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Immunology

Synthesis of immunoglobulin μ chain gene products precedes synthesis of light chains during B-lymphocyte development

(pre-B lymphocytes/fetal liver/immunoglobulin mRNA)

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ABSTRACT **Immunoglobulin (Ig) gene expression has been followed during the later stages of development of the murine fetal liver. Biosynthetic labeling and immunoprecipitation were used to isolate Ig-related polypeptides from fetal and neonatal livers. By examination of the specific immune precipitates, the earliest detectable Ig was shown to consist only of μ heavy chain. At about the time of birth, when light chain synthesis became evident, separation of surface Ig-positive cells from surface Ig-negative cells by using anti-Ig-coated dishes showed that cells lacking surface Ig (pre-B lymphocytes) synthesized only μ chains. Thus, commencement of light chain synthesis was closely coordinated with the appearance of surface Ig. Ig RNA species were examined by electrophoretic fractionation and hybridization with cloned Ig DNA sequences. The sizes and amounts of Ig mRNA were found to correlate with the pattern of μ and light chain protein biosynthesis. μ chain RNA species appeared earlier in gestation than light chain RNA did, and only after birth did light chain sequences reach levels equivalent to those of μ chain. Cell populations enriched in pre-B lymphocytes also contained an excess of μ over light chain mRNA.**

Fetal murine B lymphopoiesis is detected earliest in the placenta (1) and becomes evident in the fetal liver after 13 days of gestation (2–4). After birth, the bone marrow becomes the major site of primitive B-lymphocyte development. During the development of B lymphocytes, many cellular changes occur, the most obvious involving the types of immunoglobulin (Ig) molecules produced by the cells. Mature antibody-secreting cells make complete Ig molecules, consisting of both heavy and light chains. Virgin cells in embryonic or neonatal mice probably make Ig molecules with heavy chains restricted to the μ class and either κ or λ light chains. Their precursors, the pre-B cells, were first described as cells that also make complete Ig, μ heavy chain, and a light chain (2, 5); they were distinguished from their more mature progeny because pre-B lymphocytes lacked surface Ig whereas B lymphocytes had surface Ig.

An important difference between unstimulated B lymphocytes and their antigen-stimulated, antibody-secreting progeny involves the COOH-terminal end of the μ chain. Unstimulated cells make primarily membrane-bound Ig as a consequence of the occurrence of a membrane-binding domain at the COOH terminus of the μ chain (6–8); this μ chain protein is designated "μ_m." In secreting B lymphocytes or plasma cells, the μ chain protein has a COOH terminus that allows secretion of the protein; this protein is called "μ_s." The two forms of μ chain are encoded by distinguishable mRNAs: a 2.7-kilobase (kb) mRNA for μ_m and a 2.4-kb mRNA for μ_s (6, 7). The 3′-terminal portions of these mRNAs come from different exons in the constant μ chain region of cell DNA (8).

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A suggestion that pre-B lymphocytes might lack a light chain has come from analyses of surface Ig-negative lymphoid tumor cells (9–12). Many clonal lines of mouse bone marrow cells transformed *in vitro* by Abelson murine leukemia virus (A-MuLV) (13) make only cytoplasmic μ heavy chains but no light chains (10). To explain this observation, it was suggested that the "μ-only" phenotype might be characteristic of normal pre-B cells. Subsequently it has been found that early fetal pre-B lymphocytes (14) and hybrids derived from fusion of myeloma and fetal liver cells (15) also express only cytoplasmic μ heavy chain.

To investigate the time course of Ig gene expression in developing mouse embryos, we have analyzed Ig mRNA accumulation and protein biosynthesis throughout late ontogeny. We have found that both heavy chain protein and mRNA are detectable earlier in development than light chain gene products and are enriched in cells that do not express surface Ig.

MATERIALS AND METHODS

Buffers. Buffer I contained 8.29 g of NH_4Cl, 1.0 g of $KHCO_3$, and 0.074 g of Na_2EDTA per liter (pH 7.4). Immunoprecipitation buffer contained 0.1 M sodium chloride, 10 mM sodium phosphate buffer (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, aprotinin (20 μg/ml; Boehringer Mannheim), 1 mM N^{α}-tosyl-L-arginyl methyl ester, 1 mM EDTA, and bovine serum albumin (1 mg/ml). Labeling medium was Dulbecco's modified Eagle's medium lacking methionine and supplemented to 10% (vol/vol) with dialyzed fetal calf serum containing 10 mM Hepes buffer (pH 7.35), penicillin (50 units/ml), and streptomycin (50 μg/ml). Sample buffer contained 50 mM Tris·HCl (pH 6.8), 50 mM dithiothreitol, and 1% sodium dodecyl sulfate. Standard saline citrate was 150 mM NaCl/15 mM Na citrate.

Preparation of Fetal Liver Cell Suspensions. All experiments used BALB/cAn mice raised in our own colony. Vaginal plugs were observed on day 0 and birth occurred 19 days later. Livers were removed from washed embryos or neonates and were placed into room temperature RPMI-1640 medium/10% (vol/vol) fetal calf serum/10 mM Hepes buffer, pH 7.35. Single cell suspensions were prepared by gently squeezing the livers through nylon mesh (netex) with a plunger from a 3-ml disposable syringe. The cells were constantly flushed through the mesh with medium. Large debris was allowed to settle and the cells were washed two times with medium. Erythrocytes were

Abbreviations: Ig, immunoglobulin; A-MuLV, Abelson murine leukemia virus; kb, kilobase.

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removed either by ultracentrifugation as described below or by treatment with buffer I. To treat with buffer I, washed cells were resuspended in that buffer at 10^8 cells per ml for 1 min at 0°C. Four volumes of cold phosphate-buffered saline was quickly added, and the cells were washed two times in phosphate-buffered saline before labeling.

Fractionation of Cells with Surface Immunoglobulin. To remove erythrocytes, washed cells from 12- to 19-day livers were resuspended gently with a wide-bore pipette in 7 ml of 35% (vol/vol) Path-O-Cyte V (Miles). This cell suspension was layered under cold medium in a 2.5 × 8.9 cm nitrocellulose tube for the Beckman SW27 rotor and centrifuged at 4°C for 30 min at 10,000 rpm to pellet the mature erythrocytes. The nucleated cells that banded at the interface were washed two times with medium containing 10% fetal calf serum (GIBCO) and resuspended at 10^7 cells per ml. Three milliliters of cells were fractionated on each petri dish coated with rabbit anti-mouse Ig as described (16). The nonadherent population was washed once with labeling medium, and before labeling the adherent cells were washed once while still attached to the petri dishes.

Biosynthetic Labeling, Immunoprecipitation, and Electrophoretic Analysis. After washing, cells were resuspended at 5–50 × 10^6 nucleated cells per ml in labeling medium. [^{35}S]Methionine (New England Nuclear) was added to 100 μCi/ml (1 Ci = 3.7 × 10^{10} becquerels), and the cell suspensions were labeled in a humidified CO_2 incubator for 2 hr at 37°C. The cells were then washed once with cold phosphate-buffered saline and resuspended at 10^7 cells per ml in cold immunoprecipitation buffer. Each milliliter of lysate to be precleared was incubated with 5 μl of normal rabbit serum and 25 μl of 10% (wt/vol) fixed *Staphylococcus aureus* bacteria in immunoprecipitation buffer for 2 hr at 0°C. The lysates were then centrifuged for 1 hr at 100,000 × *g* before addition of specific antiserum. Incubation was continued for 16–24 hr at 4°C followed by precipitation of the immune complexes with whole formalin-fixed *S. aureus* as described (17). Precipitates were washed twice in immunoprecipitation buffer and resuspended in sample buffer for sodium dodecyl sulfate gel electrophoresis and boiled for 1 min. Samples were analyzed on 12.5% (wt/vol) polyacrylamide gels with sodium dodecyl sulfate as described (18). The gels were fixed in 25% (vol/vol) isopropanol/10% (vol/vol) acetic acid and fluorographed as described (19).

Isolation of Poly(A)-Containing RNA from Fetal Liver and Fetal Liver Pre-B Cells. Total RNA was extracted from intact fetal livers or adult spleens that had been stored at −70°C or from purified pre-B cells by a modification of the guanidine·HCl procedure (20). Whole livers were sonicated (four times for 15 sec each) in extraction buffer (15 ml/g of tissue) to disrupt the cells. Poly(A)-containing RNA was selected on oligo(dT)-cellulose as described (21).

Size Analysis of Immunoglobulin RNA. Poly(A)-containing RNA was fractionated by electrophoresis on 1.4% (wt/vol) agarose gels containing 5 mM methylmercuric hydroxide (22) at 100 V for approximately 4 hr and then transferred to diazotized paper (23).

Preparation of Probes. Recombinant plasmids pABμ-1, pABμ-3, and pABκ-1 were derived by A. Bothwell and used under conditions that conform with the National Institutes of Health guidelines for recombinant DNA research. The whole plasmids were labeled by nick translation (24) to specific activities of 1–4 × 10^8 cpm/μg except in the quantitation experiment, in which the 400-bp insert of κ chain sequences in pABκ-1 and a 400-bp *Pst* fragment from pABμ-3 were purified before nick translation.

Hybridization was allowed to proceed with 6 × 10^6 cpm of probe per 100 cm^2 of paper for 24 hr in the presence of 10% (wt/vol) dextran sulfate (23). Unhybridized probe was removed from the paper by washing at 37°C for 3 hr with five changes of 50% (vol/vol) formamide/0.75 M NaCl/0.075 M Na citrate; 2 hr with two changes of 50% formamide/0.15 M NaCl/0.015 M Na citrate, and finally for 1 hr at 68°C with three changes in 0.015 M NaCl/0.015 M Na citrate/1% sodium dodecyl sulfate. The paper was then blotted dry and analyzed by autoradiography.

RESULTS

Metabolic Labeling of Immunoglobulins in Fetal and Neonatal Liver Cells. To determine the pattern of Ig biosynthesis in lymphoid cells of the developing mouse embryo, cells from fetal and neonatal livers were labeled with [^{35}S]methionine, and cell extracts were analyzed by immunoprecipitation. When polyspecific anti-mouse Ig serum was used to precipitate Ig-related polypeptides from detergent-solubilized extracts of liver cells from 18-day embryos, a polypeptide with the electrophoretic mobility of an Ig μ heavy chain was evident in the precipitate, but there was no detectable light chain (Fig. 1, lane B). Nonimmune rabbit serum failed to precipitate any Ig-related polypeptides (Fig. 1, lane A). Precipitation of the apparent μ chain polypeptide could be blocked with affinity-purified IgM from myeloma MOPC-104E, indicating that it was a μ chain (Fig. 1, lane C). By contrast, extracts of cells isolated from neonatal livers (21 days of gestation) contained both μ heavy and light chains (Fig. 1, lane D). Using this methodology, we first detected μ heavy chains as early as day 17 of gestation but did not detect significant amounts of light chains until day 19.

Absence of Proteolytic Degradation in Fetal Liver Extracts. A mixture of protease inhibitors and gentle extraction conditions were used in these studies to minimize possible protein degradation (25). To test whether residual proteolytic activity in extracts of the fetal tissue was capable of specifically de-

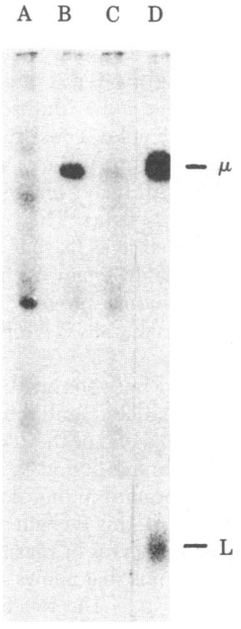

FIG. 1. Metabolic labeling of precleared extracts of 7 × 10^6 fetal and neonatal liver cells. Lanes: A, Eighteen-day embryo cells precipitated with normal rabbit serum; B, 18-day embryo cells precipitated with polyvalent rabbit anti-mouse Ig; C, 18-day embryo cells precipitated with polyvalent rabbit anti-mouse Ig and 10 μg of purified MOPC-104E IgM; D, 21-day neonatal liver cells precipitated with polyvalent rabbit anti-mouse Ig. μ, μ chain; L, light chain.

grading labeled light chains, a mixing experiment was performed. [^{35}S]Methionine-labeled extracts of 21-day livers, which contained labeled light chains, were mixed with equal amounts of unlabeled extracts of embryonic livers. The same amount of labeled light chain was recovered when the 21-day extract was mixed with unlabeled extracts from 17-day, 18-day, or 21-day liver cells (Fig. 2, lanes D, E, and F). Unlabeled extracts of 21-day liver contained no endogenous protease inhibitors that would allow detection of light chains in labeled 17-day or 18-day liver extracts (Fig. 2, lanes B and C).

Surface Immunoglobulin Phenotype of μ-Only Cells. At day 19 of gestation, liver cells synthesized both μ and light chains (Fig. 3, lane A). However, there, was a great excess of μ chain over light chain. When 19-day liver cells were fractionated by adsorption to petri dishes coated with rabbit anti-mouse Ig antibodies (16), adherent surface Ig-positive cells could be separated from the nonadherent surface Ig-negative fraction. The Ig-related polypeptides in labeled extracts of these two cell populations were compared. Light chain synthesis could not be detected in the surface Ig-negative population (Fig. 3, lane C), but light chains were synthesized by cells expressing Ig on their surface (Fig. 3, lane B). Neither of these fractions secrete significant amounts of Ig into the culture medium (data not shown).

Detection of Immunoglobulin mRNA During Ontogeny. To examine μ heavy and light chain mRNA levels in fetal liver cells, the poly(A)-containing RNA was extracted from livers taken from embryos at days 15–21 of gestation. The RNA was size-fractionated by electrophoresis through methylmercuric hydroxide-containing agarose gels, transferred to diazotized paper, and hybridized with ^{32}P-labeled DNA from molecular clones of μ chain or κ chain cDNA (Fig. 4). When a probe representing the 770 3′-terminal nucleotides of μ chain mRNA (6) was used, mRNAs 2.4–3.0 kb in length were evident (Fig. 4*A*). The 3.0- and 2.4-kb species were predominant in fetal liver cells

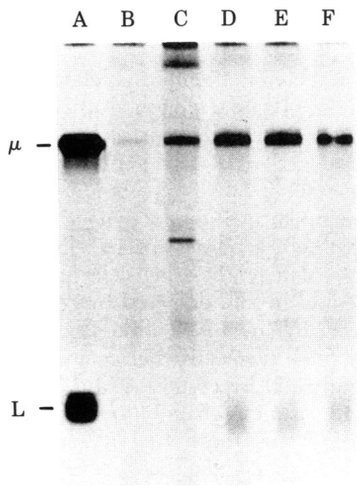

FIG. 2. Inhibition of protease activity in extracts of fetal liver cells. Detergent-solubilized extracts of 10^7 [^{35}S]methionine-labeled cells were mixed with extracts of 10^7 unlabeled cells that had been incubated similarly but without [^{35}S]methionine. After mixing, the extracts were precleared together with normal serum and fixed *S. aureus* before immunoprecipitation with rabbit anti-mouse Ig. Lanes: A, [^{35}S]Methionine-labeled cytoplasmic IgM marker from MOPC-104E myeloma; B, labeled 17-day fetal liver cell extract mixed with unlabeled 21-day neonatal liver extracts; C, labeled 18-day extract mixed with 21-day unlabeled extract; D, E, and F, labeled 21-day neonatal liver extract mixed with unlabeled 17-, 18-, and 21-day extracts respectively. μ, μ chain; L, light chain.

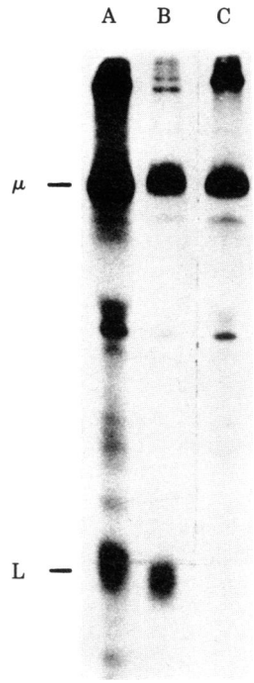

FIG. 3. Fractionation of surface Ig-positive and -negative 19-day fetal liver cells on anti-Ig-coated petri dishes. Lanes: A, Precipitation with polyvalent rabbit anti-mouse Ig from an extract of 10^7 unfractionated cells; B, precipitation from the adherent population isolated from 10^7 unfractionated cells; C, precipitation from the nonadherent population isolated from 10^7 unfractionated cells. μ, μ chain; L, light chain.

obtained between days 15 and 17; thereafter, a 2.7-kb species predominated (Fig. 4*A Inset*; Fig. 4*C*). The 2.7-kb μ chain mRNA is the same size as that encoding the membrane-bound form of μ protein (6, 7); when a DNA probe specific for the exon specifying the membrane-binding portion of μ_m protein was used as a probe (8), the 2.7-kb μ chain-specific RNA in 19-day fetal liver was labeled (data not shown), indicating that the μ_m mRNA is the predominant species in these cells. The 3.0- and 2.4-kb RNA in early fetal liver have counterparts in a variety of lymphoid cells and may not represent RNA that could encode an authentic μ protein (unpublished data). The pattern of μ chain RNA in 19-day fetal liver cells is similar to that in B lymphocytes and in A-MuLV-transformed μ chain-only cells (Fig. 4*C*, lane 12; ref. 6; unpublished data).

When a [^{32}P]DNA probe for κ chain mRNA was used, the first detectable RNA was found in 18-day liver cells (Fig. 4*B*). Both 1.2-kb mature-size κ chain nRNA and an 0.8-kb mRNA that could encode a fragment of κ chain-related protein were found in 18- and 19-day fetal liver cells. Later in development, only the 1.2-kb species was detected in both fetal liver and spleen cell RNA (Fig. 4*C*; ref. 26). A κ chain RNA pattern similar to that found in 18- to 19-day fetal liver also could be detected in certain A-MuLV-induced μ chain-negative cell lines (Fig. 4*C*, lane 11) and also is observed in certain myeloma lines (26).

To determine the relative abundance of μ_m and κ chain mRNAs during embryonic development, a mixed probe was prepared. Approximately equal amounts of DNA representing 400-base regions of the μ chain and κ chain mRNAs were mixed and labeled together with ^{32}P. To test whether the probe hybridized equivalently to the two types of RNA and whether the response of the autoradiographic detection method was linearly related to RNA concentration, serial 50% dilutions of mature

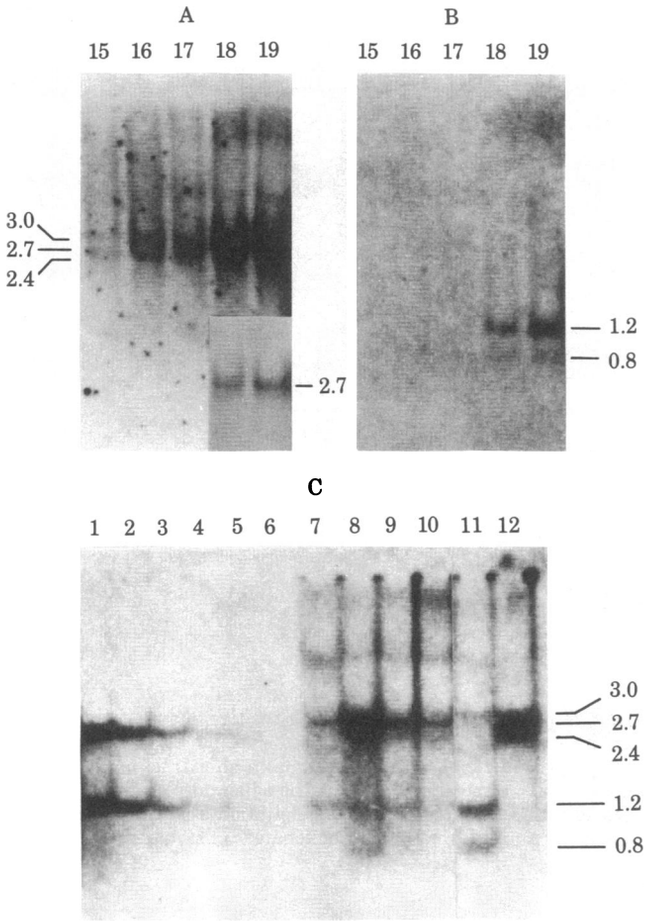

FIG. 4. Size and quantity of Ig RNA found in fetal liver cells during late gestation. Approximately 10 μg of poly(A)-containing RNA from days 15–19 of gestation (lane designation) was fractionated and hybridized with approximately 5×10^6 cpm of nick-translated probe. (*A*) μ chain RNA sequences detected by homology to labeled pABλ-1 (10^8 cpm/μg). *Inset*, shorter exposure of the same experiment. (*B*) κ chain sequences hybridizing to pABκ-1. (*C*) Amounts of various RNAs hybridized with a probe prepared by mixing equal amounts of 400-bp fragments of pABκ-1 and pABμ-3 and nick-translating these together to a specific activity of 4×10^8 dpm/μg (1 dpm = 16.7 mBq). Lanes: 1–5, adult spleen cells (220 ng, 110 ng, 55 ng, 28 ng, 14 ng, respectively); 6, blank; 7–10, fetal liver cells [21-day cells (7.5 μg), day-19 surface Ig-negative cells (6.5 μg), 19-day unfractionated cells (7.5 μg), 18-day cells (9 μg), respectively]; 11 and 12, tumor cells [cell line 18-48 (10) (3 μg), cell line 3-1 (10) (2 μg), respectively].

spleen RNA were fractionated, transferred to paper, hybridized to the probe, and autoradiographed (Fig. 4*C*, lanes 1–5). Approximately equal intensity μ chain and κ chain signals were detected at each dilution, and densitometry showed that a linear decrease in signal intensity occurred with dilution (data not shown). By day 21 of gestation, the probe showed equal amounts of 2.7-kb μ_m and 1.2-kb κ chain mRNA (lane 7). At day 18, however, the μ_m mRNA was clearly in excess (lane 10). The relative amount of κ chain-specific mRNA increased 2- to 3-fold by day 19 of development (lane 9) and began to approach the amount of μ_m mRNA.

To examine the RNAs in surface Ig-negative cells of 19-day liver, surface Ig-positive cells were removed using dishes coated with anti-Ig antibody. The surface Ig-negative cells had an increased ratio of μ_m mRNA to κ chain mRNA (Fig. 4*C*, lane 8) when compared to the unfractionated cells (lane 9). They also had enriched amounts of the 0.8-kb κ chain-related RNA relative to the 1.2-kb κ chain RNA.

DISCUSSION

These data show that at day 18 of embryonic development, when intracellular Ig-related polypeptides first become evident in fetal liver by biosynthetic labeling under our conditions, the cells make μ chains but no detectable κ chains. This result is in agreement with the evidence from Levitt and Cooper (14) who found μ chain-only synthesis in the fetal liver as early as day 15 of development. As development proceeds to birth at 19 days, κ chain synthesis becomes evident and soon equals μ heavy chain synthesis. The surface Ig-negative cells at day 19, however, still make no detectable κ chain protein. Therefore, we conclude that, at least in fetal and neonatal liver, the μ chain-only phenotype is characteristic of the majority of pre-B lymphocytes (defined as cells with cytoplasmic Ig but no surface Ig).

These changes in Ig production during B-lymphocyte development are paralleled by changes in mRNA levels. At day 18, the 2.7-kb μ_m mRNA greatly exceeds the 1.2-kb κ chain mRNA, reflecting the disparity in μ chain and κ chain proteins. By day 19, the levels of κ chain mRNA have risen, although the surface Ig-negative cells present at this time still show the preponderance of the 2.7-kb μ_m mRNA typical of the 18-day cells. The mRNA for a given Ig polypeptide becomes evident before detectable levels of the polypeptide itself appears; there is, for example, relatively more κ chain mRNA evident at day 18 than there is κ chain protein.

Our examination of light chain synthesis focused on κ chains because this isotype is known to represent the vast majority of light chain made by the adult animal. In exploratory experiments, we determined that 21-day liver extracts contain both λ chain protein, determined by radioimmunoassay, and λ chain-specific RNA, determined by quantitative hybridization (26). These comprised only about 5% of the total light chain protein and RNA (unpublished data). This result implies that κ chain-producing cells arise in the liver much more frequently than do λ chain-producing cells. γ heavy chain RNAs were only found in small amounts compared to μ chain-specific RNA (unpublished data).

Ig has been detected in the murine fetal liver as early as day 10 of gestation (25). Using techniques other than metabolic labeling, it has been claimed that surface IgM (μ chain and light chain) can be detected on fetal liver cells by day 13 of gestation (27, 28). The metabolic labeling technique we have used will detect the predominant synthetic activity of liver cells but could well miss the synthetic activities of rare cells; thus, it is possible that some light chain-synthesizing cells appear by day 18 of gestation but could not be detected by our techniques. It is evident, however, that over 90% of the Ig-related polypeptides synthesized by fetal liver cells is μ chain. This μ chain-only phenotype is mirrored by certain A-MuLV-transformed cells that contain cytoplasmic μ chain but not light chains (10).

Our results differ from those of Levitt and Cooper (14) in that we were unable to detect significant levels of secretion of μ chain peptides by pre-B lymphocytes isolated at day 18 or later in gestation. Because we cannot detect secretion of μ chains and because we find a predominance of 2.7-kb μ_m mRNA over the 2.4-kb μ_s mRNA, we believe that the majority of μ chain polypeptides made by pre-B lymphocytes are of the μ_m type. The lack of surface IgM on the cells presumably reflects a block in the normal processing scheme for IgM, possibly as a result of the lack of light chains needed to make a finished Ig molecule. Even if these early lymphocytes make some μ_s protein (and there may be some 2.4-kb μ_s mRNA in the earliest fetal liver cells), successful secretion might also be blocked.

The difference in protein and mRNA patterns between sur-

face Ig-negative and surface Ig-positive cells implies a close coordination between the onset of light chain synthesis and the appearance on the cells of sufficient surface IgM molecules to enable binding to an anti-Ig-coated petri dish. Whereas it is possible that surface μ chain could be made before surface IgM, it seems reasonable to suggest that surface Ig is deposited only after light chain synthesis is activated. Such a suggestion implies a pathway of B-lymphocyte maturation involving first activation of μ chain synthesis followed later by activation of light chain synthesis. When μ chain alone is made, the polypeptide is unable to mature to become a surface molecule and is degraded in the cell, much as it is in the A-MuLV-transformed tumor cells (10). When light chain synthesis is activated, complete IgM molecules can then assemble and be transported to the cell surface. The appearance of the human histocompatibility antigen proteins on the cell surface requires association between the heavy chain and the β_2-microglobulin light chain (29, 30). This suggests that association of multiple polypeptides may be required before surface expression of certain membrane-associated proteins can be accomplished.

The observation that the predominant form of μ chain mRNA in 18- to 21-day fetal liver is the 2.7-kb μ_m mRNA correlates with the fact that the virgin B lymphocyte has mainly surface Ig. The predominance of secreted Ig is only evident in the more mature products of B-lymphocyte activation. The 2.4-kb μ chain-related RNA found even in the earliest fetal liver cells could represent μ_s mRNA but more likely has another origin. It is always accompanied by a 3.0-kb μ chain RNA, and unpublished data suggest that this doublet of RNA represents a transcriptional activity of the μ chain constant region that does not lead to a normal μ chain protein.

In summary, the available data suggest that early B-lymphocyte development proceeds through two stages. First, μ chain-only cells (pre-B lymphocytes) appear that lack light chain or light chain mRNA. The major form of protein and mRNA in these cells is μ_m, but the protein cannot be processed to the cell surface. This cell could presumably expand as a clone until the next stage commences. Second, light chain synthesis starts, preceded by rearrangement of the light chain genes. Light chain rearrangements continue until a light chain is produced that can effectively combine with the μ chain. The resultant $(\mu L)_2$ dimers mature into surface IgM molecules. At that point, the cell becomes a resting virgin B lymphocyte awaiting antigenic stimulation.

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