ONE SYNCHRONOUS WAVE OF B CELL DEVELOPMENT IN MOUSE FETAL LIVER CHANGES AT DAY 16 OF GESTATION FROM DEPENDENCE TO INDEPENDENCE OF A STROMAL CELL ENVIRONMENT

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B lymphocyte progenitor cells arise from pluripotent stem cells in a competent environment by a set of critical steps of differentiation. During embryonic development of the mouse, progenitors of B lymphocytes arise in fetal liver at day 13 of gestation (1-4), then develop into precursor (pre) B cells, which consequently rearrange the gene segments of the sIg V regions of H chains, followed by those of the L chains (5, 6). The early stages of progenitor proliferation and induction to Ig gene rearrangements have been found to depend on interactions with cells of the environment called stroma (7-15). At least in part, the effects of stromal cells on B cell development appear to be mediated by soluble cytokines, notably IL-3 or -7 (16-21). Productive rearrangements of both H and L chain V genes allow the expression of Ig on the surface of pre-B cells, detectable from day 16 of gestation onwards in fetal liver of the mouse (22). 2 d later, these sIg$^+$ B cells become reactive to polyclonal activators (1-3), so that upon stimulation with LPS, they develop into clones of IgM-secreting plasma cells, which can be detected in a plaque assay (23, 24).

Tissue culture systems have been established in which the development of B lineage cells in fetal liver from day 13 of gestation can be followed to the final maturation into a clone of IgM-secreting plaque-forming cells (PFC)[1] (1-3). The time schedule of development in vitro follows that observed in vivo, so that mitogen-reactive B cells arise at day 19 of gestation and the peak of the IgM PFC response 5 d thereafter, no matter when the cells have been transferred from fetal liver into tissue culture at any time between days 13 and 19 of gestation (1-3).

In this report, we follow in vitro the development of B lineage cells in fetal liver from day 13 of gestation with time and in dependence of interactions with the stromal cell environment and with rIL-2 through rIL-7 (25). B lineage precursors are enriched from fetal liver at different times of gestation by FACS with the help of a recently developed mAb, G-5-2, specific for pre-B cells (26, 27). Enrichment of pre-B cells is monitored by an in situ hybridization analysis for the expression of the

pre-B cell–specific gene λ5 (28, 29) in these FACS-enriched cell populations. We interfere in the contacts of these purified B lineage precursors with the stromal cell environment with two mAbs raised against primary embryonic stromal cells from fetal liver. We terminate the interactions of precursors with stromal cells by plating them under limiting dilutions in tissue culture systems that allow the development to clones of IgM PFC in a foreign environment (1–3). Our results define two times of B lineage development in fetal liver, the first dependent, the second independent of interactions with stromal cells. They also suggest that B lineage cell development occurs in one synchronous wave in fetal liver.

Materials and Methods

Animals. Adult female C57BL/6 mice, male DBA/2 mice, and 3–6-wk-old Lewis rats were obtained from the Institute für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. (C57BL/6 × DBA/2) F_1 (BDF$_1$) embryos from timed pregnant C57BL/6 females were provided by breeding facilities at the Basel Institute for Immunology. The day of appearance of vaginal plug was counted as day 0 of gestation. Birth occurred at day 19.

Cell Lines. Sources of pre-B lymphomas 40E1, 220-8, and 204-1-8 (5), and the B lymphomas WEHI 231 (30) and WEHI 279 (31) used in this paper are provided in reference 30. The T cell hybridoma K62 was generated in our laboratory. All cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with kanamycin (100 U/ml) 5 × 10^{-5} M 2-ME, and 5% heat-inactivated FCS.

ILs. Murine rIL-2, -3, -4, and -5 were obtained as described (25) and used at a concentration of 5%, i.e., at ~5–50 U/ml. Human rIL-6, produced by a cell line transfected with the human IL-6 gene (kindly provided by Dr. W. Fiers, Biogent, Gent, Belgium), was used at a dilution of 5% (i.e., at 5–50 U/ml). IL-7 (18, 19) (kindly given to us by Drs. S. Gillis and Ch. Henney, Immunex, Seattle) was used at concentrations between 100 and 1,000 U/ml.

Tissue Culture. Fetal liver cells were prepared as described earlier (1–3, 26, 27). Total fetal liver cells and G-5-2$^+$ fetal liver cells isolated by cell sorting (see below) were cultured in serum-substituted IMDM (32), in the presence or absence of stromal cells and in the presence or absence of IL-2 through IL-7 (19, 20, 25), as indicated in Results. Cultures were set up at 0.2 ml in 96-well flat-bottomed plastic culture plates (Costar, Cambridge, MA). Primary fetal liver stromal cell layers were established from 13-d-old BDF$_1$ embryos by overnight culture at a density of 5 × 10^6 to 10^7 cells/ml in IMDM containing 5% FCS. Nonadherent cells were thereafter removed by extensive washing with serum-free medium. This stromal layer was subjected to 3,000 rad gamma irradiation before coculture with G-5-2$^+$ fetal liver cells.

Limiting dilution experiments were performed in 96-well culture plates in serum-substituted medium in the presence of 3 × 10^6 rat thymocytes/ml and 50 μg/ml LPS, (S form of *Salmonella abortus* equii; a kind gift of Drs. G. Galanos and O. Lüderitz, Max-Planck-Institut für Immunobiologie, Freiburg, FRG) as described earlier (1–3, 26, 27). Threefold dilutions of fetal liver cells between 5 × 10^6 and 5 cells/ml were set up with 24 cultures of 200 μl for every cell concentration in rat filler cells and LPS. A positive culture with a clone of IgM-secreting cells arising from one mitogen-reactive B cell contained between 30 and 120 IgM PFC at the day of assay, i.e., at the equivalent time of day 5 after birth in all fetal liver cell experiments. The peak of IgM PFC responses at that day with the various fetal liver cell suspensions cultured in various ways was ascertained in pilot experiments, and reaffirmed earlier results showing that all fetal liver cell responses, regardless from which day of gestation and regardless of treatment in tissue culture, showed a peak of IgM PFC development at the equivalent time of day 5 after birth (2, 3). Poisson's distribution was used to calculate the frequencies of mitogen-reactive, Ig-expressing cells yielding clones of IgM PFC. Cultures were scored as negative if they contained three IgM PFC or less.

Cultures of Fetal Liver Cells Containing Various Combinations of ILs. rIL-2, -3, -4, -5, -6, and -7, except for human IL-6, all of murine origin (25), were used at concentrations between

5 and 50 U/ml. 20-µl cultures were set up in moist Terasaki plates with all possible combinations, i.e., 37 different combinations of single, double, triple, quadruple, quintruple ILs and the one total combination of all six ILs with 2×10^5 mAb G-5-2$^+$ fetal liver cells of day 13 or 14 of gestation per milliliter in Iscove's medium containing either serum substitutes (see above) or 5% FCS. Cells were counted under the microscope using a Bürker hemocytometer.

Plaque Assay. The total number of IgM-secreting PFC in a population of cells was determined by the protein A SRBC plaque assay (24) using a rabbit anti-mouse IgM (MOPC 104E µ/λ) antiserum.

Production of Stromal Cell mAbs and Testing their Effects on the Adherence of the Pre-B Lymphoma 40E1 to Stromal Cells. Lewis rats were immunized on day 0 with 2×10^7 stromal cells emulsified in CFA in the hind foot pad. On day 4 and 7 the rats were boosted with 2×10^7 stromal cells in PBS in the same foot pad. On day 8 the regional lymph nodes were removed and fusion was performed as described (33).

Individual hybridoma clones were then tested for the production of antibodies that interfere with the adherence of the pre-B lymphoma 40E1 to the fetal liver stromal cell layers (prepared as described above). Stromal cell layers were therefore cocultured with 5×10^4/ml 40E1 cells and 20% final concentration of the different hybridoma clone culture supernatants. Adherence of the 40E1 cells to the stromal cells was read after 24–36 h of coculture using an inverted microscope. Two mAbs called STR4 and STR10 were found that interfered with the adherence of 40E1 to the stromal cells.

Purification of mAbs. mAbs G-5-2 (26), STR4, STR10, 14.8 (anti-B220), and 5.1 (anti-mouse µ; reference 34) were purified from hybridoma supernatant on a protein G or protein A column (35) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immunofluorescence Analysis and Cell Sorting. Immunofluorescence staining and analysis, as well as cell sorting, were done as described earlier (26) using biotinylated (Calbiochem-Behring Corp., La Jolla, CA) mAbs and FITC-streptavidin (Amersham International, Amersham, UK) as secondary reagents.

Results

Development of Precursors of LPS-reactive B Cells in Cultures of Unseparated Fetal Liver Populations. Tissue culture conditions have been developed in which resting mature B cells from a variety of lymphoid organs of the mouse can be stimulated by polyclonal activators such as LPS under conditions of limiting dilution to develop in high frequencies to clones of PFC (1–3, 26, 27). These culture conditions, in which single B cells are plated in serum-substituted medium (32), in the presence of rat thymus cells known as "fillers," are also suitable to stimulate in vitro those B cells that have developed, or will develop in this foreign environment, to the stage of a mitogen-reactive, Ig$^+$ B cell. When cells are taken from fetal liver at different days of gestation between days 13 and 19 of embryonic development of the mouse and directly cultured with LPS and rat thymus cells under limiting dilutions, the peak of the PFC response occurs always at the same time, i.e., at the day equivalent to day 5 after birth. Therefore, fetal liver cells of day 13 (Fig. 1, *circles*) and of day 14 (Fig. 1, *triangles*) of gestation were cultured in high density suspensions (see legend to Fig. 1), and then transferred after various days under limiting dilution conditions into cultures containing rat thymus filter cells and LPS. This was done for fetal liver cells of day 13 at the equivalent of days 14, 15, 16, 17, 18, and 19 (Fig. 1, *circles*) and for cells of day 14 at the equivalent of days 15, 16, 17, 18, and 19 (Fig. 1, *triangles*). All limiting dilution cultures were assayed for the development of IgM PFC at the time equivalent to day 5 after birth. High density cultures of day 14 fetal liver were also kept in the presence of IL-6 and -7, and their limiting dilutions in rat thymus
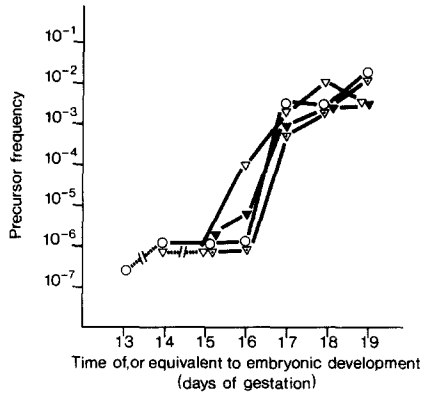
FIGURE 1. Frequencies of precursors developing to LPS-reactive B cells. Fetal liver cells of days 13 (O) and 14 (∇, $\blacktriangledown$, ∇) of gestation were plated in tissue culture at high densities (1.7 × 10^6 cells/ml) and in the presence of L-6 and IL-7 under conditions described in Materials and Methods. After various days in tissue cultures (· · ·/ /· · ·), cells were plated under limiting dilution in rat thymus filler cells and LPS and assayed for the development of IgM PFC clones at the equivalent time of day 5 after birth. In parallel tissue culture experiments, IL-6 and IL-7 were omitted from the day 14 fetal liver cell cultures ($\blacktriangledown$). Limiting dilution cultures in the presence of rat thymus cells and LPS were set up, either to contain (∇) or not to contain (∇) IL-6 and IL-7.

The frequencies of precursors developing into LPS-reactive B cells were also measured in vivo in fetal liver at various times of embryonic development, by directly plating fetal liver cells of various days of gestation under limiting dilution in rat thymus filler cells and LPS and assaying the development of IgM PFC clones at the equivalent of day 5 after birth, and were found to be 1 in 3 × 10^7 at day 13, 1 in 3 × 10^4 at day 16, and 1 in 100 at day 18.

filter cells and LPS were done in the presence of IL-6 and -7. As can be seen from the data in Fig. 1, *filled and dotted triangles*, IL-6 and -7 did not show any significant effects on the frequencies of pre-B cells capable of development to mitogen-reactive B cells and to clones of IgM PFC. The frequencies of precursors that can develop in this environment increases from ~1 in 10^7 at day 13 to ~1 in 30 at day 19 of gestation (1-3). The change in precursor frequency is particularly high, i.e., ~100-fold, between days 16 and 17 (Fig. 1).

When fetal liver cells of early times of gestation (days 13-15) are cultured at high density, e.g., when the progenitors and precursors are not diluted in rat thymus cells but kept in contact with each other until the time equivalent to day 19 and then plated in limiting dilution with LPS and rat thymus cells, the frequency of precursors of LPS-reactive cells increases. Over 100-fold more LPS-reactive B cells have developed in high density cultures in contact with each other (Figs. 1, *open circles and triangles*, at day 19). This indicates that their own environment is favorable for the development of precursors to mature, sIg⁺ B cells, while that of rat thymus cells is not. The change in precursor frequencies capable of development to IgM PFC between days 13 and 19 is, in fact, as high in the equivalent time in vitro as it is in vivo (compare Figs. 1 and 2) and reaches frequencies comparable with those in vivo, i.e., ~1 in 30 cells. The culture conditions of fetal liver cell suspensions at high densities, thus, appear as good as those of cells in vivo in fetal liver. They make it further unlikely that exogenously added IL-6 and -7 could show any stimulatory effects.

When fetal liver cells of either day 13 (Fig. 1, *circles*) or 14 (Fig. 1, *triangles*) of gestation are removed at different times of tissue culture from their own environment into that of rat thymus cells, the 100-fold change in LPS-reactive cells developing in the second environment (observed above) occurs within 24 h, between the time equivalent to days 16 and 17 of embryonic development (Fig. 1). This sudden change indicates that pre-B cells in fetal liver change from dependence to independence on their own environment to develop to LPS-reactive cells at that time.
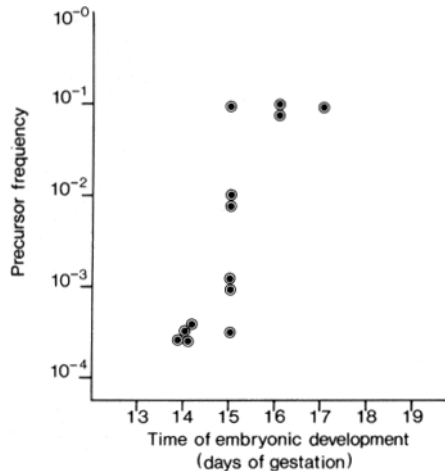
FIGURE 2. Precursor frequencies in G-5-2$^+$ fetal liver cells from different times of embryonic development. Fetal liver cells were sorted at the day of gestation indicated and directly plated under limiting dilution in rat thymus filler cells and LPS, and assayed for the development of IgM PFC clones at the equivalent time of day 5 after birth. (●) Results of individual limiting dilution experiments.

Failure of Purified Fetal Liver B Cell Precursors to Develop Outside their Own Environment.   Pre-B cells express a lineage- and developmental stage–specific glycoprotein, PB76, on their surface that is recognized by mAb G-5-2 (26, 27). B cell precursors of fetal liver can be enriched by FACS using this mAb, as shown by the concomitant enrichment of cells expressing the pre-B cell–specific gene λ5 (27, 28). Thus, mAb G-5-2$^+$ fetal liver cells of day 14 were ~30%, of day 16 ~60%, and of day 18 ~85% λ5$^+$. In the same G-5-2$^+$ cells, μ H chain mRNA-positive B cell precursors were present at 1% (at day 13), 6% (at day 14), 50% (at day 16), and 85% (at day 18). When plated in limiting dilutions with rat thymus cells immediately after FACS, these cell populations proved to be enriched for precursors of LPS-reactive B cells (26, 27). Although between 30 and 85% of all G-5-2$^+$ cells expressed λ5 at days 14–18 and could, therefore, be regarded as pre-B cells, only a very small fraction of all G-5-2$^+$ cells before days 15 and 16 of gestation could develop into clones of IgM PFC in the foreign environment of rat thymus cells. The frequencies of these precursors in the enriched populations increased from ~1 in 3 × 10^3 at day 14 to ~1 in 10 at day 19 of gestation. The highest increase in frequency was detected within 24 h of gestation, between days 15 and 16 (Fig. 2) (see also Discussion).

When high concentrations (5 × 10^4 to 5 × 10^5 cells/ml) of mAb G-5-2$^+$ day 14 fetal liver cells were cultured in rat thymus filler cells (5 × 10^6/ml) plus LPS, no significant cell death was observed between day 14 and the time equivalent to day 19 of gestation, indicating that rat thymus cells had no adverse effect on the viability of precursor cells. Lower concentrations of the fetal liver cells, as those used in the limiting dilution analyses, cannot be monitored for survival, due to the vast excess of rat thymus cells in culture.

The Influence of IL-2, -3, -4, -5, -6, and -7.   The inability of early fetal liver cells (day 14) to develop in high frequencies to LPS-reactive B cells did not change when IL-2, -3, -4, -5, -6, and -7, either alone or in all possible combinations of one, two, three, four, five, or all six ILs, i.e., 37 different sets of ILs (data not shown), were added to the cultures of G-5-2$^+$ cells (shown only for IL-6 and -7 in Fig. 1 and for IL-3 and IL-7 in Fig. 4). All ILs were used in concentrations (5–50 U/ml or more)

that would be expected to suffice for occupancy of high avidity receptors for these ILs. We conclude that neither rat thymus cells with LPS nor these six ILs, by themselves or in combinations, induce the development to mitogen-reactive B cells under conditions of high avidity IL-R occupancy.

The Influence of Embryonic Stromal Cells. Since the own environment of fetal liver precursors of the B lineage appeared beneficial for their development to mature cells, i.e., since high density suspensions of fetal liver cells allowed the development of mitogen-reactive B cells, while limiting numbers of fetal liver cells in the foreign environment of high density rat thymus filler cells and LPS did not, the influence of adherent "stromal" cells on this development was investigated.

Cultures of primary embryonic stromal cells were established from day 13 fetal liver cells. Cells that adhered to the plastic tissue culture wells in overnight culture were either trypsinized and replated for various periods of time up to 2 wk, or were used directly after removal of the nonadherent cells in the primary culture as sources of embryonic stromal cells. They were irradiated before coculture with FACS-sorted G-5-2$^+$ B cell precursors.

In the presence of such irradiated embryonic stromal cells, G-5-2$^+$ precursors were now induced to the development of mitogen-reactive B cells in high frequencies. The frequencies increased from ~1 in 3 × 10^3 at day 14 to ~1 in 30 when precursors of day 14 and stromal cells were cocultured until the time equivalent to day 19, before they were replated in LPS and rat thymus cells (Fig. 3). Addition of the various ILs led to no significant increase in the frequencies of mitogen-reactive B cells, although addition of IL-6 plus IL-7 tended to increase the size of a single LPS-reactive B cell clone by one quarter to one half (data not shown). For all subsequent experiments these two ILs were included in the cultures, unless specified otherwise. We conclude from these results that irradiated embryonic stromal cells provide an environment in culture that is inductive for B cell differentiation to mitogen-reactive B cells. Exogenously added IL-2 through IL-7 are not needed.

A Stromal Cell-dependent, Followed by a Stromal Cell-independent Period of B Cell Development. Experiments with unseparated and FACS-sorted G-5-2$^+$ fetal liver cells described above had indicated that the environment of stromal cells in fetal liver pro-
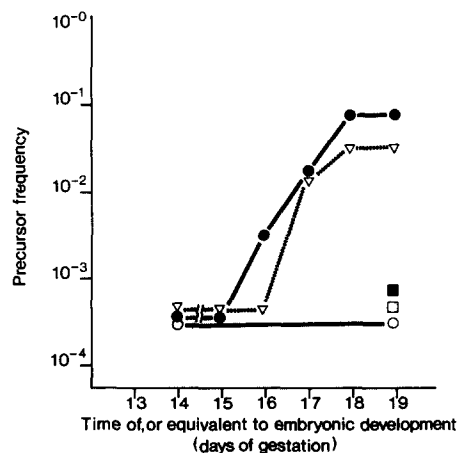

Figure 3. Frequencies of precursors in G-5-2$^+$ fetal liver cell populations of days 13 and 14 of gestation developing into LPS-reactive B cells under various conditions of culture. Fetal liver cells were cultured either in the presence (●, ▽) or absence (○) of a layer of irradiated fetal liver stromal cells, either in the presence (●) or absence (▽) of IL-6 and IL-7 (see Materials and Methods), in some cultures in the presence of the mAb, STR4 (□) or STR10 (■). Frequencies of G-5-2$^+$ precursors of LPS-reactive B cells ex vivo of fetal liver at days 14 and 19 of gestation for comparison are 1 in 7 × 10^4 and 1 in 50, respectively. The G-5-2-sorted fetal liver cells were removed at various days after coculture with irradiated fetal liver stromal cells (· · ·//· · ·) and replated under limiting dilutions in rat thymus cells and LPS. Assays for the development of clones of IgM-PFC were done at the equivalent time of day 5 after birth.

vides the conditions for development to mitogen-reactive B cells at high frequencies. Thus, we investigated how long this environment had to be present for the precursors to reach the mitogen-reactive stage as assayed in the presence of rat thymus cells and LPS in limiting dilution analysis. Previous experiments have shown that the LPS-reactive stage is reached in culture at the time equivalent to day 19 of gestation (1-3, 26, 27).

G-5-2$^+$ precursors of day 14 fetal liver were cocultured with irradiated stromal cells for either 1, 2, 3, 4, or 5 d, and then replated in limiting dilutions with LPS and rat thymus cells. The results in Fig. 3 show that the frequencies of LPS-reactive B cells remained low (between 1 in 3,000 and 1 in 1,000) until the time equivalent to day 16 when it changed to between 1 in 100 and 1 in 30 within 24 h. This indicates that B cell development is stroma cell dependent until day 16 of gestation, and becomes independent thereafter. This change occurs at the time when adherence of precursors to stromal cells is lost and when pre-B cells become sIg$^+$ in these cultures (see below). The rapid change in frequencies of precursors indicates that this development is synchronous for the majority of the precursors.

The same rapid change from stromal cell dependence to independence was observed in experiments where G-5-2$^+$ cells at various stages of development in vivo in fetal liver were cultured in limiting dilutions with LPS and rat thymus cells immediately after FACS. Results in Fig. 2 show that G-5-2$^+$ fetal liver cells from days 14 and 15 of gestation did not develop in high frequencies to mitogen-reactive B cells, while from day 16 onwards they did, indicating a change in their independence of the fetal liver cell environment, e.g., as shown above in vitro on stromal cells.

Limited Proliferation of Precursors in the Presence of Stromal Cells and/or ILs, but Change in Adherence. When G-5-2$^+$ B cell precursors from day 14 fetal liver were plated on layers of adherent irradiated embryonic stromal cells, they adhered in clusters to the stromal cells. They remained adherent for 2 d, then became enlarged and detached from the stromal cell layer. At best, only a twofold increase in the number of cells cultured in the absence or presence of either IL-3 or IL-6 plus IL-7, with or without stromal cells, was observed over a 5-d culture period (Fig. 4, *top*). These results provide evidence that G-5-2$^+$ B cell precursors do not proliferate polyclonally at a high rate. They suggest that the precursors change their interactions with stromal cells at a time equivalent to day 16 of gestation from adherent to nonadherent.

Inhibition of the Influence of Stromal Cells by Specific mAbs. Adherence to embryonic stromal cell layers could also be observed with Abelson murine leukemia virus–transformed B cell lymphomas, but not with mature B or T cells (data not shown). This indicated that transformed pre-B cell lines could be used to assay inhibition of adherence by specific ligands such as mAbs. mAbs were raised against embryonic stromal cells from day 13 fetal liver expanded for 2 wk. Two of them, STR4 and STR10, interfered with the binding of the Abelson murine leukemia virus–transformed pre-B cell line 40E1 to stromal cells and stained stromal cells as well as pre-B lymphoma cells, as detected by FACS. These mAbs were then tested in cocultures of G-5-2$^+$ precursors of day 14 fetal liver with embryonic stromal cells for their capacity to influence the development to mitogen-reactive B cells. Both mAbs inhibited the adherence of the precursor to the stromal cell layer. Both mAbs also inhibited the development to mitogen-reactive B cell, but did not influence the number of cells in culture. As controls, the isotype-matched progenitor, pre-B cell-, and B cell-specific
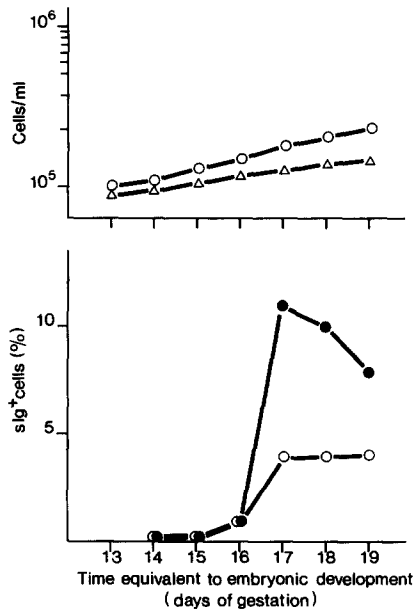
FIGURE 4. (*Top*) Growth of G-5-2$^+$ cells in the presence of mIL-3 (O) and mIL-7 (Δ) on layers of adherent, irradiated fetal liver stromal cells. No growth was detectable in the absence of mILs and/or in the absence of stromal cells. The concentration of cells in the absence of any ILs and in the absence of stromal cells was 8 x 10^4/ml at the time equivalent to day 19 of gestation. (*Bottom*) Appearance of sIg$^+$ cells in cultures of G-5-2-sorted precursors after coculture with irradiated fetal liver stromal cells. Two experiments (O, ●) are shown.

rat mAb 14.8 (anti-B220), as well as the nonmatched, but pre-B cell–specific mouse mAb G-5-2, at the same concentrations and under the same conditions, did not inhibit this stromal cell–dependent development to mitogen-reactive B cells. So far, we have not yet obtained an isotype-matched, stromal cell–binding mAb with no inhibitory effect, an antibody that could be used as another negative control in these experiments.

Development of sIg$^+$ Cells.   Cultures of G-5-2$^+$ precursor cells of day 14 fetal liver on irradiated stromal cells were assayed each consecutive day for the development of sIg$^+$ cells. The results in Fig. 4 (*bottom*) show that within 24 h, in the time between the equivalent of days 16 and 17 of gestation, 5–10% of all cultured cells became sIg$^+$ and remained in that state for the next 2 d. Both mAbs STR4 and STR10 inhibited the development of sIg$^+$ cells (data not shown). These results indicate that the change to sIg$^+$ cells occurs in a majority of G-5-2$^+$-sorted cells, and does so synchronously for the majority of them. This change occurs at the same time that precursors become independent of stromal cells in their development to mitogen-reactive B cells. Based on these results, a model of B lymphocyte lineage development (Fig. 5) is discussed below.

Discussion

Development of B lineage cells from pluripotent stem cells and committed progenitors has been found to be dependent on interactions with so called stromal cells in organs where B cells are generated, e.g., mainly in fetal liver and bone marrow of the mouse. Tissue culture conditions and stromal cells lines have been developed with which the contributions of stromal cells to B lineage development can be studied in vitro (7–15). Once B lineage development has become stromal cell independent,

single cells can be followed in limiting dilutions with rat thymus filler cells (1–3) or other feeder cells (4), and under the stimulating influence of LPS in their development to clones of IgM-secreting PFC (24). In fetal liver this later development is programmed in time, so that the majority of all cells in vivo or in vitro gain mitogen reactivity at the same time, i.e., at day 19 of gestation (1–3, 26, 27). It then takes another 5 d to stimulate these mature B cells to the peak of clonal proliferation and maturation to IgM PFC (24). The results presented in this paper confirm the observations that two phases are distinguishable, one stromal cell dependent, the other independent (36). Our results specify the time of embryonic development when this change occurs, i.e., between days 15 and 16 of gestation. They show that the change occurs in the majority of all cells at the same time. The two mAbs able to interfere with the interactions of B cell precursors and stromal cells that we have developed should be useful in studies of the molecular and cellular requirements of such interactions necessary to induce the precursor to become sIg$^+$, and then mitogen-reactive. Our results further support the idea that specific cellular contacts between the progenitors/precursors and the stromal cells mediate the induction to the development of mature B cells.

Whenever G-5-2$^+$ precursor cells of day 13, 14, or 15 fetal liver were plated immediately in rat filler cells, the change from stromal cell dependence to independence was observed to occur between days 15 and 16. On the other hand, when these purified precursor cells were first plated on stromal cells and then replated in the time equivalent until day 19 of gestation in rat filler cells, the change occurred almost 24 h later (compare Figs. 1–3). This suggests that a second handling of the precursors (washing, cooling down, etc.) might delay the normal development. Therefore, we think that the time of change to stromal cell independence in vivo is between days 15 and 16 of gestation in fetal liver.

None of the exogenously added ILs had any influence on the purified precursor cells that would replace the requirement for stromal cells in the development towards mature B cells. Similar findings have been reported for adult bone marrow precursors (21). It, however, does not exclude that ILs, notably IL-7 (18–20) and IL-1 (21), could play a role in this development. The stromal cells that we use could well synthesize these ILs and thereby provide, endogenously and locally, the necessary growth and differentiation signals.

The contacts with stromal cells, and any IL that may be produced endogenously in this interaction, do, however, not induce extensive pre-B cell proliferation (Fig. 4, top). This is in line with many observations (37, 38) that point to a very limited proliferation of precursors between days 14 and 19 of gestation in fetal liver. In this time of gestation, all rearrangements of V gene segments of H and L chain genes take place in a time-ordered fashion (5, 6). If rearrangements occur when cells divide, then it is tempting to speculate that every division at this time of development in fetal liver is critical. The first division might rearrange D$_H$ to J$_H$, the second V$_H$ to D$_H$J$_H$, the third V$_\kappa$ to J$_\kappa$, and a fourth Vλ to Jλ. A very well ordered machinery for these rearrangement processes should exist, controlled in its activity by the interactions with stromal cells (Fig. 5).

At the end of the rearrangement processes, sIg$^+$ cells appear, provided that these rearrangements were functional, and provided that the Ig could be transported to and inserted into the surface membrane. The rearrangement process is known to
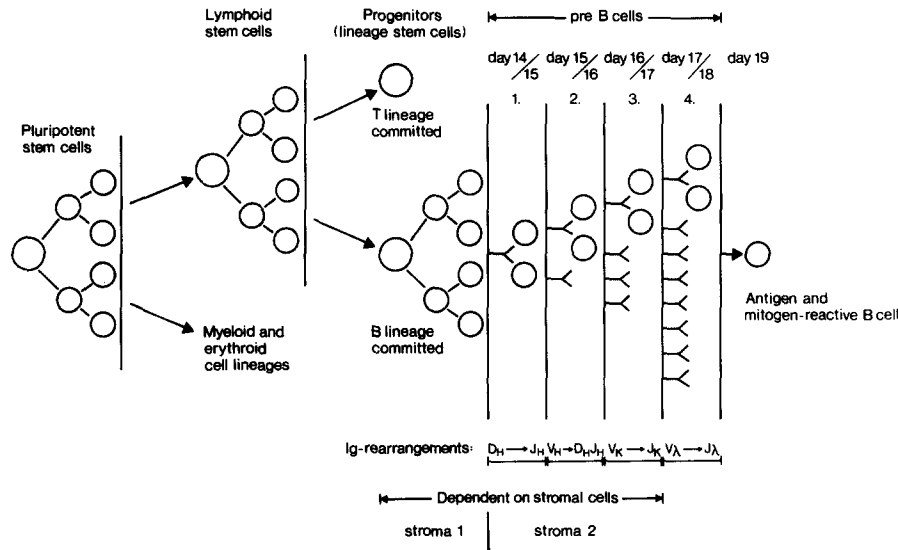
FIGURE 5. A proposed scheme of B lymphocyte lineage development in fetal liver. Stroma 1 and stroma 2 are two types of fetal liver stromal cells proposed to either induce proliferation of progenitors without differentiation to Ig gene rearrangements (stroma 1), or to induce Ig gene rearrangements in critical steps of divisions (stroma 2).

lead not only to productive, but also to nonproductive Ig genes, due to incomplete, out-of-frame, and pseudogene rearrangements in either the H and/or L chain genes. Consequently, no sIg can be made. It is difficult to estimate how high the percentage of nonproductive rearrangements should be, since we do not know whether the rearrangement processes are purely stochastic, or guided by molecular interactions that favor productive rearrangements. We also do not know how many pseudogenes participate in these rearrangement processes. Our findings that between 5 and 10% of all G-5-2$^+$ precursors develop to sIg$^+$ cells (Fig. 4, *bottom*) suggests that such nonproductive rearrangements could happen in 90% or more of all precursors. This number is, at least, within the range of frequencies of productive vs. nonproductive rearrangements expected from a stochastic process of rearrangements.

The appearance of Ig on the surface of pre-B cells at day 16 of gestation is obviously dependent on successful rearrangements of V gene segments, and on transcription and translation of the genes. The deposition of Ig in the surface membrane, however, could also be dependent on interactions with neighboring molecules, like mb-1 (39), which could have functions similar to the CD3 complex associated with TCR-α and -β, respectively, and γ and δ chains (reviewed in reference 40). mb-1 is expressed from the earliest stages of pre-B cell development, i.e., when D$_H$ segments rearrange to J$_H$ segments, and continues to be expressed to the stage of the mature B cell. While this is true for mRNA expression, a protein with CD3 line functions might only be expressed at a given, later stage of precursor development. This could, then, also control expression of surface Ig on pre-B cells and its transition from a stromal cell–dependent to an independent stage.

Particularly striking is the synchrony of B cell development in one wave in fetal

liver. This indicates that stem cells and progenitors may populate the fetal liver at any time in development, but are induced only within a time window of 24 h, around day 13 of gestation, to enter the pathway of B cell development by Ig gene rearrangements and other processes connected with this program of differentiation. Before and after this time, progenitors may exist but they are not drawn. It is reasonable to assume as a working hypothesis that the environment of stromal cells is competent only for this short period of time to convey the necessary interactions with progenitors. Synchrony of B cell development in this wave is high for a remarkably long period of time in embryonic development, i.e., for 6 d between days 13 and 19 of gestation, pointing to an ordered process all along the way. It is, therefore, not very likely that normal cells at any one of these stages of B cell development would, without transformation, be capable of extensive cell proliferation.

Other waves of lymphocyte development are known. In mice, the wave of B cell development in fetal liver is preceded by one in fetal placenta and embryonic blood (41). In sheep (and maybe in other species), a late wave has been observed in Peyer's patches (42). In chicken, colonization of the bursa occurs at a specific time before hatching (43). Development of T cells in the thymus of birds occurs in three waves (44). Initially, these waves are initiated by a similar short 24-h period of competence of the organs for immigration and induction to development of the precursors. Whether these waves of lymphocyte development use the total repertoire of V gene segments or prefer, as in fetal liver, only part of it (V_H7183), and whether different environments actually select the expressed V genes according to their binding specificities for self determinants in the special environments, remains to be seen.

In bone marrow, throughout life, the generation of B cells is continuous. At the same time, turnover of the mature, primary B cell is high (43, 44). Therefore, if pre-B cells in bone marrow are equally limited in their capacity to proliferate as they rearrange their V segments, become sIg^+ and, finally, mitogen and antigen reactive, a pool of progenitor cells before rearrangements must proliferate very actively. This progenitor pool, possibly reactive to another stromal cell and to IL-7 (45), should continuously generate by stem cell-like proliferation those cells that can then be drawn into the pathway of B cell development at rates that keep up the pool of primary mature B cells (Fig. 5).

Summary

Precursor cells of the B lineage can be enriched from mouse fetal liver by FACS with the aid of the pre-B cell-specific mAb G-5-2. The cells are concomitantly enriched for cells expressing the pre-B cell-specific gene λ5, and for cells developing to LPS-reactive mature B cells. The enriched purified precursors are not influenced by rIL-2 through -7, alone or in combination, to develop to mitogen-reactive, sIg^+ cells. Marginal proliferation of-the precursors is observed in response to IL-3 plus -4, and IL-6 plus -7, and this does not change in the presence of stromal cells. Development to mitogen-reactive, sIg^+ cells is dependent on interactions with embryonic stromal cells from fetal liver. Two mAbs raised against the stromal cells inhibit this development. Two phases of precursor cell development can be distinguished in fetal liver. Between days 13 and 15 of gestation, it is dependent on stromal cell interactions, thereafter, from days 16 to 19, it is independent. A sudden increase in the

number of mitogen-reactive, sIg^+ B lineage cells occurs within 24 h between days 16 and 17. All these results indicate that B cell development occurs in one wave with synchronous steps of changes from a mitogen-insensitive, sIg^-, stromal cell dependent to a mitogen-reactive, sIg^+, stromal cell–independent B lineage line.

References

1. Melchers, F., H. von Boehmer, and R. A. Phillips. 1975. B lymphocyte subpopulations in the mouse. Organ distribution and ontogeny of immunoglobulin-synthesizing and of mitogen-sensitive cells. *Transplant. Rev.* 25:26.
2. Melchers, F. 1977. B lymphocyte development in fetal liver. I. Development of reactivities to B cell mitogens "in vivo" and "in vitro". *Eur. J. Immunol.* 7:476.
3. Melchers, F. 1977. B lymphocyte development in fetal liver. II. Frequencies of precursor B cells during gestation. *Eur. J. Immunol.* 7:482.
4. Paige, C. J., R. H. Gisler, J. P. McKearn, and M. N. Iscove. 1984. Differentiation of murine B cell precursors in agar culture. Frequency, surface marker analysis and requirements for growth of clonable pre-B cells. *Eur. J. Immunol.* 14:979.
5. Alt, F., N. E. Rosenberg, S. Lewis, E. Thomas, and D. Baltimore. 1981. Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: rearrangement of heavy but not light chain genes. *Cell.* 27:381.
6. Maki, R., J. Kearney, C. Paige, and S. Tonegawa. 1982. Immunoglobulin gene rearrangements in immature B cells. *Science (Wash. DC).* 209:1366.
7. Kincade, P. W., G. Lee, C. J. Paige, and M. P. Street. 1981. Cellular interactions affecting the maturation of murine B lymphocyte precursors *in vitro. J. Immunol.* 127:255.
8. Whitlock, C., K. Denis, D. Robertson, and O. N. Witte. 1985. In vitro analysis of murine B cell development. *Annu. Rev. Immunol.* 3:213.
9. Dorshkind, K., A. Johnson, L. Collins, G. M. Keller, and R. A. Phillips. 1986. Generation of purified stromal cell cultures that support lymphoid and myeloid precursors. *J. Immunol. Methods.* 89:37.
10. Witte, P. L., M. Robertson, A. Henley, M. G. Low, D. L. Stiers, S. Perkins, R. A. Fleischman, and P. W. Kincade. 1987. Relationships between B-lineage lymphocytes and stromal cells in long term bone marrow cultures. *Eur. J. Immunol.* 17:1473.
11. Pietrangeli, L. E., S.-I. Hayashi, and P. W. Kincade. 1988. Stromal cell lines which support lymphocyte growth: characterization, sensitivity to radiation and responsiveness to growth factors. *Eur. J. Immunol.* 18:863.
12. Kinashi, T., K. Inaba, T. Tsubata, K. Tashiro, R. Palacios, and T. Honjo. 1988. Differentiation of an interleukin 3-dependent precursor B cell clone into immunoglobulin-producing cells in vitro. *Proc. Natl. Acad. Sci. USA.* 85:4473.
13. Palacios, R., S. Stuber, and A. Rolink. 1989. The epigenetic influences of bone marrow and fetal liver stroma liver cells on the developmental potential of $Ly1^+$ pro-B lymphocyte clones. *Eur. J. Immunol.* 19:347.
14. Kincade, P. W., G. Lee, L. E. Pietrangeli, S.-I. Hayashi, and J. M. Grimble. 1989. Cells and molecules which regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:11.
15. Gisler, R. H., A. Söderberg, and M. Kamber. 1987. Functional maturation of murine B lymphocyte precursors. II. Analysis of cells required from the bone marrow microenvironment. *J. Immunol.* 138:2433.

16. Palacios, R., G. Henson, M. Steinmetz, and J. P. McKearn. 1984. Interleukin-3 supports growth of mouse pre-B cell clones in vitro. *Nature (Lond.)*. 309:126.

17. Palacios, R., and M. Steinmetz. 1985. IL-3 dependent bone marrow clones that express B-220 surface antigen, contain Ig$^+$ genes in germline configuration and generate B lymphocytes *in vivo*. *Cell*. 41:727.

18. Palacios, R., H. Karasuyama, and A. Rolink. 1987. Lyl$^+$ Pro-B lymphocyte clones. Phenotype, growth requirements and differentiation *in vitro* and *in vivo*. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3687.

19. Namen, A. E., A. E. Schmierer, C. J. March, R. W. Overell, L. S. Park, D. L. Urdal, and D. Y. Mochizuki. 1988. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J. Exp. Med.* 167:988.

20. Namen, A. E., S. Lupton, K. Hjenild, J. Wijnall, D. Y. Moohizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, S. Gillis, D. Cosman, and R. G. Goodwin. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature (Lond.)*. 333:571.

21. Gisler, R. H., C. Schlienger, A. Söderberg, F. Ledermann, and J. D. Lambris. 1988. Functional maturation of murine B lymphocyte precursors. III. Soluble factors involved in the regulation of growth and differentiation. *Mol. Immunol.* 25:1113.

22. Owen, J. J. T., M. D. Cooper, and M. C. Raff. 1974. In vitro generation of B lymphocytes in mouse fetal liver, a mammalian "bursa equivalent." *Nature (Lond.)*. 249:361.

23. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Wash. DC)*. 140:405.

24. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type of class. *Eur. J. Immunol.* 6:588.

25. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5 using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97.

26. Strasser, A. 1988. Analysis of the repertoire of autoreactive hematopoietic cell-specific B cells in normal and in graft versus host induced auto-immune mice. Ph.D. thesis. University of Basel, Switzerland. 153 pp.

27. Strasser, A. 1988. PB:76: a novel surface glycoprotein preferentially expressed on mouse pre-B cells and plasma cells detected by the monoclonal antibody G-5-2. *Eur. J. Immunol.* 18:1803.

28. Sakaguchi, N., and F. Melchers. 1986. λ_5, a new light-chain-related locus selectively expressed in pre-B lymphocytes. *Nature (Lond.)*. 324:579.

29. Sakaguchi, N., C. Berger, and F. Melchers. 1986. Isolation of a cDNA copy of an RNA species expressed in murine pre-B cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2139.

30. Warner, N. L., M. J. Daley, J. Rahey, and C. Spellman. 1979. Flow cytometry analysis of murine B lymphoma differentiation. *Immunol. Rev.* 48:197.

31. Sibley, C. H., S. J. Ewald, H. R. Kehry, R. H. Douglas, W. C. Raschke, and L. E. Hood. 1980. Characterization of multiple immunoglobulin μ-chains synthesized by two clones of a B cell lymphoma. *J. Immunol.* 125:2097.

32. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipids in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* 147:923.

33. Rolink, A. G., F. Melchers, and R. Palacios. 1989. Monoclonal antibodies reactive with the mouse interleukin 5 receptor. *J. Exp. Med.* 169:1693.

34. Leptin, M. 1985. Monoclonal antibodies specific for murine IgM. II. Activation of B lymphocytes by monoclonal antibodies specific for the four constant domains of IgM. *Eur. J. Immunol.* 15:131.

35. Björck, L., and G. Kronvall. 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J. Immunol.* 133:969.

36. Gisler, R. H., G. Holländer, and A. Söderberg. 1987. Functional maturation of murine B lymphocyte precursors. I. Selection of adherent cell-dependent precursors from bone marrow and fetal liver. *J. Immunol.* 138:2427.

37. Melchers, F. 1978. Differentiation of normal and neoplastic hematopoietic cells. *Cold Spring Harbor Conf. Cell Proliferation.* 5:485.

38. Park, Y. H., and A. G. Osmond. 1987. Phenotype and proliferation of early precursor cells in mouse bone marrow. *J. Exp. Med.* 165:444.

39. Sakaguchi, N., S.-I. Kashiwamura, M. Kimoto, P. Thalmann, and F. Melchers. 1988. B lymphocyte-lineage-restricted expression of mb.1, a gene with CD3-like structural properties. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3457.

40. Clevers, H., B. Alarcon, T. Wileman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629.

41. Melchers, F. 1979. Three waves of B lymphocyte development during embryonic development in the mouse. *In* Cell Lineage, Stem Cells and Cell Determination, INSERM Symposium No. 10. N. Le Douarin, editor. Elsevier Science Publishers B. V., Amsterdam. 281–288.

42. Reynolds, J. D., and B. Morris. 1983. The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur. J. Immunol.* 13:627.

43. Pink, J. R. L., O. Lassila, and O. Vainio. 1987. B-lymphocytes and their self-renewal. *In* Avian Immunology. A. Toivanen and P. Toivanen, editors. CRC Press, Inc., Boca Raton, FL. 65–78.

44. Le Douarin, N. M. 1978. Ontogeny of hematopoietic organs studied in avian embryo interspecific chimeras, in differentiation of normal and neoplastic hematopoietic cells. *Cold Spring Harbor Conf. Cell Proliferation.* 5:5.

45. Takeda, S., S. Gillis, and R. Palacios. 1989. *In vitro* effects of recombinant interleukin 7 on growth and differentiation of bone marrow pro-B and pro-T-lymphocyte clones and fetal thymocyte clones. *Proc. Natl. Acad. Sci. USA.* 86:1634.