stained band identified on a 1.5% agarose gel. The agarose containing the band was excised, the DNA eluted, purified, and blunt-end cloned into the SmaI site of pBSM13$^-$ (Stratagene, San Diego, CA). Orientations of the cloned fragments were not determined. RNA transcripts of EcoRI restricted probes were made according to the manufacturers' procedure using T3 polymerase (17). Approximately 1/6 of a labeling was used per blot.

Results

$B220^+S7^+$ Cells in Bone Marrow Are Large in Size and Resolved into Three Subpopulations by Correlated BP-1 and 30F1 (HSA) Expression. As Fig. 1 shows, fluorescence activated cell sorter (FACS®) analysis demonstrates that a small fraction (3–4%) of cells in BM express low levels of both B220 (detected by RA3-6B2) and S7. These cells are larger in size compared with $B220^+S7^-$ cells (as shown by measurement of forward light scatter) and do not express cell surface IgM. The presence of B-lineage cells in this $B220^+S7^+$ cell fraction was suggested by further analysis, including determination of BP-1 expression (Fig. 2), a molecule predominantly found on early B lineage cells (10) (and some stromal lines, reference 18). BP-1 is probably the best example of a molecule whose expression is restricted to early B-lineage cells and initially was considered to be an A-MuLV cell line specific marker (18, 19, 20). Although more than 90% of the $BP\text{-}1^+$ cells in BM have been reported to be cytoplasmic μ^+ pre-B

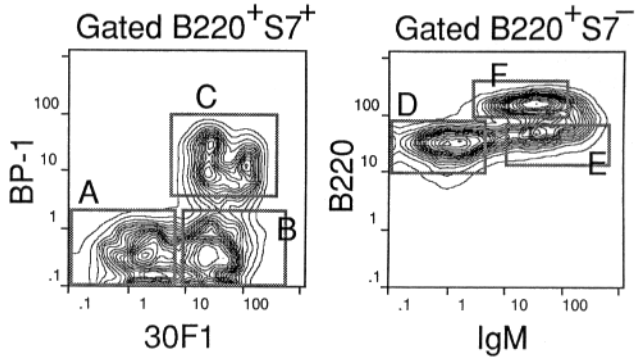

Figure 2. $B220^+S7^+$ cells can be resolved into three subsets (Fr. *A-C*) on the basis of BP-1 and 30F1 (anti-HSA) expression and $B220^+S7^-$ cells are resolved into three fractions (Fr. *D-E*) by differential expression of B220 and surface IgM. Cells were stained simultaneously either with FL-S7, PE–anti-BP-1, APC–anti-B220(6B2) and BI-30F1 (revealed by TR-Avidin) for the left panel or with FL-S7, APC–anti-B220(6B2) and PE–anti-IgM, and then analyzed as in Fig. 1. Fr. *A*, *B*, *C* represent 30%, 40%, and 30% of $B220^+S7^+$ cells; Fr. *D*, *E*, *F* represent 50%, 30%, and 20% of $B220^+S7^-$ cells (corresponding to 0.9%, 1.2%, 0.9%, 10%, 6%, and 4% of total BM, respectively). Data shown are representative of numerous analyses (>10).

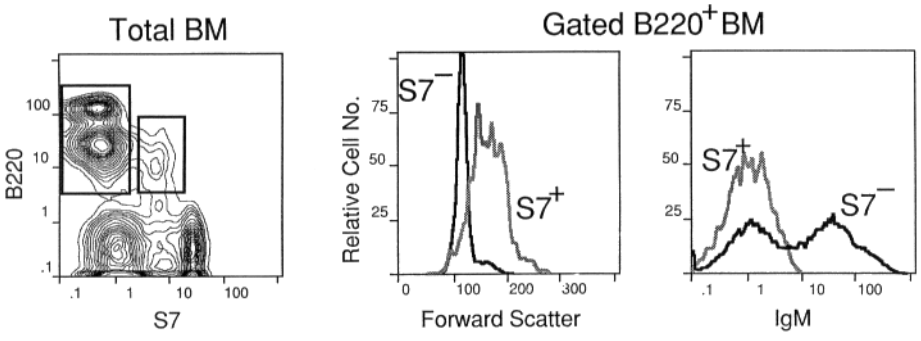

Figure 1. $S7^-$ versus $S7^+$ $B220^+$ B-lineage cells in the bone marrow (BM). BM cells were stained simultaneously with FL-S7, APC–anti-B220 and PE–anti-IgM, and then analyzed on a dual-laser multiparameter flow cytometer. In the left panel, $B220^+S7^-$ and $B220^+S7^+$ populations are boxed which correspond to 20% and 3% of total BM respectively. $B220^+S7^+$ cells have the lowest levels of B220 (*left panel*), are larger in size as measured by forward light scatter (*middle panel*), and do not bear surface IgM (*right panel*), in contrast with $S7^-$ ($B220^+$) cells.

cells (10), the extent of BP-1 expression at the earliest stages of B cell development (pro-B) has not been determined; studies with Abelson cell lines suggested that BP-1 expression does not appear to define one type of Ig gene status, in that both V-D-J and D-J rearranged cells could express BP-1 (21). We found that although most BP-1$^+$ cells are S7$^-$B220$^+$IgM$^-$ (data not shown), a proportion of S7$^+$B220$^+$ cells also express BP-1 (Fig. 2).

Furthermore, four-color FACS analysis of the correlated expression of 30F1 (recognizing the HSA) and BP-1 revealed the presence of three resolvable subpopulations in this B220$^+$S7$^+$ cell fraction (Fig. 2, left). HSA is so termed because of its characteristic resistance to heat denaturation after fixation and is widely distributed on many cell types with structurally heterogeneous forms of 30–60 kD depending on cell type (22). The three distinct phenotypes of cells in this B220$^+$S7$^+$ cell population, 30F1$^-$BP-1$^-$, 30F1$^+$BP-1$^-$, 30F1$^+$BP-1$^+$ are termed Fr. *A*, *B*, and *C* (Fig. 2, *left*). In addition, we delineate three S7$^-$ B lineage cell fractions: B220$^+$(dull)S7$^-$IgM$^-$ (Fr. *D*), B220$^+$(dull)S7$^-$IgM$^+$ (Fr. *E*) and B220^{++}(bright)S7$^-$IgM$^+$ (Fr. *F*) (Fig. 2, *right*), provisionally defined as pre-B, newly generated B and mature B cells, respectively and confirmed by Ig-gene rearrangement status, as shown below. While we use 30F1 to identify HSA, we obtain similar staining by using other anti-HSA antibodies, J11d, and M1/69 (data not shown).

Progression of Cell Surface Phenotype During Culture Predicts a Cell Differentiation Order of Fr. A, Fr. B, then Fr. C. The presence of B lineage cells in the B220$^+$S7$^+$ cell fractions was established by their ability to generate B cells and/or proliferate on a preestablished fetal liver derived stromal line (FLST2) capable of supporting limited B lymphopoiesis (13). Graded doses of cells (1–5 × 10^4) in each fraction were deposited by FACS® (Becton Dickinson and Co.) directly onto FLST2 and 4 d later B lineage cell proliferation and surface phenotype were determined by FACS®. This analysis revealed that the 30F1$^+$ cell fractions, Fr. *B* and *C*, are the most responsive during stromal culture, proliferating (described in the next section) and generating surface IgM$^+$ cells. More IgM$^+$ cells were found in cultures of Fr. *C* (48%) than Fr. *B* (20%) (Table 1), suggesting that Fr. *C* includes more differentiated B lineage cells. As might be expected, >90% of B220$^+$(dull)S7$^-$ pre-B cells (Fig. 2 Fr. *D*) became cell surface IgM$^+$ after overnight culture on FLST2 without significant cell proliferation, indicating that they are the immediate precursors for newly generated B cells (data not shown). The generation of IgM$^+$ cells from Fr. *A* was negligible (Table 1) and cultures initiated with B220$^-$ BM cells did not yield B lineage cells during short term culture.

Alteration of the S7, 30F1, BP-1, and IgM levels following short term culture provide a basis for defining relationships among the three S7$^+$ subpopulations. FACS® analysis summarized in Table 1 revealed that, although Fr. *A* did not exhibit extensive proliferation or generation of IgM$^+$ cells during 4-d culture, these cells did begin to express 30F1 and half became BP-1$^+$ (Fr. *B* and *C* phenotypes). In contrast with Fr. *A* culture, where cells retained S7 expression, a large proportion of Fr. *B* cells and essentially all of Fr. *C* cells ceased S7 expression and progressed to a pre-B cell phenotype. In summary, considering these phenotypic changes and the fractions' differential ability to generate IgM$^+$ B cells (Table 1), we postulate three early B cell differentiation stages in order from Fr. *A* to Fr. *B* to Fr. *C*, and thence to B220$^+$S7$^-$IgM$^-$ pre-B cells (Fr. *D*); that is, progressive acquisition first of 30F1 expression, then BP-1 expression and finally loss of S7 expression.

Functional Characterization of Early B Lineage Fractions: Early Dependence on a Contact Mediated Signal and Restriction of IL-7 Activity to S7$^+$ B Lineage Cells. We found that the B220$^+$S7$^+$ cell fractions in BM are the only B-lineage populations capable of proliferation (and differentiation) on the stromal cell layer in short term (4-d) culture. As shown in Fig. 3 *a*, the 30F1$^+$ fractions (Fr. *B*, *C*) exhibited very significant proliferation during 4-d stromal culture compared with the 30F1$^-$ fraction (Fr. *A*). Furthermore, other BM cell fractions (B220$^-$ non-B cells, B220$^+$S7$^-$ pre-B and B cells) showed very poor cell recoveries (Fig. 3 *a*). All three B220$^+$S7$^+$ fraction cultures (including Fr. *A*) were dependent on the presence of stromal cells, as very few viable cells (<1%) were recovered after culture in medium alone (not shown).

Furthermore, we found that the conditions required for proliferation and differentiation of S7$^+$ cells differed among the three fractions. We compared the relative contact dependence of the three B220$^+$S7$^+$ fractions by culturing them either directly on a stromal layer or instead in a diffusion chamber where the cells are prevented from coming in contact with the stromal line. Cell recovery with these different culture conditions was measured after 4 d and the results demonstrated a variation from contact dependence to contact independence. As Fig. 3 *b* shows, Fr. *A* was absolutely dependent on contact with the stromal layer for survival (exhibiting minimal proliferation) since without the stromal layer,

Table 1. *Phenotypic Change, Including Generation of Surface IgM$^+$ Cells after 4-D FLST2 Culture*

Surface Expression	Fr. A		Fr. B		Fr. C	
	d 0	d 4	d 0	d 4	d 0	d 4
S7	100	100	100	40	100	7
30F1	0	100	100	100	100	100
BP-1	0	50	0	90	100	100
IgM	0	3	0	20	0	48

1–5 × 10^4 cells were fractionated and cultured as described in Fig. 3. After culture, recovered cells (10^4-3 × 10^5) were stained with FL-S7, APC–anti-B220, BI-30F1 (revealed by TR-Avidin in a second step) and either PE–anti-BP-1 or PE–anti-IgM. Percentage of positive cells before (d 0) and after (d 4) culture is presented. Increasing percentages of surface IgM$^+$ cells were seen in order from Fr. A to Fr. C. Representative data is shown from a total of three to six independent analyses, always at day 4; individual variation was 5% or less.

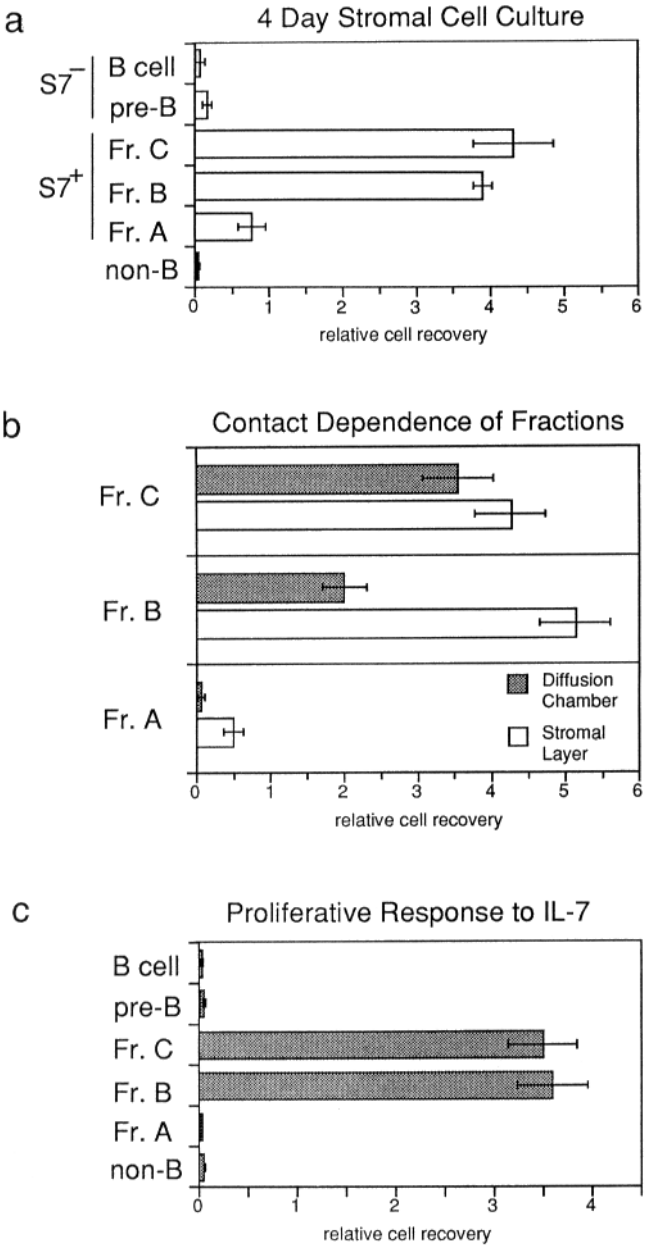

Figure 3. (*a*) B-lineage cell proliferation in short-term stromal layer culture of BM is due to B220$^+$S7$^+$ cells, largely Fr. *B* and Fr. *C*. BALB/c BM cells were stained as described in Fig. 2 and then selected populations (1–5 × 10^4 cells/well) were sorted directly onto preestablished stromal layers (FLST2) in 1 ml multiwell plates. 4 d later, cells were dissociated from the layer by pipetting, then restained with FL-S7, APC–anti-B220 and either PE–anti-BP-1 or PE–anti-IgM; propidium iodide was used to eliminate dead cells. B-lineage cell recovery was quantitated as B220$^+$ which simultaneously allowed the elimination of any stromal contaminant. Non-B are B220$^-$ BM cells (which failed to generate B220$^+$ cells). Relative recovery: 1 = the number of input cells before culture. Data is based on three independent experiments with several wells (2–3) of each phenotype per experiment. (*b*) Fr. *C* is least dependent on cell contact for proliferation in stromal culture. Cells were fractionated as described in Fig. 2, then sorted either directly onto a preestablished FLST2 layer or else onto a membrane (Millicell) suspended over the layer. 4 d later, cells were harvested and treated as for Fig. 5. Representative data from three experiments is shown. No live cells were recovered from Fr. *A* cell culture in the diffusion chamber. (*c*) IL-7 induced cell proliferation is solely restricted to B220$^+$S7$^+$ Fr. *B* and Fr. *C* in the BM. Cells were fractionated as in Fig. 2, then sorted directly into wells containing medium supplemented with 100 U/ml of rIL-7. 4 d later cells were harvested and treated as for Fig. 5. Results with 10 U/ml rIL-7 yielded comparable results (data not shown).

no live cells were recovered. On the other hand, Fr. *B* showed only modest dependence (a two- to three-fold difference) and Fr. *C* showed the least difference between contact and supernatant supported growth.

We next attempted to determine the nature of the factor in the supernatant providing a growth stimulus to the latter two fractions (Fr. *B* and *C*). One obvious candidate for this factor is IL-7 which was cloned on the basis of its ability to support the growth of a stromal-dependent B cell line (23). Responsiveness to rIL-7 was first tested by sorting the various fractions from BM into medium supplemented with this lymphokine (Fig. 3 *c*). The results show that growth in response to rIL-7 is remarkably restricted to the B220$^+$S7$^+$ cell fraction in BM. Furthermore, as expected, within this S7$^+$ cell fraction, growth in rIL-7 alone was observed with Fr. *C* and Fr. *B*, but not Fr. *A* cultures, similar to results with diffusion chambers.

While rIL-7 shows a significant proliferative effect with Fr. *B* and *C* cells, a question remains whether IL-7 is indeed the major growth factor in stromal cell culture. Furthermore, the absence of growth of Fr. *A* does not exclude a role for IL-7 at this stage. Previous work has suggested that IL-7 might be important at a contact dependent stage, since an IL-7 negative variant of a "support" stromal layer failed to support even an earlier (contact dependent) stage of B lineage differentiation (24). We tested this directly by adding neutralizing antiserum specific for IL-7 to our stromal cultures (Fig. 4). After 4-d culture, cell recoveries in the presence of the anti–IL-7 antiserum were decreased around 10-fold compared to control cultures with or without preimmune serum in Fr. *B* + *C* cultures (and also cultures of Fr. *B* or Fr. *C*, data not shown). This was also the case with the earliest fraction (Fr. *A*) that is absolutely contact dependent. Thus, we would suggest that, early in the B lineage differentiation pathway, cells progress from a requirement for both a contact dependent signal together with IL-7 to a requirement for IL-7 alone.

Fr. C Includes Cells in Cycle Which Show the Highest Level of 30F1 Among B Lineage Cells (Fr. C'). As shown above, we detect considerable cell proliferation by IL-7 with the latter two S7$^+$B220$^+$ fraction cultures (Fr. *B* and *C*). However, since the intermediate fraction (Fr. *B*) can "progress" to a phenotype similar to that of the most mature fraction (Fr. *C*) in this in vitro system, we could not decide whether the proliferation seen by day 4 of Fr. *B* culture was due to an initial ability to proliferate or instead to progress to Fr. *C* followed by proliferation. We have attempted to answer this question directly in situ by determining the degree of proliferating cells within each of the three phenotypes found in BM. Sorted cells of each phenotype from BM were analyzed by FACS® for DNA content by propidium iodide staining.

This analysis revealed that the most mature S7$^+$B220$^+$ fraction (Fr. *C*) contains significant numbers (>30%) of cells in cycle in adult BM (Fig. 5, *left*) and that such cycling cells

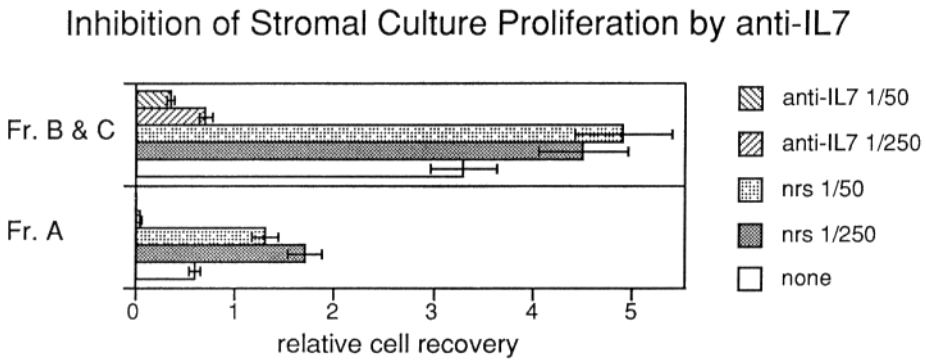

Figure 4. Inhibition of B-lineage cell growth by anti-IL-7. Cells were stained as in Fig. 2, then B220$^+$S7$^+$ cells were separated into 30F1$^-$ (Fr. *A*) and 30F1$^+$ fractions (Fr. *B* and *C*) and sorted onto FLST2 either with no additive, with nonimmune rabbit serum added (nrs) or with rabbit anti-IL-7 antiserum added. 4 d later, cells were harvested and treated as in Fig. 3. Data from three experiments.

comprise an increasing percentage in Fr. *C* from younger mice (data not shown). We could also predict this by analysis of cells size as estimated by forward light scatter on the FACS®. Although all cells in the B220$^+$S7$^+$ cell fractions are large when compared with those in the B220$^+$S7$^-$ fraction (Fig. 1), only Fr. *C* includes the largest cells (data not shown) which are also discriminated as cells with a distinctively higher level of 30F1 (Fig. 5, Fr. *C'*, *right*) and the lowest expression of S7 (data not shown). That Fr. *C'* represents cycling cells within Fr. *C* is strongly supported by data showing that elimination of 30F1^{++} cells from Fr. *C* decreases the frequency of cells with higher DNA content (Fig. 5). The frequency of Fr. *C'* cells in adult (2–4 mo) BALB/c BM is 0.1–0.2% of nucleated cells.

Thus, the proliferation found with Fr. *B* culture as described above is likely to be the result of differentiation from Fr. *B* to Fr. *C* (and *C'*) stage, promoted by stromal contact and IL-7. Therefore, in vivo, only cells in the most mature B220$^+$S7$^+$ fraction are rapidly proliferating, cells immediately before (and during) the loss of S7. Then S7$^-$B220$^+$ cells in BM (pre-B cells) lose the ability to proliferate in the bone marrow environment as shown in vitro by using either stromal layer or rIL-7 culture.

Ig Rearrangements in B Lineage Cell Fractions: Fr. B and C Are D-J Rearranged Pro-B Cells. Analysis of Ig gene rearrangement at both the heavy chain and at the κ light chain loci in sorted populations was performed via the PCR (cycling Fr. *C'* cells were excluded from Fr. *C* for this experiment). Our approach has been to amplify DNA corresponding to sequences that are lost upon rearrangement at each locus. Therefore, we have synthesized a pair of oligonucleotide primers (Table 2 and Fig. 6) that amplify a region just 5′ of J_H1 (25) lost upon D-J rearrangement, a pair of primers for a region 5′ of the most upstream D element, DFL16.1 (26), lost upon V to D-J rearrangement and a pair of primers for the region 5′ of J_k1 lost upon deletional V-J rearrangement at the κ locus. We have also generated a pair of primers that amplify an α-actin intron sequence to serve as a normalizing signal since it is not lost upon any of these rearrangements and thus allows us to correct for variation in input DNA. After amplifying each of these fragments from BALB/c liver DNA, we have cloned them into pBSM13$^-$ in order to permit generation of riboprobes which can then be used to quantitate the relative intensity of each amplified band. For heavy chain rearrangement we can coamplify the V-D, D-J and actin fragments since they generate resolvable bands on a 1.5% agarose gel. The specificity and resolution of this PCR assay was confirmed by using B lineage cell lines in which Ig gene status had already been determined. As Fig. 6 demonstrates, whereas all three cell lines show complete loss of the 5′ J_H1 intron fragment (present in DNA from liver), two still retain a fraction (50%) of the 5′DFL16-1 fragment (V-D-J/D-J) while the other shows complete loss (V-D-J/V-D-J) in agreement with previous characterization (1, 27, 28).

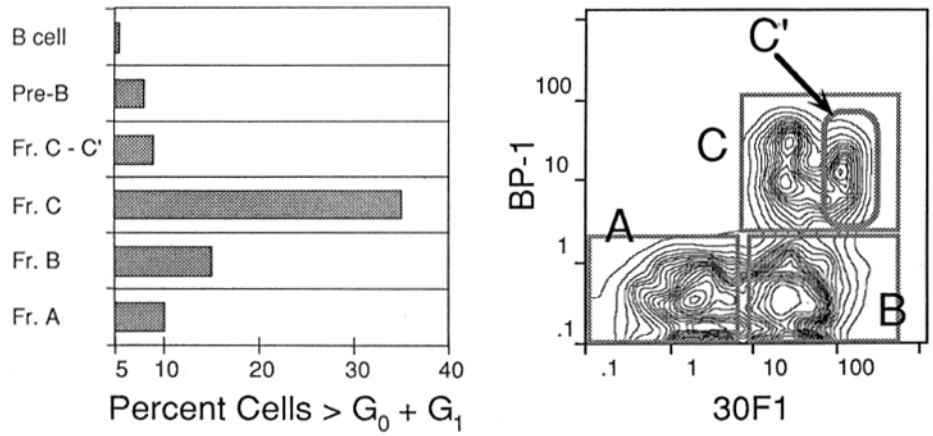

Figure 5. Fr. *C* includes the most cells in cycle which are enriched in a portion expressing higher levels of 30F1 (Fr. *C'*). Cells were stained as in Fig. 2, then 10^5 cells of indicated fractions were sorted, fixed, stained with propidium iodide and analyzed for DNA content by flow cytometry. Fr. *C'* comprises 20% of Fr. *C* (*right*). Representative data from a total of three separate analyses is shown; individual variation was <5% for animals of the same age.

Table 2. *Sets of Oligonucleotide Pairs Employed in Determination of Ig Gene Rearrangement*

Set	Fragment	5′ oligo	3′ oligo	Size
1	5′$D_{FL16.1}$	GCC TGG GGA GTC ACT CAG CAG C	GTG TGG AAA GCT GTG TAT CCC C	1,227
2	5′J_H1	CCC GGA CAG AGC AGG CAG GTG G	GGT CCC TGC GCC CCA GAC A	472
3	5′$J_κ1$	ATG TAC TTG TGG ATG CAG AGG CTG	CCT CCA CCG AAC GTC CAC CAC	435
4	Actin	GGT GTC ATG GTA GGT ATG GGT	CGC ACA ATC TCA CGT TCA G	737

Oligos are listed 5′ to 3′. Size is in nucleotides.

Our results with normal cells (Table 3 and Fig. 7) reveal that Ig genes from Fr. *A* are germline for all loci. Analysis of $30F1^+B220^+S7^+$ fractions (whether $BP\text{-}1^-$ or $BP\text{-}1^+$; Fr. *B* or Fr. *C*) show more than 50% of IgH loci (80% in Fr. *C*) have lost the fragment 5′ of J_H1 while completely retaining the sequence 5′ of DFL16.1. This clearly demonstrates the existence in BM of a significant population of B lineage cells possessing D-J, but not V-D-J, rearrangement. In contrast to $S7^+$ cells, $B220^+S7^-IgM^-$ cells (pre-B) show, in turn, significant (50%) V-D-J rearrangement. Curiously, the degree of V to DJ rearrangement of the heavy chain remains at 50% of alleles (determined within the linear PCR amplification range) even in surface IgM^+ cells, a value consistently found in all B cell populations examined (including IgM^+IgD^+ B cells in spleen, not shown). Since all IgM^+ cells must bear a productive V-D-J rearrangement at one allele, this result suggests that the unexpressed Ig heavy gene allele usually has an incomplete Ig gene rearrangement (D-J) rather than a nonfunctional V-D-J rearrangement.

As Table 4 shows, light chain gene rearrangement (V-J) is not seen in any $S7^+$ cell fraction (Fr. *A–C*). Rearrangements at the κ locus can occur by either an inversional or deletional mechanism (29). Inversional rearrangement to J_k1 would eliminate the 5′ of J_k1 PCR fragment, but such rearrangement to the other J_k segments would preserve this fragment leading to an underestimate of total κ gene rearrangement. Thus our data gives a minimum level of light chain rearrangement since it misses inversional rearrangement to J_k2–5. Bearing in mind this caveat, rearrangement is first detected in the pre-B population (30% of alleles) and by the IgM^+ stage at least 60% of alleles are rearranged (consistent with earlier studies; reference 30). In addition, our light chain data show that there are B lineage cells with V-J rearrangement before cell surface Ig expression since our pre-B cells were sorted as surface IgM^- cells. This may suggest a lag in surface expression after rearrangement or, alternatively, that these are nonfunctional.

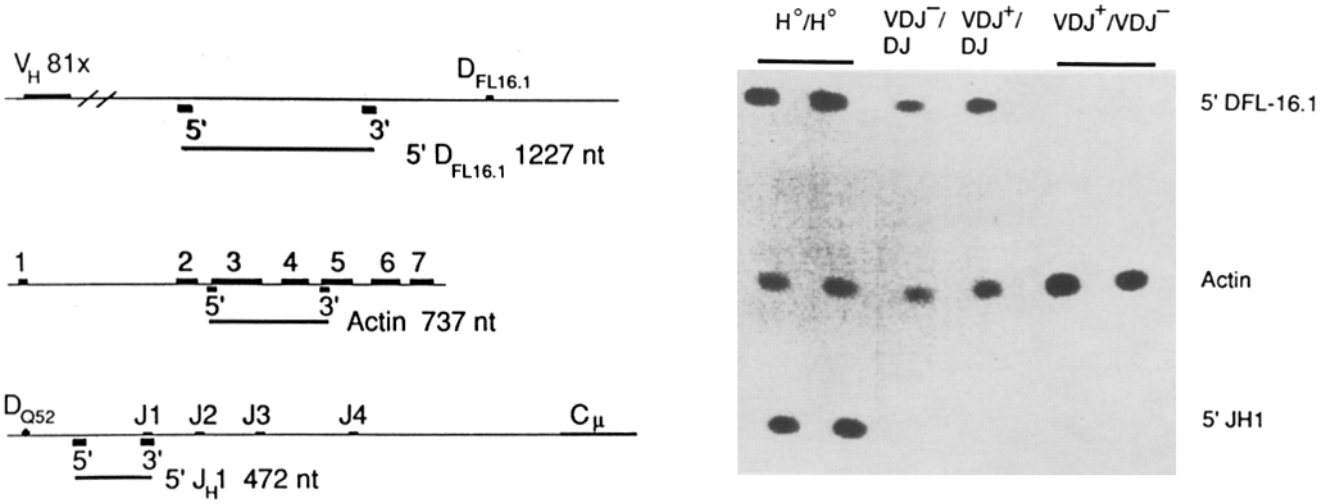

Figure 6. (*a*) Diagram of DNA loci employed in PCR amplification studies measuring V-D-J heavy chain and V-J light chain rearrangement. 5′ and 3′ oligonucleotides used for primers are shown in Table 2. (*b*) Discrimination of Ig heavy chain gene rearrangement D-J/V-D-J versus V-D-J/V-D-J in B lineage cell lines by PCR analysis. After amplification of DNA from cell line or liver (2×10^4 cells) with primer sets 1, 2, and 4 (Table 2), 1/10 of the reaction was size-fractionated by agarose electrophoresis and blotted. After incubation with probes made from the three amplified fragments, the filter was washed and autoradiographed for 2 h at −70°C. Samples were: liver (H°/H°, lanes *1* and *2*), 1–8 (V-D-J^-/D-J, lane *3*), 38C-13 (V-D-J^+/D-J, lane *4*) and 3–1 (V-D-J^+/V-D-J^-, lanes *5* and *6*). Liver and 3–1 DNA were processed in duplicate. Both 1–8 and 38C-13 DNA shows 50% retention of germ line 5′DFL16.1 whereas there is no retention in 3–1 DNA (<0.05% as determined by scintillation counting). All cell lines lack the 5′J_H1 germline fragment (<0.05%).

Table 3. *Lack of Decrease of 5'DFL16.1 or 5'J_H1 Fragments (Retention of Germline) Demonstrates that Cells in Fr. A Are Unrearranged at the Heavy Chain Locus whereas Fr. B and Fr. C Show D-J, but Not V-DJ Rearrangement*

	Fractional retention of germline							
	Fragment 5′ of D				Fragment 5′ of J			
Cell fraction	Individual samples			Mean (SE)	Individual samples			Mean (SE)
Fr.A	1.00	0.98	1.16	**1.05**	0.96	0.84	0.99	**0.95**
	0.89	1.22		(0.06)	0.91	1.03		(0.03)
Fr.B	0.98	0.98	1.07	**0.98**	0.39	0.30	0.41	**0.39**
	0.97	0.86	1.04	(0.03)	0.43	0.35	0.45	(0.02)
Fr.C	1.00	0.97	1.08	**1.00**	0.19	0.12	0.24	**0.17**
	0.94			(0.04)	0.14			(0.03)
Pre-B	0.50	0.49		**0.50**	0.06	0.05	0.04	**0.05**
				(0.01)				(0.01)
B cell	0.50	0.46	0.44	**0.50**	0.09	0.03	0.06	**0.05**
	0.60			(0.04)	0.03			(0.01)

PCR data from multiple determinations of several sorted samples of sets of each phenotype (from different mice) are shown. DNA was amplified by PCR using primer sets 1, 2, and 4 (Table 2). SE is standard error.

Discussion

The development of B lineage lymphocytes from their hematopoietic progenitors proceeds through a series of microenvironmentally regulated differentiation events. At least some of this complex gene regulation has been revealed by alteration of cell surface components on hematopoietic cells, notably that seen with carbohydrate structures on glycoproteins or glycolipids (31–34). Although the biological significance of most of this differential expression on the cell surface is not understood at present, these determinants may be important in cell-cell interactions (4) or may function as receptors for microenvironmental signals leading to the activation or inactivation of regulatory genes involved in cell differentiation. Thus, if early B lineage differentiation involves critical stepwise events, we expect the populations of early B lineage cells

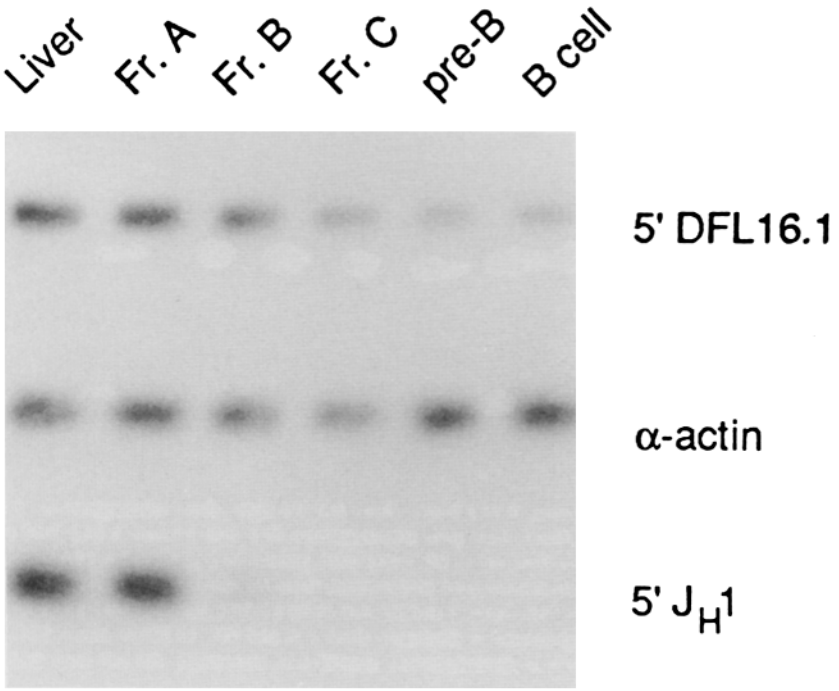

Figure 7. PCR analysis of rearrangement at the Ig heavy chain locus reveals the presence of cell populations with significant D-J rearrangement, but without V-D-J. DNA isolated from fractions sorted as in Fig. 3 was amplified, blotted, and probed as described in Fig. 6. After washing, the filter was quantitated on a two-dimensional scintillation counter (see Table 3) and autoradiographed. The figure presented is from a 2-h exposure. The $S7^+B220^+$ fractions (Fr. *A–C*) were defined as in Fig. 2, *left panel*. Pre-B and B cells were defined as in Fig. 2, *right panel*; that is, pre-B are $S7^-B220^+IgM^-$ (Fr. *D* in the figure) and B cells are $S7^-B220^+IgM^+$ (Fr. *E* in the figure).

Table 4. *PCR Analysis of Rearrangement at the κ Light Chain Locus Demonstrates Onset of Light Chain Rearrangement at the pre-B Cell Stage*

Cell fraction	Fraction of germline	SE	n
Fr. A	0.96	0.03	5
Fr. B	1.02	0.03	5
Fr. C	0.94	0.07	5
pre-B	0.71	0.03	5
B cell	0.43	0.05	5

DNA was amplified by PCR using primer sets 3 and 4 (Table 2). SE is standard error and n is the number of determinations.

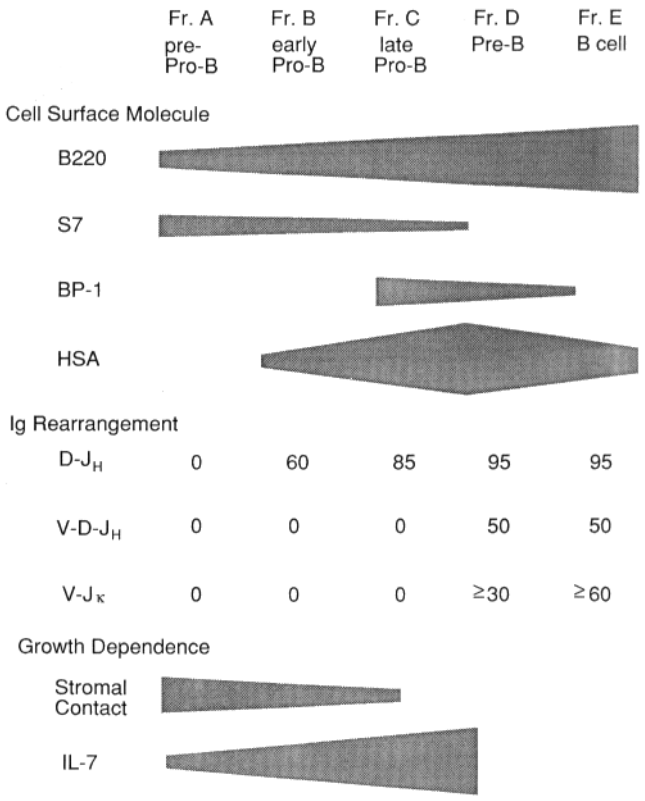

Figure 8. Summary of changes in cell surface molecule expression during early B-lineage cell differentiation stages correlated with Ig gene rearrangement status and growth dependence.

to show phenotypic distinctions. Furthermore, such study could potentially allow characterization of the signals or factors necessary for normal cell differentiation.

In this report we delineate a B cell differentiation stage with D-J rearrangement (pro-B) in BM and furthermore resolve three previously unrecognized early B cell development stages showing phenotypic and functional distinctions as summarized in Fig. 8. The use of the recently described mAb S7, together with B220 (RA3-6B2) and multiparameter FACS® analysis clearly defines a population (3–4%) in BM highly enriched for the earliest B lineage cells. Thus, our analysis shows that B-lineage cells express S7 at a very early (pre–pro-B and pro-B cell) stage, then downregulate it upon V-D-J rearrangement and transition to the pre-B cell stage. This transition is identified by the lowest expression of S7 on large cycling cells in the most mature stage of the $B220^+S7^+$ fraction.

The molecule recognized by S7 was recently demonstrated to be the murine homolog of human CD43, (also known as leukosialin or sialophorin; reference 8) since it is expressed on a cell line transfected with a cloned murine cDNA homologous with the human and rat leukosialin gene (C. M. Baecher-Allen, J. D. Kemp, and J. G. Frelinger, manuscript submitted for publication). Leukosialin has been recognized as the major sialoglycoprotein on myeloid and T lymphocytes (7, 35, 36). It is heavily glycosylated (with O-linked oligosaccharide structures accounting for >50% of weight of this molecule), and displays a Mr of 90–150 kD depending on differentiation stage within a cell lineage or on particular cell lineage. Interest in its possible biological significance in immune function has arisen from earlier work. Patients with Wiskott-Aldrich syndrome, characterized by profound immunodeficiency, eczema, and thrombocytopenia, show structurally aberrant or reduced levels of leukosialin (37). Although it is not yet clear how leukosialin expression functions in hematopoietic development, there is speculation that the carbohydrate on leukosialin is important for the maintenance of cell morphology or adherence (8). Furthermore, it has been reported that leukosialin is phosphorylated upon cell activation (38) and that anti-CD43 induces both aggregation and proliferation of T cells, suggesting that cell surface leukosialin is involved in an activation pathway (39–41). In this context, it is intriguing that S7 expression is restricted to distinct B cell developmental stages, early (and again at the terminal stage) in the pathway.

Our study also demonstrates that three distinct early B lineage stages with S7 expression can be recognized by the ordered alteration of cell surface BP-1 and HSA (30F1) expression. Acquisition of 30F1 expression is indicative of cells at the stage of D-J rearrangement as distinguished from $30F1^-$ cells completely lacking rearrangement. Subsequently, such cells further differentiate to express BP-1 and this coincides with alteration of the physiological characteristics of the B-lineage cells. Previous work with stromal cell cultures led Dorshkind to suggest that the earliest B lineage progenitors are absolutely dependent on signals mediated by direct contact with the layer while latter stages are relatively contact independent (42). Lee et al. (43) showed recently that rIL-7 alone leads to proliferation of large early stage $B220^+IgM^-$ cells in BM. We confirm and extend these observations by resolving a fraction of normal BM B lineage cells which is absolutely cell contact dependent and another fraction which is completely contact independent. Our data clearly show that IL-7 operates at a restricted stage before

the (late-)pre-B cell and plays a major role in early B cell differentiation promoted by stromal cells.

Interestingly, while the cells proliferating in IL-7 come from fractions initially bearing only incomplete rearrangements, they evidently rapidly reach the V-D-J stage since Lee et al. (43) previously reported that the majority of cells responding in this fashion express cytoplasmic μ. We presume that complete V-D-J rearrangement can be first detected in Fr. *C'*, rapid cycling cells with an intermediate phenotype similar to that of Fr. *C*, but with extremely high expression of 30F1 and decreased levels of S7. This in situ proliferation is likely a response to de novo IL-7 as we found in Fr. *B* and Fr. *C* culture. In response to IL-7, Fr. *B* cells progress to Fr. *C* (and *C'*) stage (which occurs rapidly during in vitro short term culture), acquiring the ability to proliferate in IL-7 alone. We postulate that this stage (late Fr. *C*) at which extensive proliferation occurs in response to IL-7 must immediately follow V-D-J rearrangement (μ^+) since this would allow expansion of clones of B lineage cells before light chain rearrangement, resulting in greater combinatorial diversity (several light chains with the same heavy chain). The proliferative response to IL-7 is completely absent in the $S7^-$ pre-B cells, so that clones of B cells with identical (V_H/V_L) combining sites will not be expanded.

Our hypothesis may also explain why only Fr. *C* proliferates extensively in the presence of IL-7 alone. If our prediction is indeed the case, one candidate to regulate IL-7 induced proliferation is the μ chain produced after productive rearrangement. If the presence of μ chain before complexing with light chain leads to a proliferative response after occupancy of the IL-7 receptor, then lack of response in the absence of productive V-D-J rearrangement would provide a mechanism for eliminating (or holding) cells with nonfunctional V-D-J rearrangements at this early stage. Indeed we have recently found that while Fr. *C* cells from SCID mice (that lack the ability to complete functional Ig rearrangement) fail to proliferate in IL-7 alone, cells from such mice bearing a productively rearranged immunoglobulin μ transgene can respond (M. Reichman-Fried, M. Bosma, R. Hardy, manuscript in preparation). This model would also account for cessation of IL-7 responsiveness following production of light chain (which would complex the μ chain). Since our data reveal that Fr. *C* does not show detectable levels of V-D-J rearrangement, whereas, in contrast, $S7^-$ pre B cells do, it will be of interest to isolate and determine the Ig gene status of cycling Fr. *C'* cells and, in addition, to determine IL-7 receptor levels on these B lineage cell populations in BM.

Although resolution of the $S7^+B220^+$ population into three fractions based on expression of the HSA and BP-1 has demonstrated important functional distinctions, roles for the determinants used to separate these populations are yet to be defined. BP-1 (also recognized by the antibody 6C3) is a 140 kD glycoprotein homodimer whose expression has been reported to be restricted to early B lineage cells in BM (10, 18, 19). Our finding that it is first expressed on a transitional stage of pro-B cells demonstrates its highly ordered regulation during B cell differentiation. Recently BP-1 has been cloned and sequenced and shown to be the second member (along with CALLA/CD10/neutral endopeptidase) of the zinc-dependent metallopeptidase family expressed on early B lineage cells (20). Its sequence shows highest homology with aminopeptidase N (APN) and its biochemical function is thought to be similar to APN. Since APN plays an important role in the degradation of peptides, such membrane associated peptidases may have an effect on the factors which regulate the progression of cell differentiation.

A recent cDNA cloning and sequencing study showed that the HSA (recognized by 30F1) is a phosphatidylinositol-linked glycoprotein consisting of a very short peptide with extensive N- and O-linked glycosylation (44). HSA is found on numerous cell types, often at different levels depending on differentiation state (22). The various mAbs that react with HSA (J11d, M1/69, B2A2, 30F1) may recognize differentially glycosylated forms, although their potential fine specificities for distinct HSA epitopes have not been investigated. In general, these HSA epitopes, while absent from peripheral T cells, are expressed on early T lineage cells in the $CD4^-CD8^-$ and $CD4^+CD8^+$ subsets of immature thymocytes (45, 46). In B cell differentiation, as we show here, HSA expression becomes detectable at the D-J rearranged stage, then reaches highest levels at the large cycling stage between pro-B to late pre-B stage. HSA expression is downregulated thereafter and is found at a lower level on most peripheral B cells, but rapidly increases upon B cell activation (as reported previously; reference 12).

Molecular analysis by PCR of deletion of DNA resulting from rearrangements at the heavy chain (D-J and V-D-J) and light chain (V-J) loci has permitted correlation of phenotype with Ig gene rearrangement status. Thus we find that two resolvable fractions of early B lineage cells show increasing rearrangement at the D-J locus before any V to D-J activity. These data demonstrate clearly that the order of recombination is tightly regulated in normal B lineage cells. Since the entire Ig region (V-D-J) is thought to be relatively open for access to the recombinase system at this stage (47), there must be a further level of control that distinguishes between D to J and V to D-J rearrangement as has been noted previously (48). Thus, T cell lines often have D-J (but never V-D-J) rearrangement (49). Studies of the rearrangement of transgenic constructs have also found that V-D-J, but not D-J rearrangement is lineage specific (50). Based on our result that Fr. *C* is the stage immediately before V-D-J rearrangement, it will be informative to determine whether germline V transcripts (thought to be hallmarks of initiation of V gene rearrangement; reference 47) are restricted to this population. Fig. 8 summarizes our correlation of surface phenotype with change in Ig gene status and growth response.

A surprising observation in our study is that complete heavy chain rearrangement (V to D-J) appears to occur predominantly only on one allele per cell. That is, IgM^+ cells (where all cells have a productive V-D-J rearrangement on at least one allele) still show retention of 50% of the germline level of the 5'DFL16.1 sequence. Much previous work on IgH gene rearrangement has employed J probes to determine Ig gene status so that the extent of complete nonfunctional rearrangement on the nonexpressed allele has not been thoroughly in-

vestigated. However careful studies of Abelson transformed lines have revealed that significant numbers are VDJ^+/VDJ^- (1). Whether this degree of nonfunctional rearrangement is a result of transformation remains to be determined. There is also prior evidence in the literature of incomplete rearrangement at nonexpressed alleles in normal B cells, but at significantly lower levels than we find here (1). Considering that the generally accepted model of Ig rearrangement assumes significant error in the joining mechanism (generating nonfunctional products), it is important to investigate further the extent of VDJ^- rearrangements in normal populations of B cells (51).

Finally, a question remains as to the degree of heterogeneity of Fr. *A* and the degree of "commitment" to the B cell lineage. At least some cells in this fraction, lacking detectable Ig gene rearrangement, show progression to Fr. *B* (and Fr. *C*) phenotype during a limited (4-d) culture period, so this population is the only candidate we find for a committed (but unrearranged) B cell progenitor. In fact, in ongoing studies we have found that Fr. *A* shows progenitor activity, reconstituting B cells in irradiated SCID recipient mice using a relatively high dose of transferred cells (10^5 mouse). While this requirement for reconstitution is large compared with similar work using stem cell enriched fractions ("Thy-1^{low} Lineage$^-$" BM; reference 3), it is still 5–10 times more efficient, as determined by number of reconstituted peripheral B cell, than transfers of the more differentiated B lineage fraction, Fr. *C* (data not shown). However, the extremely variable expression of Ly-6A ("Sca-1"; references 52 and 53) in Fr. *A* and sporadic T cell reconstitution at higher doses (data not shown) argues for the presence of more than one type of restricted progenitor (or even of a less restricted cell type), which must be resolved by further work.

The ability to delineate stages of early B cell differentiation provides a powerful new approach for further study of important unresolved questions. Our study clearly delineated significant physiological distinction between pro-B cell and pre-B cell stages. It will now be possible to investigate the effects of Ig transgenes on B cell development, comparing the timing of expression in relation with Ig gene rearrangement and changes of physiological function. Similarly, the nature of B cell immunodeficiencies can be probed at these earlier stages of development to define the point at which the defect becomes manifest. Furthermore, the question of the divergence of B cell progenitors generating distinct B cell lineage can be pursued. Finally, our work provides a foundation for molecular studies of genes thought to be differentially regulated during B cell differentiation.

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