REGULATED PROGRESSION OF B LYMPHOCYTE DIFFERENTIATION FROM CULTURED FETAL LIVER

By KATHLEEN A. DENIS, KENNETH DORSHKIND,* AND OWEN N. WITTE

*From the Molecular Biology Institute and Department of Microbiology, and Howard Hughes Medical Institute, University of California at Los Angeles, Los Angeles, California 90024; and the *Division of Biomedical Sciences, University of California at Riverside, Riverside, California 92521*

The fetal liver is a principal site of B lymphopoiesis before the initiation of B cell development in the bone marrow (reviewed in references 1, 2). The study of the differentiation events that occur in these tissues has been confounded by the large number of cell types and developmental stages present in these biological compartments. We have previously described (3–5) a method for long-term culture of murine fetal liver that produces cells that are morphologically and functionally lymphoid and appear to be restricted to the B lymphocyte lineage. The lymphoid fetal liver culture (LFLC)[1] cells retain characteristics of the early B lineage cells found in fetal liver (3) and provide an enriched source of these cells for study. The cultured cells are predominantly pre–pre-B lymphocytes, before Ig expression, and cytoplasmic μ^+ pre-B lymphocytes. ~10% of the LFLC cells have not undergone Ig gene rearrangement. These LFLC cells do not secrete Ig and only ≤1% are $sIgM^+$ (3).

The failure of LFLC cells to express sIgM could be due to an in vitro developmental block or an intrinsic defect in the B lineage cells derived from fetal liver (6). Investigations that would distinguish between these possibilities would be dependent upon providing the cells from LFLC with an optimal environment in which the entire B cell differentiative pathway could proceed. To achieve this aim, cells from LFLC have been transplanted into mice with severe combined immunodeficiency disease (SCID) (7). SCID mice provide an ideal environment for such studies, as they lack mature T and B cells, are serum Ig^-, and are congenic with the BALB/c strain used for the initiation of these long-term cultures.

The results show that LFLC cells can reconstitute splenic B cells and serum IgM upon transplantation into SCID mice. However, they require a source of exogenous T cells to mediate the full range of B cell function such as complete class switching and responsiveness to T-dependent antigens. The use of this

This work was supported by National Institutes of Health grant AI-21256 to K. Dorshkind, and by grants from NIH and the March of Dimes to O. N. Witte. K. A. Denis was a Lievre Senior Fellow of the American Cancer Society, California Division. O. N. Witte is a Faculty Scholar of the American Cancer Society. Address correspondence to K. A. Denis, Molecular Biology Institute, UCLA, Los Angeles, CA 90024.

[1] *Abbreviations used in this paper:* LFLC, lymphoid fetal liver culture; SCID, severe combined immunodeficiency disease; sIg, surface Ig.

combination in vitro/in vivo system has provided a means by which B lymphopoiesis can be studied in a controlled manner from early fetal liver precursors to mature end cells. The ability to regulate the progression of B lymphocyte differentiation at discrete stages will enable characterization of the genetic, molecular, and cellular influences upon this process.

Materials and Methods

Mice. BALB/c mice, originally obtained from Cumberland View Farms (Clinton, TN), and BAB-14 mice, obtained from Dr. I. L. Weissman, Stanford University, Stanford, CA, were bred and maintained at the University of California, Los Angeles. Mice homozygous for the *scid* mutation (7), IgH congenic to BALB/c mice, are designated as CB-17 scid and will be referred to as SCID mice. These mice were obtained from a colony at the Ontario Cancer Institute, Toronto, Canada (a gift of Drs. R. A. Phillips and M. J. Bosma) and were bred and maintained at the University of California, Riverside. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Long-Term Cell Cultures. Bone marrow stromal cell feeder layers were prepared from the femoral bone marrow cells of BAB-14 mice in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (Gibco Laboratories) plus 50 μM 2-ME, as detailed in Whitlock et al. (8). Fetal liver cells from midgestational BALB/c mice (13–16 d after the appearance of a vaginal plug) were suspended in the above medium and placed upon 4–6-wk-old feeder layers at a cell density of 5×10^5 cells/ml as described by Denis et al. (3). Nonadherent cells were transfered to a fresh feeder layer on day 7 and the LFLC were maintained as previously described (3).

SCID Mice Reconstitution. 4–6-wk-old SCID mice received an intravenous injection of 5×10^6 cells of either fresh BALB/c fetal liver or nonadherent cells harvested from 6–8-wk-old LFLC. For experiments that augmented T-lymphocyte function, 10^6 fresh BAB-14 thymocytes that had been passed over nylon wool were added to the inoculum. Mice were housed in microisolator cages (Lab Products Inc., Maywood, NJ) during the 8–12-wk reconstitution period. After this, animals were killed by cervical dislocation and serum and tissues were collected for analysis.

B and T Lymphocyte Analysis. Colony-forming B cells (9) and surface Ig (sIg)-positive B cells were assayed as previously described (10). Cytotoxic T cells were assayed in one-way mixed lymphocyte cultures with stimulation by C57BL/6 splenocytes and cytotoxic activity against ^{51}Cr-labeled EL4 cells as detailed by Dorshkind et al. (10).

Serum Ig Analysis. Ig present in the serum of the reconstituted mice was analyzed by both solid-phase ELISA and two-dimensional gel electrophoresis. For ELISA, polyvinyl chloride plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight with either goat anti–mouse Ig (BioRad Laboratories, Richmond, CA) for total Ig quantitation or TNP-transferrin for TNP-specific antibody quantitation at 10 μg/ml in borate-buffered saline (BBS), pH 8.4. After blocking the plates with 1% OVA in BBS, dilutions of normal mouse serum, SCID serum, reconstituted SCID serum, or purified mouse Ig (Litton Bionetics, Charleston, SC) were added. After a 2-h incubation at room temperature, the plates were washed with BBS + 0.02% Tween-20 (Sigma Chemical Co., St. Louis, MO) and incubated for an additional 2 h with peroxidase-conjugated Ig class-specific antisera (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Positive wells were detected after extensive washing using 2,2′-azino-di-(3-ethyl-benzthiazline-sulfonic acid (Kirkegaard & Perry Laboratories) and reading the absorbance at 414 nm using a BioRad Laboratories reader (No. 2550 E1A). Subisotype determination was done using the BioRad Laboratories Mouse Typer Panel.

Two-dimensional gel analysis was performed with ^{125}I-labeled serum that was immunoprecipitated with either rabbit anti–mouse Ig (Dako Corp., Santa Barbara, CA) followed by *Staphylococcus aureus* or TNP-transferrin coupled to Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). The precipitates were washed extensively and subjected to isoelectric focusing followed by SDS–10% PAGE as previously described (11, 12).

Immunization for Specific Immune Responses. BALB/c, SCID, or reconstituted SCID

TABLE I
B Cell Reconstitution of SCID Mice with Fresh and Cultured Fetal Liver

Reconstituting cells	Spleen			Serum	
	Cell	CFU-B	sIgM$^+$	IgM	IgG
	$\times 10^6$	$\times 10^5$	%	*mg/ml*	*mg/ml*
None	6.6 (2–10)	0	0	<0.01	<0.01
Fresh FL	77.9 (25–110)	1,206 (425–1725)	18.1 (4.0–29.9)	1.7 (1.0–2.5)	14.0 (10–16)
LFLC	57.5 (11.2–200)	71.4 (1–250)	3.4 (0.4–16.7)	0.51 (0.1–1.0)	0.93 (0.1–2.0)

4-wk-old SCID mice received an intravenous injection of 5×10^6 of the indicated cells. Sera and tissues were harvested 8–12 wk later. Assays were performed as detailed in Materials and Methods, data are expressed as the mean and range (in parentheses) of the determinations from at least eight individual animals.

mice were immunized with either TNP-ficoll (70 μg i.p.) or TNP-BSA (100 μg i.p. in CFA; Biosearch, San Rafael, CA). A secondary immunization was given 2 wk later and the mice were bled via the tail vein 6 d later. Serum was spun out and stored at −70°C before analysis.

Antiallotype Immunoblotting. Serum from reconstituted or control animals was immunoprecipitated with rabbit anti-mouse Ig (Dako Corp.) followed by *S. aureus*. The reduced immunoprecipitates were run upon SDS–10% polyacrylamide gels and subsequently blotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose was blocked with 10% nonfat milk in Tris-buffered saline and incubated with sheep anti-mouse IgG2a (1a allotype-specific; Nordic Laboratories, El Toro, CA) followed by peroxidase-conjugated rabbit anti-sheep (Kirkegaard & Perry Laboratories) to detect specific allotype production. Total Ig production was monitored using peroxidase-labeled goat anti-mouse Ig.

Results

Both Fresh Fetal Liver and LFLC Can Reconstitute Splenic B Lymphocytes and Serum Ig in SCID Mice. Long-term cultures derived from fetal liver were composed of cells from very early in the B lymphoid lineage. <1% of these cells bore sIgM and a subpopulation retained a germline configuration of the Ig heavy chain JH locus (3). The distribution of early B lymphoid cells in these cultures has been previously described (3, 4). These cells could represent either defective B lymphocytes that would not survive in vivo or a normal intermediate in development that lacked further differentiation signals. To distinguish between these possibilities, reconstitution of SCID mice was done with both fresh and cultured fetal liver. 5×10^6 of fresh fetal liver or LFLC cells were injected into SCID mice and 8–10 wk later sera and tissues were collected for analysis. Both reconstituting cell sources raised the spleen cell number ~10-fold (Table I), indicating cellular reconstitution and proliferation. Although a few mice had splenomegaly, no tumor formation was observed in any of the reconstituted mice that were all healthy at the time they were killed. Mature B lymphocytes, as measured by CFU-B and sIgM$^+$ cells, were present in the spleens of all mice injected with either fresh fetal liver or LFLC. Fresh fetal liver reconstitution gave levels of CFU-B and sIgM$^+$ cells comparable to normal BALB/c mice (data

for normal mice not shown). LFLC reconstitution yielded only 5–20% of normal CFU-B and sIg^+ cells. However, these numbers were very significant when compared with the total absence of these cell types in unreconstituted SCID mice and were in the range observed with reconstitution of these mice with lymphoid bone marrow cultures (10).

SCID mice reconstituted with fresh fetal liver and LFLC cells had demonstrable levels of IgM and IgG in their serum (Table I). Those animals reconstituted with fresh fetal liver had levels of serum IgM and IgG that equaled or exceeded those found in normal BALB/c mice. LFLC-reconstituted SCID mice had IgM levels ~50% of normal BALB/c serum but the IgG concentration was usually <10% of normal. None of the unreconstituted SCID mice that were tested, including those controls that underwent immunization with defined antigen, had serum Ig within the range of detection of the ELISA assay used (<2 μg/ml).

The relatively long reconstitution period made it possible that a limited number of pre-B or B cells committed to specific VDJ_H arrangements had proliferated and were mediating a mono- or pauciclonal reconstitution. To examine this directly, the heterogeneity of the Ig heavy and light chains synthesized by the reconstituted mice was examined using two-dimensional gel electrophoresis of iodinated serum Ig isolated by immunoprecipitation. In this manner, both size and charge diversity of the Ig chains were evaluated. Serum from fresh fetal liver and LFLC-reconstituted SCID mice displayed μ and light chain heterogeneity (Fig. 1) similar to that seen with normal BALB/c serum when evaluated by this method (10). This strongly argues against the theory that reconstitution of these mice is mediated by one or a small number of committed B lymphocytes.

The size differences of the heavy chain subclasses allows visualization of the isotype representation in the sera. Reconstitution of serum γ Ig heavy chains was very poor in the SCID mice reconstituted with LFLC cells and two-dimensional analysis showed these γ chains to be very restricted in both quantity and isoelectric diversity (Fig. 1, *C* and *D*). Glycosylation of the heavy chain molecules results in heterogeneity of the charge of each individual heavy chain and could only be used as a rough guide of variable region diversity. The number of light chain species served as a more reliable measure of Ig diversity in these sera, since they are not altered by glycosylation.

Isotype-specific ELISA was used to further examine the γ heavy chains synthesized in the serum of the reconstituted mice. SCID mice reconstituted with fresh fetal liver had a distribution of IgG subisotypes in their serum very similar to BALB/c controls (Fig. 2). In contrast, only IgM and IgG3 were at levels within 50% of normal in the serum of the LFLC-reconstituted mice. The remainder of the IgG subisotypes were present at <10% of normal levels (Fig. 2). Serum IgA levels were 25% of normal or less in all reconstituted animals, perhaps reflecting the sterile environment and lack of gastrointestinal stimulation.

Functional T Lymphocytes Are Not Detected in LFLC-reconstituted Mice. Studies in vivo and in vitro of antibody responses have demonstrated T cell mediated enhancement of Ig class switching (13–16). In the absence of mature T lymphocytes or T cell–derived factors, antibody production of each isotype correlated directly with the 5′ to 3′ Igh-C gene order (M-D-G3-G1-G2b-G2aA; reference 17). The inefficient class switching of the LFLC derived B lymphocytes that

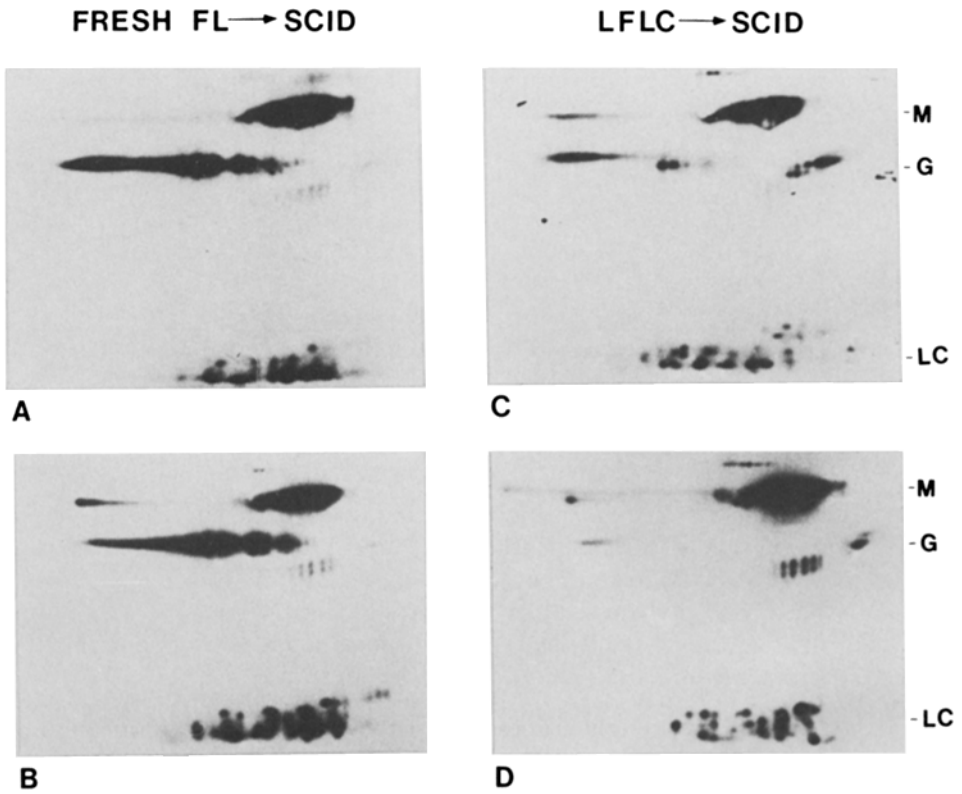

FIGURE 1. Two-dimensional gel electrophoresis of ^{125}I-labeled, immunoprecipitated Ig from the sera of SCID mice reconstituted with either fresh fetal liver (*A* and *B*) or LFLC (*C* and *D*). Separation was by isoelectric focusing in the horizontal direction and by SDS–10% polyacrylamide gel in the vertical. μ (*M*) and γ (*G*) heavy chains and light chains (*LC*) are as indicated.

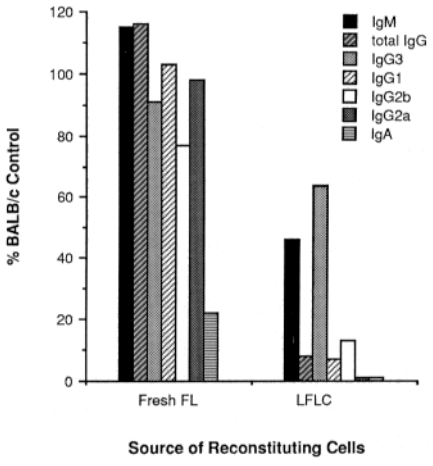

FIGURE 2. Serum isotypes of reconstituted SCID mice. The distribution of isotypes present in the sera of SCID mice reconstituted by the cells indicated was determined by ELISA (See Materials and Methods). Data represent the averages of at least six individual mice and are expressed as a percentage of the level of isotype found in normal BALB/c serum.

reconstituted the SCID mice, resulting primarily in IgG3, was perhaps due to a similar lack of T lymphocytes.

Two independent methods were used to detect T cells in LFLC reconstituted

TABLE II

T Cell–dependent Responses in Fresh and Cultured Fetal Liver Reconstituted SCID Mice

Reconstituting cells	Spleen cytotoxicity	Serum anti–TNP-Ficoll		Serum anti–TNP-BSA	
		IgM	IgG	IgM	IgG
		μg/ml		*μg/ml*	
None	0	<0.01	<0.01	<0.01	<0.01
Fresh FL	55.1	30	25	212.5	331.2
	(17–80)	(10–50)	(20–30)	(100–300)	(125–400)
LFLC	0	28	5	22	0.5
		(2–80)	(3–10)	(<0.1–80)	(<0.01–5)

4-wk-old SCID mice received an intravenous injection with 5×10^6 of the indicated cells; sera and tissues were harvested 8–12 wk later. Assays were performed as detailed in Materials and Methods, data are expressed as the mean and range (in parentheses) of the determinations from at least eight individual animals.

SCID mice. Cytotoxic T lymphocytes were measured in the spleens of the reconstituted animals after sensitization in mixed lymphocyte culture. The fresh fetal liver–reconstituted SCID mice had splenic CTLs comparable to normal BALB/c mice (Table II). In contrast, the LFLC-reconstituted mice were devoid of measurable CTLs. Evidence of gross morphological thymus reconstitution was seen in the fresh fetal liver–reconstituted animals but not in the LFLC–reconstituted animals (data not shown).

T-dependent antibody responses were used as a more sensitive measure for the presence of T lymphocytes in these animals. The mice were immunized 8–10 wk after reconstitution with either TNP-ficoll, a T lymphocyte–independent antigen (18) or TNP-BSA, a T lymphocyte–dependent antigen. Fresh fetal liver–reconstituted mice gave excellent serum antibody responses to both these antigens, as monitored by hapten-specific ELISA (Table II). LFLC-reconstituted mice were responsive to TNP-ficoll but gave a very poor, predominantly IgM response to TNP-BSA (Table II). These data indicated the absence of helper T lymphocytes in the LFLC-reconstituted animals and confirmed the inference made from the heavy chain class-switching data.

Exogenous T Lymphocytes Can Complement the Immune Functions of LFLC-derived B Lymphocytes in SCID Mice. The paucity of T lymphocytes in the LFLC-reconstituted SCID mice did not allow us to assess whether the LFLC-derived B lymphocytes present could respond to T cell–mediated differentiation signals. Perhaps the lack of T lymphocyte–derived differentiation signals was solely responsible for the failure to observe efficient class switching and select antibody production rather than a defect in the LFLC-derived B lymphocytes themselves.

To clarify this, an exogenous source of T lymphocytes was added to the LFLC cells for injection into SCID mice. Thymocytes from BAB-14 mice were used to provide a broad population of T lymphocyte lineage cells that could repopulate the SCID mice. BAB-14-derived cells differ from the BALB/c-derived LFLC cells by Ig allotype markers and a restriction fragment length polymorphism in their Ig heavy chain genes (19). These markers allow the positive identification of both the antibodies produced and the cell populations present in the reconstituted mice.

TABLE III
Serum Reconstitution of SCID Mice with LFLC Plus Thymocytes

Reconstituting cells*	Total		Percent of BALB/c control with subisotypes:				Anti-TNP	
	IgM	IgG	G1	G2a	G2b	G3	IgM	IgG
	mg/ml						*μg/ml*	
LFLC alone								
A.1	0.5	0.5	2	0	5	20	20	0.5
A.2	0.5	0.5	10	1	1	10	30	0.1
B.1	1.0	1.0	1	1	1	10	20	8
B.2	1.5	0.5	2	1	1	10	4	2
C.1	2.0	0.2	1	1	5	80	5	0.1
C.2	1.5	0.1	1	1	1	20	1	0.1
C.3	1.5	0.1	2	1	10	20	1	0.1
Mean	1.2	0.4	2.1	0.9	3.4	24.3	11.7	1.5
LFLC + thymocytes								
A.1	0.8	10.0	80	80	40	40	250	500
A.2	0.5	12.0	80	100	20	50	300	500
B.1	1.0	5.0	80	90	100	90	50	2,000
B.2	0.5	5.0	90	100	90	90	10	1,000
C.1	1.5	8.0	100	100	100	80	50	20
C.2	2.0	5.0	80	80	50	20	20	100
C.3	2.0	5.0	90	70	90	50	50	1,000
Mean	1.2	7.1	85.7	88.6	70.0	60.0	104.2	731.4
Thymocytes alone								
A.1	0.01	0.1	2	0	20	0	0.001	0.001
A.2	0.01	0.1	5	0	10	1	0.001	0.001
B.1	0.05	0.5	5	0	20	0	0.2	0.1
B.2	0.01	0.5	2	0	30	0	0.2	0.1
C.1	0.1	1.0	5	0	20	0	0.2	0.1
C.2	0.1	2.0	10	0	80	0	0.8	0.1
C.3	0.05	0.5	2	0	10	0	5.0	0.1
Mean	0.04	0.7	4.4	0	27.1	0.1	0.9	0.07

* Letters represent individual experiments and numbers represent individual mice in that experiment.

8–10 wk after the coinjection of LFLC and BAB-14 thymocytes into SCID mice, these animals were assayed for reconstitution. As seen previously (Table II), measures of T lymphocyte presence were absent in mice reconstituted with LFLC alone. No CTLs were detected in these animals and none had greater than the background level of 1% $L3T4^+$ cells in their spleen. Mice reconstituted with thymocytes alone possessed both cytotoxic and $L3T4^+$ (range, 2–19%) T lymphocytes in their spleens. No CFU-B were detected in such mice and only 4 of 17 demonstrated very low levels of $sIgM^+$ cells (range, 0.4–1.6%). When the SCID mice were reconstituted with both LFLC and thymocytes, both B and T cell parameters were consistently positive and, importantly, these mice were able to synthesize normal levels of all Ig isotypes and mount a significant humoral response to TNP-BSA.

The serum data from the individual mice in three such experiments are shown in Table III. SCID mice reconstituted with LFLC alone synthesized normal

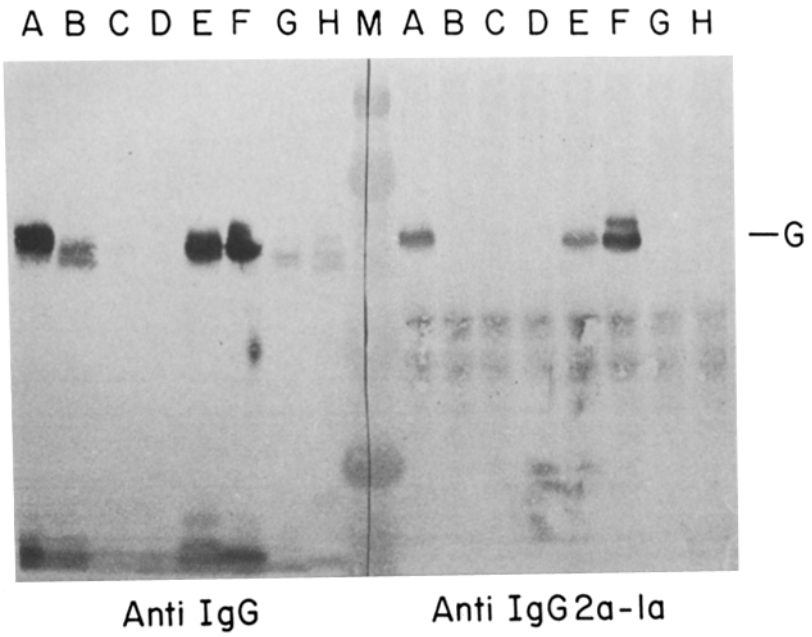

FIGURE 3. Immunoblots of serum Ig from reconstituted SCID mice. Serum Ig was immunoprecipitated, run on an SDS–10% polyacrylamide gel, and blotted onto nitrocellulose as detailed in Materials and Methods. The *left* panel was probed for total IgG and the *right* panel was probed for IgG2a-1a allotype. Molecular weight markers in center were used for realignment. (*A*) BALB/c serum; (*B*) BAB-14 serum; (*C* and *D*) LFLC-reconstituted SCID serum; (*E* and *F*) LFLC plus thymocyte–reconstituted SCID serum; (*G* and *H*) thymocyte alone–reconstituted SCID serum.

levels of serum IgM, were very low in IgG of all subisotypes except G3, and responded poorly to TNP-BSA immunization. Mice reconstituted with both LFLC and thymocytes had normal levels of serum Ig, normal isotype distribution, and made 10–10,000-fold higher IgM and IgG antibody responses to TNP-BSA than mice reconstituted with LFLC alone. Thymocytes alone injected into SCID mice reconstituted low levels of IgG, mainly of the G2b subisotype. IgM and anti-TNP antibody levels were usually just slightly above the threshold of detection by ELISA. We believe that this minimal serum reconstitution in the thymocyte-reconstituted mice (as well as the very low level of sIg^+ cells detected in 4 of 17 of such mice) was mediated by the expansion of mature B lymphocytes present in the blood supply of the thymus or in associated lymph nodes. The observed lack of IgM synthesis and specific antibody responses reinforces this.

To verify that the enhanced Ig production in the doubly reconstituted mice originated from the LFLC-derived B lymphocytes and not passenger B cells in the thymus, antiallotype immunoblots were performed. Total anti-Ig immunoprecipitated serum samples were probed with either total anti-IgG or anti-IgG2a (1a allotype specific). As shown in Fig. 3, *left* panel, high levels of total IgG were detected in the BALB/c and BAB-14 controls (Fig. 3, *A* and *B*) and in the doubly reconstituted mice (*E* and *F*). Low levels of total IgG were detected in the serum of SCID mice reconstituted with LFLC (*C* and *D*) or thymocytes (*G* and *H*) alone. Probing with anti–IgG2a-1a–specific antisera showed the Ig synthesized

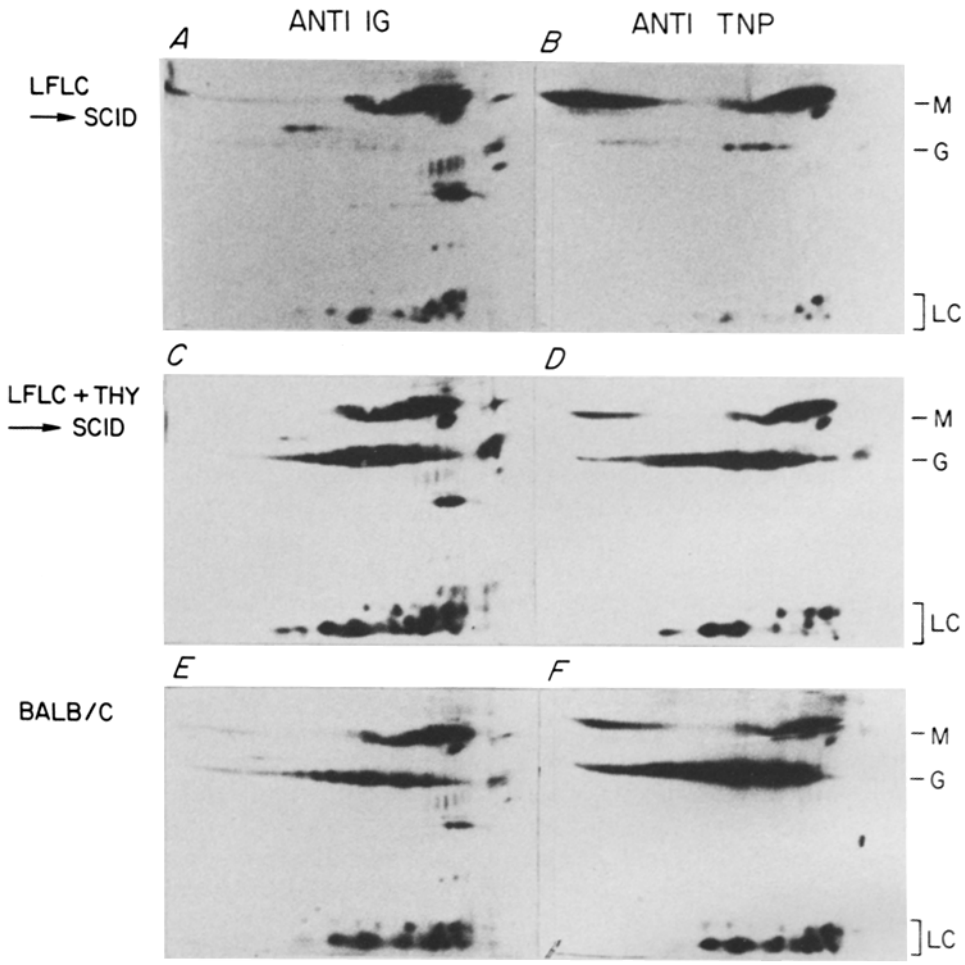

FIGURE 4. Heterogeneity of total serum Ig (*left* panels) and anti-TNP-specific Ig (*right* panels) from SCID mice reconstituted with LFLC (*A* and *B*), LFLC plus thymocytes (*C* and *D*), or normal BALB/c mice (*E* and *F*). See Materials and Methods and Fig. 1 for additional details.

by the SCID mice reconstituted with LFLC plus thymocytes to be of the 1a allotype and thus of BALB/c origin. This demonstrated that the LFLC-derived B lymphocytes were synthesizing the high levels of Ig present in these doubly reconstituted mice.

To show that the increased Ig production in the LFLC plus thymocyte–reconstituted mice was the result of general stimulation of B lymphocyte responsiveness and not the outgrowth of a few Ig-producing clones, two-dimensional gel analysis was performed on these sera. Analysis of the total Ig and the anti-TNP antibody produced by these mice showed them both to be very heterogeneous (Fig. 4, *C* and *D*). As previously seen, mice reconstituted with LFLC alone synthesized predominantly μ heavy chains (Fig. 4, *A* and *B*). When thymocytes were coinjected with the LFLC cells, total Ig and specific antibody synthesis were indistinguishable from that seen in normal BALB/c mice (Fig. 4, *E* and *F*).

Thymocytes appear able to complement the LFLC-derived B lymphocytes to elicit full serum reconstitution and a diverse antibody response from these long-term cultured cells when placed in vivo.

Discussion

Isolation of the B lymphocyte pathway of development in studies with neoplastic or short-term cultured cells has yielded much information about B lymphocyte differentiation and Ig gene expression (2, 20, 21). These cells are limited to a small range of the total pathway, however, making relationships between early and later events difficult to study. In this paper we describe a system to control B lymphocyte development from early progenitors derived from fetal liver to mature, immune-responsive B lymphocytes able to reconstitute SCID mice.

We have experimentally separated three broad B lymphocyte developmental phases with this system. The first occurs in vitro in LFLC that is predominantly progenitor cells, pre–pre-B cells that have just begun to rearrange their Ig genes, and cytoplasmic μ^+ pre-B lymphocytes. These cultures appear to lack the necessary differentiation signals that would produce $sIgM^+$ B lymphocytes efficiently (3). The same bone marrow stromal layers which support $sIgM^+$ cells in lymphoid marrow cultures are present in LFLC. In addition, however, the bone marrow cultures have been shown (10) to contain low levels of cells that can reconstitute T cell activity upon transplantation into SCID mice. It is possible that exposure to these T lineage cells in vitro accounts for the maturational differences between the culture types. T lymphocyte precursors are not encountered by LFLC cells until they are injected into SCID mice (see below). Inherent defects in the LFLC cells themselves seem unlikely to account for the in vitro differences, in light of their ability to fully differentiate and function in the proper in vivo environment. The presence in the LFLC of an inhibitory cell derived from fetal liver cannot be ruled out.

The second phase is reached when LFLC cells alone are used to reconstitute SCID mice. Further differentiation signals, lacking in vitro, are provided by the environment of the SCID mouse. These mice have been shown to provide a normal setting for lymphocyte differentiation, despite their lack of functional B and T lymphocytes (22). Presumably, this second level of growth and differentiation signals involves interactions with macrophages and other accessory cells, growth factors, and perhaps bone marrow stromal elements that do not survive in our in vitro culture. It is also possible that immature T lymphocyte precursors could provide or enhance some of these differentiation signals. SCID mice appear to possess both B and T lymphocyte precursors that are unable to properly rearrange their antigen receptor genes (23). These T lymphocyte precursors are absent in LFLC but could be present in sufficient numbers in SCID mice to provide signals to the cultured cells. However, as indicated by our findings, these signals are not sufficient to provide B cells with stimuli sufficient for them to exhibit their full range of responsiveness.

The third phase of B lymphocyte maturation in this system proceeds only in the presence of mature T lymphocytes. The events involved include complete class switching to IgG subisotypes other than G3 and responsiveness to TNP-

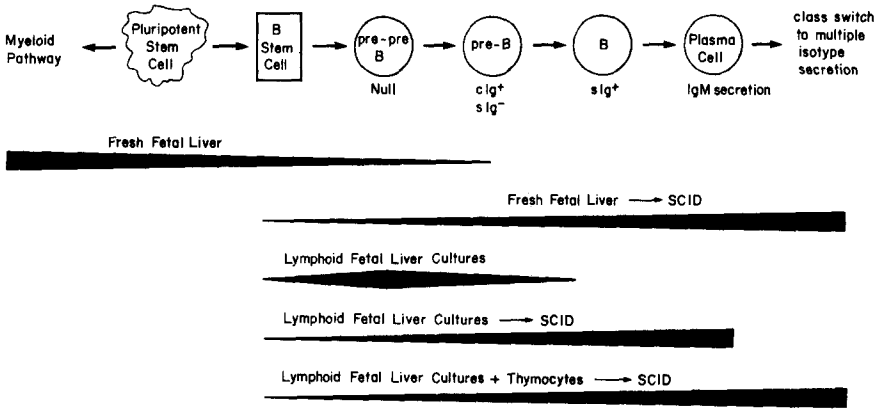

FIGURE 5. Schematic representation of the stages of B lymphocyte development represented in the in vitro and in vivo systems used in this paper. The width of the line is proportional to the approximation of cells present in that developmental stage.

BSA. The resultant serum responses appear indistinguishable from normal BALB/c serum responses. The B lymphocyte precursor present in the LFLC does not have any intrinsic developmental block when placed in the proper environment. These studies indicate that a similar B lymphocyte precursor is present in long-term cultures derived from both bone marrow and fetal liver. This precursor can proceed to reconstitute a diverse immune response in the SCID environment. We have no direct evidence for distinct B lymphocyte lineages from bone marrow and fetal liver at this time.

This regulated progression of B lymphocyte development is summarized in Fig. 5. Fresh fetal liver contains cells from all branches of hematopoiesis, and when injected into SCID mice it is able to fully reconstitute the lymphoid pathway. Lymphoid fetal liver cultures are devoid of CFU-S and other myeloid pathway cells (3) and contain early elements of the B lymphoid lineage. These LFLC, blocked developmentally in culture, are able to proceed through many but not all stages of B lymphocyte differentiation when placed in the environment of the SCID mouse. Addition of a source of mature T lymphocytes enables the final differentiation steps.

Thymocytes, an easily obtainable source of broad T lymphocyte potential, were chosen to augment LFLC reconstitution in the SCID mice. Low-level contamination of B lymphoid cells in our thymocyte preparations could be eliminated by further treatment of these cells. However, we have shown that the LFLC-derived B lymphocytes can cooperate with exogenous T lymphocytes in a functional immune response. Future experiments will involve more defined sources of T lymphocytes and their products to determine their effect upon this third phase of B lymphocyte differentiation. Specific control of antigen responses and class switching may be possible through this approach.

We have not precisely determined how many cells of the donor inoculum are mediating the observed reconstitution of the SCID mice. Marker genes, introduced by retroviral vectors (24), are being used to address this issue. In addition, a larger number of antigens will be used to more fully characterize the scope of the immune response in these animals.

The system for the study of B lymphocyte development presented here allows us to follow normal B lymphocyte precursors through all stages of differentiation. Investigation of B lymphocyte lineages through retrovirally introduced marker genes (24) and manipulation of these cells are now possible. Evaluation of responses to a broad panel of antigens as well as examination of the V region genes used in these responses should yield much information on the development of the immune system.

Summary

Lymphoid fetal liver cultures (LFLC) are long-term, nontransformed cultures of early B lymphoid lineage cells which appear developmentally blocked at the pre-B stage in vitro. When injected into severe combined immunodeficient (SCID) mice, cells from LFLC could reconstitute splenic B lymphocytes and serum IgM. T lymphocyte reconstitution was not observed and serum IgG levels were very low. IgG3 was the predominant γ subisotype in the serum of the LFLC-reconstituted mice, indicating impaired class switching in these B lymphocytes. When thymocytes were coinjected with LFLC, the B lymphocytes were able to class switch fully and respond to T-dependent antigens. These serological responses were heterogeneous. This experimental system allows separation of three B lymphocyte developmental stages: (*a*) early differentiation in vitro, (*b*) progression to IgM secretion in vivo, and (*c*) late differentiation dependent upon mature T lymphocytes in vivo. The unique advantage of this system is the ability to regulate the B lymphocyte developmental pathway in a defined, stepwise manner.

The authors would like to thank Audrey Davis and Linda Collins for excellent technical help; Carol Crookshank for expert manuscript preparation; and Drs. Jonathan Braun, Kathryn Calame, and Randy Wall for their comments and criticisms.

Received for publication 19 December 1986 and in revised form 20 April 1987.

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 mitogen-sensitive cells. *Transplant. Rev.* 25:26.
2. Kincade, P. W. 1981. Formation of B-lymphocytes in fetal and adult life. *Adv. Immunol.* 31:177.
3. Denis, K. A., L. J. Treiman, J. I. St. Claire, and O. N. Witte. 1984. Long term cultures of murine fetal liver retain very early B lymphoid phenotype. *J. Exp. Med.* 160:1087.
4. Whitlock, C., K. Denis, D. Robertson, and O. N. Witte. 1985. *In vitro* analysis of murine B cell development. *Annu. Rev. Immunol.* 3:213.
5. Denis, K. A., and O. N. Witte. 1987. Long-term culture systems for analysis of early B lymphocyte development. *Int. Rev. Immunol.* 2:285.
6. Paige, C. J., P. W. Kincade, M. A. S. Moore, and G. Lee. 1979. The fate of fetal and adult B cell progenitors grafted into immunodeficient CBA/N mice. *J. Exp. Med.* 150:548.
7. Bosma, G. C., R. P. Custer, and M. J. Bosma. 1983. A severe combined immunodeficiency mutation in the mouse. *Nature (Lond.).* 301:527.

8. Whitlock, C. A., D. Robertson, and O. N. Witte. 1984. Murine B cell lymphopoiesis in long term culture. *J. Immunol. Methods.* 67:353.
9. Kincade, P. W., C. J. Paige, R. Michael, E. Parkhouse, and G. Lee. 1978. Characterization of murine colony-forming B cells. I. Distribution, resistance to anti-immunoglobulin antibodies, and expression of Ia antigens. *J. Immunol.* 120:1289.
10. Dorshkind, K., K. A. Denis, and O. M. Witte. 1986. Lymphoid bone marrow cultures can reconstitute heterogenous B and T cell dependent responses in severe combined immunodeficient mice. *J. Immunol.* 137:3457.
11. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
12. Whitlock, C. A., S. F. Ziegler, L. J. Treiman, J. I. Stafford, and O. N. Witte. 1983. Differentiation of cloned populations of immature B cells after transformation with Abelson murine leukemia virus. *Cell.* 32:903.
13. Taylor, R. B., and H. H. Wortis. 1968. Thymus dependence of antibody: variation with dose of antigen and class of antibody. *Nature (Lond.).* 220:927.
14. Mongini, P. K. A., W. E. Paul, and E. S. Metcalf. 1982. T cell regulation of immunoglobulin class expression in the antibody response to trinitrophenyl-ficoll: evidence for T cell enhancement of the immunoglobulin class switch. *J. Exp. Med.* 155:884.
15. Mongini, P. K. A., K. E. Stein, and W. E. Paul. 1981. T cell regulation of IgG subclass antibody production in response to T-independent antigens. *J. Exp. Med.* 153:1.
16. Vitteta, E. S., K. Brooks, Y. W. Chen, P. Isakson, S. Jones, J. Layton, G. C. Mishna, E. Pure, E. Weiss, C. Word, D. Yuan, P. Tucker, J. Uhr, and P. Krammer. 1984. T cell derived lymphokines that induce IgM and IgG secretion in activated murine B cells. *Immunol. Rev.* 78:137.
17. Honjo, T. 1983. Immunoglobulin genes. *Annu. Rev. Immunol.* 1:499.
18. Mosier, D. E., I. M. Zitron, J. J. Mond, A. Ahmed, I. Scher, and W. E. Paul. 1977. Surface immunoglobulin D as a functions receptor for a subclass of B lymphocytes. *Immunol. Rev.* 37:89.
19. Nottenberg, C., and I. Weissman. 1981. Cμ gene rearrangement of mouse immunoglobulin genes in normal B cells on both the expressed and non-expressed chromosomes. *Proc. Natl. Acad. Sci. USA.* 78:484.
20. Wall, R., and M. Kuehl. 1983. Biosynthesis and regulation of immunoglobulins. *Annu. Rev. Immunol.* 1:393.
21. Yancopoulos, G. D., and F. W. Alt. 1986. Regulation of the assembly and expression of variable region genes. *Annu. Rev. Immunol.* 4:339.
22. Dorshkind, K., G. M. Keller, R. A. Phillips, R. G. Miller, G. C. Bosma, M. O'Toole, and M. J. Bosma. 1984. Functional status of cells from lymphoid and myeloid tissues in mice with severe combined immunodeficiency disease. *J. Immunol.* 132:1804.
23. Schuler, W., I. J. Weiler, A. Schuler, R. A. Phillips, N. Rosenberg, T. W. Mak, J. F. Kearney, R. P. Perry, and M. J. Bosma. 1986. Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. *Cell.* 46:963.
24. Keller, G., C. Paige, E. Gilboa, and E. F. Wagmer. 1985. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature (Lond.).* 318:149.