The EMBO Journal vol.11 no.2 pp.593–601, 1992

Enrichment and characterization of uncommitted B-cell precursors from fetal liver at day 12 of gestation

Ana Cumano[1] and Christopher J.Paige[2]

The Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Canada M4X 1K9

[1]Present address: Pasteur Institut, Rue du Dr Roux, 75724 Paris Cedex 15, France

[2]Corresponding author

Communicated by P.-A.Cazenave

We describe an assay system that allows precursor cells, uncommitted for heavy and light chain immunoglobulin expression, to develop into B lymphocytes that can differentiate to antibody-producing cells. Some precursors have the immunoglobulin loci in germ-line configuration. Approximately 200–1500 precursor cells are present in one fetal liver by day 12 of gestation; they express the surface marker AA4.1. Most precursors do not express the B220 marker. Commitment to heavy chain immunoglobulin expression occurs after an average of two cell divisions; commitment to light chain expression takes place after two additional rounds of division. DNA analysis from the progeny of single precursor cells shows that: (i) most B220$^-$ precursor cells have not completed D–J rearrangement (9/11) and some were in germ line configuration (4/11); and (ii) most B220$^+$ precursor cells exhibit two D–J rearrangements (4/5 samples). These experiments define two types of B-lymphocyte precursor cells in fetal liver: the first, B220$^+$ AA4.1$^+$, acquires the capacity to respond to mitogens only after 5 days in culture, and does not have productive V–D–J rearrangements but might exhibit two stable D–J rearrangements; the second, B220$^-$ AA4.1$^+$, acquires the capacity to respond to mitogens only after 9 days in culture and can be in germ-line configuration in the Ig loci, and undergoes rearrangement of heavy and light chain genes *in vitro*. Both precursor types require interaction with stromal cells before becoming responsive to interleukin 7.

Key words: limiting dilution/surface markers/uncommitted B-cell precursors

Introduction

Hematopoiesis occurs, during embryonic life, in the yolk sac blood islands and subsequently in fetal liver. *In vivo*, murine B lymphocytes develop from progenitors first detected in fetal liver by day 11–12 of gestation. Much of our knowledge of lymphopoiesis has been based on the development of culture systems that allow the expansion and differentiation of B-cell progenitors. These include short term assays which permit clonal analysis of B cell progenitors developing into mature cells (Melchers, 1977; Paige, 1983; Kincade, 1988); long term growth of primary pre-B cells (Whitlock and Witte, 1982); and studies based on transformed cell lines (Rosenberg *et al.*, 1979). These studies have yielded three criteria which are useful for delineating the sequence of events leading from stem cells to mature, Ig-secreting B cells. These are Ig gene rearrangements, cell surface markers and growth factor responsiveness.

Immunoglobulin gene assembly generally proceeds in a sequence of events starting with heavy chain $D-J_H$ rearrangement on both chromosomes. This is one of the first recognizable indicators of B lineage commitment. Subsequently, V_H joins to $D-J_H$ and then light chain V–J rearrangement occurs (Alt *et al.*, 1981, 1986). The ability to distinguish cells based on the status of their immunoglobulin loci provides an important tool for assessing progression along the B lineage pathway. Cell surface marker studies have also been used to define stages of B cell development. Particularly important for the studies presented in this paper have been the monoclonal antibodies designated AA4.1, which recognizes an uncharacterized determinant expressed on cells of the myeloid and lymphoid lineage (McKearn *et al.*, 1985), and 14.8, which recognizes the B220 form of CD45 (Kincade *et al.*, 1981). Finally, differential response to growth factors and mitogens has also been used to characterize the stage of differentiation of B lineage cells (Kincade *et al.*, 1988). For example, interleukin 7 (IL-7) (Namen *et al.*, 1988), the most important pre-B cell growth factor identified to date, appears to act only on pre-B cells that are at a relatively late stage of differentiation (Welch *et al.*, 1990; Cumano *et al.*, 1990; Hayashi *et al.*, 1990).

The association between surface marker expression, a given stage of immunoglobulin gene rearrangement and the ability to grow after stimulation with a particular growth factor is not yet clear. In addition, little is known about the genetic events and growth requirements in B-cell precursors before immunoglobulin gene rearrangement, in committed or uncommitted precursors. Assay systems that would allow primary cells with the immunoglobulin locus in germ-line configuration to develop into a mature functional B-lymphocyte *in vitro* would facilitate such studies, but have been difficult to develop. Ideally, such a system should: (i) detect single cells and allow their clonal progeny to be examined, by both morphological and genetic characterization; (ii) detect a high frequency of the progenitor avoiding selection of rare cellular events; (iii) allow cells to differentiate *in vitro* in a time frame similar to that found in normal development; (iv) not require transforming agents; and (v) be widely reproducible. In this paper we describe a system that allows such primary cells to generate B cells that respond to mitogens and secrete immunoglobulin.

The cells which initiate clonal expansion express the AA4.1 surface antigen (McKearn *et al.*, 1985; Sudo *et al.*, 1989). They are unable to grow when stimulated by IL-7 alone and require exposure to stromal cells. Cells differentiate to AA4.1$^+$ B220$^+$ cells *in vitro* and become mitogen responsive. Analysis of the immunoglobulins secreted by

© Oxford University Press

individual clones indicate that precursor cells have not yet stably rearranged the heavy chain immunoglobulin locus. Moreover, Southern blot analysis of DNA isolated from some clones shows unrearranged heavy chain bands corresponding to both chromosomes. This result indicates that the precursor cell which initiates clonal expansion was in germ-line configuration at the immunoglobulin loci.

Results

All B-cell precursors detected in a fetal liver in day 12 of gestation express the AA4.1 marker

In vivo reconstitution assays suggest that fetal liver at day 12 of gestation harbors many progenitors capable of generating B lymphocytes (Paige *et al.*, 1979). However, these cells respond poorly in an agar-based, colony-forming pre-B cell assay (Paige *et al.*, 1984). Our previous estimates show that only 5–10 precursor cells per fetal liver develop into Ig-secreting plaque-forming colonies (<1 in 3 × 10^5 cells). Our goal was to identify B-cell precursors before immunoglobulin gene rearrangement and analyze the genetic changes occurring during early stages of differentiation. We therefore attempted to establish a culture system which would permit the growth and differentiation of more primitive progenitor cells.

Fetal liver cells from day 12 of gestation were separated based on adherence to plates coated with the AA4.1 monoclonal antibody. Panning procedures for enrichment of AA4.1$^+$ cells resulted in a 0.8–2% recovery of fetal liver cells. The positive and negative populations were seeded at 1–2 × 10^4 cells/well in 24-well plates under various culture conditions. The AA4.1$^-$ population, which comprises 98% of the fetal liver cells, did not expand under any of the conditions tested (Table I). Table I also shows that we failed to detect cell expansion when IL-7 alone was added to cultures containing AA4.1$^+$ cells. In contrast, we had previously found that B220$^+$ fetal liver populations at day 15 of gestation proliferate extensively in response to IL-7 alone (Cumano *et al.*, 1990). The stromal cell line S17 has been previously shown to induce the expression of B220 in cells isolated from long term bone marrow cultures (Collins and Dorshkind, 1987). Table I shows that S17 cells alone failed to induce proliferation of the AA4.1$^+$ cells. However, we found that the combination of IL-7 and S17 cells induced a strong proliferative response. Cell numbers increased 100-fold in 6 days and surface staining revealed that >80% of those cells express B220. Moreover, cells obtained from these cultures could now be expanded in IL-7 alone (data not shown). The addition of interleukin-3 (IL-3), which has been implicated in the growth of B-cell precursor lines (Palacios and Steinmetz, 1985), did not alter the absolute number of B220$^+$ cells recovered. We conclude that stromal cells allow the expansion of a population of precursors, which precede the IL-7 responsive, pre-B cell stage.

Table I. Selection of culture conditions for 12 day fetal liver B-cell precursors

Growth conditions	Cell recovery (× 10^3) on day 6/10^3 input cells	Surface staining (% positive)		
		B220$^+$	AA4.1$^+$	sIgM$^+$
IL-7	<5	ND	ND	ND
S17[a] cells + IL-7	95	80	82	<5
S17[a] cells	<5	ND	ND	ND
S17[a] cells + IL-7 + IL-3	145	56	54	<5

AA4.1$^+$ fetal liver cells were set in culture in 24-well Costar plates at 10^4 cells in 1 ml of medium. On day six, cells were harvested and viable cells were counted. Cells were then resuspended at concentrations of 10^6 cells/ml and stained for surface markers. Cells in culture with IL-7 alone and S17 cells alone contained virtually no viable cells.

[a]S17 cells were irradiated with 2000 rads.

The frequency of B-cell precursors in the AA4.1$^+$ fetal liver population

The next experiments were designed to determine the frequency of B-cell precursors in AA4.1$^+$ 12 day fetal liver cells. Cells were panned on plates coated with either AA4.1 or 14.8. Cells bound by the antibody-coated plates were cloned under limiting dilution conditions and incubated for 7 days in the presence of IL-7 and S17 cells. On day 6, plates were examined microscopically. Two cell types were observed: (i) medium sized cells that became adherent by day 6–9 and, presumably, are macrophage precursors stimulated by CSF-1 produced by S17 cells; and (ii) small mononucleated cells which proliferated extensively in cultures containing IL-7 in the absence of S17 cells. Twenty individual clones of small non-adherent cells were stained with 14.8 antibody and >95% were B220 positive. Cells which generated the B220$^+$ small mononuclear progeny were scored as B-cell precursors. As discussed below, >90% of these cells give rise to progeny undergoing Ig gene rearrangement. The frequency of this cell type was determined in limiting dilution experiments and wells containing such cell types were scored positive for the presence of B-cell precursors (Figure 1). The frequency of B-cell precursors from three independent experiments is shown in Table II. Also shown are the percentage of cells recovered in the various fractions. We calculated the total number of precursors per fetal liver from the frequency of B-cell precursors in the population, the fraction of fetal liver cells this population represents and the mean number of cells recovered per

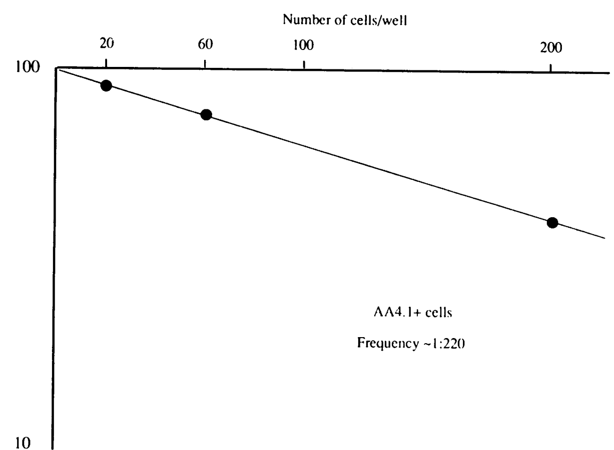

Fig. 1. Frequency of B-cell precursors in AA4.1$^+$ cells. Enriched AA4.1$^+$ cells were seeded at 20, 60, 100 and 200 cells per well in 96-well plates containing 1000 irradiated S17 cells and IL-7. 48 cultures were used for each experimental point. On the logarithmic scale (*y* axis) the percentage of cultures showing no lymphocyte growth was plotted and on the linear scale (*x* axis) the number of cells seeded per culture was plotted. The frequency of responding cells was calculated as the number of cells seeded, corresponding to 37% of non-responding microcultures.

fetal liver. We estimate that there are 200–1500 B-cell precursors per 12 day fetal liver and the frequency of these precursors in the selected population is between 1 in 250 and 1 in 50. Nearly all of these cells express AA4.1 while ~20% express B220 as well. This was demonstrated by a two-step panning separation. In this experiment enrichment for AA4.1$^+$ cells by positive selection was followed by a negative selection on 14.8 antibody-coated plates (this population is designated AA4.1$^+$ B220$^-$). As shown in Table II, most B-cell precursors detected in this assay belong to this cell population. The reciprocal separation was also done by selecting B220$^+$ cells followed by a negative selection on AA4.1 antibody-coated plates (AA4.1$^-$ B220$^+$). This cell population does not contain detectable numbers of B-cell precursors and probably contains the cells that adhere non-specifically to the coated plates.

Cells that did not bind to the AA4.1 or 14.8 antibody-coated plates are designated AA4.1$^-$ or B220$^-$ respectively, and their properties are listed in Tables II and III. AA4.1$^-$ cells recovered constitute 70% of fetal liver cells and do not contain detectable numbers of B-cell precursors (<20 per fetal liver). Incubation of those cells with IL-7 and stromal cells did not result in significant expansion and very few of the surviving cells expressed the B220 marker (Table III). These results confirm our previous conclusion that most B-cell precursors found in day 12 fetal liver express the AA4.1 surface marker. In contrast, the B220$^-$ precursor population contains most B-cell precursors and the staining data confirm that after 9 days in culture cells become B220$^+$. The specificity of the panning procedure was tested by panning cells in mouse anti-rat IgG-coated plates which resulted in the recovery of less than five precursors per fetal liver.

These results show that: (i) there are 50–100 times more B-lymphocyte precursor cells in fetal liver at day 12 of gestation than previously estimated based on a pre-B cell colony assay (Paige, 1983) or by limiting dilution analysis (Melchers, 1977); (ii) most precursor cells detected in the assay described express the AA4.1 surface marker but not the B220 marker (cells acquire the B220 surface marker in culture); (iii) ~20% of precursors detected express the B220 antigen and are a subpopulation of the AA4.1$^+$ cells; and (iv) neither population is capable of proliferating in IL-7 alone as shown in Table II.

Both AA4.1$^+$ B220$^-$ and B220$^+$ B-cell precursors develop into mitogen responsive cells and differentiate to immunoglobulin secreting plasma cells

We used two parameters to define B-cell precursors in the culture system described above, the acquisition of the B220 surface antigen and the ability of cells to proliferate in response to IL-7. Neither of these characteristics are unique for B-cell precursors. Confirmation of B lymphopoiesis requires the fulfilment of two additional expectations, the capacity to respond to lipopolysaccharide mitogen (LPS), by proliferation, differentiation and immunoglobulin secretion, and the rearrangement of immunoglobulin gene segments.

We therefore investigated the capability of the precursors which develop under these culture conditions to respond to LPS. We have previously reported that stromal cells are required for efficient maturation of B-cell precursors to acquire mitogen responsiveness (Cumano *et al.*, 1990).

Cells were obtained from 8 day cultures of single AA4.1$^+$ precursors and independently stimulated with LPS and S17 cells (Table IV). Detectable amounts of immunoglobulin were found in 40% of the 36 clones tested. The failure of some clones to produce Ig could be explained by: (i) cells that rearranged the Ig genes nonproductively either early in the culture period or that overgrew cells with productively rearranged Ig genes; (ii) absence of immunoglobulin gene rearrangement; or (iii) conditions allowing only a fraction of the precursors to respond to LPS. We tested the latter possibility by separating AA4.1$^+$ cells into B220$^+$ and B220$^-$ populations. These clones were stimulated with LPS, after 6 days in culture. As seen in

Table II. Frequency as determined by limiting dilution analysis of B-cell precursors from AA4.1$^+$ fetal liver cells

Exp. no.	Phenotype of enriched fetal liver cells	% of cells recovered after enrichment	Frequency of proliferating lymphocytes	Number of B-cell precursors/fetal liver
I	Mouse anti-rat Ig$^+$	0.15	<1:2000	<5
	AA4.1$^+$	2.6	1:240	195
	AA4.1$^-$	70.0	<1:83000	<20
	B220$^+$	0.4	1:220	36
	B220$^-$	65.0	1:4800	240
	AA4.1$^+$ B220$^-$	1.3	1:150	156
	AA4.1$^-$ B220$^+$	0.2	<1:1780	<13
	B220$^+$ (IL-7)	0.4	<1:1000	<8
	AA4.1$^+$ (IL-7)	2.6	<1:15000	<30
II	AA4.1$^+$	1.2	1:60	920
	AA4.1$^+$ B220$^-$	0.96	1:70	788
	B220$^+$	0.23	1:70	151
III	AA4.1$^+$ B220$^-$	1.5	1:42	1320
	B220$^+$	0.6	1:125	197

The different cell fractions were isolated by panning as described in the text. Plates coated with the first antibody alone (affinity purified mouse anti-rat IgG) were used as a control for the specificity of the panning procedure. Less than 0.2% of the cells adhered to this plate and no B-cell precursors were recovered in this population. The number of precursors/fetal liver was calculated as a function of frequency, cell recovery after separation and total numbers of cells isolated per fetal liver. The numbers of cells isolated per fetal liver were 1.8×10^6 cells, in the first experiment, 4.6×10^6 cells in the second and 4.4×10^6 in the third experiment. In the first experiment, cells were also stimulated with IL-7 alone (IL-7) at the same dilutions as conditions with IL-7 + S17 cells. No growing cells were observed after seven and 20 days of incubation.

Table IV none of the clones derived from B220$^-$ cells produced immunoglobulin. In contrast, 70% of clones derived from B220$^+$ cells produced Ig. However, after an additional 4 days in culture immunoglobulin production was observed in 88% of clones derived from B220$^+$ cells and 66% of clones derived from B220$^-$ cells.

In order to establish the kinetics of LPS responsiveness from the progeny of B220$^+$ and B220$^-$ cells, we performed a bulk culture experiment. We isolated B220$^+$ and AA4.1$^+$ B220$^-$ cells from fetal liver of Balb/c × C57BL/6 mice at day 12 of gestation. Cells were incubated with irradiated S17 stromal cells and IL-7 at 10^4 cells/ml. On days 5–10, 10^4 cells were removed daily and tested for their ability to respond to LPS. After a 12 day stimulation period, the culture supernatant was collected and the amount of immunoglobulin was determined in an ELISA assay. Figure 2 shows the concentration of immunoglobulin present in the supernatant of LPS stimulated cells from B220$^+$ and AA4.1$^+$ B220$^-$ precursors. B220$^+$ precursors can respond to LPS stimulation by secretion of immunoglobulin between days 5 and 10 of culture. In contrast, AA4.1$^+$ B220$^-$ cells cannot respond to LPS until days 8–9 after the beginning of culture: from day 10 to 12 the amount of immunoglobulin secreted by the same number of cells is comparable with that of B220$^+$-derived precursors. The results described above suggest that AA4.1$^+$ B220$^-$ cells require a longer period of time in culture than B220$^+$ cells from day 12 fetal liver to become mature, functional lymphocytes.

Table III. Properties of AA4.1$^-$ and B220$^-$ fetal liver cells

Cell phenotype	Cell recovery on day 9/10^4 cells	Surface staining (% positive)
		B220$^+$
AA4.1$^-$	2.58×10^4	4.4
B220$^-$	20.83×10^4	68

The negative populations for AA4.1 and B220 were cultured for nine days with IL-7 and S17 irradiated cells at 10^4 cells/well in 24-well plates. After this period, cells were counted and resuspended at 10^6 cells/ml and stained with 14.8 antibody followed by mouse anti-rat IgG FITC-labelled.

Table IV. Immunoglobulin production by single clones of AA4.1$^+$ B220$^-$ and B220$^+$ precursors

Cell phenotype (days in culture)	Number of clones tested	Number of clones secreting Ig	% of clones secreting Ig
AA4.1$^+$ (Day 8)	36	15	41.6
AA4.1$^+$ B220$^-$ (Day 6)	17	0	0
B220$^+$ (Day 6)	18	12	66.6
AA4.1$^+$ B220$^-$ (Day 10)	15	9	70
B220$^+$ (Day 10)	17	15	88

After 6, 8 or 10 days in culture with IL-7, clones were resuspended in 50 μl of medium and divided between two wells of a 96-well plate with 25 μg LPS/ml and irradiated S17 cells, in a final volume of 250 μl/well. The supernatants were harvested after 12 days in culture and the immunoglobulin concentration measured by an enzyme-linked immunosorbent assay (ELISA). Clones considered positive for Ig secretion produced more than 100 ng Ig/ml in 400 μl. The detection limit of our assay was 5 ng/ml. Most clones considered negative did not secrete detectable levels of Ig.

Clonal analysis of immunoglobulin secreted by 12 day fetal liver cells

Mature B cells in the spleen of Balb/c × C57BL/6 F1 animals express only one of the immunoglobulin heavy chain allotypes, either the Igh6a (IgM) allotype encoded by the Balb/c chromosome or the Igh6b allotype encoded by the C57BL/6 chromosome. Because of allelic exclusion a single B cell will express immunoglobulin encoded by either allele but not both. By analyzing the allotype in the Ig secreted by a clone of cells, we can obtain information on the differentiation stage of the cell that originated the clone. The progeny of an uncommitted precursor will independently rearrange the Ig heavy chain locus as they divide resulting in the appearance of two allotypes in a single clone. Likewise, κ and λ light chain expression can be studied to determine whether the cell that originated the clone was already restricted to light chain isotype.

We tested the clones generated from B220$^+$ and B220$^-$, AA4.1$^+$ precursor cells for expression of immunoglobulin heavy chain allotype and light chain isotype. The limiting dilution culture conditions in 96-well plates were such that one-third of the wells are positive for cell growth. Based on coincidence, it would be expected that ~15% of the positive wells are the product of two or more precursors. Thus, of 50 wells examined, at least 42 would be expected to be derived from a single cell. Microscopic inspection of the plates allowed us to identify most wells containing multiple precursor cells. The numbers found coincided with the expected values and such clones were excluded from our analysis. Individual clones were then transferred to 96-well plates with S17 cells and LPS, and supernatants were collected after 10–12 days of stimulation. The immuno-

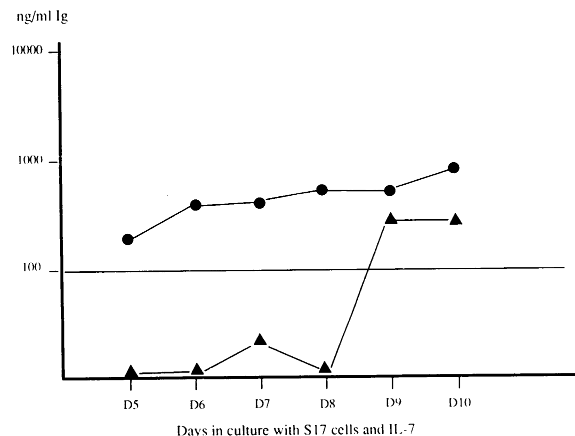

Fig. 2. Titration of immunoglobulin secreted by the progeny of B220$^+$ and B220$^-$, AA4.1$^+$ fetal liver cells. Cells were cultured at 10^4 cells/ml with IL-7 and irradiated S17 cells. From days 5–10, 10^4 cells were transferred daily to two wells of a 96-well plate with LPS and S17 irradiated cells. After 12 days of stimulation the supernatant was harvested and IgM and IgG concentrations were determined by an ELISA assay. The results are expressed in ng/ml of IgM in 400 μl of supernatant. Supernatants contained comparable amounts of IgM and IgG. ▲ designates the progeny of B220$^-$ cells and ● the progeny of B220$^+$ cells. Supernatants expressing <100 μg/ml were considered negative for immunoglobulin secretion.

Table V. Heavy chain allotype and light chain isotype secreted by clones of AA4.1$^+$ B220$^-$ and B220$^+$ precursors

Cell phenotype	Number of clones secreting immunoglobulin						
	IgM	Igh6^{a+b}	Igh6^b	Igh6^a	IgM$^{\kappa+\lambda}$	IgM$^\kappa$	IgM$^\lambda$
AA4.1$^+$ (Day 8)	15	13	0	2			
AA4.1$^+$ B220$^-$ (Day 6)	0	0	0	0	0	0	0
B220$^+$ (Day 6)	12	10	0	2	12	0	0
AA4.1$^+$ B220$^-$ (Day 10)	9	8	1	0	8	1	0
B220$^+$ (Day 10)	15	13	1	1	14	1	0

The same clones shown in Table IV were analyzed for allotype heavy chain and isotype light chain expression. Clones considered positive produced more than 100 ng/ml of immunoglobulin. Only clones which secreted Ig are shown.

globulin concentrations were measured and the heavy chain allotype and light chain isotype of the secreted antibodies were determined (Table V). The clones in Table V are the same as in Table IV. Most clones analyzed express both Igh6a- and Igh6b-derived immunoglobulins. Single allotype expression was observed in seven out of 51 clones. This result is consistent with our hypothesis that B-cell clones develop from an immunoglobulin heavy chain uncommitted precursor. Moreover, most clones expressed both κ and λ light chains. Two out of 31 clones expressed κ chain only whereas none expressed λ only. These results indicate that most precursor cells are not yet committed to heavy or light chain immunoglobulin gene expression. In control experiments we analyzed the immunoglobulin produced by B220$^+$ 15 day fetal liver cells and splenic B cells from Balb/c $\times$ C57BL/6 F1 mice. We isolated 11 random clones from day 15 fetal liver growing in the conditions we described for day 12 fetal liver and stimulated them with LPS. All clones produced detectable amounts of immunoglobulin although three had <100 ng of Ig/ml. Of the remaining eight clones, five showed heavy chain allotype commitment and two showed light chain isotype commitment. LPS-stimulated splenic B cell clones were analyzed as a control for the specificity of the allotype-specific mAb; only one out of 33 showed reactivity to both allotype-specific antisera. Finally, B-cell precursors, expanded as described and subcloned after 12 days in the presence of S17 cells and LPS, were found to have undergone commitment to heavy chain expression, giving rise to progeny expressing only a single allotype.

Analysis of immunoglobulin heavy chain rearrangements in DNA isolated from individual clones

One of the unique characteristics of B-cell precursors is their ability to rearrange their DNA during development, bringing together V_H, D and J_H elements which, along with the constant region, encode for the heavy chain immunoglobulin protein. Table IV shows that 70% of the B220$^-$ and 90% of the B220$^+$ derived clones can respond to LPS and secrete immunoglobulin, proving that they are indeed B-cell precursors. However, a fraction of clones does not secrete Ig. It is possible that they constitute a hematopoietic lineage different from B-lymphocytes. To address this question we analyzed the Ig-gene rearrangement of DNA from individual Ig-secreting and non-secreting clones.

If the progeny from AA4.1$^+$ cells developed from B-cell precursors capable of undergoing Ig gene rearrangement, we would expect to detect multiple rearrangement restriction fragments in the DNA isolated from individual clones. For this analysis, cells from individual clones were expanded to $2-6 \times 10^6$ cells, harvested and stained with 14.8 mAb (cells were all $>98\%$ B220$^+$). DNA was prepared according to conventional methods; between 15 and 60 μg per clone was obtained. We probed the filters successively with the different J_H probes. Each $D-J_H1$ or V_H-D-J_H1 rearrangement should result in a band of a unique size that can be detected by all four probes. A rearrangement of a $D-J_H2$ or V_H-D-J_H2 should result in bands that cannot be detected by a J_H1 probe but can be detected by all other three probes. The same reasoning applies to J_H3 and J_H4 rearrangements.

Figure 3B shows an example of this analysis. Samples A, B, C and F were prepared from clones derived from B220$^-$ precursors and show discrete germ-line bands with all four J_H probes as well as multiple J_H rearrangements. Samples D and E were obtained from clones derived from B220$^+$ precursors. Sample E shows a germ-line band and several J_H rearrangement bands. Sample D shows evidence for two J_H rearrangements and no detectable germ-line band. Samples E and F produced immunoglobulin of both Igh-6a and Igh-6b allotypes. Sample G was obtained from an Abelson transformed pre-B cell line known to have two J_H2 and one J_H4 rearrangement and was used as an internal control on all filters. DNA samples from a total of 19 clones were analyzed. In eight of the DNA samples, rearranged bands hybridizing to three or more of the J_H segments could be detected, indicating ongoing $D-J$ rearrangements. The samples analyzed consisted of 11 from AA4.1$^+$ B220$^-$, five from B220$^+$ and three from unseparated AA4.1$^+$ precursors. Of the 11 B220$^-$ samples, seven showed evidence of more than two rearranged J_H segments and two showed a diffuse pattern of hybridization (9/11). Of the B220$^+$ samples, only one showed evidence of more than two rearranged J_H segments (sample E in Figure 3B). Of the three unseparated AA4.1$^+$ precursors two showed all four J segments rearranged while the other showed a diffuse pattern. Since allotype analysis indicated that B220$^+$ precursors are heavy chain immunoglobulin uncommitted, the results suggest that most B220$^+$ cells, in contrast to B220$^-$, already have two $D-J$ rearrangements.

Seven DNA samples showed a strong germ-line band. To determine whether this band was derived from one or both alleles, DNA was digested with *Kpn*I, a restriction enzyme with polymorphism in the J_H locus which distinguishes between the Igh6b allele (14.3 kb) and the Igh6a allele (13.1 kb) (Notenburg and Weissman, 1981). Southern blot analysis of *Kpn*I digested DNA, probed with J_H1 revealed two bands corresponding to the size of the germ line from both alleles. This result indicates that the precursor of the clones analyzed was in germ-line configuration in both immunoglobulin loci (Figure 3C). Of 11 DNA samples isolated from B220$^-$ precursors, seven showed germ-line bands corresponding to both chromosomes. This number is a minimum estimate as it relies on some cells retaining the original germ-line bands during expression. Clones which undergo loss of germ-line on both chromosomes early in culture expansion might be missed. We consider it unlikely

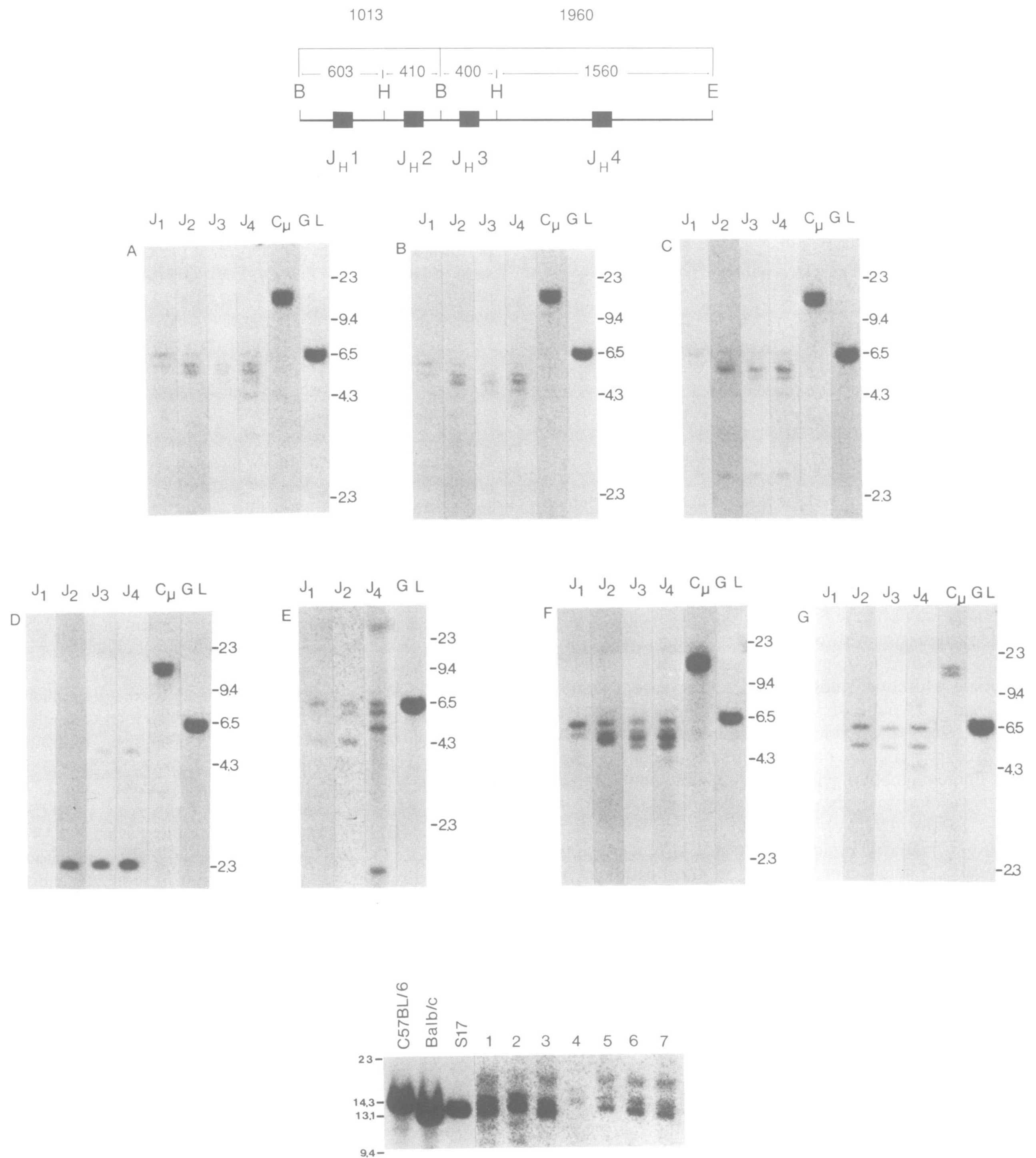

Fig. 3. (**A**) Schematic diagram of the restriction sites in the J_H locus. The numbers represent the size of restriction fragments in base pairs. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; J_H joining gene segment. (**B**) Southern blot analysis of DNA isolated from clones developed from B220 positive and negative precursor cells. 10 μg genomic DNA was digested with the enzyme *Eco*RI, blotted onto nylon membranes and hybridized to the J_H1, J_H2, J_H3 and J_H4 probes labelled with [^{32}P]CTP by random priming. All probes were labelled to comparable specific activities. The intensity of the signal decreased with successive reprobing as judged by the intensity of the bands detected in liver DNA. DNA samples labelled F and E were isolated from clones which could express immunoglobulin. Sample G corresponds to an Abelson transformed pre-B cell line from a DBA/2 × C57BL/6 mouse having two D$-J_H2$ rearrangements and one D$-J_H4$ rearrangement. After 3–4 days exposure the probe was removed and the filter was hybridized to the next probe. The last of five hybridizations was with the $C\mu$ probe which recognizes the constant region of the immunoglobulin locus and which should be identical to all DNA samples as a control for DNA partial degradation (the $C\mu$ bands for Balb/c, C57BL/6 and DBA/2 are 13 kb, 14.1 kb and 12.3 kb respectively). All filters had a germ-line control included and the composed figure was aligned according to the germ-line bands and to the Abelson line DNA. Similar rearrangement patterns could correspond to D$-J_H$ rearrangements. (**C**) Southern blot analysis of *Kpn*I-digested DNA isolated from clones developed from B220+ (lane 4) and B220$^-$ (all other lanes) precursor cells. Filters were hybridized with the J_H1 probe and exposed for 1 week (lanes 1–7) or 2 days (C57BL/6, Balb/c and S17 DNA). Lane 1 corresponds to lane A in Figure 3B, lane 2 to B, lane 5 to F and lane 7 to C. The polymorphic restriction fragments are 14.3 kb for the b allotype and 13.1 kb for the a allotype. S17 cells were derived from a Balb/c mouse bone marrow (Collins and Dorshkind, 1987).

that the S17 cells (Balb/c origin) used to expand the B-cell precursors, contributes detectable amounts of DNA to our preparations. Based on cell numbers S17 contamination would represent $<0.8\%$ of the samples used for DNA preparation. Moreover, all cells were expanded under the same conditions and DNA samples from $B220^+$ precursors did not show detectable germ-line bands.

Discussion

The results presented document a culture system which provides the requirements for the generation of mature B cells from progenitors that are immunoglobulin heavy chain uncommitted. Utilizing an enrichment protocol for fetal liver derived B-cell precursors, we followed the development of 50 clones to the terminal stage of differentiation. This analysis led us to establish several significant stages in the development of B lymphocytes: (i) B-cell precursors in fetal liver originate from $AA4.1^+$ $B220^-$ cells which have the immunoglobulin genes in germ-line configuration; (ii) the expression of the B220 marker occurs at the time of $D-J_H$ rearrangement; (iii) two or three generations separate heavy and light chain commitment; (iv) precursor cells with both immunoglobulin loci in germ-line configuration can develop *in vitro* to mature B cells.

The identification of cell surface markers that allow the isolation of heavy chain uncommitted B-cell precursors, has never been clearly established. Muller-Sieburg *et al.* (1986) have shown that a population of $Thy\text{-}1^{lo}$ $B220^-$ cells can generate lymphoid clones under Wittlock–Witte culture conditions. The stage of Ig rearrangement of the precursor cells has not been determined and their capacity to differentiate into mature cells was not reported. Collins and Dorshkind (1987) have also shown that $B220^-$ cells from Dexter long-term bone marrow cultures can differentiate to $B220^+$ cells under certain culture conditions. The stage of differentiation of the precursor detected in this system is, however, unknown. Hardy *et al.* (1991) described a population of B-cell precursors in bone marrow with the Ig loci in germ-line configuration expressing detectable levels of the B220 antigen. This result conflicts with our finding that most fetal liver derived B-cell precursors with the Ig loci in germ-line configuration are $B220^-$. There are several possible explanations for this discrepancy including a different source of cells (fetal liver versus bone marrow), different reagents (RA3-6B2 versus 14.8 to detect B220) or different cell separation techniques (panning versus cell sorting).

Several long-term cell lines have been described which also make this transition (Palacios and Steinmetz, 1985). The fact that such lines respond directly to IL-3, unlike primary cells cultured under the conditions described here and in previous reports (Paige *et al.*, 1984), suggests that different populations are being studied. More recently, Rolink *et al.* (1991) generated pre-B cell lines from $PB76^+$ mouse fetal liver cells, under growth conditions similar to those described here. The cell lines described by Rolink *et al.* have $D-J_H$ rearrangements in both loci, suggesting that AA4.1 is expressed earlier than PB76 in B-cell precursors.

We present evidence that a cell surface marker, AA4.1, is present and can enrich for B-cell precursors that are uncommitted for heavy chain expression and possibly still multipotent in their differentiation potential. The cells are unresponsive to IL-7 alone, as shown either in bulk culture or limiting dilution conditions. This result contrasts with bone marrow and fetal liver $B220^+$ cells at later stages of development, which will proliferate after addition of identical concentrations of IL-7 alone. We suggest, therefore, that stromal cells have a fundamental role in inducing maturation of B-cell precursors to a stage of IL-7 responsiveness. A similar sequence of events has been suggested by Hayashi *et al.* (1990) based on the study of stromal cell-dependent bone marrow-derived cell lines.

An analysis of immunoglobulin heavy chain allotype and light chain isotype showed that in most cases the two allotypes and the two isotypes were secreted by cells within a clone from F1 mice. Allelic exclusion, as defined by the inability to express the product of both heavy chain immunoglobulin loci, has been consistently observed in mature B lymphocytes. Therefore, the detection of secreted antibody from both alleles indicates that the progenitor cell was uncommitted for heavy chain expression. The same interpretation is applied to light chain double producers. Light chain analysis is somewhat complicated because of reports of double producer mature cells. These cells are, however, rare ($\sim 0.1\%$) and go on to secrete predominantly λ chain (Gollahon *et al.*, 1988). We find that double producing clones are common in the analyzed population and produce five times more κ than λ chain. We conclude, therefore, that if we are detecting mature light chain double producers, they constitute a minority of the clones analyzed.

Our data allow us to estimate the minimum number of cell divisions required for the progeny of an Ig uncommitted precursor to express both heavy chain allotypes or light chains. If two cells undergo heavy chain recombination there is a 50% chance that both express the product of the same chromosome. If rearrangement occurs after two generations, the chance that all four cells utilize the same chromosome drops to 12.5%. This number comes close to the observed incidence (13.6%) of clones expressing a single allotype. Assuming that all cells have the same probability of expressing immunoglobulin, we infer that cells became heavy chain committed after two divisions *in vitro*. Application of the same analysis to light chain expression, where the chance of expressing κ and λ light chain is unequal (95:5), leads to the conclusion that at least four cell divisions are required to generate the observed data [95% of analyzed clones expressing both light chain isotypes (Table V)]. Our study of 50 heavy chain uncommitted clones is consistent with the interpretation that heavy and light chain rearrangement are separated by two or more generations and that clonal expansion can occur in cells that have rearranged the heavy chain but not yet the light chain genes (Caton, 1990). This conclusion is consistent with observations originally made in Abelson transformed pre-B cell lines (Alt *et al.*, 1981, 1986) that $D-J_H$, V_H-D-J_H and light chain gene rearrangement occur sequentially. Analysis of hybridomas derived from siblings in a bone marrow colony also support this concept (Yoshida *et al.*, 1987).

Genomic DNA was analyzed to examine heavy chain gene rearrangement in clones that could not react to LPS. We found that these cells contained several heavy chain rearrangements, demonstrating that virtually all clones we identified were potential B-cell precursors whether or not they secrete Ig upon LPS stimulation. In most cases more than four rearranged bands were identified and in some cases a diffuse pattern of hybridization was observed. This reflects multiple rearrangements occurring as we would expect from

the progeny of uncommitted precursors. Another conclusion from the Southern blot analysis is that more than two J_H segments are rearranged in some clones. This result could only be possible if the precursor had not yet undergone two stable D−J rearrangements, either because it has the Ig loci in germ-line configuration or because it undergoes multiple $D-J_H$ replacement events. The presence of germ-line bands in some of the clones corresponding to both chromosomes is an indication that the precursor isolated is, at least in some cases, in germ-line configuration in the Ig loci. Although most samples analyzed were derived from LPS-unresponsive clones, we show evidence that one multiple D−J and one germ-line clone differentiate to Ig-secreting plasma cells. More than 80% $B220^-$ precursor-derived clones showed detectable multiple ongoing D−J rearrangement restriction fragments. $B220^+$ precursor-derived clones are less heterogeneous and in only 20% of the cases can we detect more than two different J_H rearrangements. This observation suggests that most $B220^+$ cells at day 12 of gestation have already started D−J rearrangement although V_H genes are still unrearranged. This is in contrast with $B220^-$ precursors which are predominantly unrearranged.

Materials and methods

Cell preparation

C57BL/6 and Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal colony of the Ontario Cancer Institute. Timed pregnancies were determined as previously described (Cumano *et al.*, 1990). Fetal liver cell suspensions were made by standard procedures and viable cells were determined using trypan blue exclusion.

Media and culture conditions

Opti-MEM (Gibco, Canada) was prepared as previously described (Cumano *et al.*, 1990). LPS (*Salmonella typhosa* WO901; Difco) was used at 25 μg/ml. IL-7 was obtained by transient transfection of plasmid pSRa IL-7 (kindly given by Dr Shin-Ichi Nishikawa, Kumamoto University, Japan) into COS1 cells and subsequently harvesting after 3 days as described (Sudo *et al.*, 1989). Alternatively, IL-7 produced by stably transfected cells (a gift from Dr Fritz Melchers, Basel, Switzerland) according to a previously described protocol (Karasuyama *et al.*, 1988) was used. The activity of IL-7 was determined in a thymidine incorporation assay as previously described (Sudo *et al.*, 1989). The limiting dilution cultures were set up by culturing overnight 500−1000 S17 cells in each well of a 96-well plate (Coster, Cambridge, MA). The plates were irradiated with 2000 rads using a cesium source. IL-7 was used at concentrations of 50−100 U/ml based on the optimum concentrations titrated in a pre-B cell colony assay (Cumano *et al.*, 1990). Enriched fetal liver cells at a specified dilution were subsequently plated at a final volume of 200 μl/well. Replicates (36, 48 or 96) were used for each cell dilution. On day 5, 100 μl of media was removed and replaced by 100 μl of fresh IL-7-supplemented media. For LPS stimulation, cells obtained from a single well of a 96-well plate were resuspended in 50 μl and divided into two wells containing irradiated S17 cells and LPS (25 μg/ml). The final volume was 250 μl/well and supernatants were collected after 12 days.

Panning procedure, ELISA assay and fluorescence surface staining

Panning was done as described (Kincade *et al.*, 1981) in Optilux 100 mm plastic Petri dishes (Falcon No. 1001, Oxnard, CA). We used a two step procedure to coat plates with antibodies. First we applied an affinity purified mouse anti-rat IgG (50 μg/plate; Jackson Immunoresearch Laboratories, Jackson, ME). After blocking with 5% FCS, hybridoma supernatant was applied. We used the AA4.1 rat antibody (McKearn *et al.*, 1985), which recognizes the antigen of the same name, and the 14.8 rat antibody, which recognizes the B220 antigen (Kincade *et al.*, 1981). Cells were allowed to bind to the antibody-coated plates for 1 h at 4°C. Non-adherent cells were recovered by three gentle washes with Earls balanced salt solution (EBSS)−5% FCS. After eight washes with 3 ml of cold EBSS−5% FCS the adherent fraction was obtained by cell removal with a rubber policeman. $AA4.1^+$ cells were stained with 14.8 mAb and shown to be 6% positive for B220. After 12 h of incubation at 37°C, to allow the re-expression of blocked receptors, the cells were stained with the AA4.1 mAb, as described later in this section, and shown to be 78% positive. Although it is possible that some cells acquired the AA4.1 marker *in vitro*, we conclude that, in agreement with the panning data, most $AA4.1^+$ cells are clearly $B220^-$.

ELISAs were done by coating Nunc-Immuno Plate MaxiSorp (Intermed, Canada) with 5 μg/ml of either affinity purified anti-mouse μ chain antibody (Sigma, St Louis, MO), goat anti-mouse κ or λ chain (Southern Biotechnology, Birmingham, AL) or protein-G purified AF6 (anti-mouse Igh6a) (Schuppel *et al.*, 1987) or RS3.1 (anti mouse Igh6a) (Stall and Loken, 1984) antibodies. Plates were blocked for 1 h with PBS−2% BSA followed by eight washes with water. Serial dilutions of the supernatants in PBS−2% BSA were applied to the plates and incubated for 1 h at 37°C. Plates were again washed eight times with H_2O and appropriate dilutions of anti-mouse μ or anti-mouse γ chains coupled to horse-radish peroxidase (HRPO; Sigma, St Louis) was applied and incubated for an additional 1 h at 37°C. After washing, the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was applied and the absorbance read at 405/630 nm. In all plates negative and positive controls were used. A blank readout consisting of PBS−2% BSA alone instead of culture supernatants was also routinely included.

Fluorescence staining was done by incubating 10^5 cells in 100 μl with appropriated dilutions of either 14.8 or AA4.1 mAb culture supernatant for 20 min on ice and subsequently with a second antibody (mouse anti-rat IgG) coupled to FITC (Jackson Immunoresearch). For anti-μ staining cells were incubated with Fab_2 fragments of a goat anti-mouse μ chain coupled to FITC (Jackson Immunoresearch). All samples were incubated with the second antibody alone as a negative control. Fluorescence was measured using a FACScan (Becton-Dickinson, CA) and cells were gated by forward scatter and propidium iodide staining.

Southern blot analysis, genomic DNA and probe preparation

Rearrangements of the immunoglobulin locus were analyzed using standard methods of DNA preparation, enzyme digestion, Southern blot transfer and hybridization (Sambrook *et al.*, 1989). All washes were done under stringent conditions and the final wash after probing was always 0.1 × SSC, 0.1% SDS, at 65°C. Probes were stripped by incubation for 15 min at 65°C with 50% formamide, 1% SDS and 0.5 × SSC, followed by 15 min at 65°C with 0.5 × SSC.

J_H3 and J_H4 probes were isolated from a plasmid pGW78 containing the *Bam*HI−*Eco*RI genomic fragment and digested with *Eco*RI, *Bam*HI and *Hin*dIII. J_H1 and J_H2 were isolated from the plasmid pGW46 containing the 1 kb *Bam*HI genomic fragment and digested with *Bam*HI and *Hin*dIII. Fragments were separated in low melting temperature agarose gels, and bands were cut out and eluted from the gel by phenol extraction (Sambrook *et al.*, 1989). Purity and concentration of the fragments was accessed by a agarose gel. The fragments were labelled to specific activity of $\sim 5 \times 10^8$ c.p.m./μg using the random priming method and tested. Testing of probes was done by digesting 15 μg of liver DNA with *Bam*HI + *Hin*dIII and *Bam*HI + *Hin*dIII + *Eco*RI. Four samples of digested DNA were separated in an agarose gel and blotted. Each of the samples was hybridized to an individual probe. Single fragments of the expected size were observed confirming the specificity of the probes. Autoradiograms were exposed for 3−8 days at −70°C with intensifying screens.

Acknowledgements

Ian McDermott and Caren Furlonger provided excellent technical assistance. We thank Drs Gerard Brady, Norman Iscove, Arumugavadivel Narendran, Fred Sablitzky and Gillian E.Wu for helpful discussion and critical reading of the manuscript; Drs Fritz Melchers and Shin-Ichi Nishikawa provided an IL-7 producing cell line and a cDNA clone containing the IL-7 coding sequence; Dr Kenneth Dorshkind provided the S17 stromal cell line. A.C. is a fellow of the Medical Research Council of Canada. This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.

References

Alt,F.W., Rosenberg,N., Lewis,S., Thomas,E. and Baltimore,D. (1981) *Cell*, **27**, 381.

Alt,F.W., Blackwell,T.K., DePinho,R.A., Reth,M.G. and Yancopoulos,G. (1986) *Immunol. Rev.*, **89**, 5.

Caton,A.J. (1990) *J. Exp. Med.*, **172**, 815.

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

Cumano,A., Dorshkind,K., Gillis,S. and Paige,C.J. (1990) *Eur. J. Immunol.*, **20**, 2183.

Gollahon,K.A., Hagman,J., Brinster,R.L. and Storb,U. (1988) *J. Immunol.*, **141**, 2271.
Hardy,R.R., Carmack,C.E., Shinton,S.A., Kemp,J.D. and Hayakawa,K. (1991) *J. Exp. Med.*, **173**, 1213.
Hayashi,S.-I., Kunisada,T., Ogawa,M., Sudo,T., Kodama,H., Suda,T., Nishikawa,S. and Nishikawa,S.-I. (1990) *J. Exp. Med.*, **171**, 1683.
Karasuyama,H. and Melchers,F. (1988) *Eur. J. Immunol.*, **18**, 97.
Kincade,P.W., Lee,G., Watanabe,T., Sun,L. and Scheid,M.P. (1981) *J. Immunol.*, **127**, 2262.
Kincade,P.W. (1988) *Adv. Immunol.*, **41**, 181.
McKearn,J.P., McCubrey,J. and Fagg,B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7414.
Melchers,F. (1977) *Eur. J. Immunol.*, **7**, 482.
Muller-Sieburg,C.E., Witlock,C.A. and Weissman,I.L. (1986) *Cell*, **44**, 653.
Namen,A.E., Schmierer,A.E., March,C.J., Overell,R.W., Park,L.S., Urdal,D.L. and Mochizuky,D.Y. (1988) *J. Exp. Med.*, **167**, 988.
Notemburg,C. and Weissman,I.L. (1981) *J. Immunol.*, **78**, 484.
Palacios,R. and Steinmetz,M. (1985) *Cell*, **41**, 727.
Paige,C.J., Kincade,P.W., Moore,M.A.S. and Lee,G. (1979) *J. Exp. Med.*, **150**, 548.
Paige,C.J. (1983) *Nature*, **302**, 711.
Paige,C.J., Gisler,R.H., McKearn,J.P. and Iscove,N.N. (1984) *Eur. J. Immunol.*, **14**, 979.
Rolink,A., Kudo,A., Karasuyama,H., Kikuchi,Y. and Melchers,F. (1991) *EMBO J.*, **10**, 327.
Rosenberg,N., Siden,E. and Baltimore,D. (1979) In Cooper,M., Mosier,D.E., Scher,I. and Vitetta,E.S. (eds), *B Lymphocytes in the Immune Response*. Elsevier/North Holland, New York, p. 379.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schuppel,R., Wilke,J. and Weiler,E. (1987) *Eur. J. Immunol.*, **17**, 739.
Stall,A.M. and Loken,M.R. (1984) *J. Immunol.*, **132**, 787.
Sudo,T. *et al.* (1989) *J. Exp. Med.*, **170**, 333.
Welch,P.A., Burrows,P.D., Namen,A., Gillis,S. and Cooper,M.D. (1990) *Int. Immunol.*, **2**, 697.
Whitlock,C.A. and Witte,O.N. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3608.
Yoshida,N., Radbruch,A. and Rajewsky,K. (1987) *EMBO J.*, **6**, 2735.

Received on September 11, 1991; revised on October 31, 1991