The EMBO Journal vol.10 no.2 pp.327–336, 1991

Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells *in vitro* and *in vivo*

Antonius Rolink, Akira Kudo, Hajime Karasuyama, Yuji Kikuchi and Fritz Melchers

Basel Institute for Immunology, CH-4005 Basel, Switzerland

Communicated by F.Melchers

Cell lines and clones were established from PB76-positive mouse fetal liver at day 13 and 14 of gestation, which proliferated with division times of a day in serum-substituted cultures under the stimulatory influence of adherent stromal cells and the cytokine IL-7 for periods longer than half a year. These lines expressed varying levels of the B lymphocyte lineage related markers PB76, B220, BP-1, $V_{pre}B$ and λ_5, but no surface Ig or MHC class II molecules. All clones expressed PB76, $V_{pre}B$ and λ_5 in a high percentage of cells, while B220 and/or BP-1 expression was low or undetectable in some. A cell line, and several clones established from it, all had κ and λ light chain genes in germ-line configuration. Either one or both of their H-chain-gene containing chromosomes carried a D_H to J_H. These pre B cell lines and clones could be induced to V_H to D_H and V_L to J_L rearrangements. This resulted in the development of varying percentages of sIg-positive surface, MHC class II negative, LPS-reactive B cells within 2–3 days, in the absence of contacts with stromal cells and/or IL-7. When injected into SCID mice, the cultured pre B cells populated the spleen of these mice to 5% with surface Ig-, MHC class II-positive LPS-reactive cells for >25 weeks. The long-term *in vitro* proliferative capacity of these D_H-J_H rearranged pre B cell clones makes them major candidates for committed stem cells of the B lineage.

Key words: pre B cell clones/Ig gene rearrangements/stromal cells/IL-7/transition to mature B cells

Introduction

B lymphocyte development in the mouse from pluripotent stem cells and from lymphocyte and B lineage committed progenitors (Phillips, 1989) occurs during embryonic development in waves in embryonic blood and fetal liver (Melchers, 1979), and continues throughout life in bone marrow, so that 5×10^7 Ig^+ B cells are generated until birth, and continue to be generated daily thereafter (Park and Osmond, 1987). The process of B cell development can be most clearly dissected into different stages in fetal liver between days 13 and 19 of gestation, since there it occurs in one wave and synchronously for all cells (Strasser *et al.*, 1989). Early stages of progenitor proliferation and inductions to Ig gene rearrangements in fetal liver between day 13 and 16 of gestation have been found to depend on contacts with stromal cells (Kincade *et al.*, 1981a; Whitlock *et al.*, 1985; Dorshkind *et al.*, 1986; Gisler *et al.*, 1987; Witte *et al.*, 1987; Kinashi *et al.*, 1988; Pietrangeli *et al.*, 1988; Palacios *et al.*, 1989; Strasser *et al.*, 1989).

In the stromal cell-dependent phase of B cell development, gene segments of the Ig gene loci are rearranged in an ordered fashion, so that first D_H segments are rearranged to J_H segments, followed by V_H to D_HJ_H, then by V_K to J_K, and finally by V_λ to J_λ rearrangements (Tonegawa, 1983). Since rearrangement processes produce in-frame '(1/3)' and out-of-frame '(2/3)' joints, the majority of all B cells generated from progenitors are expected to be sIg-negative, nonfunctional cells because they have either nonfunctionally rearranged H- and/or L-chain genes. The different cellular stages of development can be distinguished by a set of markers (Park and Osmond, 1987; Phillips, 1989), including the expression of the pre B cell-specific genes $V_{pre}B$ and λ_5 (Sakaguchi and Melchers, 1986; Kudo *et al.*, 1987; Kudo and Melchers, 1987).

Only a limited number of cell divisions can be measured either *in vivo* or *in vitro* when B cells are generated from progenitors (Paige *et al.*, 1984; Strasser *et al.*, 1989; Melchers *et al.*, 1990), indicating that the proliferating pool of stem cells which feed the assembly lines of Ig gene rearranging cells must be before, or at the beginning of these Ig gene rearrangements, involving at least 5×10^7 progenitors which feed the daily production of this number of B cells in a mouse (Melchers *et al.*, 1990). We have searched for these progenitors in fetal liver at early stages (days 13 and 14 of gestation) of this development, and have found them in the population of cells expressing early B lineage-related markers such as PB76, recognized by mAb G-5-2 (Strasser, 1988), $V_{pre}B$ and λ_5 (Kudo *et al.*, 1987; Melchers *et al.*, 1990).

B cell progenitors and precursors have been cultured for extended time periods (Whitlock and Witte, 1982). Such long-term cultured cells are capable of repopulating the B cell compartment of SCID mice (Bosma *et al.*, 1983; Phillips, 1989). The stromal cell layer mandatory for such long-term cultures can be provided by clones of stromal cell lines (Kodama *et al.*, 1984; Collins and Dorshkind, 1987; Hunt *et al.*, 1987; Whitlock *et al.*, 1987; Nishikawa *et al.*, 1988; Ogawa *et al.*, 1988). IL-7 has been identified as the cytokine with growth factor activity in these cultures (Namen *et al.*, 1988). By using these culture conditions in serum-substituted media (Iscove and Melchers, 1978) with mAb G-5-2 FACS enriched populations of progenitor and precursor B cells from fetal liver, we have been able to establish lines and clones of progenitor B cells which continue to proliferate for several months. They can be induced to differentiate *in vitro* within 2–3 days to sIg^+, LPS-reactive (Andersson *et al.*, 1972) B cells, at which point they lose their proliferation capacities. They can also populate the B cell compartment of SCID mice with sIg^+ mitogen-reactive B cells.

© Oxford University Press

Results

Establishing cell lines from fetal liver growing on a layer of stromal cells in the presence of IL-7

Fetal liver cells of BDF1 mouse embryos at day 13 or 14 of gestation were enriched for mAb G-5-2-positive, i.e. PB76-positive precursor B cells by FACS. The positive fraction, representing between 1.2 and 4.7% of all fetal liver cells, were put into culture in serum-substituted medium in the presence of various interleukins (ILs) (IL-2, IL-3, IL-4, IL-5, IL-6 and IL-7) in the presence or absence of 3000 rad γ-irradiated stromal cells from different sources. The stromal cell lines PA-6 and ST-2, 3T3 fibroblasts and Ltk$^-$ cells were used as stromal cell layers.

In the absence of stromal cells only IL-3, or IL-3 plus IL-4 yielded long-term proliferating cell lines. Since these cell lines did not express the PB76 surface antigen, nor any other B cell lineage-specific markers (sIg, intracytoplasmic μ, BP-1, B220), we did not study them any further.

In the presence of any of the adherent cell lines (PA-6, ST-2, 3T3, Ltk$^-$) and IL-7, cells of the PB76-positive populations of fetal liver cells proliferated. A cell line, termed PA-B, was established from day 14 fetal liver cells which proliferated in the presence of PA-6 cells and IL-7, with division times near 24 h (Figure 1A). Proliferation was dependent on the presence of both PA-6 cells and IL-7. IL-7 could not be replaced by IL-2, 3, 4, 5 or 6 at the concentrations tested, while PA-6 cells could be replaced by ST-2, 3T3 or Ltk$^-$ cells.

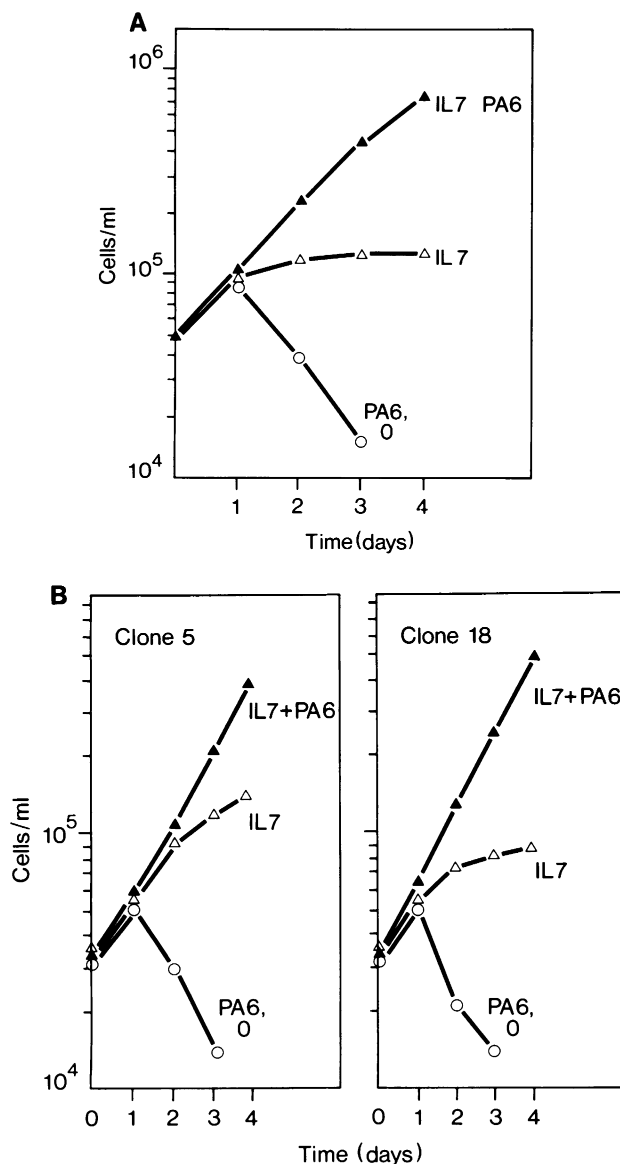

Fig. 1. Growth requirements of the PA-B line (**A**) and clones 5 and 18 (**B**). Cultures were initiated at densities between 3 and 5 × 10^4/ml in the presence of PA-6 (○), IL-7 (△) or PA-6 + IL-7 (▲). IL-7 was used at a concentration of 100 U/ml. Cell growth was determined by counting the number of cells in the cultures with the use of a Buerker hemocytometer at different times after initiating the cultures.

Cloning of cells enriched for PB76-positive cells, and of the PA-B cell line

Fetal liver cells of day 14 embryos enriched for PB76-positive cells, as well as cells of the PA-B line at different times of long-term culture, were cloned on PA-6 cells in the presence of IL-7. At the time of original isolation of the cells from fetal liver, the cloning frequency was ~1 in 60 cells. This frequency was indistinguishable when cloning was done either on PA-6, ST-2, 3T3 or Ltk$^-$ cell layers. After 10 days in culture, cells of the PA-B line could be cloned with a frequency of 1 in 10 to 1 in 20. After 2 months this frequency was increased to nearly 100%. Plated at 0.5 cells per well, 34 of 384 cultures contained growing cells. According to Poisson's distribution ~90% of these positive cultures could be expected to be derived from a single cell, i.e. constitute a clone. From this cloning experiment, 18 clones were raised, of which clones 1, 2, 3, 4, 5, 8, 9, 12 and 18 were recloned and studied in greater detail.

All clones, as well as the PA-B line, were dependent in their growth on the presence of stromal cells and IL-7, shown for clones 5 and 18 in Figure 1B. The PA-B line and the clones derived from it have now been in continuous culture for several months without alteration of their growth properties or surface marker phenotypes.

Expression of surface markers on the PA-B line and on the clones

The PA-B line and clones 1, 2, 3, 4, 5, 8, 9, 12 and 18 derived from it were assayed for surface expression of Ig μ and κ chains, B220, BP-1, PB76 and MHC class II molecules (Table I). The FACS patterns of the PA-B line are included as one example for these analyses (Figure 2).

Table I. Surface phenotype of the PA-B line and clones derived from it (percent, surface-positive)

Cells	Marker					
	κL	μH	B220	BP.1	PB76	MHC class II
PA-B line	0	0	87	17	96	0
Clone 1	0	0	0	12	71	0
2	0	0	33	15	73	0
3	0	0	98	12	100	0
4	0	0	0	20	98	0
5	0	0	25	100	100	0
8	0	0	0	49	92	0
9	0	0	65	5	100	0
12	0	0	41	7	86	0
18	0	0	90	0	89	0

All cells negative for: CD5, IL-5R, IL-2R (p55), Mac1, thy1, CD4, CD8.

Ig κ and μ chains, and MHC class II molecules were not expressed on any of the cells. PB76 was expressed on all of them, though on varying numbers of cells for different clones. Clones 1 and 8 did not express detectable levels of B220, while between 25 and 90% of all cells of the other clones, and of the PA-B line, were B220-positive. Clone 18 did not express detectable levels of BP-1. In the PA-B line, and in clones 1, 2, 3, 4, 9 and 12, only a minor fraction of all cells (between 5 and 20%) were BP-1-positive. Only clones 5 and 8 expressed BP-1 on a majority of cells. The pattern of surface marker expression on the clones and the percentage of cells expressing a given marker appeared to remain stable over several weeks of continuous culture on PA-6 in the presence of IL-7 (data not shown). All cells were negative for CD5, the IL-5 receptor, the IL-2 receptor p55 chain, Mac1, thy1, CD4 and CD8.

In conclusion, the PA-B cell line and the clones derived from it appeared to express B lineage-related surface markers and were dependent on IL-7, all characteristics of cells at earlier stages of B cell development before expression of sIg.

Expression of mRNA corresponding to $V_{pre}B$, λ_5 and μH genes

$V_{pre}B$ and λ_5 are two genes which have been found to be expressed specifically on the B lineage at the pre B, but not at the B cell stage. RNA prepared from the PA-B line and from the clones were analyzed by Northern blotting for the expression of these genes. Results in Figure 3B and C show that the PA-B line and all clones expressed the 0.85 kb mRNA for $V_{pre}B$ and the 1.2 kb mRNA for λ_5. Results in Figure 3A also show that the PA-B line and its clones expressed a mixture of μH transcripts ranging in size between 2.3 and 3.0 kb which, most likely, indicate Iμ, μm and DJCμ transcripts (Lennon and Perry, 1990). The control pre B and B cell lines expressed μH, $V_{pre}B$ and λ_5 transcripts as expected from their stage of differentiation. We conclude that, by these markers, the PA-B line and the clones appear to be pre B cells.

A variant subline of clone 5 which does not need PA-6 cells for growth

After ~2 months in continuous culture on PA-6 in the presence of IL-7, clone 5 developed a subline which proliferated in the presence of IL-7 only, i.e. which had become independent of stromal cells. Examination of the surface markers of this subline showed that the cells had become sIg^+ (95% μ^+,κ^+), while remaining $B220^+$, $BP\text{-}1^-$, $PB76^+$ and MHC class II negative (data not shown).

Status of Ig gene rearrangement

DNA prepared from the PA-B line and from the clones were digested with *Eco*RI or *Hin*dIII, and analyzed by Southern blotting with probes for the genes of the constant region of λL chain, and for the regions 3′ of J_H segments and J_K segments, as well as regions 5′ of D_{FL} regions (for exact location of the probes see Materials and methods). Results in Figure 4A, B and C show that both L chain loci (κ and λ) are in germ-line configuration in all clones.

The same filters were then tested with a J_H-region specific probe. Results in Figure 4D show that the PA-B line and all clones had rearranged to the J_H-region on either one or both chromosomes. The PA-B line showed several bands, indicating the oligoclonal nature of the cell line. All clones showed two bands, expected from the two chromosomes of a diploid cell carrying two H chain genes. This confirms the clonal nature of the cells analyzed here.

DNA of the PA-B line and clones 1, 4, 5, 6, 8 (and 18, not shown in Figure 4) showed rearranged bands with mobilities coinciding with one (clones 1, 4, 6 and 18) or two (clones 5 and 8) of the J_H hybridizing DNA fragments (compare Figure 4D with E) when hybridized with a probe covering the 5′ end of D_{FL}, indicating that a D_{FL} element has been rearranged to one of the J_H segments on one or both chromosomes of these clones. Since the regions 5′ of D_{FL} were detectable on these D−J-joined DNA segments, the corresponding chromosomes are D_HJ_H-, but not yet $V_HD_HJ_H$-rearranged, In clone 12, a $V_HD_HJ_H$-rearranged chromosome was found, which uses the V_H7183 family for expression on the RNA level (data not shown).

A summary of all rearrangements identified in the PA-B cell line and the clones derived from it (Figure 4A−E) is given in Table II. Eight of the 16 chromosomes analyzed from the PA-B clones used D_{FL} as the gene segment for D_HJ_H rearrangement.

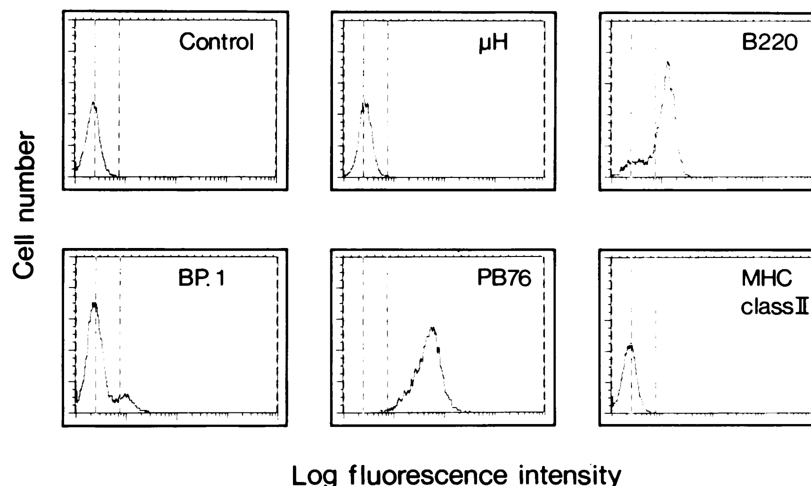

Fig. 2. Analysis of cell surface markers of the PA-B cell line and clones. The surface phenotype of PA-B cells was determined for μH, B220, BP.1, PB76 and MHC class II by FACS as described in Materials and methods.

In vitro differentiation of the PA-B line and its clones to sIg$^+$, PA-6/IL-7-nonreactive, mitogen-reactive B cells

Transfer of the PA-B line and of the clones into cultures containing only stromal cells (like PA-6, or ST-2 or 3T3 or Ltk$^-$), or only IL-7, or neither of the two, resulted in growth arrest of the cells (Figure 1A and B). On stromal cells alone growth ceased immediately; this was followed by rapid cell death. Those cells which were clonable 2 days after differentiation on PA-6 only appeared to retain the phenotype of the original pre B cell surface markers, $V_{pre}B/\lambda_5$ expression, and had the two L chain loci (κ and λ) in germ-line configuration, and the H chain gene segments in their clone-characteristic D_HJ_H rearranged form (data not shown). Cells in only IL-7 proliferated for 2–7 days, then ceased to divide. Concomitant with growth arrest was a loss in the capacity of the cells to clone on PA-6 and IL-7. Results in Figure 5A show that the frequency of clonable B cells dropped to ~1% within 2 days.

When cells of the PA-B line and of clones 1, 3, 4, 5, 8, 9 and 12 were removed from the joint stimulating influence of PA-6 and IL-7 and grown in only IL-7 or on PA-6, they were induced to rearrange V_H to D_HJ_H and V_L to J_L so that a fraction of all cells became sIg$^+$, mostly $\mu^+\kappa^+$ (Table III and Figure 6). In these experiments surface expression of λ L chains has not been tested. Other clones (like clone 18) never yielded sIg$^+$ cells, although they lost the capacity to grow on PA-6 and IL-7 upon differentiation, just as fast as the other clones. Differentiation to sIg$^+$ cells *in vitro* did not induce the expression of MHC class II molecules on the surface.

The kinetics of development of sIg$^+$ cells *in vitro* on PA-6 cells only is shown (Figure 5B). Kinetics suggest that V_H to D_HJ_H and V_L to J_L rearrangements can occur in the PA-B cell line and in the clones within 2 days, and reach a maximum at 3 days. During this transition from pre B to early B cells, many of the cells die (Figure 1), and they do so by apoptosis, detectable by degradation of DNA (Figure 7).

Introduction of differentiation to sIg$^+$ cells also leads to the development of LPS-reactive cells. This was tested by limiting dilution of the cells into cultures containing LPS and rat thymus 'filler' cells at day 3 after induction of differentiation. The higher the percentage of sIg$^+$ cells developing from a given pre B cell clone, the higher was the frequency of LPS-reactive cells observed in these experiments (Table III). The absolute numbers of the LPS-reactive cells, however, did not correlate linearly with the number of sIg$^+$ cells.

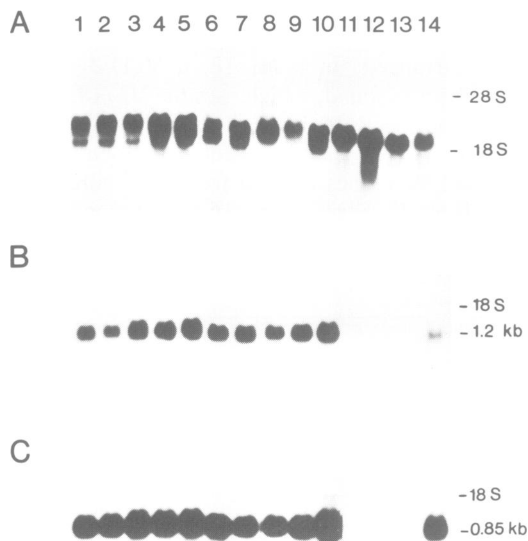

Fig. 3. Northern blot analyses of RNA from the PA-B cell line and clones. Approximately 10 μg of total RNA was separated by gel electrophoresis and transferred to a nylon filter. The same filter was then successively hybridized with ^{32}P oligolabeled Cμ (**A**) and λ_5 (**B**) and $V_{pre}B$ (**C**) probes. Lanes 1–9, PA-B, clones 1, 2, 3, 4, 5, 6, 8, 12 and 18; lane 10, the PA-B cell line; lanes 11 and 12, mouse mature B cells WEHI 231 WEHI 279; lane 13, mouse plasma cell hybridoma Hyb.19.9; lane 14, the mouse pre B cell lymphoma 18.81 (for origins of cell lines see Materials and methods and Sakaguchi and Melchers, 1986).

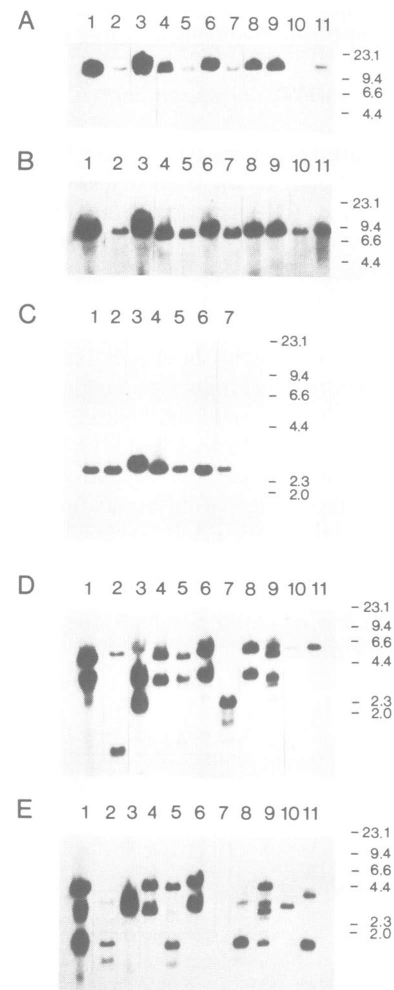

Fig. 4. Southern blot analysis of DNA from the PA-B line and its clones. Approximately 10 μg of DNA was digested with *Eco*RI (A,B,D,E) and *Hin*dIII (C), applied to 0.8% agarose gels and transferred to nylon filters. Filters were thereafter hybridized with ^{32}P oligolabeled probes for C_λ (**A**), J_K (**B**,**C**), 3' of J_H (**D**) and 5' of D_{FL} (**E**). In (A,B,D,E) lanes 1–8 contain DNA of clones 1, 3, 4, 5, 6, 8, 12 and 15; lane 9 the PA-B line; lane 10 C57BL/6 liver and lane 11 DBA/2 liver. In (C) lane sl–5 contain DNA of clones 1, 3, 5, 6 and 8; lane 6 the PA-B line and lane 7 DBA/2. The second restriction enzyme digestion with *Hin*dIII, probed with the J_K probe, was done to ascertain the germ-line configuration of the K_L loci by a shorter restriction enzyme fragment.

In vivo differentiation of the PA-B cell line into mature, sIg$^+$, LPS-reactive B cells

When 5 × 10^6 cells of the PA-B line (H-2^{bxd}) were injected into CB17 SCID (H-2^d) mice, they gave rise to ~5% sIg$^+$ B cells in spleen within 6–8 weeks and remained at this level for at least 25 weeks. The Ig$^+$ cells were H-2^b-positive, MHC class II-positive and B220-positive (Table IV). Donor H-2^{bxd}-positive T cells and myelopoietic cells did not develop in the spleen of these mice to any measurable level. The total number of nucleated cells in the spleens of these SCID mice did not change over the 24 weeks and, moreover, did not differ significantly from noninjected SCID mice.

The cells of sIg$^+$ B cell-containing spleens of these reconstituted mice could be stimulated with LPS *in vitro* to IgM-secreting, plaque-forming cells in frequencies of ~1 in 100 spleen cells, indicating that every fifth sIg$^+$ B cell developing from the donor PA-B cell line *in vivo* was LPS-reactive (Table III).

We conclude that the PA-B pre B cell line has the capacity to populate some of the B cell compartment of SCID mice; and can thus be regarded as a source of B lineage committed precursor cells capable of differentiation to sIg$^+$, mitogen-reactive B cells *in vivo*.

Table II. H chain gene rearrangements in the PA-B line and its clones

PA-B clones	J_H (*Eco*RI) fragment size (kb)	Rearrangement	D-assignment
Clone 1	5.5	DJ	D_{FL}
	4.0	DJ[a]	?
Clone 3	6.4	?	?
	1.2	?	?
Clone 4	3.5	DJ	D_{FL}
	2.6	?	?
Clone 5	5.5	DJ	D_{FL}
	3.5	DJ	D_{FL}
Clone 6	5.5	DJ	D_{FL}
	3.8	DJ[a]	?
Clone 8	5.5	DJ	D_{FL}
	3.5	DJ	D_{FL}
Clone 12	2.6	VDJ	V_H7183
	2.6	?	?
Clone 15	6.4	?	?
	3.8	DJ[a]	?
Clone 18	4.0	?	?
	3.5	DJ	D_{FL}
PA-B line	6.4	?	?
	5.5	DJ	D_{FL}
	3.8	DJ[a]	?
	3.5	DJ	D_{FL}
	3.2	?	?
	2.6	?	?

A probe specific for the region 5′ of D_{SP} (see Materials and methods) was used to search for additional DJ rearrangements. In the cases marked [a] such a rearrangement could be assigned. The germ-line pattern of D_{SP} hybridizing bands are so heterogeneous and different in C57BL/6 and DBA/2 that the remaining hybridization patterns in all the clones and the PA-B line were not interpreted (data not shown).

Discussion

B lymphocyte development in mouse fetal liver occurs in one synchronous wave between days 13 and 19 of gestation (Strasser *et al.*, 1989). Between days 13 and 16, while rearrangements of the H and L chain gene segments take place, this wave of development is dependent on interactions with stromal cells. Only a few divisions can be measured during this time and until the cells reach the stage of surface expression of Ig. At these stages of B cell differentiation, precursor B cells express PB76 (Strasser *et al.*, 1988), $V_{pre}B$ (Kudo and Melchers, 1987) and λ_5 (Sakaguchi and Melchers, 1986). Between days 16 and 19, sIg$^+$ cells develop to mitogen-reactive cells, and this development is independent of the stromal cell environment needed for the earlier development, and again is not accompanied by much

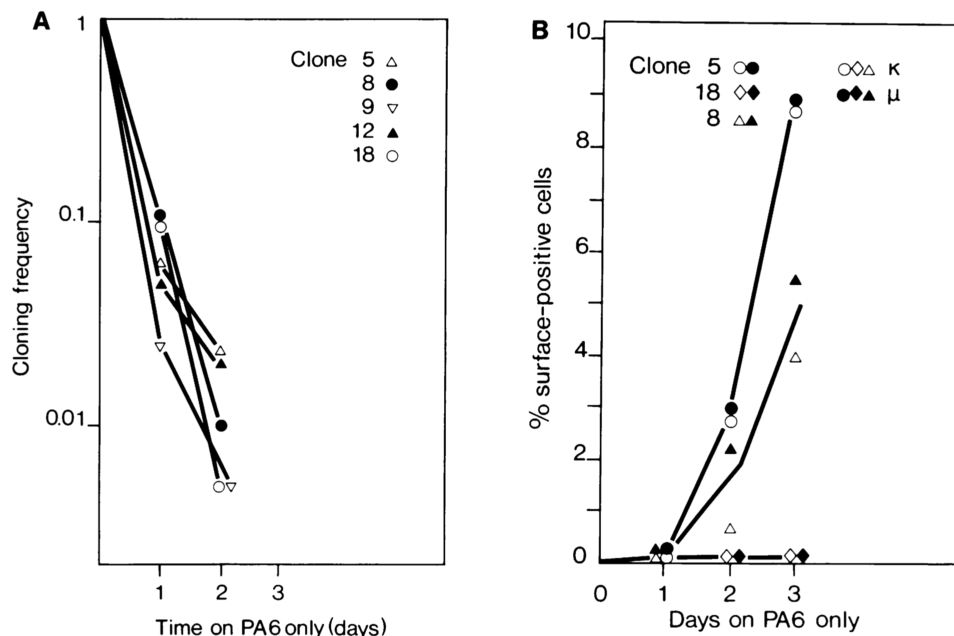

Fig. 5. (**A**) Cloning frequencies of clones 5, 8, 9, 12 and 18 on PA-6 + IL-7 (100 U/ml) after culture for 0, 1 and 2 days on PA-6 alone. Cloning frequencies were determined by limiting dilution analysis with live cells. Positive pre B cell growth of clones was scored by the use of an inverted microscope at 5–6 days after onset of the cultures. (**B**) Kinetics of development of sIg$^+$ of clones 5, 8 and 18 cells cultured for different days on PA-6 only. Surface μH and K_L expression was determined as described in Materials and methods.

cell division. In order to produce the 5×10^7 sIg^+ B cells of the newborn mouse, it is likely that at last 10^6 progenitor or precursor cells exist at day 13 to feed the lines of B cell differentiation on stromal cells, which leads to Ig gene rearrangements and to sIg expression. This study was initiated with the aim of identifying these early progenitors or precursors.

Table III. Surface Ig and MHC class II expression and LPS reactivity of the PA-B cell line and its clones after 3 days of culture on PA-6 stromal cells alone and of B cells from SCID mice injected with PA-B cells

Cells	μH	κL	MHC class II	LPS reactivity[a]
PA-B line	5	4.5	0	1 in 1500
Clone 1	5.2	3.2	0	<1 in 1000
3	0.4	1.0	0	<1 in 1000
4	1.8	1.2	0	1 in 800
5	18.0	13.6	0	1 in 30
8	4.8	3.6	0	1 in 800
9	1.3	1.4	0	<1 in 1000
12	3.0	3.0	0	<1 in 1000
18	0	0	0	<1 in 1000
In vivo transfer into SCID mice[b]	+	+	+	1 in 100

[a]LPS reactivity was determined in limiting dilution experiments as described in Materials and methods.
[b]See Table IV.

In the hope that these early progenitors would express these markers, we purified PB76-expressing cells from fetal liver by FACS at a time of gestation (days 13–14) when the synchronous wave of development could be expected to be shortly before, or at the beginning of H chain gene segment rearrangements, and certainly before L chain rearrangements.

Early progenitors or precursors of the B lineage could be expected to have extensive proliferation capacity with stem cell-like properties since Whitlock and Witte (1982) had established tissue culture conditions which allowed long-term and continuous development of bone marrow-derived B lineage cells from the precursor cell to the sIg^+ cell level of differentiation. This long-term *in vitro* development, dependent on the interactions between stromal cells and B cell progenitors, could also be maintained in the presence of IL-7 (Namen *et al.*, 1988) by the proadipocytic cell line PA-6 (Kodama *et al.*, 1984) not producing IL-7, and by the bone marrow-derived stromal cell line ST-2 (Ogawa *et al.*, 1988), producing and secreting IL-7 upon induction

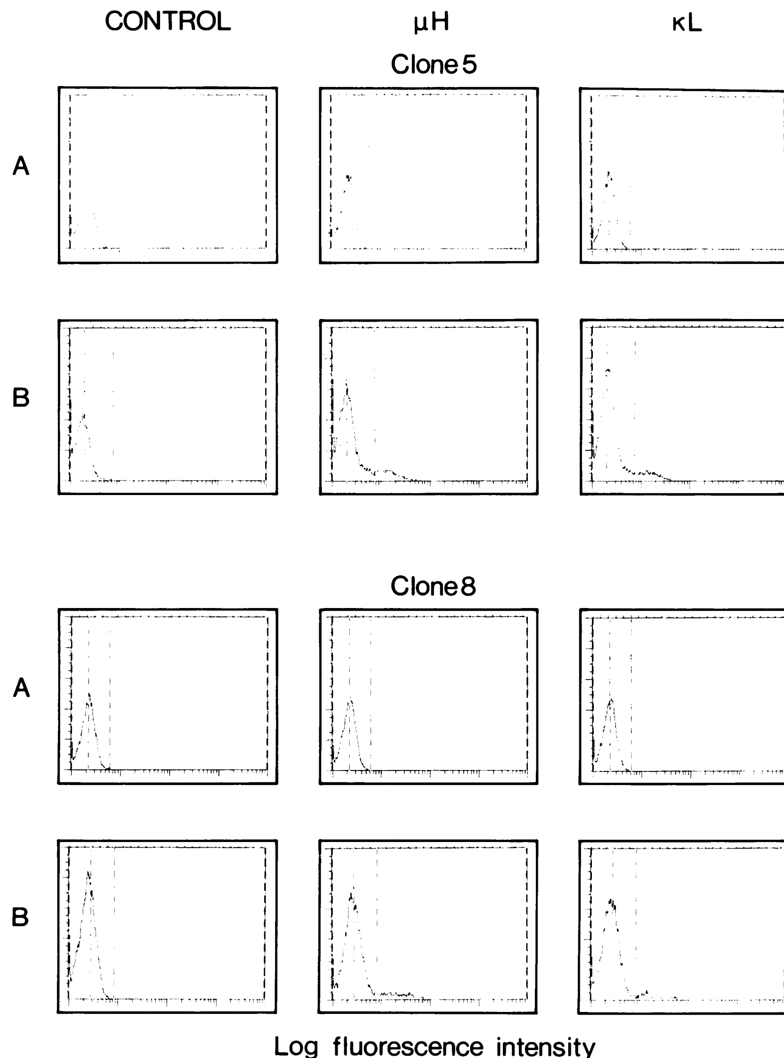

Fig. 6. Analysis by FACS of sIg (μH and κ light chains) expressed on cells of clone 5 (top) and clone 8 (bottom) after a 3 day culture period of PA-6 cells in the absence of IL-7. Control staining was done without specific antibody (see Materials and methods).

(Nishikawa *et al.*, 1990). Other, as yet unidentified cytokines may participate in the induction of proliferation of such cells.

Our results show that we can establish cell lines and clones representing an early pre B-stage which proliferate *in vitro* for long periods of time (>6 months) under the influence of stromal cells and IL-7. The specificity of the stromal cells in their contacts with the precursor B cells is not yet apparent, since PA-6, ST-2, 3T3 and Ltk^- cells were all equally efficient in primary cloning and continuous proliferation of pre B cells. While it is surprising how efficiently cells of the established pre B cell lines can be cloned in these stromal cells in the presence of IL-7, only 1–2% of all PB76-positive cells initiate a clone, and even after 10 days on PA-6 and IL-7, only 5–10% of all cells do so. We would also like to emphasize that we have not yet been able to establish all of the original clones as long-term growing clones. It is possible that we are selecting a subpopulation of all pre B cells as long-term proliferating cells by our method of cell culture. The PA-B line and the clones derived from it appear not to be a random population of pre B cells, since 50% of all D_HJ_H rearrangements can be assigned to D_{FL}, which detects only two or three of all the germ-line encoded D_H segments (Kurosawa and Tonegawa, 1982).

Our results confirm for fetal liver the findings for bone marrow by others (Nishikawa *et al.*, 1990) that pre B cells can grow on stromal cells and IL-7. The PB76-positive population of fetal liver cells did not, however, contain any cells at detectable levels (i.e. >1 in 1000) which proliferated

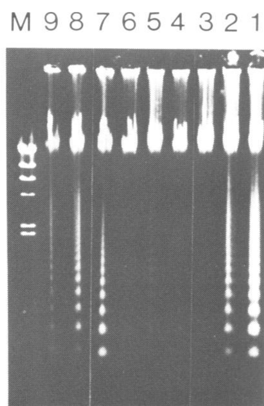

Fig. 7. Agarose gel electrophoresis of DNA from the PA-B line line and its clones (taken at day 0) visualized by UV light. Approximately 1 μg high mol. wt DNA was loaded onto an agarose gel. Lanes 1–6, 8 and 9 contain DNA of clones 4, 5, 8, 9, 12, 18 1 and 3; lane 7 the PA-B cell line. DBA/2 liver DNA showed very little, if any, degradation (not shown in the figure).

either with stromal cells alone, or with IL-7 alone. We could have missed the cells which proliferate on PA-6 alone, either because they are not PB76 positive, or because the wave of differentiation might already have passed the time of development where such cells were present in fetal liver, if the scheme of B cell differentiation proposed by Nishikawa *et al.* (1990) turns out to be correct. A search for earlier progenitors is now under way at earlier times of embryonic development.

We have observed with one clone (clone 5) the spontaneous development of a stromal cell-independent, IL-7-dependent cell clone that may be analogous to IL-7-CFU (in the nomenclature of Nishikawa and colleagues). In contrast to the IL-7-CFU, our cell clone is sIg^+. Since it needs several weeks in culture to develop, we favor the idea that they represent transformed cell lines, rather than a normal step in development, which retain part of their normal phenotype, i.e. capable of induction to Ig-secreting plaque-forming cells by mitogens (A.Rolink and F.Melchers, in preparation). Interestingly, our 'transformed' cell line was sIg^+ while retaining pre B cell-type markers like $V_{pre}B$, λ_5, PB76 and IL-7 reactivity. Surface Ig^+ pre B cell-type tumors and transformed cell lines have been identified (Cooper *et al.*, 1986; Reth *et al.*, 1986a,b), indicating that the status of rearrangements and expression of Ig genes can be disconnected from other markers and functions depicting an early pre-B cell phenotype.

Pro B cell lines with Ig genes in germ-line configuration have been established from bone marrow which proliferate under the stimulating influence of IL-3 (Palacios *et al.*, 1984; Palacios and Steinmetz, 1985). We have established IL-3 dependent cell lines from the PB76-positive population of fetal liver, but have not characterized these cells further. Our IL-3 dependent cells do not proliferate on stromal cells and/or IL-7, nor can our cells, which proliferate on stromal cells and IL-7, continue to grow with IL-3. In contrast, IL-3 dependent pro B cell clones established by others were shown to differentiate into sIg^+ cells upon interacting with stromal cells and IL-7 (Takeda *et al.*, 1989).

The differentiation of our stromal cells/IL-7-dependent pre B cells to sIg^+ cells occurs with the same rapid time schedule of 2–3 days, as it does *in vivo* and in primary cell cultures of fetal liver (Melchers, 1977; Strasser *et al.*, 1989). We think that the majority of all cells rearrange both V_H to D_HJ_H and V_L to J_L, although this is difficult to measure quantitatively, since differentiation is accompanied by cell death. Differentiation from the pre B cell line or from the clones did result in cells becoming sIg^+. It is very likely, but needs to be shown, that this differentiation also leads

Table IV. Appearance of donor IgM^+ B cells in the spleen of CB17 SCID mice injected with PA-B pre B cells

Weeks after injection of pre B cells	Percentage of nucleated spleen cells positive for*					
	$H\text{-}2K^b$	$H\text{-}2K^b + \mu H$**	$H\text{-}2K^b$ + B220**	$H\text{-}2K^b$ + MHC class II**	$H\text{-}2K^b$ + CD4 + CD8**	$H\text{-}2K^b$ + Mac1**
6	4.1	3.3	3.9	ND	0.1	0.1
9	5.2	5.1	5.0	ND	0.2	0.2
9	10.1	9.6	11.0	ND	0.4	0.3
21	3.3	2.9	2.9	2.0	0.2	0.1
25	5.8	4.2	4.7	4.5	0.3	0.1

*Percentage of positive cells was determined by two color FACS analysis using the PE-labeled anti-$H\text{-}2K^b$ mAb Y-3, and biotin–FITC–streptavidin-labeled secondary mAbs specific for the second determinants.
**$H\text{-}2K^b$ negative μH positive cells were undetectable in these CB17 SCID mice.

to sIg^- cells. One clone (18) never yielded any detectable level of sIg^+ cells. A low number of sIg^+ cells arising from precursors which all rearrange their H and L chain loci is expected if the gene segments of the loci rearrange randomly, i.e. in two-thirds of all events out of frame. In the periphery of a normal lymphoid system, as well as in the pre B cell-reconstituted SCID mice, sIg^- B cells with appropriate lineage markers are notably absent. sIg^+ cells appear to have a survival advantage over sIg^- cells.

Cell death by apoptosis is particularly evident in pre B cell cultures differentiating to fully rearranged H and L chain loci, although it is difficult at the present time to rule out the possibility that this cell death can also occur at earlier stages of differentiation. It is conceivable that apoptosis occurs preferentially in the H/L-nonproductively rearranged sIg^- population. Future experiments should clarify whether surface deposition of Ig-like or Ig molecules (on pre B or B cells) turns off apoptosis, or whether the lack of surface deposition turns on apoptosis in mature B cells.

The variation in the expression of B lineage related phenotypes in the PA-B line and in different cell clones, in particular that of B220, is remarkable. Even more surprising is the fact that not all cells within a clone express a given marker. This apparent 'nonclonal' behavior is retained even after recloning where Poisson's distribution of the cloning experiments and the analysis of the context of D_H and J_H segments both clearly support the clonal nature of these cells. It points to a complex hierarchy in the expression of these markers. At the present time we see no relation to the function of different pre B cell clones in their capacity to rearrange H and L chain gene segments to functional genes, to lose their capacity to grow in stromal cells and IL-7 upon differentiation, and to become sIg^+, mitogen-reactive cells. Most remarkable is the difference in surface marker differentiation *in vitro* and *in vivo*. Particularly striking is the difference in expression of MHC class II on the surface of sIg^+ cells, absent from cells differentiated *in vitro*, but present on those differentiated *in vivo*. We have not yet been able to induce MHC class II expression *in vitro*, either by adding IL-4 or by adding γ-IFN (A.Rolink and F.Melchers, unpublished). We think that sIg^+ MHC class II-negative cells precede in differentiation those which turn MHC class II-positive. This transition might now be studied with our cell lines.

Our work has defined a B-lineage committed progenitor cell, with long-term (at least 6 months of proliferation) renewal capacity *in vitro*, to be at the stage of D_HJ_H re-arranged H chain chromosomes before V_H to D_HJ_H, and before V_L to J_L rearrangements have taken place. Commitment of the B lineage might, thus, be expected to include D_H to J_H rearrangements, which should occur in cells possibly before they become reactive to stromal cells and IL-7. A rigorous test of a possible committed stem cell character of these pre B cells still needs to be done by secondary transfer experiments, and studies on the turnover of the established B cell compartments in SCID mice.

D_HJ_H rearranged H chain loci in the PA-B line and in the clones appear to be transcribed, in agreement with findings by others, such that a rearranged D_HJ_H locus can yield a 2.3 kb long transcript (Reth and Alt, 1984; Lennon and Perry, 1990). In some instances such a transcript can be translated into a $D_HJ_HC_\mu$-protein (Reth and Alt, 1984). Recently it has been shown that the proteins encoded by the pre B cell specific genes $V_{pre}B$ and λ_5 can form a complex with normal ($VDJC_\mu$) μH chain and, moreover, it was shown that this complex can be expressed on the surface of cells (Pillai and Baltimore, 1987; Kerr *et al.*, 1989; Karasuyama *et al.*, 1990; Tsubata and Reth, 1990). In analogy to this one could imagine D_HJ_H-rearranged pre B cells to express the $V_{pre}B$ and λ_5 protein and maybe also the protein encoded by a V_H gene segment specifically expressed by pre B cells (Yancopoulos and Alt, 1985), together with the $D_HJ_HC_\mu$ protein on the surface. We speculate that, when expressed, such a complex could be involved in the specific interaction of stromal cells and pre B cells, keeping pre B cells at the D_HJ_H rearrangement state.

Finally, pre B cell lines of the type described in this paper have been established from a series of mouse strains, including SCID. This opens the possibility for studying possible genetic differences and defects in B cell development, and to monitor possible changes to (partial) transformation towards malignant states of the B cell lineage. Transfections of genes and selection of the transfected pre B cells, followed by functional analysis of the transfected genes before and after induction of differentiation to sIg^+ cells and plasma cells appear possible. It should be possible to establish similar cell lines from other species, notably from man. Finally, the transplantation of these cells into SCID mice promises exciting possibilities to study B cell generation and turnover, and the influences of the environment (in particular antigens and T cells) on them.

Materials and methods

Animals

Adult female C57BL/6 mice, male DBA/2 mice and 3- to 6-week-old Lewis rats were obtained from the Institut für Medizinische Forschung AG, Füllinsdorf, Switzerland. (C57BL/6 × DBA/2)F_1 (BDF_1) embryos from time pregnant C57BL/6 females were provided by breeding facilities at the Basel Institute for Immunoloty. The appearance of vaginal plugs was counted as day 0 of gestation. Birth occurred at day 19. CB17 SCID mice were bred at our own animal facilities from breeding pairs originally obtained from M.Bosma (Institute for Cancer Research, Fox Chase, Philadelphia, PA, USA).

Cell lines

The stromal cell line PA-6 was obtained from Dr Kodama, the ST-2 cell line from Dr S.I.Nishikawa, Kumamoto University, Kumamoto, Japan. All cell lines were grown in IMDM containing 100 U/ml kanamycin, 5×10^{-5} M 2-ME and 10% heat inactivated FCS.

Interleukin

IL, murine r-IL-2, -3, -4, -5 and human r-IL-6 were obtained as described (Karasuyama and Melchers, 1988) and were used at 50–100 U/ml. Murine rIL-7, produced by a cell line transfected with the mouse IL-7 gene (kindly provided by Dr S.I.Nishikawa), was used at a concentration of 100–200 U/ml.

Monoclonal antibodies

The mAbs G-5-2 [pre B cell related (Strasser, 1988)], 14.8 [anti-B220 (Kincade *et al.*, 1981b)], BP-1 [pre B cell specific (Cooper *et al.*, 1986)], Ly-1 53.7.3 [anti-mouse CD5 (Ledbetter and Herzenberg, 1978)], M1/70 [anti-mac1 (Springer *et al.*, 1979)], R52.120 [anti-mouse IL-5R (Rolink *et al.*, 1989)], M5/114 [anti-mouse MHC class II (Bhattacharya *et al.*, 1981)], GK 1.5 [anti-mouse CD4 (Dialynas *et al.*, 1983)], Lyt-2 53.6.7 [anti-mouse CD8 (Ledbetter and Herzenberg, 1979)], M41 [anti-mouse-H (Leptin, 1985)], 187.1 [anti-mouse κ-L (Yelton *et al.*, 1981) and Y-3 anti-H-$2K^b$ (Hämmerling *et al.*, 1982)] were purified from culture supernatant or ascites on protein C (Pharmacia, Uppsala, Sweden) as recommended by the supplier. The mAbs 7D4 anti-IL-2R p55 (Malek *et al.*, 1983) and J.I.J. [anti-mouse thy1 (Bruce *et al.*, 1981)] were purified from ascites

fluid by precipitation at 35% $(NH_4)_2SO_4$ saturation followed by FPLC on Superose 12 (Pharmacia).

Immunofluorescence and flow cytometric analysis

For immunofluorescence analysis we routinely used purified mAbs either coupled to biotin and visualized with an appropriate dilution of FITC-labeled streptavidin (Radiochemical Centre, Amersham, UK) or directly coupled with PE (Molecular Probes, Inc., Junction City, OR). Coupling of PE to mAbs was done as described by the supplier. Staining of the cells was done as described by Rolink *et al.* (1989). Fluorescence intensity was measured with a FACScan instrument or with a FACS 440 (Becton Dickinson, Mountain View, CA) equipped with an argon laser tuned to 488 nm, operating at 200 mW. Positive cells were sorted using the FACS 440.

Establishment of pre B cell lines and clones

At days 13 and 14 of gestation, fetal liver cells were enriched for pre B cells by cell sorting using mAb G-5-2 and were cultured at $5 \times 10^4-2 \times 10^5$/ml in serum-substituted IMDM (Iscove and Melchers, 1978) on a semi-confluent layer ($1-3 \times 10^4$/ml) of 3000 rad γ-irradiated stromal cells or fibroblasts in the presence of 100–200 U/ml IL-7. Cultures were set up at 0.2 ml in 96-well flat bottomed microculture plates (Costar, Cambridge, MA). After 3–4 days when the pre B cells were grown to a semi-confluent layer on the stromal cells or fibroblasts, they were transferred to a new stromal cell or fibroblast layer in a 24-well culture plate (Costar). Cells were then once again grown in the presence of 100–200 U/ml IL-7 into a semi-confluent layer (3–4 days) in 1 ml serum-substituted IMDM, whereafter they were transferred to PA-6/IL-7 cultures in a 25 cm^2 tissue culture flask. The cells were harvested after a 3–4 day culture period and recultured in PA-6/IL-7 cultures in 75 cm^2 tissue culture flasks at a density of 2×10^6 pre B cells/flask. From then on the cells were fed every 3–4 days as described above. Cloning of a pre B cell line established under these conditions was done by culturing the cells at 0.5 cells/well in PA-6/IL-7 cultures in 96-well flat bottomed microculture plates in serum-substituted IMDM. After 5–6 days the plates were checked for cultures showing positive growth of clones of pre B cells by the use of an inverted microscope. Clones of pre B cells were then transferred to 24-well culture plates and thereafter to tissue culture flasks under conditions as described above.

LPS reactivity

LPS reactivity was determined in limiting dilution experiments (Andersson *et al.*, 1977). These were performed in 96-well culture plates in serum-substituted IMDM in the presence of 3×10^6 rat thymocytes/ml and 50 μg/ml LPS (S form of *Salmonella abortus equi*, a kind gift of Drs G.Galanos and O.Lüderitz, MPI, Freiburg, Germany). IgM-secreting cells were determined on day 5 of culture using the protein A plaque assay (Gronowicz *et al.*, 1976). A positive culture with a clone of IgM-secreting cells arising from one LPS-reactive B cell contained between 15 and 50 IgM PFC.

Transfer of pre B cells to CB17 SCID mice

CB17 SCID mice were irradiated with 300 rad and, 4–6 h after irradiation, i.v. injected with 5×10^6 pre B cells. At different times after the injection the spleen cells of these mice were analyzed with a panel of mAbs in FACS and for LPS reactivity in limiting dilution.

Southern blot analysis

High mol. wt DNAs were extracted from established pre B cell lines, clones and from livers of C57BL/6 and DBA/2 mice. After restriction enzyme digestion, 10 μg of DNA fragments were separated by electrophoresis on 0.8% agarose gel and transferred to the Zetaprobe membrane (BioRad) using 0.4 N NaOH as a transfer buffer (Reed and Mann, 1985). After transfer, filters were rinsed twice in $2 \times$ SSC and dried at 80°C under vacuum. Prehybridization and hybridization were carried out with $1.5 \times$ SSPE, 1.0% SDS, 0.5% non-fat powdered milk, 0.5 mg/ml carrier salmon sperm DNA and 10% dextran sulfate at 65°C by using ^{32}P oligolabeled J_H (*Xba*I–*Eco*RI 0.7 kb fragment), J_K (*Xba*I–*Hin*dIII 1.7 kb fragment), 5′ D_{FL} 16.1 (*Bam*HI 0.6 kb fragment; Reth *et al.*, 1986a), 5′ $D_{SP}2$ probe (*Pst*I 0.4 kb fragment; Reth *et al.*, 1986a) and $C\lambda 1$ (420 bp *Xho*I–*Pst*I fragment; Bothwell *et al.*, 1982) as probes. Filters were washed finally with $0.2 \times$ SSC, 0.1% SDS at 65°C. Filters were stripped with $0.1 \times$ SSC, 0.1% SDS at 95°C for 30 min and rehybridized.

Northern blot analysis

Total RNA for Northern blot analysis was prepared by phenol extraction in the presence of guanidine thiocyanate (Chomczynski and Sacchi, 1987). Total RNA (10 μg) was electrophoresed by the agarose–formaldehyde method and transferred to the Zetaprobe membrane using 50 mM NaOH as a transfer buffer. Prehybridization and hybridization were performed with 50% formamide, $5 \times$ SSC, 0.2 M Na-phosphate buffer, pH 6.8, 0.5% non-fat powdered milk, 0.5 mg/ml carrier salmon sperm DNA and 10% dextran sulfate at 50°C by using ^{32}P oligolabeled C_μ (1.2 kb *Pst*I fragment), $V_{pre}B1$ (0.9 kb *Eco*RI fragment of pz121; Kudo and Melchers, 1987) and λ_5 (0.7 kb *Hin*cII–*Xho*I fragment of pz183-1a; Kudo *et al.*, 1987) as probes.

Acknowledgements

The able technical assistance of Ms Anita Imm and Fabienne Fisson is gratefully acknowledged. We thank Drs Klaus Karjalainen, Jean-Claude Weill and Ronald Palacios for critically reading the manuscript. The Basel Institute for Immunology was found and is supported by F.Hoffmann-La Roche Ltd, Basel, Switzerland.

References

Andersson,J., Sjöberg,O. and Möller,G. (1972) *Eur. J. Immunol.*, **2**, 349–357.

Andersson,J., Coutinho,A. and Melchers,F. (1977) *J. Exp. Med.*, **145**, 1511–1522.

Bhattacharya,A., Dorf,M.E. and Springer,T.A. (1981) *J. Immunol.*, **127**, 2488–2496.

Bosma,G.C., Custer,R.P. and Bosma,M.Y. (1983) *Nature*, **302**, 527–530.

Bothwell,A.L.M., Paskind,M., Reth,M., Imanishi-Kari,T., Rajewsky,K. and Baltimore,D. (1982) *Nature*, **298**, 380–382.

Bruce,J., Symington,F.W., McKearn,T.J. and Sprent,J. (1981) *J. Immunol.*, **127**, 2496–2501.

Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.

Collins,L.S. and Dorshkind,K. (1987) *J. Immunol.*, **138**, 1082–1087.

Cooper,M.D., Mulvaney,D., Coutinho,A. and Cazenave,P.A. (1986) *Nature*, **321**, 616–618.

Dialynas,D.P., Quan,Z.S., Wall,K.A., Pierres,A., Quintas,J., Loken,M.K., Pierres,M. and Fitch,F.W. (1983) *J. Immunol.*, **131**, 2445–2451.

Dorskind,K., Johnson,A., Collins,L., Keller,G.M. and Phillips,R.A. (1986) *J. Immunol. Meth.*, **89**, 37–45.

Gisler,R.H., Söderberg,A. and Kamber,M. (1987) *J. Immunol.*, **138**, 2433–2470.

Gronowicz,E., Coutinho,A. and Melchers,F. (1976) *Eur. J. Immunol.*, **6**, 588–590.

Hämmerling,G.J., Rausch,E., Tada,N., Kimura,S. and Hämmerling,U. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4737–4741.

Hunt,P., Robertson,D., Weiss,D., Rennick,D., Lee,F. and Witte,O.N. (1987) *Cell*, **40**, 991–1007.

Iscove,N.N. and Melchers,F. (1988) *J. Exp. Med.*, **147**, 923–930.

Karasuyama,H. and Melchers,F. (1988) *Eur. J. Immunol.*, **18**, 97–107.

Karasuyama,H., Kudo,A. and Melchers,F. (1990) *J. Exp. Med.*, **172**, 969–972.

Kerr,W.G., Cooper,M.D., Feng,L., Burrows,P.D. and Hendershot,L.M. (1989) *Int. Immunol.*, **1**, 355–361.

Kinashi,T., Inaba,K., Tsubata,T., Tashiro,K., Palacios,R. and Honjo,T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4473–4482.

Kincade,P.W., Lee,G., Paige,C.J. and Street,M. (1981a) *J. Immunol.*, **127**, 255–260.

Kincade,P.W., Lee,G., Watanabe,T., Sun,L. and Scheid,M.P. (1981b) *J. Immunol.*, **127**, 2262–2268.

Kincade,P.W., Lee,G., Pietrangeli,L.E., Hayashi,S.-I. and Grimble,J.M. (1989) *Annu. Rev. Immunol.*, **7**, 11–25.

Kodama,H., Sudo,H., Koyama,H., Kasai,S. and Yamamoto,S. (1984) *J. Cell Physiol.*, **118**, 233–240.

Kudo,A. and Melchers,F. (1987) *EMBO J.*, **6**, 2267–2272.

Kudo,A., Sakaguchi,N. and Melchers,F. (1987) *EMBO J.*, **6**, 103–107.

Kurosawa,Y. and Tonegawa,S. (1982) *J. Exp. Med.*, **155**, 201–218.

Ledbetter,J.A. and Herzenberg,L.A. (1979) *Immunol. Rev.*, **47**, 63–80.

Lennon,G.G. and Perry,R.P. (1990) *J. Immunol.*, **144**, 1973–1987.

Leptin,M. (1985) *Eur. J. Immunol.*, **15**, 131–138.

Malek,T.R., Robb,R.J. and Shevach,E.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5694–5698.

Melchers,F. (1977) *Eur. J. Immunol.*, **7**, 476–481.

Melchers,F. (1979) *INSERM Symp.*, **10**, 281–290.

Melchers,F., Strasser,A., Bauer,S.R., Kudo,A., Thalmann,P. and Rolink,A. (1990) *Cold Spring Harbor Symp. Quant. Biol.*, **54**, 183–189.

Namen,A.E., Lupton,S., Hjenild,K., Wynall,J., Mochizuki,D.Y.,

Schmierer,A., Mosly,B., Mach,C.J., Urdal,D., Gillis,S., Cosman,D. and Goodwin,R.G. (1988) *Nature*, **333**, 571–574.
Nishikawa,S.I., Ogawa,M., Nishikawa,S., Kunisada,T. and Kodama,H. (1988) *Eur. J. Immunol.*, **18**, 1767–1777.
Nishikawa,S.-I., Hayashi,S.-I., Ogawa,M., Kunisada,T., Nishikawa,S., Sudo,T., Nakaguchi,H. and Suda,T. (1990) *Cold Spring Harbor Symp. Quant. Biol.*, **54**, 171–181.
Ogawa,M., Nishikawa,S., Ikuta,K., Yamamura,F., Naito,M., Takahashi,K. and Nishikawa,S.I. (1988) *EMBO J.*, **7**, 1337–1343.
Paige,C.J., Gisler,R.H., McKearn,J.P. and Iscove,M.N. (1984) *Eur. J. Immunol.*, **14**, 979–988.
Palacios,R. and Steinmetz,M. (1985) *Cell*, **41**, 727–734.
Palacios,R., Henson,G., Steinmetz,M. and McKearn,J.P. (1984) *Nature*, **309**, 126–131.
Palacios,R., Stuber,S. and Rolink,A. (1989) *Eur. J. Immunol.*, **19**, 347–356.
Park,Y.H. and Osmond,D.G. (1987) *J. Exp. Med.*, **165**, 444–458.
Phillips,R.A. (1989) *Prog. Immunol.*, **7**, 305–315.
Pietrangeli,L.E., Hayashi,S.-I. and Kincade,P.W. (1988) *Eur. J. Immunol.*, **18**, 863–877.
Pillai,S. and Baltimore,D. (1987) *Nature*, **329**, 172–174.
Reed,K.C. and Mann,D.A. (1985) *Nucleic Acids Res.*, **13**, 7207–7221.'
Reth,M.G. and Alt,F.W. (1984) *Nature*, **312**, 418–423.
Reth,M.G., Jackson,S. and Alt,F.W. (1986a) *EMBO J.*, **5**, 2131–2138.
Reth,M., Gehrmann,P., Petrac,E. and Wiese,P. (1986b) *Nature*, **322**, 840–842.
Rolink,A.G., Melchers,F. and Palacios,R. (1989) *J. Exp. Med.*, **169**, 1693–1701.
Sakaguchi,N. and Melchers,F. (1986) *Nature*, **324**, 579–582.
Springer,T., Galfré,G., Sacher,D.S. and Milstein,C. (1979) *Eur. J. Immunol.*, **9**, 301–306.
Strasser,A. (1988) *Eur. J. Immunol.*, **18**, 1803–1811.
Strasser,A., Rolink,A. and Melchers,F. (1989) *J. Exp. Med.*, **170**, 1973–1986.
Takeda,S., Gillis,S. and Palacios,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1634–1638.
Tonegawa,S. (1983) *Nature*, **302**, 575–578.
Tsubata,T. and Reth,M. (1990) *J. Exp. Med.*, **172**, 973–976.
Whitlock,C.A. and Witte,O.N. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3608–3612.
Whitlock,C., Dennis,K., Roberston,D. and White,O.N. (1985) *Annu. Rev. Immunol.*, **3**, 213–235.
Whitlock,C.A., Tidmarsh,G.F., Müller-Siegburg,C. and Weissman,I.L. (1987) *Cell*, **48**, 1009–1021.
Witte,P.L., Robertson,M., Henley,A., Low,M.G., Stiers,D.L., Perkins,S., Fleischman,R.A. and Kincade,P.W. (1987) *Eur. J. Immunol.*, **17**, 1473–1480.
Yancopoulos,G.D. and Alt,F. (1985) *Cell*, **40**, 271–277.
Yelton,D.E., Desaymarol,C. and Scharff,M.D. (1981) *Hybridoma*, **1**, 5–10.

Received on October 5, 1990; revised on November 26, 1990