RELATIONSHIPS BETWEEN B CELL AND MYELOID DIFFERENTIATION

Studies with a B Lymphocyte Progenitor Line, HAFTL-1

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Studies of transformed cell lines (1–3) and normal bone marrow cells (4) have suggested that the earliest cells committed to the B lymphocyte differentiation pathway, termed pro-B (1) or pre-pre-B cells (4), have a Thy-1$^-$, Mac-1$^-$, 8C5$^-$, Ly-5(B220)$^-$, Lyb-2$^+$ phenotype (1–4) and are descendants of rare Thy-1$^+$ pluripotent stem cells (4, 5). Both the Thy-1$^+$ and Thy-1$^-$ populations of normal cells undergo limited expansion in culture and spontaneously differentiate into Ly-5(B220)$^+$ pre-B cells and surface Ig (sIg)[1]-positive B cells (4). Before the discovery of normal pro-B cells, the earliest well-characterized differentiation stage in the B lineage was the large pre-B cell (6). Normal and transformed cells at this stage express Ly-17, the Fc receptor (FcR; reference 7), and the B lineage-specific markers Lyb-2 and Ly-5(B220), but not ThB, sIg, or Ia, and undergo D to J_H rearrangements of at least one Ig heavy (IgH) chain gene (1–3, 6, 8–10). Normal large pre-B cells subsequently differentiate into functional B cells by a series of sequential steps involving productive VDJ_H rearrangement (6, 10, 11), production of cytoplasmic μ (cμ) chains (12, 13), expression of ThB (6, 8), productive rearrangement of Ig light (IgL) chain genes (10), and finally, expression of sIgM followed by surface Ia (14, 15). This series of events is presumed to occur in the development of both the Ly-1$^-$ and Ly-1$^+$ B cell lineages (1–3).

Although the stages of differentiation within the B cell lineage are increasingly well understood at both the phenotypic and molecular levels, little is known about the timing and mechanisms signaling irrevocable commitment to this lineage, or those controlling the orderly progression of cells from one stage of differentiation to the next. The study of regulatory genes in normal B cell differentiation is limited by difficulties in identifying, isolating, and propagating early precursors. As an alternative approach to these questions, we have chosen to study spontaneously or virally transformed cell lines. Our initial goal was to identify lines with IgH genes in the germline configuration that expressed at least one B lineage-specific marker and were able to differentiate in vitro, either spontaneously or after stimulation with LPS and/or lymphokines.

Over 30 transformed cell lines were examined for organization of IgH and IgL

[1] *Abbreviations used in this paper:* CAS, supernatant from Con A-stimulated rat spleen cells; cμ, cytoplasmic μ chain; FMF, flow microfluorometry; sIg, surface Ig; Sμ, sterile IgM.

genes and B lineage-specific surface antigens. One of these lines, HAFTL-1, a fetal liver cell line transformed by v-Ha-*ras*, had the hallmarks of a progenitor for B cells of the Ly-1$^+$ lineage. HAFTL-1 cells were uniformly Ly-1$^+$, Lyb-2$^+$, Lyb-8$^+$, Ly-17(FcR)$^+$, Ly-5$^+$, ThB$^-$, Mac-1$^-$, Ia$^-$, 6C3$^-$, and expressed low levels of Ly-5(B220) (3). In addition, the cells produced terminal deoxynucleotidyl transferase, were cμ^-, and were heterogeneous with respect to IgH gene rearrangements; some cells had unrearranged IgH genes (16) and others had one or more DJ_H rearrangements (3, 16). In the present study, we show that HAFTL-1 cells stimulated with LPS differentiate along either the lymphoid pathway into pre-B-like cells, or along the myeloid pathway into functional macrophages. The results of this study and a previous report (3) lead us to predict the existence of a normal, bipotential progenitor cell capable of giving rise to both Ly-1$^+$ B cells and Ly-1$^+$ macrophages.

Materials and Methods

Cell Lines. The HAFTL-1 cell line was derived by in vitro transformation of NFS/N fetal liver cells with Harvey murine sarcoma virus as previously described (17). The progenitor cell line and its derivatives were maintained in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) containing 10% heat-inactivated fetal calf serum FCS, 2 mM glutamine, 50 U/ml penicillin and streptomycin, 16 mM Na_2HCO_3, 25 mM Hepes, and 5×10^{-5} M 2-ME. For some cultures, *Escherichia coli* 0111 LPS (Difco Laboratories Inc., Detroit, MI) was added at a final concentration of 20 μg/ml.

Cell Sorting. Surface Ia$^+$ cells from HAFTL-1 progenitor cells and Mac-1$^+$ cells from macrophage-containing cultures were sorted and single cell-cloned in 96-well round-bottomed microtiter plates using an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL).

Flow Microfluorometry (FMF) Analyses. FMF analyses were performed on a FACS II or FACS 440 (Becton Dickinson & Co., Sunnyvale, CA) as described previously (3). Viable cells were electronically gated by exclusion of propidium iodide and narrow forward-angle light scatter. Cells were stained with a panel of FITC-conjugated or unlabeled mAbs specific for the following cell surface antigens: Ly-1, Lyb-2.1, Lyb-8.2, Ly-17(FcR), Ia, ThB, Ly-5, Ly-5(B220), Mac-1, Mac-2, Mac-3, and IgM. Bound unlabeled antibodies to Lyb-8.2 were stained with FITC-labeled goat anti-mouse IgG as a second-stage reagent, and binding of unlabeled anti-Mac-2 or -Mac-3 was detected with FITC-labeled rabbit anti-rat Ig. Surface Ig-bearing cells were detected with FITC-labeled goat anti-mouse κ antibodies. The clone designations, nominal specificities, and origin of these reagents have been detailed previously (3).

Southern Blots. High molecular weight DNA was prepared, digested with restriction enzymes, separated on 0.8% agarose gels, and transferred to nitrocellulose using established techniques. Southern blots were hybridized with ^{32}P-labeled nick-translated DNA probes at 65°C in a buffer containing 3× SSC, 10% dextran sulfate, 1× Denhardt's solution, 0.1% SDS, 5 mM EDTA, and 50 μg/ml salmon sperm DNA. Blots were washed successively in 3× SSC at 37°C for 20 min, 1× SSC at 65°C for 10 min, and 0.2× SSC at 65°C for 30 min, and were analyzed by autoradiography. Three probes were used: J_{H1}, a 1.96-kb Bam HI/Eco RI fragment of the BALB/c germline J_H region (18); J_K, a 2.8-kb Hind III/Hind III fragment from clone pEC_K containing all J_K sequences (19); and clone BS-9, a v-Ha-*ras* fragment (20). Rearranged J_H-containing fragments that hybridize at lower intensities than a single rearranged J_H fragment represented in all cells are referred to as submolar fragments in the text. The appearance of submolar fragments in the HAFTL-1 foundation line indicates the emergence of subsets of cells within the population that have undergone recent D_H to J_H rearrangement.

Northern Blots. RNA was extracted from cells by the guanidinium hydrochloride method (21, 22). Briefly, cells were homogenized with a polytron in 4 M guanidinium thiocyanate containing 0.5% sodium *N*-laurylsarcosine, 25 mM trisodium EDTA, and 0.1 M 2-ME. The homogenate was layered onto 5.7 M CsCl and spun at 30,000 rpm for 24 h. The RNA pellet was dissolved, extracted twice with phenol/chloroform, and precipitated. Poly(A)$^+$ RNA was

prepared by passing the RNA over an oligo-dT-cellulose column and eluting with low salt buffer. Aliquots of poly(A)$^+$ RNA (5 μg) were adjusted to 50% formamide, 20 mM MOPS, 5 mM Na acetate, 1 mM EDTA (1× MOPS buffer), and 2.2 M formaldehyde, heated at 65°C for 10 min, and loaded onto a 1.4% agarose, 2.2 M formaldehyde gel. Electrophoresis was performed at 80–100 mA for 5–6 h in 1× MOPS buffer. Gels were blotted onto nitrocellulose paper, and the blots were baked and prehybridized in a buffer containing 50% formamide, 5× SSC, 10% dextran sulfate, and 50 μg/ml denatured salmon sperm DNA. Blots were hybridized at 45°C with DNA probes labeled with ^{32}P by nick translation and then were washed in 5× SSC at 45°C. All Northern blots were hybridized with a ^{32}P-labeled GAPDH probe (23) to ensure that equivalent amounts of RNA were loaded on the gels. Other probes used included Ly-5-68, a 4.7-kb Bam HI fragment containing the complete sequence of Ly-5 (24); pMu 3741 for IgM (25); and MD-10, a 2.1-kb cDNA fragment coding for Ly-1 (26).

Stimulation of Cell Lines with LPS and Lymphokines. HAFTL-1 progenitor cells were grown for varying lengths of time in medium containing 20 μg/ml LPS alone or in combination with 10% supernatant from rat spleen cells cultured with Con A (CAS). To determine the effects of IL-4 on surface Ia expression, HAFTL-1 progenitor cells and their derivatives were cultured for 72 h or longer with 10 U/ml rIL-4 (a gift from Dr. R. Coffman, DNAX Research Institute, Palo Alto, CA) and then examined by FMF for staining with FITC-labeled monoclonal anti-Ia.

Assays for Cytoplasmic IgM. Cytoplasmic IgM (cμ) was detected by competition RIA as described previously (3).

Assays for Macrophage Function. Lysozyme production was assayed by the lysoplate assay using egg white lysozyme as a standard (27). Levels of lysozyme were expressed in micrograms per 10^6 cells per 24 h. The presence of nonspecific esterase or myeloperoxidase was determined by cytochemical staining using Sigma Chemical Co. (St. Louis, MO) research kits. Cells were tested for nonspecific phagocytosis by incubating 10^5 cells in a 0.1% suspension of 1-μm latex beads (15711; Polysciences, Inc., Warrington, PA) in 24-well plates for 3 h at 37°C. The cells were then harvested and washed, and aliquots were removed for cytocentrifugation. Slides were stained with Wright's Giemsa, and the cells were scored for bead ingestion at × 100 magnification.

Results

Derivation and Characteristics of HAFTL-1 Lines.

In a previous study, the twice-cloned HAFTL-1 foundation line was examined for evidence of Ig gene rearrangements and shown to have a single predominant rearranged 4.8-kb J_H-containing fragment and a less than equimolar (submolar) J_H-containing fragment in the germline configuration (3). Studies by Alessandrini et al. (16) of individual clones derived from the foundation line revealed that some of the cells had both IgH alleles in the germline configuration and that these clones subsequently underwent spontaneous D to J_H joining in vitro. The original HAFTL-1 population therefore appeared to comprise a minor population of cells that had not yet undergone rearrangement of the IgH loci and a major population that had undergone D to J_H rearrangement of one IgH allele with the possible exclusion of J_H-containing sequences on the other allele.

For the studies detailed below, we compared the foundation HAFTL-1 population and one of the clones (No. 14) derived by Alessandrini et al. (16). These two populations were designated lines A and B, respectively. In initial studies, the lines were evaluated for cell surface phenotype, somatically-acquired v-*ras* integration sites, and IgH and IgL gene rearrangements. Both lines were morphologically and phenotypically indistinguishable. The cells were uniformly Ly-1$^+$, Ly-17(FcR)$^+$ and expressed the B cell lineage-specific markers Ly-5(B220), Lyb-2, and Lyb-8, but were Ia$^-$,

ThB$^-$, sIg$^-$, Mac-1$^-$, and cμ^-, (3). Both lines also had identical patterns of v-*ras* integration sites, and thus arose from the same transformed target cell (data not shown). Southern blots of genomic DNA from lines A and B, digested with Eco RI and probed with a J_H-specific probe, are shown in Fig. 1.

As reported previously (3), the foundation line A had a single 4.8-kb rearranged J_H-containing fragment and a less than equimolar germline fragment (Fig. 1 *A*, lane *2*; Fig. 1 *B*, lanes *2* and *3*). After long-term exposure to film, additional faint bands were also detected, suggesting that further D_H to J_H rearrangement of germline DNA was possible (Fig. 1 *C*, lane *1*). Further evidence for spontaneous rearrangement of the IgH locus was obtained with DNA extracted from line A cells maintained in culture for 20 passages. As shown in Fig. 1 *C*, lane *2*, almost all of the germline fragment disappeared and a number of new fragments not observed in the foundation line were readily apparent.

Similar observations were also made with the line B cells. This line originally had both IgH loci in the germline configuration (16) but in our laboratory underwent rapid spontaneous rearrangement during the culture interval required to expand the line for DNA extraction (Fig. 1 *A*, lane *3*). Maintenance of line B cells for 20 passages before DNA isolation also resulted in the loss of the germline band and the appearance of new rearranged fragments (data not shown). For both lines A and B, no rearrangements of the IgL loci were detected (data not shown). The results obtained with the line A and B cells are consistent with the view that HAFTL-1 cells containing IgH loci in the germline configuration are able to undergo spontaneous rearrangement in vitro. However, the data do not exclude the alternative possibility that germline cells are lost from the cultures over a period of time, while previously undetected minor populations of cells with new rearrangements are expanded.

On the basis of the foregoing phenotypic and molecular criteria, we classified the HAFTL-1 cell line as the transformed equivalent of an early B lineage progenitor or pro-B cell.

LPS-and IL-4-induced Differentiation of Line A and B HAFTL-1 Cells.

To determine whether the HAFTL-1 progenitor B cell lines could differentiate spontaneously or after stimulation with LPS and lymphokines, the cells were carried either long term in culture medium alone or were stimulated for various time intervals with LPS or LPS in combination with lymphokine-containing CAS. The cells were monitored by FACS analysis for surface Ig (using both anti-IgM and anti-κ antibodies), Ia, ThB, Ly-5(B220), and Mac-1 expression and by competition RIA for cytoplasmic μ. After a year of continuous culture in medium alone, neither cell line changed significantly in growth characteristics, morphology, or phenotype, although, in some experiments, a small subpopulation of Ia$^+$ cells (<10%) was observed in both lines. However, both cell lines underwent apparent spontaneous rearrangement of the IgH chain loci as demonstrated for line A in Fig. 1 *C*, lane *2*. No rearrangements of the IgL κ loci were detected (data not shown).

By contrast, the cell surface phenotype of lines A and B treated for 1 wk or longer with LPS changed significantly, with *de novo* expression of both Ia and ThB. The fluorescence profiles for untreated HAFTL-1 line A and lines A and B treated for 8 wk with LPS and stained with mAbs specific for Ia, ThB, and Ly-5(B220) are

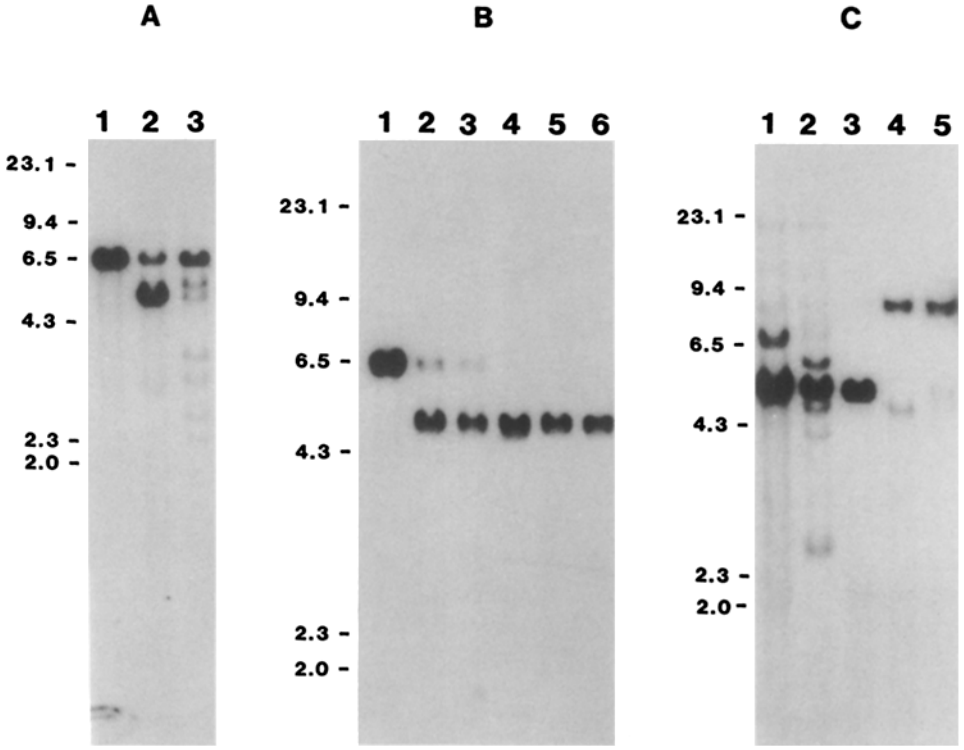

FIGURE 1. Rearrangements of the IgH chain gene locus in HAFTL-1 progenitor cells and derivatives. (*A, B,* and *C*) Southern blots of DNA digested with Eco RI and hybridized with a ^{32}P-labeled 1.96-kb J_H fragment containing J_3 and J_4 (J_{11}). Each panel was exposed to film long term to reveal submolar bands. (*A*) Lane *1*, NFS liver showing the 6.5-kb germline band; lane *2*, HAFTL-1 foundation line A; lane *3*, HAFTL-1 line B. (*B*) Lane *1*, NFS liver; lanes *2* and *3*, DNA prepared from stocks of HAFTL-1 cells frozen soon after the initial derivation of the cloned line; lanes *4, 5,* and *6*, HAFTL-1 macrophage clones 3G4, 2B7, and 1G4 isolated from LPS-stimulated line A. (*C*) Lane *1*, HAFTL-1 foundation line A; lane *2*, line A cells maintained in culture for 20 passages; lanes *3, 4,* and *5*, HAFTL-1 pre-B cell–like clones 6, 7, and 14 isolated from LPS-stimulated line A.

shown in Fig. 2. Treatment with LPS resulted in Ia expression on 55% of the line A and 90% of the line B and ThB expression on 7% and 76% respectively, of the line A and B cells (Fig. 2, *A* and *B*). Expression of ThB required the continuous presence of LPS, whereas surface Ia expression was not affected by withdrawal of LPS from the cultures (data not shown). For both cell lines, the addition of CAS to the LPS-stimulated cells did not significantly alter the proportions of Ia^+ or ThB^+ cells (data not shown). Stimulation with LPS also caused a significant increase in the level of Ly-5(B220) expression, as indicated by the increased fluorescence intensities of the treated line A and B cells compared with the untreated line A control (Fig. 2 *C*).

Induction of surface Ia expression on a significant proportion of cells was also observed for both A and B lines after treatment with rIL-4. After 3 d of treatment with rIL-4, the proportion of Ia^+ cells rose from 3 to 16% for line A (not shown)

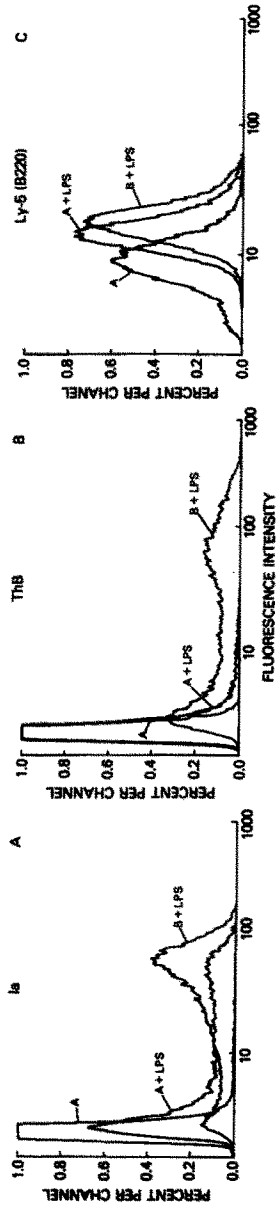

FIGURE 2. Effects of LPS treatment on cell surface phenotype. FMF profiles of untreated HAFTL-1 line A cells and LPS treated line A and line B cells stained with F1-labeled antibodies to Ia (*A*), ThB (*B*), and Ly-5(B220) (*C*).

and 2 to 46% for line B (Fig. 3 *A*). Extension of the culture interval to 11 d resulted in Ia expression by 46% of line A (not shown) and 89% of line B cells (Fig. 3 *B*). Unlike LPS, rIL-4 treatment did not alter the level of Ly-5(B220) expression and did not induce expression of antigens other than Ia. Finally, no cells expressing cytoplasmic IgM, Mac-1, surface μ or κ chains were detected in cell populations stimulated short or long term with any combinations of LPS, CAS, or rIL-4. Both the A and B lines, therefore, were capable of expressing high levels of the B lineage-specific marker Ly-5(B220), as well as antigens, such as ThB and Ia, that

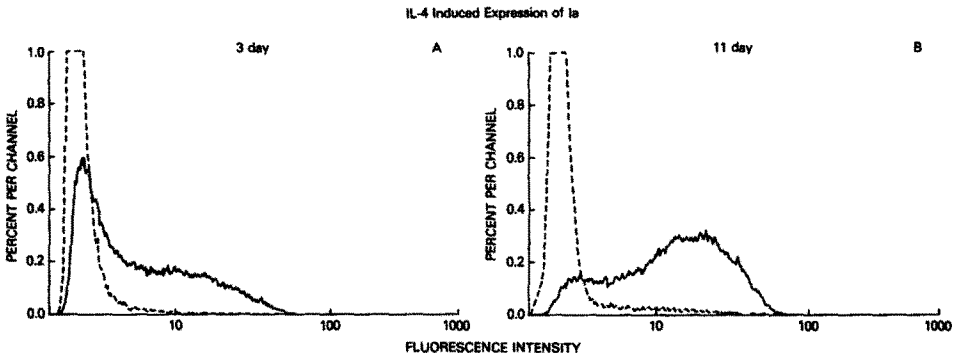

FIGURE 3. Induction of Ia expression with rIL-4. FMF analyses of untreated HAFTL-1 line B cells (*dashed lines*) and HAFTL-1 line B cells treated rIL-4 (*solid lines*) for 3 d (*A*) or 11 d (*B*) stained with Fl-labeled anti-Ia mAb.

are normally expressed by B cells or their immediate precursors, but cells that expressed cytoplasmic or surface IgM, and therefore had undergone productive IgH and IgL chain gene rearrangements, were not detected in the bulk cultures.

Single Cell Cloning of Ia$^+$ HAFTL-1 Cells.

Since surface Ia is thought to be expressed shortly after sIg in normal B cell differentiation (14, 15), the pool of Ia$^+$ cells in the LPS-stimulated progenitor HAFTL-1 cultures possibly contained a small subpopulation of sIg$^+$ cells that escaped detection by FACS analyses of the bulk cultures. To explore this possibility, LPS-stimulated HAFTL-1 line A cells expressing high levels of Ia were single cell–cloned using the cell sorter, and the expanded LPS-stimulated clones were examined for cell surface antigen expression. Of 64 clones analyzed, none expressed sIgM. 15 clones with high proportions of Ia$^+$ cells were chosen for further study. Eight of these clones had the same lymphoid morphology as the HAFTL-1 progenitor cells and did not express the putative myeloid marker Mac-1. Three of the eight clones, 6, 7, and 14, also had a subpopulation (16–28%) of ThB$^+$ cells, and were classified as pre-B cell clones. The remaining seven clones selected were, unexpectedly, heterogeneous both morphologically and phenotypically. Three cell types, namely lymphoblastic cells, larger refractile cells with granular cytoplasm, and occasional adherent, macrophage-like cells were observed in variable proportions. Phenotypically, the mixed clones were unlike any HAFTL-1 populations examined previously, and contained different proportions of Ia$^+$ and Mac-1$^+$ cells. A representative heterogeneous clone (No. 5) containing 38% Ia$^+$ cells and 18% Mac-1$^+$ cells was used for the isolation of the Mac-1$^+$ subpopulation.

Stable Mac-1$^+$ lines were established from clone 5 by two additional rounds of single cell cloning. The cells were first cloned in soft agar, and individual diffuse myeloid colonies were harvested, expanded, and analyzed for Mac-1 expression. Cultures containing a high proportion of Mac-1$^+$ cells were then recloned using the cell sorter. Three representative Mac-1$^+$ lines (3G4, 1G4, and 2B7) were chosen for further analysis. As shown below, these cells had clearly differentiated along the monocyte/macrophage lineage.

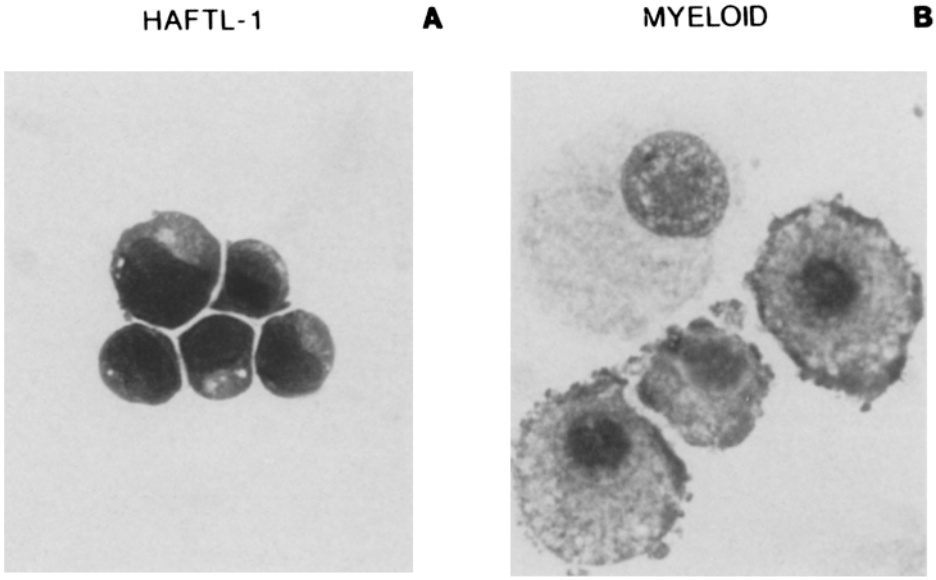

FIGURE 4. Morphology of HAFTL-1 progenitor and myeloid lines. Cytocentrifuge preparations of HAFTL-1 progenitor cells (*A*) and HAFTL-1-derived macrophage clone 3G4 (*B*) stained with Wrights-Giemsa. × 1,000

Comparisons of HAFTL-1 Progenitor Cells, LPS-induced Ia$^+$ Pre-B Cells, and Mac-1$^+$ Monocyte/Macrophage Clones

Morphology, Growth Characteristics, and Tumorigenicity. The HAFTL-1 progenitor cells and Ia$^+$, ThB$^{+/-}$ pre-B cell clones differed significantly from the Mac-1$^+$ HAFTL-1 clones in both growth characteristics and morphology. The progenitor and pre-B cells were nonadherent, 2-ME dependent, and had a doubling time of 12–15 h. Both populations had a distinctive lymphoblastic morphology and a variable proportion had discrete clear cytoplasmic vacuoles (Fig. 4 *A*). By comparison, the Mac-1$^+$ clones grew independently of 2-ME as adherent and nonadherent cells with a doubling time of several days. Microscopic examination revealed a mixture of large macrophage-like cells with eccentric nuclei and vacuolated cytoplasm and smaller, less mature monocytic cells. The cultures were enriched for macrophages by passaging only adherent cells. An example of typical adherent 3G4 macrophages is shown in Fig. 4 *B*.

Subcutaneous injection of 10^7 HAFTL-1 progenitor or 3G4 cells into neonatal NFS mice resulted in the development, within 1 wk, of large solid tumors that metastasized to the spleen and lymph nodes. The HAFTL-1 progenitor line gave rise to tumors composed of lymphoblastic cells that morphologically resembled the injected cells, while the tumors arising from the 3G4 line were composed almost exclusively of cells with the morphology of mature macrophages. Both the absence of monocytic cells in the 3G4 tumors and the large size of the tumors suggested that factors were present in vivo that allowed the 3G4 cells to undergo rapid growth and further differentiation.

Phenotype. The cell surface phenotypes of the pre-B cell clone, 14, and the monocyte/macrophage clone, 3G4, are compared in Figs. 5 and 6. Clone 14, as well as

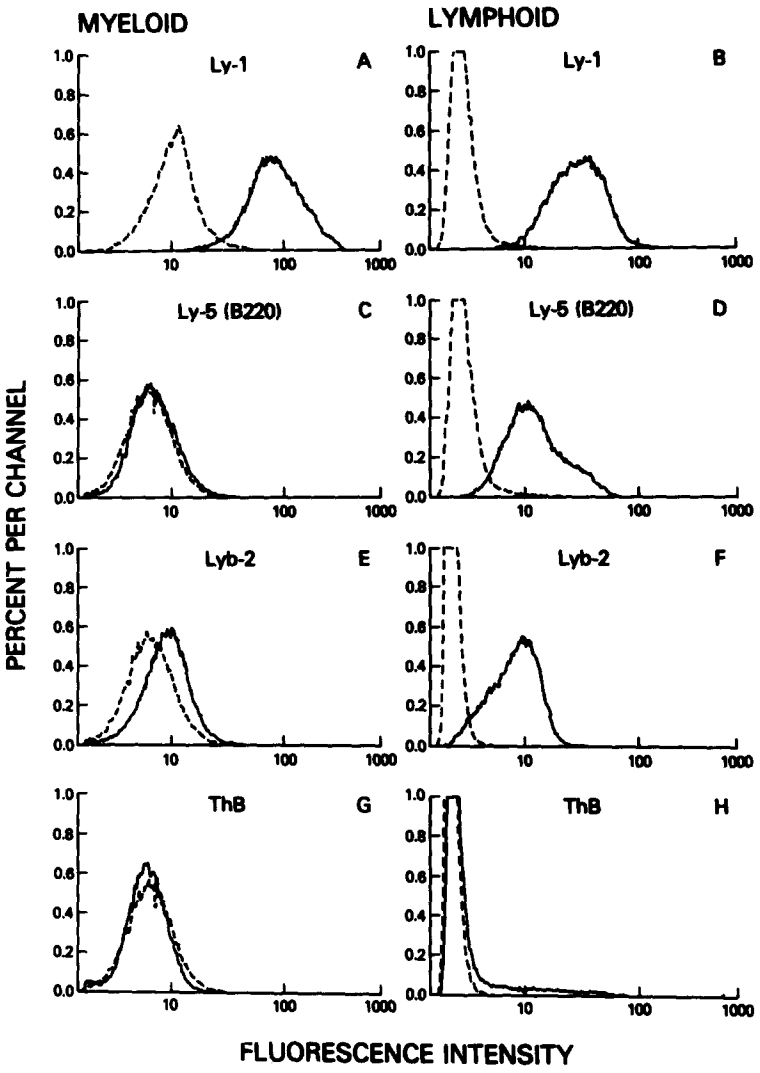

FIGURE 5. Cell surface phenotype of myeloid and lymphoid clones derived from LPS-treated HAFTL-1 line A. FMF profiles of the myeloid clone 3G4 (*A, C, E, G*) and pre-B cell-like clone 14 (*B, D, F, H*) stained with Fl-labeled anti-rat Ig (*dashed lines*) or FITC-labeled antibodies to Ly-1, Ly-5(B220), Lyb-2, and ThB (*solid lines*).

clones 6 and 7, was uniformly Ly-1$^+$, Ly-5(B220)$^+$, Lyb-2$^+$, and Ia$^+$, had a subpopulation (28%) of ThB$^+$ cells, but did not express Mac-1 or the mature macrophage marker, Mac-2. By comparison, clones 3G4, 1G4, and 2B7 did not express the B cell markers Ly-5(B220) or ThB, expressed very low levels of Lyb-2 and Ia, but expressed both Mac-1 and Mac-2. Importantly, the macrophage lines, like the progenitor and pre-B cell lines, expressed high levels of Ly-1 (Fig. 5 *A*). In addition, both the pre-B and macrophage lines were Ly-17(FcR)$^+$, Thy-1$^-$, κ^-, and Mac-3$^-$ (data not shown).

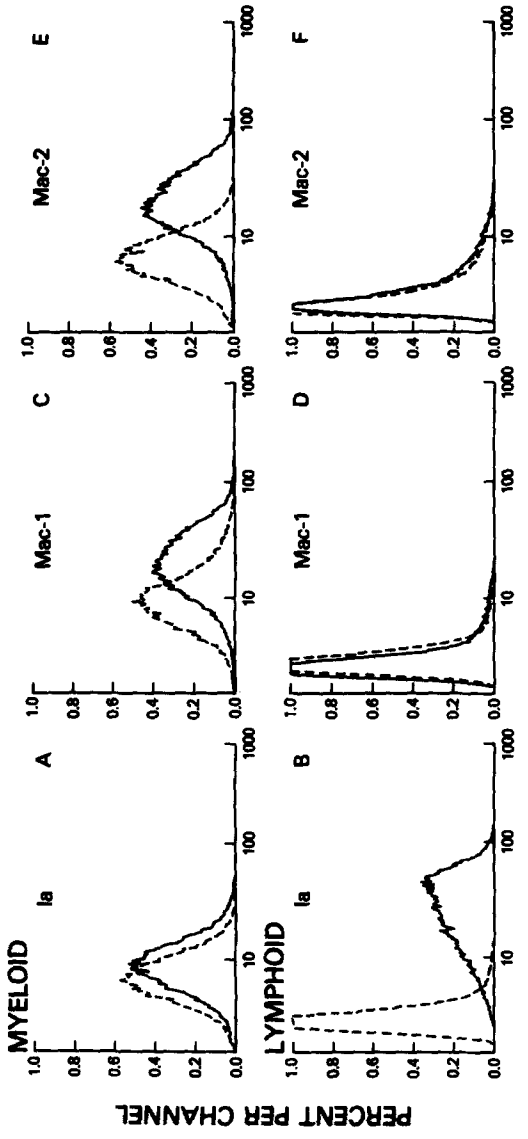

FIGURE 6. Cell surface phenotype of HAFTL-1-derived myeloid and lymphoid phoid clone 14 cells (*B, D, F*) stained with F1-labeled anti-rat Ig (*dashed lines*) lines. FMF profiles of the myeloid clone 3G4 (*A, C, E*) and pre-B cell-like lym- or FITC-labeled antibodies to Ia, Mac-1, and Mac-2 (*solid lines*).

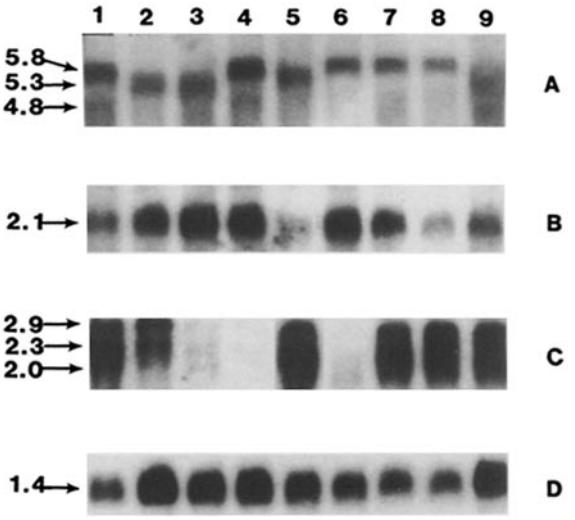

FIGURE 7. Northern blots of poly(A)$^+$ mRNA hybridized with ^{32}P-labeled probes specific for Ly-5 (*A*), Ly-1 (*B*), cμ (*C*), and GAPDH (*D*). (*A, B,* and *D*) Lanes 1–9 represent RNA extracted from the HAFTL-1 foundation line A (lane *1*); the HAFTL-1-derived macrophage lines 3G4 and 1G4 (lanes *2* and *3*); HAFTL-3 (lane *4*); the HAFTL-3-derived macrophage line HAFTL-3A (lane *5*); the HAFTL-1-derived pre-B cell lines 6, 7, and 14 (lanes *6–8*); and EL4 thymoma cells (lane *9*). (*C*) Lanes *1–9* represent HAFTL-1 (lane *1*); the heterogeneous clone 5 from which the HAFTL-1 macrophages were isolated (lane *2*); 3G4 (lane *3*); 1G4 (lane *4*); HAFTL-3 (lane *5*); HAFTL-3A (lane *6*); and clones 6, 7, and 14 (lanes *7–9*).

Further studies of Ly-5 expression on the macrophage lines showed that, while the cells did not express the 220-kD B cell form of Ly-5 recognized by the mAb 6B2, they expressed high levels of the core Ly-5 protein detected by the mAb F-11 (data not shown). Examination of mRNA (see below) revealed that the transcript for the Ly-5 gene was smaller in the macrophage lines than in the progenitor cell or pre-B cell lines, providing further evidence that the macrophage lines expressed a different form of Ly-5 antigen to the lymphoid cells (Fig. 7 *A*).

The macrophage lines were also treated for 3 or 14 d with rIL-4 to determine whether the levels of Ia antigen expressed on the cells could be enhanced. No significant changes in Ia expression were observed at either time point (data not shown).

v-Ha-ras Integration Sites and Ig Gene Rearrangements. To demonstrate that the pre-B cell and macrophage cell lines were descendants of v-Ha-ras–transformed HAFTL-1 cells and did not arise from a contaminating cell population, DNA from the clones was examined for v-*ras* integration sites. Fig. 8 shows that all of the lines had identical integration sites to the progenitor cells and, therefore, clearly were derived from them. Further evidence for the clonal relatedness of the various lines was obtained in studies of IgH gene rearrangements. The myeloid clones (Fig. 1 *B*, lanes *4, 5* and *6*) and the pre-B cell clone 6 (Fig. 1 *C*, lane *3*) had a single J_H-containing fragment of similar size (4.8 kb) to the predominant rearranged fragment of the progenitor cells, with apparent loss of J_H-containing sequences from the opposite chromosome. Further restriction enzyme analyses of the predominant DJ rearrangement indicated that rearrangement occurred close to the J_H3 locus (data not shown). Clones 7 and 14 had unique, submolar J_H-containing fragments and several weakly hybridizing bands, suggesting additional rearrangements in these lines (Fig. 1 *C*, lanes *4* and *5*). None of the myeloid or pre-B cell clones underwent further J_H rearrangements during long-term culture, and no rearrangements of Ig κ or λ light chain genes were observed in any of the cell lines (data not shown).

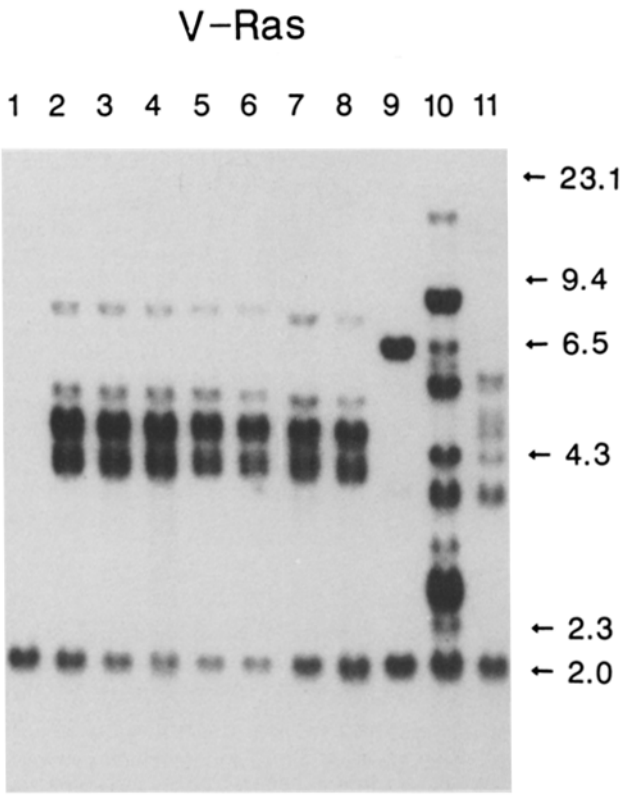

FIGURE 8. Clonality of HAFTL-1 progenitor, myeloid, and pre-B cells. Southern blot of Pst I-digested DNA extracted from NFS liver (lane *1*); HAFTL-1 foundation line (lane *2*); the HAFTL-1-derived myeloid clones 3G4, 1G4, and 2B7 (lanes *3–5*); the HAFTL-1-derived pre-B cell clones 6, 7, and 14 (lanes *6–8*); and three unrelated v-Ha-*ras*-transformed cell lines HS1C5 (lane *9*), HRC3 (lane *10*), and HAFTL-2 (lane *11*) (3). Blots were hybridized with a ^{32}P-labeled v-*ras* probe.

RNA Analyses. Sterile IgM (Sμ) mRNA transcripts of various sizes have been reported in pre-B cell lines and some macrophage lines (28–30). To determine whether Sμ transcripts were produced in early B cell progenitors and their more differentiated progeny, Northern blots of poly(A)$^+$ RNA extracted from the panel of HAFTL-1 clones, HAFTL-3, and HAFTL-3A were hybridized with a cμ probe. HAFTL-3 is a v-Ha-*ras*-transformed pre-B cell line that differentiates spontaneously into monocytes and macrophages, and HAFTL-3A is a mature macrophage line derived from HAFTL-3 (3). As shown in Fig. 7 *C*, the HAFTL-1 progenitor cells, the pre-B cell clones 6, 7, and 14 (lanes *7, 8,* and *9*), and HAFTL-3 (lane *5*) produced high levels of three mRNA species ~2.9, 2.3, and 2.0 kb in length that corresponded in size to Sμ transcripts (28, 29). By comparison, lower levels of these transcripts were present in the mixed lymphoid/myeloid clone 5 (lane *2*), and the transcripts were barely detectable or completely absent in the macrophage clones 3G4 and 1G4 and HAFTL-3A (Fig. 7 *C*, lanes *3, 4,* and *6*, respectively). These observations implied that, in differentiating from precursors into mature macrophages, HAFTL-1 and HAFTL-3 cells lost their ability to spontaneously transcribe the Ig μ constant region gene and, in this respect, were different from the myeloid lines WEHI-265.1 and RAW8.2 shown by Kemp et al. (29) to contain Sμ transcripts of 3.0, 2.3, 2.1, and 1.9 kb.

In a previous section it was shown that HAFTL-1 and clones 6, 7, and 14 ex-

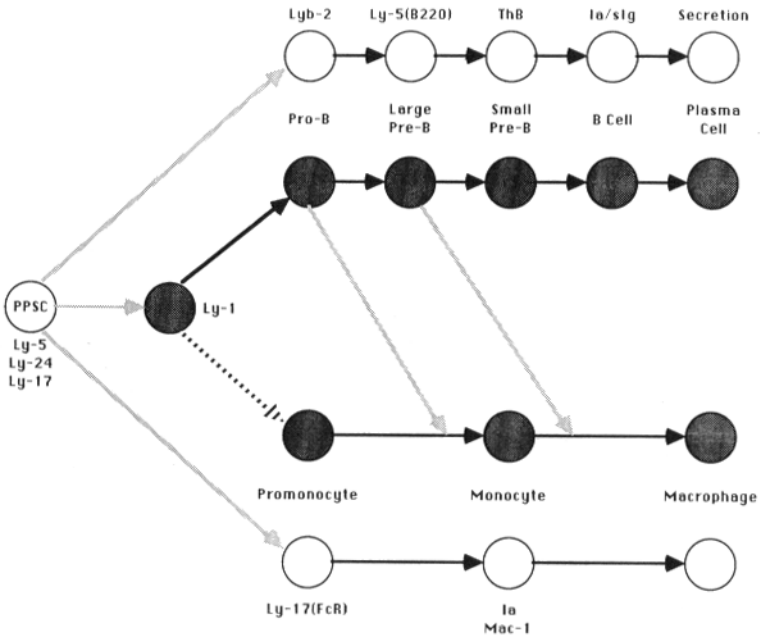

FIGURE 9. A model for the development of Ly-1$^+$ B cells and a proposed subset of Ly-1$^+$ macrophages. Ly-1$^+$ B cells and macrophages depicted by the shaded circles are drawn as related lineages arising from a common bipotential progenitor cell. By comparison, the major Ly-1$^-$ B cell and macrophage lineages (*open circles*) arise independently from separate lineage-restricted progenitor cells. Solid lines represent established pathways, shaded lines represent proposed pathways for which there is some experimental evidence, and the broken line represents a hypothetical pathway. Also shown are the stages during normal development at which different cell surface antigens are first displayed.

pressed the B lineage isoform of Ly-5 detected by the monoclonal 6B2 (6), whereas the macrophage lines expressed an alternative isoform. Similar differences were also observed between the lymphoid and myeloid HAFTL-3 cells (Davidson, W. F., unpublished observation). To determine whether the lymphoid and macrophage Ly-5 isoforms arose from the same or different sized mRNA species, Northern blots were hybridized with the Ly-5-specific probe pLy-5-68 (24). HAFTL-1, HAFTL-3, and HAFTL-1 clones 6, 7, and 14 had abundant 5.8-kb transcripts and a minor 4.8-kb species, whereas the macrophage lines had a single 5.3-kb mRNA (Fig. 7 *A*). The 5.8- and 5.3-kb Ly-5 mRNA forms presumably are translated into the 220-kD B lymphocyte-specific (6, 31) and 204-kD macrophage-specific (31) Ly-5 isoforms, respectively. Significant levels of a 2.1-kb Ly-1 transcript were observed in poly(A)$^+$ RNA samples from the progenitor, macrophage, and pre-B cell HAFTL-1 lines, HAFTL-3, and HAFTL-3A (Fig. 7 *B*). These data provided further confirmation for the expression of Ly-1 on HAFTL-1 and HAFTL-3 macrophage lines. No mRNA transcripts for IL-1 or IL-3 were detected in any of the lymphoid or myeloid HAFTL-1 lines (data not shown).

Functional Assays. To determine whether the macrophage-like HAFTL-1 clones exhibited functions associated with normal monocytes and macrophages, the panel was examined for lysozyme and esterase production and phagocytic ability. As shown

TABLE I

Functional Characteristics of HAFTL-1 Progenitor, Myeloid, and Pre-B Cell Lines

Line	Lysozyme*	NSE‡	Phagocytosis§
HAFTL-1 progenitor	<0.5	-	-
Myeloid clone 3G4	3.9 ± 0.7	+ + +	+ + +
1G4	2.7 ± 0.8	+ + +	+ + +
2B7	3.6 ± 0.4	+ + +	+ + +
Pre-B clone 6	<0.2	-	-
7	<0.2	-	-
14	<0.2	-	-

* Micrograms of lysozyme (± SE) produced by 10^6 cells in 24 h.

‡ Relative amounts of nonspecific esterase compared with positive (HAFTL-3A [3]) and negative (HAFTL-1) controls.

§ Phagocytosis of latex beads. Greater than 95% of the cells contained at least 10 beads/cell and the majority contained at least 50 beads/cell. No beads were detected inside the HAFTL-1 cells or pre-B cell clones.

in Table I, the three myelomonocytic clones spontaneously produced high levels of nonspecific esterase and lysozyme, and >95% of the cells phagocytosed large numbers of latex beads. These functions were not evident in the HAFTL-1 progenitor cells or the Ia^+ lymphoid clones 6, 7, and 14. None of the clones produced detectable amounts of myeloperoxidase.

Discussion

The data presented here demonstrate that the cloned and expanded HAFTL-1 foundation line (line A) is a dynamic cell population that comprises at least two clonally related subsets. One population, exemplified by line B cells, had both IgH loci in the germline configuration and underwent spontaneous D to J_H joining during cultivation (reference 16, Fig. 1). A second, predominant population had a single D to J_H rearrangement and, in some cases, had lost J_H-containing sequences from the second allele (reference 3, Fig. 1). Both line A and line B cells were Ly-1^+, Ly-5(B220)$^+$, Lyb-2^+, and Lyb-8^+. On the basis of this combination of properties, HAFTL-1 was classified as a progenitor of Ly-1^+ B lymphocytes and, as such, antedated in ontogeny other previously described B lineage precursor lines, including the Abelson virus-transformed lines in which D to J_H joining occurred on both alleles before isolation (32, 33). During prolonged cultivation, neither line A nor line B changed significantly in cell surface phenotype or morphology, or underwent productive rearrangements of the IgH or IgL loci. By contrast, stimulation of either line with LPS resulted in significant changes in cell surface phenotype, with increased levels of Ly-5(B220) expression and *de novo* Ia and ThB expression. LPS-stimulated cells maintained their lymphoid morphology and continued to express Ly-1, Lyb-2, and Lyb-8. Ia expression also was induced on both cell lines after stimulation for 72 h or more with rIL-4. Single cell cloning of LPS-stimulated parental cells expressing high levels of surface Ia gave rise to two distinct types of clones, Ia^+ lymphoid clones and Ia^+ mixed lymphoid and myeloid clones from which stable Ly-1^+, Mac-1^+, Mac-2^+ clones were established that had morphologic and functional

properties of monocytes and macrophages. Both the parental line A and the macrophage lines were tumorigenic and gave rise to metastatic lesions in neonatal NFS/N mice.

The LPS-induced changes in surface antigen expression on HAFTL-1 cells deserve comment, since LPS reportedly does not induce Ia expression on cultured normal pre-B cells (15) or pre-B cell lines (34, 35). There are several possible explanations for our results. First, HAFTL-1 cells may be the transformed counterparts of a progenitor population that is rare or only transiently responsive to LPS and therefore difficult to detect in normal pre-B cell populations. A correlation between the level of maturity of the progenitor population and the ability to respond to LPS may also explain why the less mature HAFTL-1 line B had higher proportions of Ia^+ and ThB^+ cells than the parental line after LPS treatment. Alternatively, LPS responsiveness may be an exclusive property of *ras*-transformed progenitor cells. Interestingly, another *ras*-transformed cell line, HAFTL-3 (a pre-B cell line that spontaneously differentiates into Ia^+, $Mac\text{-}1^+$, $Ly\text{-}1^+$ monocytes and macrophages [3]) also developed a significantly increased subpopulation of Ia^+ cells and a small subpopulation of ThB^+ cells after stimulation with LPS (Davidson, W. F., unpublished data).

In normal B cell ontogeny, ThB is usually not detected on the surface until after cells produce cμ (6), and Ia is not expressed until after the cells acquire sIgM (14, 15). These results are consistent with the view that expression of IgM in the cytoplasm or on the cell surface may regulate a number of ordered steps in B cell differentiation. The observation that HAFTL-1 and HAFTL-3 cells stimulated with LPS or IL-4 can express ThB and/or Ia in the absence of cμ or sIgM suggests that there may be alternative mechanisms for regulating the expression of these surface molecules.

Reports by Polla et al. (35, 36) have shown that Ia^- pre-B cell lines, such as 70/Z, and the less mature Abelson virus–transformed line R8205, were induced to express surface Ia after stimulation with IL-4-containing EL-4 supernatant. More recently, IL-4-induced Ia expression was also demonstrated for Ia^- adherent peritoneal exudate cells (37). Our studies of the effects of rIL-4 on HAFTL-1 cells extended these findings by showing that Ia expression could be induced on apparent B cell progenitors with the capacity of differentiating into macrophages. Thus, IL-4 can interact with precursors as well as differentiated cells of both the B and myelomonocytic cell lineages.

Despite the lymphoid morphology and phenotype of LPS-stimulated HAFTL-1, single cell cloning of cells expressing high levels of surface Ia gave rise to approximately equal proportions of lymphoid clones and heterogeneous clones containing both lymphoid and $Mac\text{-}1^+$ monocytic cells. The $Mac\text{-}1^+$ monocytic cells were considered to be the differentiated progeny of individual Ia^+ lymphoid cells for the following reasons: $Mac\text{-}1^+$ cells were not detected in the Ia^+ population used for sorting; cloning was performed at limiting dilution; no pure monocytic clones were obtained; and the macrophage clones derived from the mixed clones had v-Ha-*ras* integration sites and IgH gene rearrangements identical to the mixed clones.

In differentiating from lymphoid-like precursors into monocytes and macrophages, HAFTL-1 cells ceased to express the B lineage–specific antigens Ly-5(B220) and Lyb-8, expressed significantly reduced levels of Lyb-2, and no longer made sterile μ transcripts. Similar results were also obtained for HAFTL-3 macrophages (Fig.

7 and data not shown). In lieu of Ly-5(B220), the differentiated monocytes and macrophages expressed an alternative form of Ly-5 on the cell surface encoded by a 5.3-kb macrophage-specific mRNA species and detected by the pan-Ly-5-reactive mAb F-11. Immunoprecipitation studies will reveal whether this Ly-5 isoform corresponds to the 204-kD protein precipitated from other macrophage lines (31). Our studies with HAFTL-1 and HAFTL-3 cells represent the first demonstration of an alteration in Ly-5 isoform expression in a differentiating cell population and offer a unique opportunity to study the regulation of expression of Ly-5 lineage-specific isoforms in clonally related cells.

The close developmental relationship between Ly-1$^+$ B cell precursors and Ly-1$^+$ macrophages observed for HAFTL-1 and HAFTL-3 is not an isolated instance and is not restricted to v-Ha-*ras*-transformed cells. The pre-B cell line BAMC1, established from neonatal bone marrow cells transformed with BALB/c sarcoma virus, like HAFTL-3, also spontaneously differentiated into Mac-1$^+$, Ia$^+$, Ly-1$^+$ macrophage-like cells (3). Another example of pre-B cells and macrophages that shared a common precursor was the methylcholanthrene-induced Ly-1$^+$ pre-B cell line, P388, and the related Ly-1$^-$ macrophage line, P388D1 (38). Finally, two pre-B cell lines, ABLS 8.1 and RS4:11, whose Ly-1 phenotype was not examined, have been described that could be induced chemically to differentiate along the myelomonocytic pathway. A small proportion of murine ABLS 8.1 pre-B cells differentiated into macrophages after treatment with 5-azacytidine (39) and a human pre-B cell line RS4:11 differentiated into monocytoid cells in the presence of PMA (40). These independent observations suggest a biologically relevant relationship between Ly-1$^+$ B cells and macrophages.

Two postulated pathways for generating Ly-1$^+$ macrophages are depicted by the shaded circles in the model outlined in Fig. 9. In one pathway, we predict that the unique Ly-1$^+$ B cell progenitor described by Hayakawa and colleagues (41) and its recent Ly-1$^+$ pre-B cell descendants may not be B lineage-restricted, but instead have the potential to differentiate into Ly-1$^+$ B cells or Ly-1$^+$ macrophages. Alternatively, Ly-1$^+$ pre-B cells derived from a Ly-1$^+$ B lineage-restricted progenitor may differentiate under some circumstances into myeloid-like cells. Interestingly, normal Ly-1$^+$ B cells do share some properties with myeloid cells. For example, compared with normal resting Ly-1$^-$ B cells, Ly-1$^+$ B cells are larger and have greater internal granularity (42), are weakly adherent to glass (42), and when isolated from the peritoneal cavity, express the putative myeloid marker Mac-1 (43).

In conclusion, HAFTL-1, a cell line with the hallmarks of an early B cell progenitor, can be induced by treatment with LPS to differentiate with high frequency into Ly-1$^+$, Ia$^+$, ThB$^{+/-}$ pre-B-like cells or, with low frequency, into Ly-1$^+$ monocytes and macrophages. The mature morphology, phenotype, and functional characteristics of the HAFTL-1 macrophages, as well as the presence of intermediate monocytic cells, suggest that well-coordinated, lineage-specific programs of differentiation are activated in the progenitor cells. Both HAFTL-1 and the previously described HAFTL-3 progenitor and macrophage lines, therefore, offer a unique opportunity to study genes that regulate myeloid differentiation in well-defined clonal systems. It remains to be determined whether myelomonocytic programs of differentiation also can be operative in normal Ly-1$^+$ B cell progenitors or precursors.

Summary

A cell line, HAFTL-1, derived by in vitro transformation of fetal liver cells with v-Ha-*ras*, was found to have molecular and phenotypic characteristics of pro-B cells recently committed to the Ly-1$^+$ B cell differentiation pathway. Stimulation of these cells with LPS resulted in their differentiation within either the B or myelomonocytic lineages. Thus, lines derived from LPS-stimulated HAFTL-1 cells were shown to be clonally related, as evidenced by common v-*ras* integrations, but to exhibit characteristics of pre-B cells (ThB expression, continuing DJ heavy chain rearrangements) or mature macrophages (expression of Mac-1 and Mac-2, lysozyme and nonspecific esterase production, phagocytosis) while maintaining their Ly-1$^+$ phenotype. These results suggest that events resulting in the irrevocable commitment to a single lineage occur late in differentiation, at least within the pathway yielding Ly-1$^+$ B cells and a proposed subpopulation of Ly-1$^+$ monocytes and macrophages. Final commitment to these lineages is carefully orchestrated, as evidenced by restricted expression of Ly-5 isoforms and production of IgH transcripts.

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