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# LONG-TERM CULTURES OF MURINE FETAL LIVER RETAIN VERY EARLY B LYMPHOID PHENOTYPE

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Studies of the early events in B lymphocyte differentiation are complicated by the numerous hematopoietic lineages present in the in vivo compartments where these events occur. The generation of the B lymphocyte from the multipotential hematopoietic stem cell is first observed in the murine fetal liver and subsequently in the bone marrow (1–4). However, the numbers of B cells and their progenitors are very low and they are difficult to isolate and study from these in vivo organs without the ability to select and expand these cells in vitro.

The first identifiable cells of the B lymphocyte lineage, pre-B cells containing cytoplasmic mu immunoglobulin heavy chains (cIgM),[1] appear in the fetal liver at day 10–12 of gestation and are the progenitors of surface immunoglobulin (sIg)-bearing B cells (reviewed in 5). The events preceding the appearance of the pre-B cell are poorly understood. The intermediate cell types and differentiation factors involved are unknown.

It is possible that the various transitions in the fetal liver resulting in B cell formation are distinct from those that occur in adult bone marrow (6, 7) and may only occur once in an individual's lifetime. Unfortunately, this developmental stage is brief and the number of cells available for study is small. An in vitro system that would allow the lymphoid elements in the fetal liver to grow and develop in culture while maintaining traits characteristic of cells from that stage of development would be of great value in the study of the events of early B cell differentiation.

To aid in our study of early B cell development, we began to examine the ability of the murine fetal liver to grow and differentiate in culture. Previous studies of fetal liver have involved short-term systems (8, 9) or have used hybridomas or transformed cell lines derived from fetal tissue (10–12). These studies have been useful for characterization of the molecular basis of the pre-B cell phenotype and for insights into allelic exclusion and the mechanisms of Ig

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; cIg, cytoplasmic immunoglobulin; FCS, fetal calf serum; M-MuLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sIg, surface immunoglobulin.

gene rearrangements. However, with rare exceptions (13), these cells are frozen in their stage of development and neither their progenitors nor their more differentiated progeny can be examined.

Our laboratory has previously described a long-term culture system for murine bone marrow (14, 15) in which B cell precursors, pre-B cells, and IgM-expressing B cells are grown in vitro. This culture system has now been modified to successfully culture lymphoid elements of fetal liver. Several important differences, such as Ig gene expression and the type of Abelson murine leukemia virus (A-MuLV) transformants obtained, exist between the B cell lineage cells seen in bone marrow cultures and fetal liver cultures. The cultured fetal liver cells maintain traits similar to fresh fetal liver B lymphocytes even after several months in culture and provide further evidence for the hypothesis of the distinctiveness of these two lymphoid populations. In addition, these fetal liver cultures retain presumptive B cell precursor populations with unrearranged Ig genes, thus providing a system for the study of Ig gene rearrangement and B cell development in vitro.

## Materials and Methods

*Mice.* BALB/c mice were obtained from Cumberland View Farms, Clinton, TN; BAB-14 mice were obtained from Dr. I. L. Weissman, Stanford University. Both strains were subsequently bred and maintained in our colony.

*Long-term Cell Cultures.* Femoral bone marrow plugs from 3–4-wk-old mice were flushed into RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) plus 5% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA). After vigorous pipetting and centrifugation, a single-cell suspension was made in RPMI 1640 medium supplemented with 5% FCS, 50 $\mu$M 2-mercaptoethanol, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin (complete medium). High density and low density bone marrow cultures were established on 10-cm or 6-cm tissue culture dishes (Corning Medical & Scientific, Palo Alto, CA) as detailed in Whitlock et al. (28).

Fetal liver cells were obtained from midgestational fetuses (14–17 d after the appearance of a vaginal plug) and a single-cell suspension prepared as above. Fetal liver cells, at a density of $1 \times 10^6$ cells/ml were placed upon preestablished, low density, bone marrow feeder layers (15) in 6-cm or 10-cm petri dishes (6 and 15 ml of cell suspension, respectively). BALB/c fetal liver cells were plated onto BAB-14-derived feeder layers which provided a means for unambiguous determination of the origin of the lymphoid cells, using a restriction enzyme polymorphism of the mu heavy chain genes (16). 3–4 d later, a small volume of complete medium was added. At 1 wk, all nonadherent cells were transferred to a fresh preestablished feeder layer. The cultures were then maintained as the long-term bone marrow cultures by feeding every 3–4 d and replacing 80% of the spent culture medium every 7 d. Care was taken not to aspirate the nonadherent cells during the removal of the spent medium. Once the cells were growing well, with doubling times of ~48 h, they were split 1:2 onto new feeder layers by careful resuspension of the nonadherent cells. This process was repeated until sufficient cells were obtained for analysis.

*Immunoperoxidase Cell Staining.* Cells were stained using a peroxidase-conjugated goat anti-mouse IgM reagent (Boehringer-Mannheim Biochemicals, Indianapolis, IN). For surface staining, viable cells were incubated with 50 $\mu$l of a 1:100 dilution of the antiserum to phosphate-buffered saline (PBS) for 30 min on ice. After two washes, the cells were cytocentrifuged onto a microscope slide and allowed to air dry overnight. After an 8-min incubation in PBS, the slides were exposed to the 3-amino-9-ethyl carbazole substrate and hydrogen peroxide for 20 min at room temperature (17). The slides were then rinsed with $H_2O$ and counterstained with hemotoxylin (Sigma Chemical Co., St. Louis, MO). Cytoplasmic staining was performed by incubation of the antiserum dilution on the

cytocentrifuged air-dried cell button for 30 min at room temperature. Washing, staining, and counterstaining were then performed as above. Stained cells were counted under 400× magnification using a Nikon microscope.

*Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis.* Metabolic labeling of the cultured cells with [$^3$H]leucine (ICN Pharmaceuticals, Inc., Irvine, CA) was done as detailed in Whitlock et al. (15). The labeled cells were washed and lysed and the supernatant clarified according to Witte et al. (18). The clarified lysate was immunoprecipitated overnight with a polyvalent rabbit anti-mouse Ig (a gift of I. L. Weissman) or, for the A-MuLV transformants, monoclonal anti-gag antibodies. These antibodies recognize the A-MuLV-specific P160 and the Moloney MuLV (M-MuLV)-specific pr65. The immunoprecipitates were collected on the Cowan I strain of *Staphylococcus aureus* (19) and the samples were subjected to sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (20). Radioactively labeled protein bands were visualized by fluorography with sodium salicylate (21). Two-dimensional gel electrophoresis was done by the method of O'Farrell (22) as previously described (15).

*A-MuLV Transformation.* Cultured cells were transformed with P160 A-MuLV (23, 24) as described previously (15). After transformation, the cells were plated in agar (15) and colonies were visible in 10–15 d. Individual colonies of clonal origin were picked using a pasteur pipette and resuspended in 1 ml complete medium. The clones of A-MuLV-transformed cells were assayed ~3–4 wk after transformation.

*DNA Analysis.* High molecular weight DNA was extracted from various fresh tissues and cell lines and digested with the appropriate restriction enzymes noted in the figure legends. The samples (~10 μg) were electrophoresed in a 0.8% agarose gel in Tris-phosphate and transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) (25). The filters were hybridized to a $^{32}$P nick-translated probe corresponding to the $J_{H2}$, $J_{H3}$, and $J_{H4}$ regions for heavy chain gene analysis (26) or to the $J_\kappa$ region for kappa chain gene analysis (27). Bands that hybridized to the radioactively labeled probes were visualized by autoradiography.

## Results

*Fetal Liver Cultures Require a Preestablished Feeder Layer.* Lymphoid cultures derived from fetal liver, much like those derived from bone marrow (14), have a strict dependency upon an adherent layer of feeder cells to support their growth and provide factors for their proliferation. Bone marrow cells, when placed into tissue culture dishes, will set down their own adherent layer to support the growth of nonadherent cells. Fetal liver cells, under similar conditions, establish a mixed cellular adherent layer that is unable to support the long-term growth of the nonadherent lymphoid cells (J. St. Claire and O. Witte, unpublished observations). Therefore, suspensions of fresh fetal liver cells were seeded upon established adherent layers derived from low density bone marrow cultures (Fig. 1). 3–4 wk before the start of fetal liver cultures, bone marrow from BAB-14 mice was seeded at $3.5 \times 10^5$ cells/ml (see 28) in 6- or 10-cm tissue culture dishes. These low density cultures gave rise to good adherent layers but not a nonadherent B lymphoid layer. After screening the feeder layer cultures for the absence of nonadherent cells, the BALB/c fetal liver cell suspension at $1 \times 10^6$ cells/ml was added to the dish. A restriction enzyme polymorphism between BALB/c and BAB-14 mice in the Ig mu heavy chain locus allows unambiguous determination of the source of the nonadherent cells in the cultures (16; J. Kurland, S. Ziegler, and O. Witte, submitted for publication). Fresh medium was added to the cultures after 3–4 d, and at the end of 1 wk all nonadherent cells in the cultures were transferred to a fresh feeder layer. This

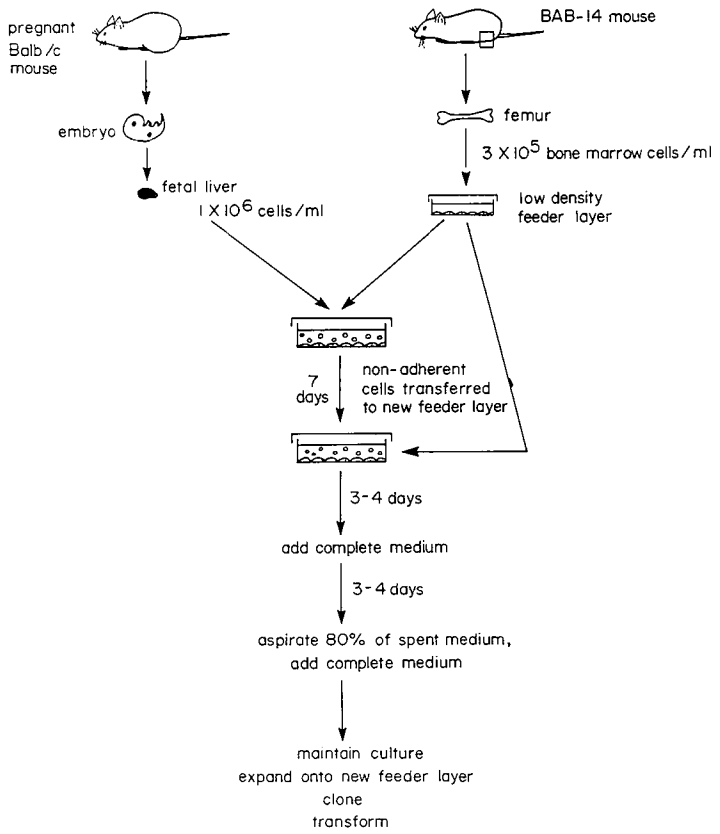


FIGURE 1. Protocol for the initiation and maintenance of long-term fetal liver cultures. Single-cell suspensions of BALB/c fresh fetal liver cells were plated upon adherent feeder layers made from BAB-14 bone marrow 3–5 wk previously. After 7 d in vitro, all non-adherent cells were transferred to a new adherent feeder layer. The cells were maintained on this plate by feeding every 3–4 d, with 80% of the spent medium being aspirated every other feeding.

transfer of cells was crucial for the establishment of these cultures. After this transfer, the cultures were maintained much as the bone marrow cultures; fresh medium was added every 3–4 d with 80% of the spent culture medium aspirated only once a week. Care was taken to avoid aspiration of the nonadherent cells.

Because the fetal liver cultures were begun on the established feeder layers, these cultures did not pass through a "crisis phase" as observed with the bone marrow cultures (14, 28). Nonadherent lymphoid cells were always visible in these cultures, even during the first several weeks, but real increases in cell number did not occur for 3–5 wk. The fetal liver cells passed through a dormant phase after which an increase in the number of lymphoid cells and cells possessing cIgM was seen. After the cells began active proliferation, they were maintained at densities of 2–7 $\times$ $10^5$ cells/ml. Above these densities, cell growth slows considerably. The doubling time for such established populations was ~36–48 h. Cells were expanded in numbers by the transfer of nonadherent cells to new feeder layers. $>10^6$ cells could be harvested from each established 6-cm plate

every 7–14 d for protein or DNA analysis. The cultured fetal liver cells were successfully frozen and recovered, and cloned by limiting dilution using the methods detailed in Whitlock et al. (14, 15). These cultures have been maintained for 4 mo and longer.

*Fetal Liver Cultures Contain Cells Early in the B Lymphocyte Lineage.* We characterized the nonadherent cells generated in the fetal liver cultures and compared them with fresh fetal liver and with nonadherent cells generated in a similar manner from bone marrow B lymphoid cultures. Culture morphology (Fig. 2*A*) and cell morphology (Fig. 2*B*) of the fetal liver cultures appeared very similar to the bone marrow cultures. The nonadherent cells were relatively homogeneous in size and grew in patches or clusters upon the adherent cell layer. Wright's staining of the nonadherent cultured fetal liver cells showed them to be lymphoid in morphology.

Cytolasmic mu staining of fetal liver cultures and bone marrow cultures was also similar. Both nonadherent cell types had a low percentage of $cIgM^+$ cells until 4–6 wk of culture when the percentage of $cIgM^+$ cells begins to rise (Table I). The fresh fetal liver cells from which the cultures were derived had <1% $cIgM^+$ cells, suggesting that a population of the B lymphocyte progenitors present in fetal liver appeared to have undergone further maturation or expansion in culture. However, cells bearing surface IgM were very infrequent in the fetal liver cultures, whereas 30–70% of the $cIgM^+$ cells in bone marrow cultures were $sIg^+$ (Table I). Thus, the cultured fetal liver cells appeared to have a shift in

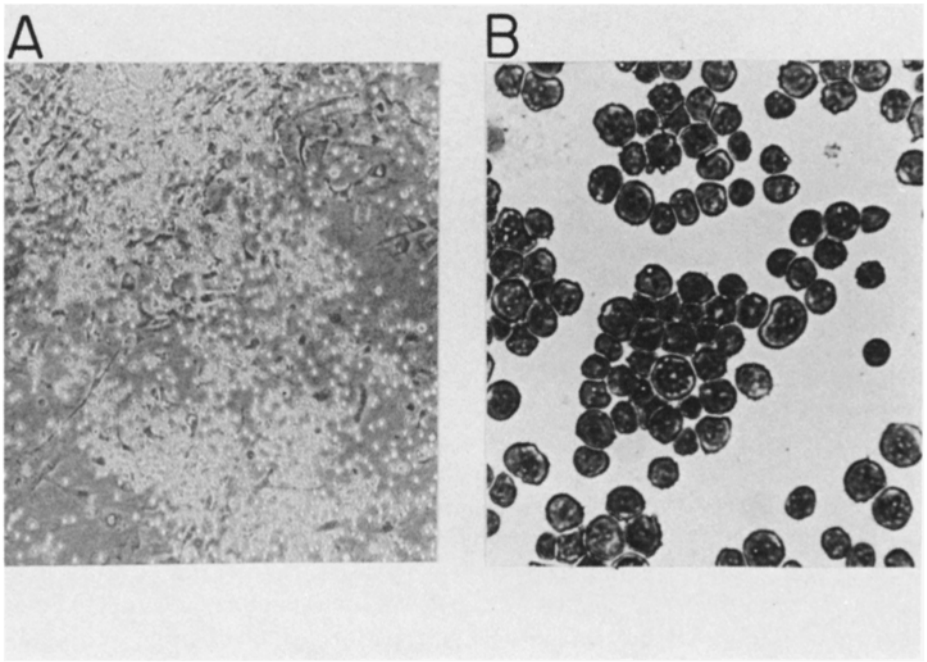


FIGURE 2. Morphology of fetal liver cells after 6 wk in vitro. (*A*) Culture morphology showing adherent stromal cell layer with clusters of nonadherent cells. Inverted phase; × 100. (*B*) Cell morphology showing lymphoid appearance of cells. Wright's stain; × 400.

TABLE I
*Cytoplasmic and Surface IgM Staining of Cultured Cells*

| Culture source | Age in weeks | Percent cIgM$^+$ | Percent sIgM$^+$ |
|---|---|---|---|
| Fetal liver | 2* | 8–10 | ND‡ |
| | 6 | 1–3 | ND |
| | 8 | 4–6 | 0.1–0.3 |
| | 11 | 14–17 | 0.5–1.0 |
| Bone marrow | 3 | 7–12 | 10 |
| | 6 | 2–4 | ND |
| | 8 | 4–8 | 3–4 |
| | 15 | 15–29 | 6–14 |

Immunoperoxidase-conjugated anti-IgM was used to stain either fixed (cIgM) or viable (sIgM) cells as described in Materials and Methods. Between 200 and 1,000 cells were scored on each slide.
* Data compiled from assays done at various time points on two different fetal liver cultures and three different bone marrow cultures.
‡ Not determined.

their phenotypic distribution toward less mature cells of the B lymphocyte lineage. The fetal liver cultures did not produce large numbers of mature sIg$^+$ B lymphocytes as seen in the bone marrow cultures. Neither bone marrow nor fetal liver cultures produced cells that secreted Ig as assayed by metabolic labeling and immunoprecipitation (data not shown). The cells from these cultures were negative for the Thy-1 and Mac-1 surface antigens by immunoperoxidase surface staining. However, it is possible that the level of these surface antigens was below that of our control cell line, WEH1-3, and thus undetectable. Both bone marrow and fetal liver cultured cells failed to produce spleen colonies when injected into lethally irradiated mice (data not shown).

*Both Rearranged and Germline Heavy Chain Gene Configurations Are Seen in the Cultured Cell Populations.* The cIgM$^+$, surface IgM–negative pre-B cell is the earliest phenotypic characterization of a cell in the B lymphocyte lineage. The ability to generate large numbers of cells in the fetal liver cultures by expansion onto new feeder layers enabled analysis of this cell population's DNA configurations at the Ig gene loci. Very early cells of the B cell lineage can be detected in this manner due to a known sequence of events involving the heavy chain gene locus (26, 29, 30). Several characteristic rearrangements take place at this locus before heavy chain protein production and pre-pre-B cell types can be detected in this manner.

The first molecular event described in the commitment of a precursor cell to the B cell pathway is the rearrangement of the heavy chain variable region and diversity (D) segment genes from their germline location to a location 5′ to a heavy chain joining ($J_H$) segment (26). This rearrangement event results in a change in the restriction enzyme digest patterns immediately 5′ to the $J_H$ segments. Using the enzyme EcoRI in combination with a $J_H$ region probe (Fig. 3), DNA with a germline arrangement at the $J_H$ locus will have a 6.2 kilobase (kb) band that hybridizes with the $J_H$ probe (Fig. 3, lane *1*). Any rearrangements

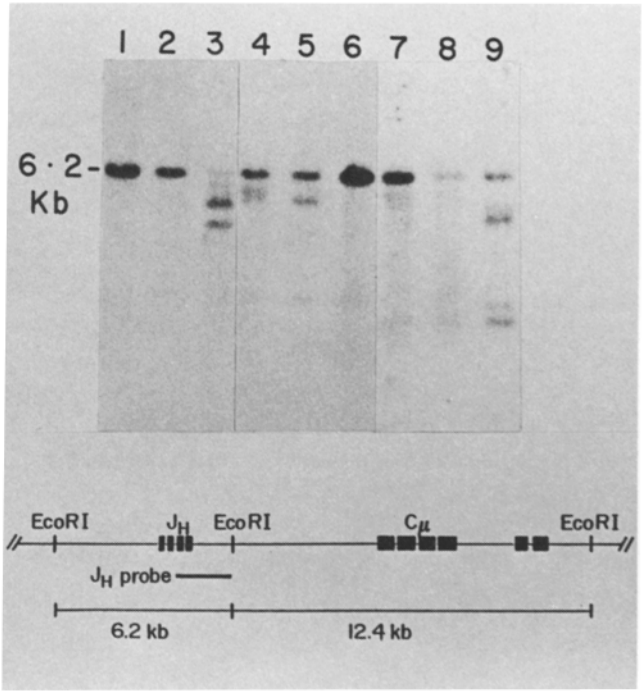


FIGURE 3. Southern blot analysis of Ig heavy chain gene loci in DNA from long-term cultures. EcoRI-digested DNA was electrophoresed in agarose and transferred to nitrocellulose filters. These filters were hybridized to a $J_H$ probe (*bottom*); autoradiographs are shown. The 6.2 kb hybridizing fragment of mouse liver DNA indicates the size of the unrearranged heavy chain gene. All of the long-term cultures analyzed showed germline heavy chain genes in their DNA as well as many discrete DNA rearrangements. (Lane *1*) liver DNA; (*2*) fresh bone marrow DNA; (*3–5*) DNA samples from long-term bone marrow cultures (16, 8, and 6 wk in culture, respectively); (*6*) fresh fetal liver DNA; (*7–9*) DNA samples from long-term fetal liver cultures (3, 4, and 12 wk in culture, respectively).

5′ to the $J_H$ locus would perturb the 5′ EcoRI site and the resulting hybridizing band would be of altered size. As illustrated in Fig. 3, lanes *2* and *6*, normal lymphoid tissue such as fresh fetal liver, bone marrow, and spleen had such a diversity of $J_H$ rearrangements that no one is present in the population in high enough frequency to be observed as a discrete band of hybridization. Hybridization was only seen with the 6.2-kb germline fragment, despite the known presence of pre-B and/or B cells. Nonadherent cells from both fetal liver and bone marrow cultures also retained some germline configuration in their DNA as evidenced by hybridization of the $J_H$ probe to the 6.2-kb fragment (Fig. 3, lanes *3–5* and *7–9*). Care was taken during nonadherent cell collection to avoid the harvest of adherent layer cells, a source of Ig gene germline DNA contamination. The BALB/c origin of the nonadherent cells was confirmed with Southern blot analysis of the mu constant region fragment (data not shown). At least one chromosome remains in the unrearranged state at the heavy chain locus in a subpopulation of the cells from these cultures for 3 mo in vitro or longer. These cultures may therefore contain cells capable of undergoing Ig gene rearrangement, presumably very early B cell progenitors able to generate more

mature members of the B lymphocyte lineage. In addition, populations of cells existed in these cultures that shared several predominant restriction fragment bands of rearranged $J_H$ regions. The predominant rearrangements that could be visualized on Southern blots decreased in number and increased in intensity as the length of culture increased. This paralleled the observed shift to pauciclonality of the Ig synthesized by the bone marrow (15) and the fetal liver cultures (data not shown) as they age. DNA analysis of a mixed cell population (nonclonal) such as this could not determine the existence of cells with both chromosomes in germline configurations at the heavy chain loci nor could it detect minor populations with unique $J_H$ rearrangements.

Southern blot analysis of kappa gene rearrangements in the fetal liver cultures did not show predominant bands of hybridization other than germline. In addition, the intensity of the germline $J_\kappa$ hybridizing band did not significantly decrease when compared with a constant region band. From this it appears that very few of these cells have rearranged kappa genes or that little expansion of such rearranged cells has occurred.

*Abelson Transformants of Cultured Fetal Liver Are Phenotypically Similar to Those From Fresh Fetal Liver.* As an aid in the analysis of the cell types present in the fetal liver cultures, transformed clonal lines were derived from the mass population by infection with A-MuLV rescued with M-MuLV helper virus (23, 24) and immediately cloned in soft agar. These independent clonal isolates were then expanded and examined at the cellular and molecular levels to further characterize the cell types present in the fetal liver cultures. Cultured cells from fetal liver were transformed after 6–8 wk in vitro and 42 transformed clones were picked and expanded into liquid culture. Eight of the transformed clones produced mu heavy chain (Table II) by immunoperoxidase staining, these eight cloned transformants were $cIgM^+$ in a range from 10 to 80% of the cells. This is a typical finding in A-MuLV-transformed clones (33). The production of mu chains was confirmed using immunoprecipitation of [$^3$H]leucine-labeled lysates (Fig. 4). All transformants were producing the A-MuLV-specific P160, which demonstrates the cells were adequately labeled. Light chain production was not observed in any of the 42 transformants. In contrast, a much higher percentage of mu positive clones was obtained when long-term-cultured bone marrow was transformed with A-MuLV, and light chain production was occasionally seen

TABLE II

*Phenotype of A-MuLV Transformants Obtained from Cultured Populations*

| Culture source | $Mu^+$/total (%) | Light $chain^+$/total (%) |
|---|---|---|
| Fetal liver | 8/42 (19) | 0/42 (0) |
| Bone marrow* | 6/11 (55) | 1/11 (9) |

Cultured cells derived from fetal liver or bone marrow were infected with A-MuLV after 6–8 wk in vitro as described in Materials and Methods. Agar colonies were expanded in liquid culture and the cells metabolically labeled with [$^3$H]leucine. Immunoprecipitated proteins were run on SDS-PAGE and visualized by autoradiography (see Materials and Methods and Fig. 4).

* Data from Whitlock and Witte (14).

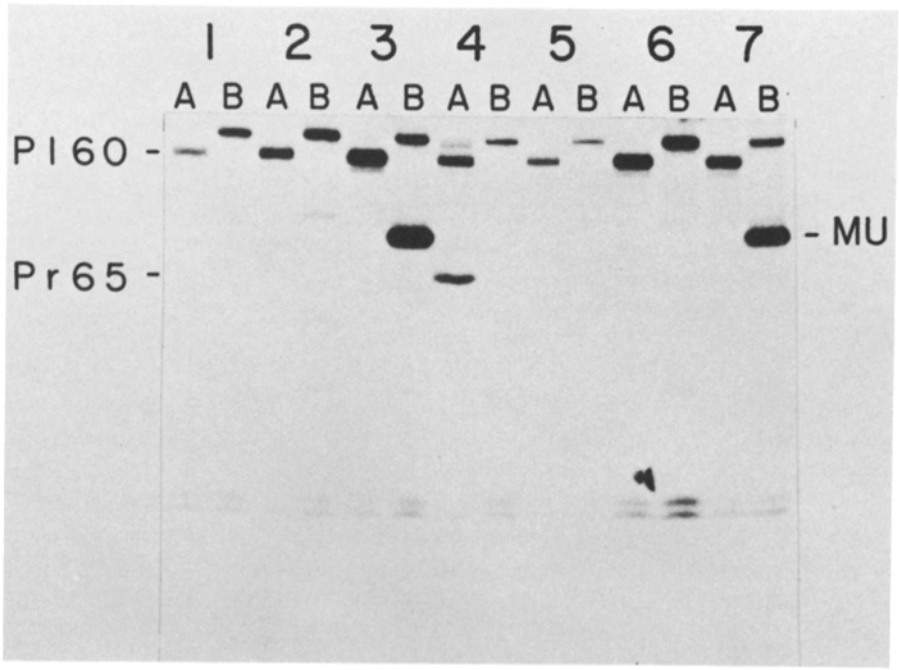


FIGURE 4. Ig production by A-MuLV-transformed clones. [$^3$H]leucine-labeled cell lysates were immunoprecipitated by anti-*gag*–specific monoclonal antibodies (*A*) or anti-Ig (*B*) and run on SDS-PAGE as detailed in Materials and Methods. Fluorogram is shown. Only clones 3 and 7 were shown to be producing mu heavy chain; no light chain production was seen. All cell lines produced the A-MuLV-specific P160 protein; clone 4 also produced the M-MuLV-specific pr65.

(Table II and 15). The distribution of phenotypes of the transformants obtained from these two cultured populations closely paralleled those obtained from the A-MuLV transformation of fresh early fetal liver and bone marrow (12, 31). A low percentage of A-MuLV transformants from early fetal liver were mu producers; no light chain production was seen. Similar to cultured bone marrow transformants, >50% of fresh bone marrow transformants produced mu, and 5–10% produced light chain.

*Abelson Transformants of Cultured Fetal Liver Have Rearrangements of Both the Heavy and Light Chain Gene Loci.* DNA from these cultured fetal liver A-MuLV-transformed clonal lines was analyzed for rearrangements at the heavy chain gene locus characteristic of B lymphocyte lineage cells. These rearrangements always involve the $J_H$ region, so an EcoRI digest was used in combination with a $J_H$ region probe (Fig. 5) to compare the $J_H$ regions of the transformed lines with the unperturbed germline configuration seen in the DNA of nonlymphoid tissues. EcoRI-digested DNA from liver showed a single, 6.2 kb band of hybridization to the $J_H$ probe, characteristic of the germline configuration of the heavy chain locus (Fig. 5*L*). In contrast, EcoRI-digested DNA from the transformed cell lines showed no germline 6.2 kb band, indicating that DNA rearrangements involving the $J_H$ region had taken place on both chromosomes of all of these cell lines (Fig. 5). This included the 34 transformants that did not produce any mu protein.

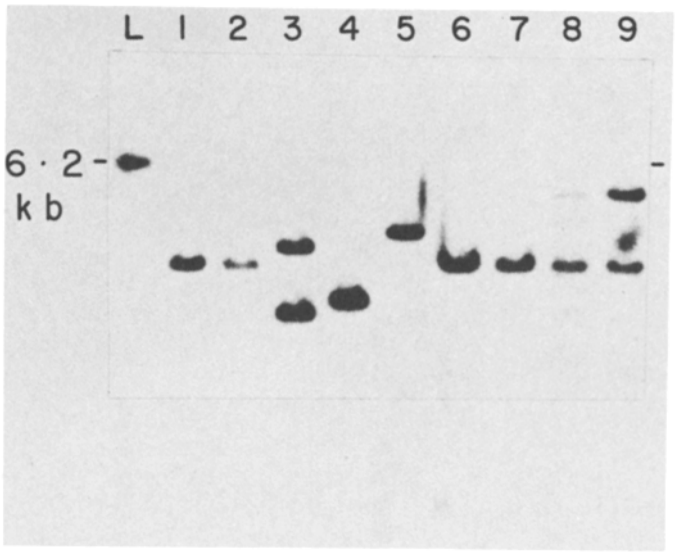


FIGURE 5. Southern blot analysis of Ig heavy chain gene loci in A-MuLV-transformed clone DNA. Digest and probe details are given in Fig. 3. The unrearranged heavy chain gene 6.2 kb hybridizing band was seen only in liver DNA (*L*); all transformed clones had DNA rearrangements at both loci. Mu heavy chain protein was synthesized by the clones shown in lanes *4* and *8*.

Several rearranged EcoRI fragments were seen multiple times among the clonal cell lines and certain of these transformants appeared to have both chromosomes identically rearranged (Fig. 5, lanes *2*, *6–8*) or to have lost one chromosome.

After rearrangement of the heavy chain genes and mu protein expression, rearrangements of a similar nature occur at the kappa light chain locus (27, 30). It had been previously noted (12) in transformants from fresh fetal liver that, although all heavy chain loci were rearranged, only a few kappa chain locus rearrangements had occurred in mu-producing clones. Kappa gene rearrangements in the cultured fetal liver transformants were analyzed by digesting the DNA with a combination of the restriction enzymes EcoRI and BamHI and using a $J_\kappa$ probe to detect changes 5′ to the $J_\kappa$ region (12, 27). Of the six mu-producing clones analyzed, only one had both chromosomes in the germline configuration in the $J_\kappa$ region (Fig. 6, lane *5*). The remaining five had one germline and one rearranged kappa gene locus (Fig. 6). These six cultures did not produce kappa chain protein as monitored by immunoprecipitation analysis (data not shown).

## Discussion

*Potential of Long-term Fetal Liver Cultures.* Long-term fetal liver cultures have many uses in the study of early B lymphocyte development and differentiation. The earliest committed B lymphocytes and their progenitors, which reside in the fetal liver for a brief time in ontogeny, play a pivotal role in the development of the immune system. The ability to culture and expand lymphocyte populations derived from fetal liver allows biochemical and molecular analyses of these cell types.

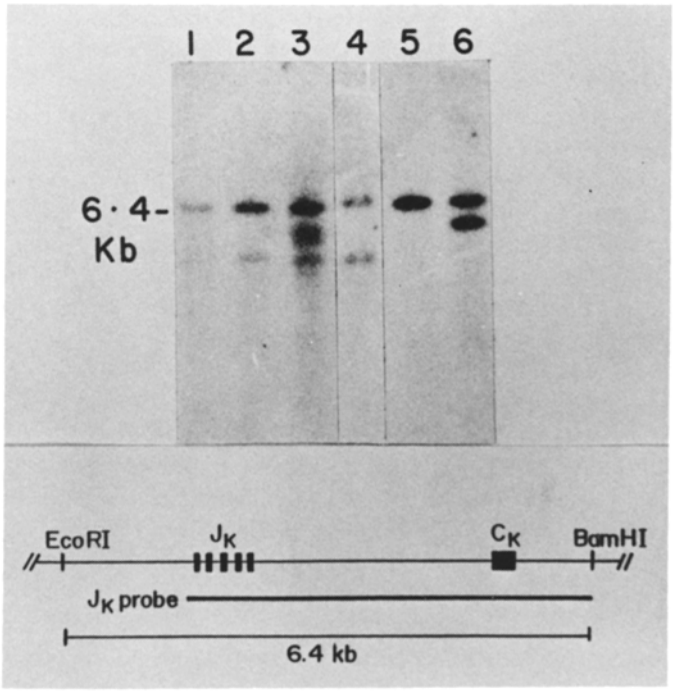


FIGURE 6. Southern blot analysis of Ig kappa chain gene loci in A-MuLV-transformed clone DNA. The DNA was digested with EcoRI and BamHI and examined by Southern blot analysis for hybridization to a $J_\kappa$ probe (*bottom*). The 6.4 kb hybridizing fragment of mouse liver DNA indicates the size of the unrearranged kappa light chain gene (lane *1*). The clones represented in lanes *2*, *3*, *4*, and *6* showed rearrangements on one chromosome at this locus. The clone represented in lane *5* had the germline configuration at both loci.

Modification of the long-term bone marrow culture system (14, 15) was necessary for the successful in vitro growth of fetal liver cells. Long-term cultures of fetal liver cells could only be established upon adherent stromal cells derived from bone marrow. Transfer to a second such feeder layer after 7–10 d of culture was also crucial. We have not established whether this was due to the presence of an inhibitory cell type that is lost upon passage, the toxic effect of an adherent cell in the fetal liver, or another cause. Other important parameters (as detailed in Materials and Methods) for the success of the cultures include: (*a*) the use of RPMI 1640 medium supplemented with 2-mercaptoethanol, (*b*) the use of 5% FCS, (*c*) careful screening of the batch of FCS for its ability to support growth of both adherent and nonadherent cell layers without overgrowth of the adherent layer and, (*d*) rigorous adherence to correct cell densities for starting cultures and feeding schedules (28).

It is important that after 3 mo or more in culture, the cultured fetal liver cells retained traits similar to those of the fresh fetal liver lymphocytes. The paucity of $sIg^+$ cells in these fetal liver cultures (Table I) and the early B cell phenotypes obtained upon Abelson transformation (Table II) are evidence of a similar phenotype. This contrasted with the results obtained with bone marrow–derived B lineage lymphocytes cultured for a similar length of time. It appears that the

culture conditions used, although selective for growth of cells from the B lineage, do not select out a specific subpopulation of B lymphocytes. The types of B lineage cells obtained appears dependent upon the tissue source. In this way we are now able to characterize the types of cells transiently present in the fetal liver and compare them with B lineage cells from other sources.

In keeping with the shift to earlier cells of the B lymphocyte lineage, the cultured fetal liver cells expressed very little sIg. This could have been due to the lack of a positive signal in the fetal liver cultures such as a specific cell type or maturation factor (36) for this event. Alternatively, the expression of Ig surface receptors could have been selected against in these very early B cells by the clonal abortion mechanism of tolerance known to function in fetal and neonatal B lymphocytes (37, 38).

Although the majority of nonadherent cells in these fetal liver cultures do not express cIg or sIg, they appear to be of the B lymphocyte lineage. After 6–8 wk of culture, all but ~10–30% of the Ig heavy chain loci were rearranged in these cells (see Fig. 3). The cultured fetal liver cells lacked Thy-1 and Mac-1 surface markers and contained no spleen colony-forming cells. The phenotype of these cultured fetal liver cells is that of a very early population of the B lymphocyte lineage.

The presence in these long-term cultures of cells with characteristics of precursors to B lymphocytes has potential importance. This was initially supported (14) by the ability of the bone marrow cultures to give rise to a heterogeneous population of pre-B and early B cell types. Additional evidence is presented here with the finding of germline Ig heavy chain genes in a subpopulation of the cells in these long-term cultures. Further confirmation of the presence of B cell progenitors in the bone marrow cultures has been obtained by the demonstration that these long-term cultured cells could repopulate the defective B cell compartment in CBA/N immunodeficient mice (J. Kurland, S. Ziegler, and O. Witte, submitted for publication).

*Characterization of the Early B Lymphoid Cells in Long-term Fetal Liver Cultures Using A-MuLV.* Clonal cell lines were obtained by transformation with A-MuLV to study the phenotypic distribution of these long-term fetal liver cultures in detail at the biochemical level. >80% of these transformants produced no heavy chain protein. This was in agreement with the phenotypic distribution obtained from transformation of fresh fetal liver (12). However, all transformants were rearranged at both mu heavy chain loci. The absence of germline configuration at the heavy chain locus after A-MuLV transformation has been noted by other investigators (12, 15, 33). This is despite the known presence of germline Ig genes in the various fresh and cultured tissues before transformation. It is not known if the heavy chain loci rearrangements seen in these A-MuLV transformants were due to a target preference of the A-MuLV for this type of cell. Alternatively, the rearrangements could have been facilitated by the growth rate stimulation of the cells by A-MuLV or other posttransformation events.

The rearrangements that were seen in the non–mu-producing clones could have been either the D-J only or the nonproductive V-D-J type (34) characterized by Alt et al. (35) in transformants from fresh fetal liver. Preliminary results using D region probes indicate that a number of the rearrangements were of the D-J

only type (L. Treiman, unpublished data). Several of the mu heavy chain producers also had rearrangements at the kappa locus; these clones did not produce kappa chains detectable by immunoprecipitation. We failed to obtain any transformants from these long-term fetal liver cultures that had multiple heavy chain rearrangements as were found with fresh liver transformants (12). This is perhaps due to the 6–8 wk culture period of the fetal liver cells before transformation in our experiments. Any heavy chain rearrangements that were the result of growth stimulation or preprogrammed events would occur during this time. Indeed, the number of heavy chain gene rearrangements (Fig. 3) and the percent of $cIgM^+$ cells (Table I) in the long-term fetal liver cultures far exceeds that found in fresh fetal liver.

We have demonstrated that early B lineage cells are present in long-term fetal liver cultures. The majority of these cells appear to be earlier in the B lineage than the cells present in long-term bone marrow cultures. However, both of these cultures contain cell populations that possess unrearranged Ig genes which may be B cell precursors and should prove useful in both in vivo reconstitutions and in vitro analysis of Ig rearrangements.

## Summary

Long-term cultures of murine fetal liver have been successfully established using a modification of our in vitro bone marrow culture system (14, 15). Fetal liver cells from midgestation BALB/c embryos were plated onto BAB-14 bone marrow stromal cell-adherent layers. After a 3–5 wk period, cell growth began to increase and these cells were expanded in number on fresh feeder layers. The cultured fetal liver cells were lymphoid in morphology, 5–20% cytoplasmic Ig–positive, but <1% surface Ig–positive. Southern blot analysis of the cultured fetal liver cells, as well as cultured bone marrow–derived B cells, demonstrated a population with germline Ig heavy chain loci, possibly representing very early B cell precursors. Abelson murine leukemia virus (A-MuLV) clonal transformants of such cultured fetal liver cells had a phenotypic distribution similar to that seen with fresh fetal liver transformants but distinct from those obtained with the transformation of either cultured or fresh bone marrow. All A-MuLV transformants isolated had rearrangements at the mu heavy chain locus of both chromosomes, irrespective of Ig production. In addition, most mu heavy chain producers had at least one rearranged kappa gene locus. These long-term fetal liver cultures provide large numbers of cells for studying events early in the B lymphocyte lineage. The cultured fetal liver cells retained phenotypic traits similar to fresh fetal liver B cells and distinctive from bone marrow cells cultured under similar conditions.

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