B CELL PROGENITORS HAVE DIFFERENT GROWTH REQUIREMENTS BEFORE AND AFTER IMMUNOGLOBULIN HEAVY CHAIN COMMITMENT

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Differentiation along the B lymphocyte developmental pathway is characterized by well-defined molecular changes resulting in mature cells committed to the production of antibody molecules (1). During this process cells undergo chromosomal rearrangements as the genetic elements that make up the Ig H and L chain genes are assembled. In this study we investigate whether the progressive commitment of cells to the expression of a particular H chain and a particular L chain is associated with differential growth properties. First, we provide evidence that clonable pre-B cells are able to undergo H and L chain commitment during the in vitro culture period. Moreover, we describe selective growth conditions associated with H chain commitment.

The clonable pre-B cell assay detects single B cell progenitors that are able to expand and differentiate into colonies containing mature Ig-producing B cells (2–4). Although the Ig gene configuration of clonable pre-B cells cannot be directly studied, the commitment of these cells to Ig expression can be inferred by analysis of the resultant colonies (4). Using the replicate nitrocellulose protein blotting method, we have previously distinguished clonable pre-B cells committed to the expression of a single L chain isotype from those not yet restricted in this regard (4). This approach has now been applied to the analysis of commitment to Ig H chain expression based on the phenomenon of allelic exclusion.

Materials and Methods

The mice used in this study were purchased from the Institut für Biologisch-Medizinische Forschung (Fullinsdorf, Switzerland) and were housed and bred in the mouse colony at the Basel Institute for Immunology, Basel, Switzerland. These included BALB/c, C.AL20, and (BALB/c × C.AL20)F₁ hybrids. These mice were chosen for study because they are congenic, differing only at the Ig H chain locus. BALB/c mice are classified as Igh-6a, while the C.AL20 mice are classified as Igh-6e.

Antibodies used in these studies to characterize the Ig produced by B cell colonies include: Bet-1 (5; rat anti–mouse Igh-6a allotype); MB86 (6; mouse anti–mouse Igh-6e allotype); 3704 (7; rat anti-total IgM); 187.1 (8; rat anti–mouse κ L chain); and L22.12.4 (9; rat anti–mouse γ L chain). Second-step antibodies used to detect these antibodies were β-galactosidase con-

jugated as described (4) and included: K2530 (5; mouse anti-rat λ); MaR-18.5 (10; mouse anti-rat κ); and goat anti-mouse λ 1 (Southern Biotechnology, Birmingham, AL). In all cases the specificities of the reagents were tested on either cell lines or B cell colonies from appropriate mouse strains. Positive colonies were visualized by incubating filters with 6-bromo-2-napthyl β-D-galactopyranoside (Fluka, Buchs, Switzerland) and Fast Garnet GBC Salts (Sigma Chemical Co., St. Louis, MO) as described (4).

The growth factors used included: recombinant CSF-1 obtained from the supernatant of transfected COS cells (S. Clark, Genetics Institute, Boston, MA); rIL-3 obtained from X63Ag8-653 cells (11; H. Karasuyma and F. Melchers, Basel Institute for Immunology, Basel, Switzerland); L cell–conditioned medium (12; LCM: 72 h culture of confluent L cells grown in Iscove's Modified Dulbeccos' Medium [IMDM] + 2% FCS and concentrated 8 X on a YM 10 amicon filter); and WEHI-3-conditioned medium (13; WEHI-3CM: 72 h culture of dense WEHI-3B [D-] cells grown IMDM and FCS and concentrated 6-8 times on a PM 10 amicon filter).

The double agar layer in vitro clonal method for growing B cell progenitors from fetal liver has been described in detail in previous publications (2-4, 14). The Ig isotype and allotype secreted by cells developing in these colonies were determined using the replicate nitrocellulose protein blot assay. The details of this method have been reported previously (4). The principal advantage of this procedure is that it permits analysis of Ig produced by large numbers of single colonies. Replicate blotting further distinguishes colonies that produce only one Ig allotype from those that produce Ig of both allotypes.

Results and Discussion

Clonable pre-B cells were assayed using fetal liver cells from a hybrid of congenic mouse strains ([BALB/c × C.AL20]F$_1$), in which the H chain alleles can be distinguished using allotype-specific anti-IgM mAbs, namely Bet-1, which recognizes the Igh-6a allele contributed by BALB/c, and MB86, which recognizes the Igh-6e allele contributed by C.AL20.

Using these reagents, along with anti-total IgM, we found that 15-d fetal liver contained 3,170 ± 800 clonable pre-B cells of which 1,850 ± 510 and 1,450 ± 340 gave rise to Igh-6a- and Igh-6e-producing B cells, respectively. Values determined for 16-d fetal liver were: 10,600 ± 1,640 total IgM$^+$; 5,400 ± 1,350 Igh-6a$^+$; and 4,500 ± 1,520 Igh-6e$^+$. A similar allotype ratio has also been observed in B cell colonies derived from sIg$^+$ B cells: 10^3 spleen cells from adult (BALB/c × C.AL20)F$_1$ mice gave rise to 34.2 ± 6.1 IgM-secreting colonies. Of these, 18.6 ± 3.7 secreted IgM of the Igh-6a allotype, while 14.4 ± 4.0 secreted IgM of the Igh-6e allotype. These control experiments show that the reagents used detect virtually all IgM-secreting colonies and that the two allotypes are distributed at similar levels, whether derived from fetal or adult tissues. To investigate whether any clonable pre-B cells were not yet committed to H chain allotype, replicate nitrocellulose blots were developed with the anti-allotype-specific mAbs. We found that ∼10% of all IgM-secreting colonies from 15-d-old fetal liver expressed both H chains simultaneously (Table I). This number decreased to ∼5% by day 16 of gestation, although the absolute number of H chain mixed colonies increased (Table I). We have rigorously excluded the possibility that double-producing colonies are dependent upon limiting accessory cells (14), rare couplets (4), or simple coincidence (4). Therefore, we conclude that mixed allotype colonies are derived from B cell progeny uncommitted to H chain allotype expression at the initiation of culture.

The frequency of allotype-mixed colonies observed are minimum estimates of the number of allotype-uncommitted B cell progenitors in the population. Allotype-

TABLE I

Detection of Colonies Secreting Antibodies of both Igh-6a and Igh-6e Allotypes

Day of development	Number of IgM-secreting colonies per fetal liver, positive for:		
	Igh-6a allotype	Igh-6e allotype	Igh-6a + Igh-6e allotype
15	1,365 ± 455	1,307 ± 246	258 ± 166
16	4,321 ± 1,422	3,568 ± 812	357 ± 51

Replicate nitrocellulose blotting from the same agar culture was performed by incubating the agar layer with the first nitrocellulose filter for 30 min and with the second filter for 4.5 h (4). Data shown are mean values and SEM from three independent experiments, in each of which 10–20 replicate cultures were assayed. Since allotype-mixed colonies can be generated by coincidence of two colonies, cell concentrations were chosen that minimize this possibility. An accurate estimate of coincidence is derived based upon the following formula: $c = (a/A)(x)(y)$; where a is the maximal colony size (3.46 mm^2); A is the area of the filter (1,104 mm^2); and x and y are the number of colonies per plate secreting IgM of the Igh-6a or Igh-6e allotype, respectively. Based upon this formulation, we chose cell concentrations that yielded ∼10 IgM-secreting colonies per plate (5–10 × 10^4/ml). At these concentrations the number of allotype-mixed colonies experimentally observed was 0.89/plate, whereas the number of allotype-mixed colonies due to coincidence was estimated to be <1 in 20 plates. Since most colonies are smaller than the maximal colony size used in this calculation, this represents an overestimate of the number of coincident colonies.

unrestricted progenitors escape detection if their daughter cells all productively rearrange the same allele. The likelihood of this event is highest when the decision to undergo H chain commitment occurs after the first cell division. The extent to which our frequency estimate could be affected by cryptic-uncommitted cells was determined by application of the same statistical treatment reported in detail previously for double L chain–producing colonies, except that H chain allotype data were substituted for L chain isotype results (4). Assuming that the experimentally observed frequencies of allotype usage reflect the probabilities of productive rearrangement of the relevant alleles, the fraction of allotype-uncommitted clonable pre-B cells in day 15 fetal liver cells rises to 22% and to 9% in 16-d fetal liver. Obviously, if the decision to undergo H chain commitment occurs after two or more cell divisions, the likelihood of allotype homogenous colonies derived from uncommitted progenitors becomes negligible.

Growth and differentiation of clonable pre-B cells depend on soluble growth factors provided by fetal liver adherent cells. It has been suggested by cell titration experiments that these growth factors act directly on the pre-B cells (2). Thus, linear regression analysis of data obtained in such experiments reveal a slope of 1 after log/log transformation (2–4, 14). In contrast, when adherent cells are replaced by conditioned media, such as WEHI-3B supernatant (WEHI-3CM), as a source of IL-3 or L cell–conditioned medium (LCM) as a source of CSF-1, differentiation of pre-B cells is supported but requires the presence of accessory cells. This is revealed by a slope of >1 and indicates an indirect effect of these factors on pre-B cells. This interpretation has been confirmed by using populations highly enriched for B cell progenitors that did not respond in the presence of CSF-1 or IL-3 alone but still responded to support provided by adherent fetal liver cells (14).

In the experiments described here, the differentiation stage of pre-B cells responding

under these different growth conditions was determined. In addition to H chain expression, we also examined L chain expression using the existence of (κ + λ)-secreting colonies as a measure for L chain gene commitment (4). Stimulation of fetal liver cells by LCM or WEHI-3CM supported the growth of colonies expressing simultaneously κ and λ L chains indicating that in both cases, L chain uncommitted pre-B cells were able to respond (Table II). However, a significant difference between these factors was observed when their ability to stimulate the development of H chain mixed colonies was tested. In the presence of WEHI-3CM, but not LCM, H chain allotype-mixed colonies were generated. This is the first suggestion that Ig commitment occurs in cells that can be distinguished based on non-Ig encoded products; in this case, most likely, a growth factor receptor.

To demonstrate that the active ingredient in these cultures was CSF-1 and IL3, conditioned media were replaced by recombinant factors. In both cases the recombinant growth factors stimulated the fetal liver cells in a manner similar to that of the cell line–conditioned media (Table III). To rule out an inhibitory effect of LCM, fetal liver cells were cultured in the presence of both LCM and WEHI-3CM. Addition of LCM to WEHI-3CM-stimulated fetal liver cells affected neither the number of IgM-secreting colonies nor the number of H chain mixed colonies (Table III). This suggests that the B cell progenitors detected after stimulation with CSF-1 or IL-3 are highly overlapping precursor populations, but that in the presence of IL-3, additional pre-B cells of a different developmental stage are stimulated. One interpretation of these findings is that at least two types of accessory cells exist that can influence the differentiation of pre-B cells: one cell type that supports the differentiation of H chain–committed pre-B cells upon stimulation with CSF-1, and a second type of accessory cell that stimulates differentiation of H chain–uncommitted pre-B cells after stimulation with IL-3. An alternative explanation is that both CSF-1 and IL-3 stimulate the same accessory cell to make different factors needed by B cell progenitors at different stages of development.

The experiments described in this report demonstrate that the clonable pre-B cell assay allows the growth of a heterogeneous population of B cell progenitors, some

TABLE II

Influence of Culture Conditions on the Expression of H and L Chains in Colonies Derived from 15-d Fetal Liver Pre-B Cells

| | Absolute number of colonies secreting: | | | |
| | | (Igh-6a + Igh-6e) | | |
Stimulus	IgM	allotype	Light chains	(κ + λ)
Fetal liver				
adherent cells	1,822 ± 437	126 ± 43	1,878 ± 397	129 ± 37
WEHI-3CM	1,585 ± 80	83 ± 20	1,548 ± 218	98 ± 6
LCM	1,261 ± 143	<1	1,239 ± 497	89 ± 15

WEHI-3B and L cell–conditioned medium were prepared as described (14). Optimal concentration was determined by titration and ranged usually between 5 and 10% final concentration. Data shown are mean values and SEM from two to three independent experiments, in each of which, 10–40 replicate cultures were analyzed in the replicate nitrocellulose transfer technique using the mAbs Bet1 and MB86 as anti-IgM reagents and the mAbs 187.1 (8) and L22.12.4. (9) as anti-κ and anti-λ chain reagents.

TABLE III

Factor-induced Development of Allotype Mixed Colonies Derived from
15-d Fetal Liver Pre-B Cells

| | Number of colonies secreting: | | |
| | | | |
Stimulus	IgM	(Igh-6a + Igh-6e) Allotype	Percent of allotype mixed colonies
WEHI -3CM	1,925 ± 115	112 ± 43	5.8
LCM	1,512 ± 300	<1	0
WEHI-3CM + LCM	1,672 ± 396	100 ± 27	6.0
rIL-3*	1,854 ± 274	132 ± 24	7.1
rCSF-1‡	1,618 ± 107	<1	0

* Supernatant from the cell line X63 Ag8 was provided by Dr. H. Karasuyama
(11) and was used at a final concentration of 1%.
‡ Supernatant from COS cells transfected with the human CSF-1 gene, provid-
ed by Dr. S. Clark, Genetics Institute, Boston, MA, was used at a concentra-
tion of 1%. Data shown are mean values and SEM from two independent
experiments, in each of which, 30–40 replicate cultures have been assayed.

of which are not yet committed to the expression of a particular H or L chain gene.
Moreover, we have discovered that pre-B cells have different growth requirements
at different stages of their development. In particular, the commitment to H chain
but not L chain is associated with the ability to grow under growth conditions that
are inadequate for uncommitted cells. Whether this association is based upon a di-
rect relationship between the commitment to Ig H chain and the expression of specific
growth factor receptors or represents concomitant expression of stage-specific genes
in the B cell developmental pathway, remains to be investigated.

Summary

    Using the clonable pre-B cell assay, we have identified B cell progenitors that are
not yet committed to the production of a particular H chain allele. These cells repre-
sent ~10–20% of clonable pre-B cells found in 15-d fetal liver. The clonable pre-B
cell assay provides an environment adequate for the expansion and differentiation
of these cells into mature, Ig-secreting, cells. Using the same methodology, we have
also identified progenitors that are uncommitted to the production of a particular
L chain isotype. Moreover, investigating the growth requirements for clonable pre-
B cells has led to the discovery of selective growth conditions that distinguish cells
before and after commitment to H chain allotype.

References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature (Lond.). 302:576.
2. Paige, C. J. 1983. Surface Ig-negative B cell precursors detected by formation of antibody-
secreting colonies in agar. Nature (Lond.). 302:711.

3. Paige, C. J., R. H. Gisler, J. P. McKearn, and N. 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.

4. Sauter, H., and C. J. Paige. 1987. Detection of normal B-cell precursors that give rise to colonies producing both κ and λ light immunoglobulin chains. *Proc. Natl. Acad. Sci. USA.* 84:4989.

5. Kung, J. T., S. O. Sharrow, D. G. Sieckmann, R. Lieberman, and W. E. Paul. 1981. A mouse IgM allotypic determinant (IgH 6-5) recognized by a monoclonal rat antibody. *J. Immunol.* 127:873.

6. Nishikawa, S. I., Y. Sasaki, T. Kina, T. Amagai, and Y. Katsura. 1986. A monoclonal antibody against Igh 6-4 determinant. *Immunogenetics.* 23:137.

7. Leptin, M., M. J. Potash, R. Grützmann, C. Heusser, M. Shulman, G. Kohler, and F. Melchers. 1984. Monoclonal antibodies specific for murine IgM. I. Characterization of antigenic determinants on the four constant domains of the μ-heavy chain. *Eur. J. Immunol.* 14:534.

8. Yelton, D. E., C. Desaymard, and M. D. Scharff. 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma.* 1:5.

9. Weiss, S., K. Lehmann, and M. Cohn. 1983. Monoclonal antibodies to murine immunoglobulin isotypes. *Hybridoma.* 1:49.

10. Lanier, L. L., G. A. Gutman, D. E. Lewis, S. T. Griswold, and N. L. Warner. 1982. Monoclonal antibodies against rat immunoglobulin kappa chains. *Hybridoma.* 2:125.

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

12. Stanley, E. R., and P. M. Heard. 1977. Factors regulating macrophage production and growth. *J. Biol. Chem.* 252:4305.

13. Ralph, P., M. A. S. Moore, and K. J. Nilsson. 1976. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. *J. Exp. Med.* 143:1528.

14. Paige, C. J., H. Skarvall, and H. Sauter. 1985. Differentiation of murine B cell precursors in agar culture. II. Response of precursor-enriched populations to growth stimuli and demonstration that the clonable pre-B cell assay is limiting for the B cell precursor. *J. Immunol.* 134:3699.