The EMBO Journal vol.6 no.12 pp.3687–3693, 1987

Ly1$^+$ PRO-B lymphocyte clones. Phenotype, growth requirements and differentiation *in vitro* and *in vivo*

Ronald Palacios, Hajime Karasuyama and Antonius Rolink

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland

Communicated by E.Klein

Several clones obtained from the bone marrow of a BALB/c mouse were found to contain the heavy and light chain Ig genes in the germline configuration, to express Ly1 and to carry the B cell lineage markers B-220, Lyb8 and BP-1; these clones are Pgp-1$^+$, LFA-1$^+$, J11d$^+$, Mac-1$^+$ and Thy1$^-$, Lyt2$^-$, L3T4$^-$, GM1.2$^-$ and Ia$^-$. Three clones analyzed in detail (Lyd9, LyH7 and Lyb9) have receptors for interleukin (IL) 2 and IL3 as assessed with the 7D4 and CC11 monoclonal antibodies respectively. They grow in rIL3 but not in rIL2 or rIL1; both rIL4 and rIL5 also promote their proliferation, albeit to a much lesser extent than rIL3. None of the interleukins tested alone or in various combinations promoted the clones to differentiate *in vitro* along the B cell pathway. Treatment with 5-Azacytidine (5-Aza) induced cell surface Ia expression but not rearrangement or expression of Ig genes. However, 5-Aza-treated Lyd9, LyH7 and Lyb9 cells co-cultured with X-ray irradiated accessory cells and LPS gave rise to Ly1$^+$, IgM$^+$ B lymphocytes (range 14–51%) including $\mu^+\kappa^+$ (78–93%), and $\mu^+\lambda^+$ (9–25%) B lymphocytes. *In vivo*, the Lyd9, LyH7 and Lyb9 clones gave rise to IgM$^+$ B lymphocytes (8.5–17%) including $\mu^+\kappa^+$, and $\mu^+\lambda^+$, but not to Lyt2$^+$ or L3T4$^+$ T lymphocytes after 4–6 weeks of transfer into Scid mice. Our results indicate that Ly1$^+$ IgM$^+$ cells comprise a subpopulation of B lymphocytes that is derived from IL3-responsive Ly1$^+$ PRO-B lymphocytes.

Key words: B cell development/cell growth/cell differentiation/B cell precursor

Introduction

The stages of B lymphocyte differentiation are marked by differences in the organization of the immunoglobulin (Ig) heavy (H) and light (L) chain genes, by the kinds of Ig chains expressed and by the levels of Ig expression (reviewed in Tonegawa, 1983; Alt *et al.*, 1986). The earliest stage of B cell development identified is represented by PRO-B lymphocytes, i.e. a cell that contains the H and L Ig chain genes in the germ line configuration, may express B cell lineage characteristic surface markers and gives rise to mature B lymphocytes (Palacios and Steinmetz, 1985). As PRO-B lymphocytes differentiate to pre-B cells, there is μ heavy chain gene rearrangement, transcription, translation and expression of cytoplasmic μ protein (Raff *et al.*, 1976). Pre-B cells proliferate, rearrange L chain genes and express them together with the H chain (Raff *et al.*, 1976; Cooper, 1981) giving rise to mature surface Ig$^+$ B lymphocytes (Raff *et al.*, 1970).

Recent studies have described a subset of normal and neoplastic B lymphocytes that carry the Ly1 antigen on the cell membrane (Manohar *et al.*, 1982; Hayakawa *et al.*, 1983; Davidson *et al.*, 1984). It has been proposed that the Ly1$^+$ and the Ly1$^-$ populations of B lymphocytes belong to separate lineages of development (Hayakawa *et al.*, 1985). This stems from the observations that bone marrow cells from adult mice (>8 weeks old) were found to give rise to Ly1$^-$ B cells but not to Ly1$^+$ B cells. In contrast, cells from the peritoneal cavity of adult mice or bone marrow of newborn mice could reconstitute the Ly1$^+$ B cell poulation after transfer into irradiated mice (Hayakawa *et al.*, 1985).

By using the CC11 monoclonal antibody (Mab) specific for interleukin-3 (IL3)-sensitive mouse cells (Palacios *et al.*, 1986) we found two major populations of B cell precursors in the bone marrow: CC11$^+$ cells that proliferate in IL3, and CC11$^-$ cells that do not proliferate in IL3, IL2 or IL1 (Palacios and Leu, 1986); the latter resembles the B cell precursors maintained in Whitlock–Witte cultures in the absence of IL3, IL2 or IL4 (Hunt *et al.*, 1987; Whitlock *et al.*, 1987).

We have previously reported the establishment of CC11$^+$, Ly1$^+$ IL3-dependent B cell precursor lines from the bone marrow of a 5-week-old CBA/J mouse (Palacios and Leu, 1986). However, there was no proof that the Ly1$^+$ B cell precursors generate Ly1$^+$ mature B lymphocytes, i.e. a precursor–product relationship. Also, it remained to be defined at what stage of development the Ly1$^+$ B cell population separates from the Ly1$^-$ B cell population. In other words, is there a Ly1$^+$ PRO-B lymphocyte or is the Ly1 antigen only expressed from the pre-B cell stage of development onwards?

Here we describe the establishment of CC11$^+$ Ly1$^+$ cell lines derived from the bone marrow of 4-week-old BALB/c mice, and show that they represent PRO-B lymphocytes that can give rise to mature Ly1$^+$ B cells.

Results

Development and establishment in culture of PRO-B cell clones

A cell line (Ly) was established from BALB/c mouse (4 weeks old) bone marrow cells depleted of granulocytes, Ia$^+$ cells and Thy1$^+$ cells using procedures summarized in Materials and methods and described in detail elsewhere (Palacios and Leu, 1986). The Ly cell line was established in culture in April 1986 and was chosen for further study because of its phenotype. In May 1986, 26 clones were derived from the Ly cell line by micromanipulation; 10 clones were selected for further work and the other 16 clones were frozen. All 10 clones were found to have a similar phenotype and to have the Ig genes in the germline configuration. In the following we describe the experiments performed with three clones (Lyd9, LyH7 and Lyb9).

Phenotype

The phenotype of the Ly clones was determined by immunofluorescence staining and flow cytometry (FACS) using a panel of monoclonal antibodies.

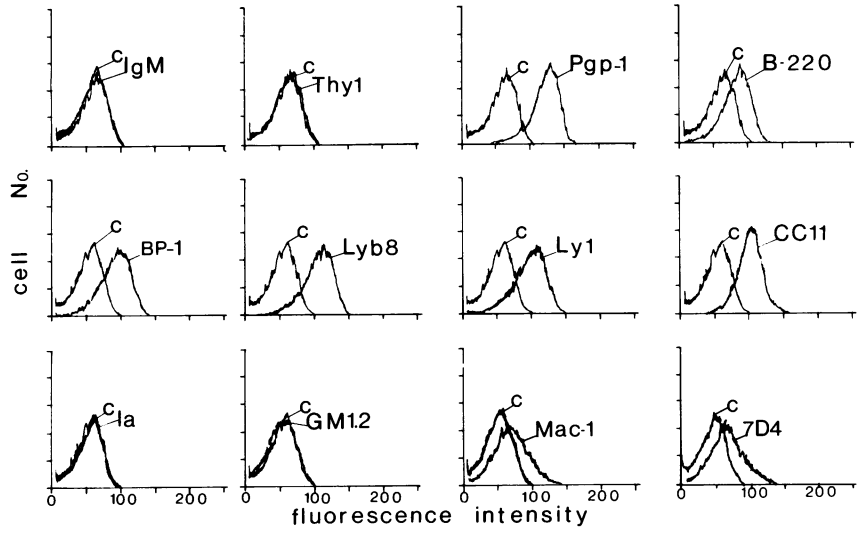

Fig. 1. Phenotypic characteristics of the Ly clones. The presence of several cell surface antigens on the Lyd9 clone was determined by direct or indirect immunofluorescence staining and flow cytometry. C = negative controls which were the cells stained with second-step reagent only. Very similar results were obtained with the LyH7 and Lyb9 clones.

Figure 1 shows the results obtained with the Lyd9 clone. These cells express the B cell lineage marker B-220, Lyb8 and BP-1, but do not have surface IgM; they are positive for the Ly1 antigen and negative for the T cell lineage markers Thy1, Lyt2 and L3T4; they express low levels of Mac-1 antigen and do not carry the granulocyte surface antigen GM1.2 or Ia molecules on the cell membrane; they bind the 7D4 anti-IL2R Mab, and the CC11 Mab specific for IL3-sensitive mouse cells and they also are Pgp-1^+, LFA-1^+, J11d$^+$, H-2^{d+}. The phenotype of the LyH7 and Lyb9 clones is very similar to that of the Lyd9 clone. All three clones have preserved this phenotype up to the present.

Configuration of the Ig genes

The state of the Ig genes in the Lyd9, LyH7 and Lyb9 clones was assessed by Southern blot analysis using a J_H-specific and a c kappa-exon-specific probe and DNAs digested with *Eco*RI or *Bam*HI restriction enzymes. All three clones contain the H chain gene in the germline configuration (Figure 2A), and there is no evidence for rearrangement of the κ light chain gene in the clones (Figure 2B).

Differentiation

The experiments described so far suggest that the Ly clones represent PRO-B lymphocytes. To test this possibility, we tried to induce their differentiation both *in vitro* and *in vivo* into more mature B lymphocytes.

In vitro studies

All attempts to induce differentiation of these clones with soluble factors (rIL1, rIL2, rIL3, rIL4, rIL5, crude supernatants from a variety of cells, plus LPS or PWM) were unsuccessful.

Since we previously found that the DNA-demethylating drug 5-Azacytidine (5-Aza) was able to induce marrow PRO-T lymphocyte clones to differentiate (Palacios *et al.*, 1987a), we studied the effect of 5-Aza on the Lyd9, LyH7 and Lyb9 clones. We found that following treatment with 5-Aza, a significant proportion (range 35–72%) of the Lyd9, LyH7 and Lyb9 cells expressed Ia but not IgM on the cell membrane (Figure 3). The levels of Ly1, BP-1 and B-220 surface antigens tended to increase in some but not in all the experiments (Figure 3 and data not shown). In two experiments 5-Aza-treated Lyd9, LyH7 and Lyb9 cells were assayed for the presence of rearrangements of Ig genes

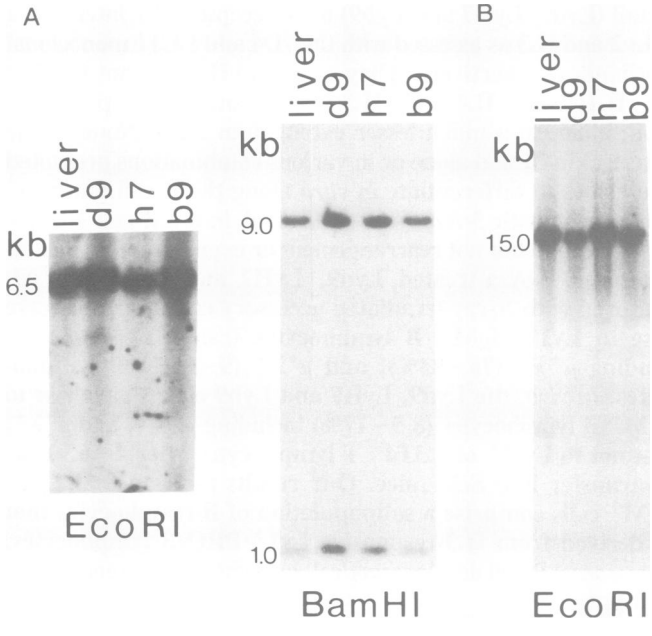

Fig. 2. Analysis by Southern blot hybridization of the configuration of the IgH and kappa light chain genes. (**A**) Hybridization with J_H-specific probe. (**B**) Hybridization with c kappa-exon-specific probe. The same result was obtained with DNAs digested with *Bam*HI.

by Southern blot analysis. We found no evidence for rearrangement of the Ig heavy chain gene in the cells. Nor could we find cells expressing cytoplasmic μ chain in 5-Aza-treated cells as assessed by immunofluorescence microscopy.

We thought that the Lyd9, LyH7 and Lyb9 clones perhaps required further stimulation after treatment with 5-Aza to differentiate into IgM$^+$ B lymphocytes. Non-lymphoid accessory cells (AC) are known to infuence the differentiation and maturation of B cell precursors (Whitlock and Witte, 1982; Paige, 1983; Corbel and Melchers, 1984; Dorskind *et al.*, 1985; Spalding and Griffin, 1986). Therefore we tested the possibility that AC together with the B cell stimulator LPS would promote differentiation of the Ly clones into mature B lymphocytes.

We found that the 5-Aza-treated Lyd9, LyH7 and Lyb9 cells

co-cultured with Scid AC and LPS at 37°C for 6–8 days gave rise to IgM$^+$ B lymphocytes as assessed by FACS analysis (Figure 4). A critical issue in this experiment is to be able to be certain that the Ig$^+$ B cells are indeed generated by the precursor cells under study and not by the AC. Therefore we have used X-ray-irradiated (2500 rad) spleen cells from the T cell- and B cell-deficient Scid mice (Bosma *et al.*, 1983) as AC. In addition, we employed the MB86 anti-IgM allotype Mab to detect putative IgM$^+$ B cells generated by the X-ray-irradiated Scid AC. The MB86 Mab reacts with IgM of the b (Scid mice) but not with the IgM of the a (BALB/c mice, Ly clones origin) allotype (Nishikawa *et al.*, 1986). None of the IgM$^+$ B lymphocytes generated in the cultures were MB86$^+$ (i.e. they were not of the b allotype) and, hence, the IgM$^+$ B lymphocytes were the progeny of the Ly clones and not of the X-ray-irradiated AC

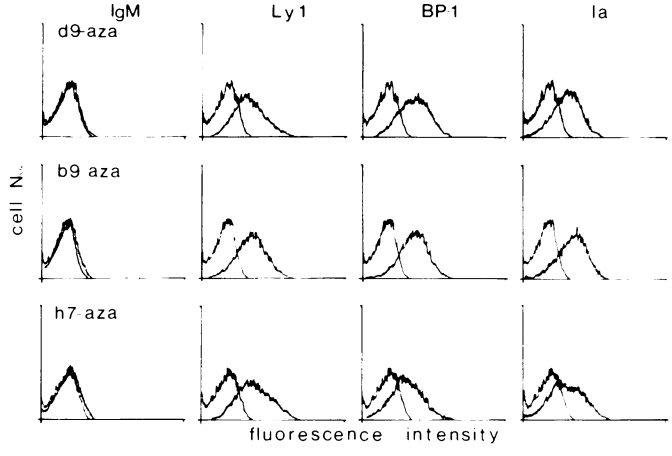

Fig. 3. 5-Aza induces the Lyd9, LyH7 and Lyb9 cells to express Ia molecules but not IgM on the cell membrane. The clones were treated with 5-Aza as detailed in Materials and methods. The presence of the various surface antigens on the cells was assessed by FACS analysis. The results shown were obtained after 14 days of completion of the 5-Aza treatment. Similar results were obtained 7 days or 21 days following treatment with 5-Aza. Negative controls were the cells stained with second-step reagent only. All cells also have B-220$^+$ and Lyb8 molecules on the cell membrane (not shown).

(Figure 4). Parallel experiments showed that the MB86 Mab reacts with B cells from C57BL/6 (IgH^b allotype) and not with splenic B cells from BALB/c (IgH^a allotype) mice in agreement with previous work (Nishikawa *et al.*, 1986). The number of IgM$^+$ B lymphocytes generated by the Ly cells varied from experiment to experiment (range 14–51%). The IgM$^+$ B cells were κ^+ (78–93%) or λ^+ (9–25%). Virtually all cells in the induced cultures were Ly1$^+$, B-220$^+$, Lyb8$^+$ and ~50–80% of them were Ia$^+$ (Figure 4 and data not shown). Similar results were obtained using enriched populations of AC from spleen of C57BL/6 and CBA/J normal mice, in which we additionally employed anti-H-2-specific Mabs to ensure that the IgM$^+$ B lymphocytes were the progeny of the Lyd9, LyH7 or Lyb9 clones (H-2^d) and not of the AC (H-2^b or H-2^k respectively). The Ly clones are able to give rise to IgM$^+$ B lymphocytes without previous treatment with 5-Aza under the influence of AC plus LPS stimulation, but under such conditions they generate lower numbers of IgM$^+$ B lymphocytes (up to 18%) (R.Palacios, unpublished observations). We did not detect secretion of Ig at any stage of the cultures as assessed by the protein A plaque assay and by ELISA.

In vivo studies

We also tested the Lyd9, LyH7 and Lyb9 clones for their capacity to give rise to mature B lymphocytes *in vivo*. The Ly clones were transferred into sublethally X-ray-irradiated (250 rad) Scid mice and the presence of B cell precursors, Ig$^+$ B cells, T lymphocytes and Mac-1$^+$ cells in the spleens was determined by FACS analysis 4–6 weeks later. In these experiments we also used the MB86 anti-IgM allotype Mab to monitor the presence of putative B cells of host (Scid mice) origin. Three Scid mice died, one from the control group (that received no Ly cells), one from the group that received the LyH7 cells and the third one from the group that received the Lyb9 cells. Figure 5 shows the data in the form of histograms to illustrate the results obtained in the experiments and Table I summarizes the data obtained from all the mice studied. All three Ly clones generated B-220$^+$, IgM$^+$ B lymphocytes including $\mu^+\kappa^+$ and $\mu^+\lambda^+$ B cells (Table I). All IgM$^+$ B cells were MB86$^-$ [i.e. they were not of the b allotype (Scid mice)] and, therefore, they were generated by the Ly clones

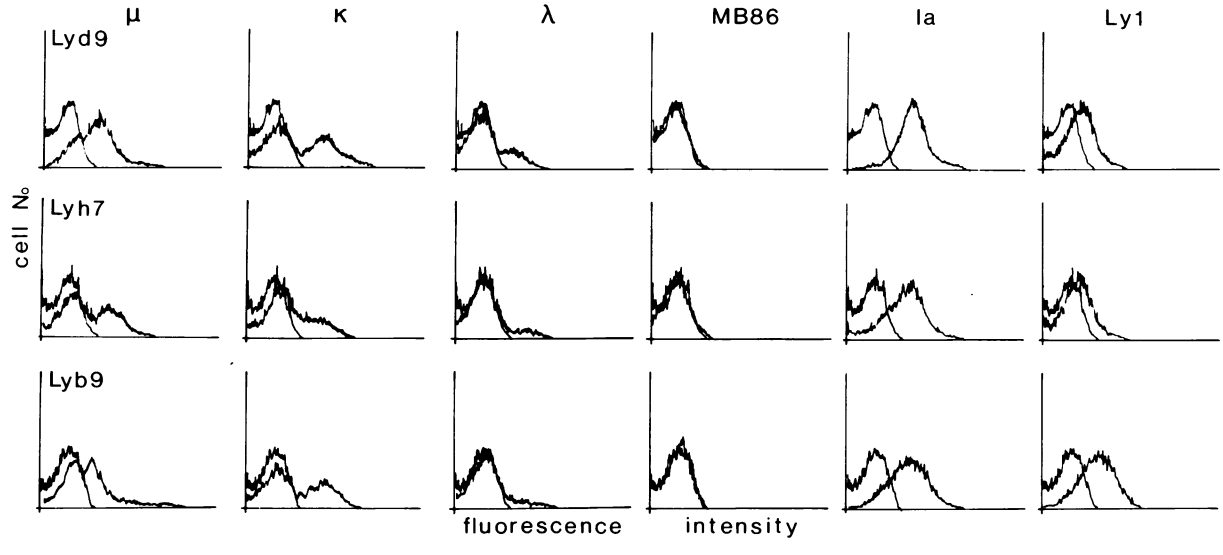

Fig. 4. Lyd9, LyH7 and Lyb9 cells give rise to IgM$^+$ B lymphocytes. 5-Aza-treated Lyd9, LyH7 or Lyb9 cells were co-cultured with X-ray-irradiated AC from Scid mice and LPS at 37°C for 8 days. The presence of the markers indicated was assessed by FACS analysis. Negative controls were cells stained with second reagent only. The MB86 Mab reacts with IgM of the b allotype (Scid mice) but not with IgM of the a allotype [expressed by the Lyd9, LyH7 and Lyb9 cells (BALB/c origin)]. All cells were also B-220+ and Lyb8$^+$ (not shown).

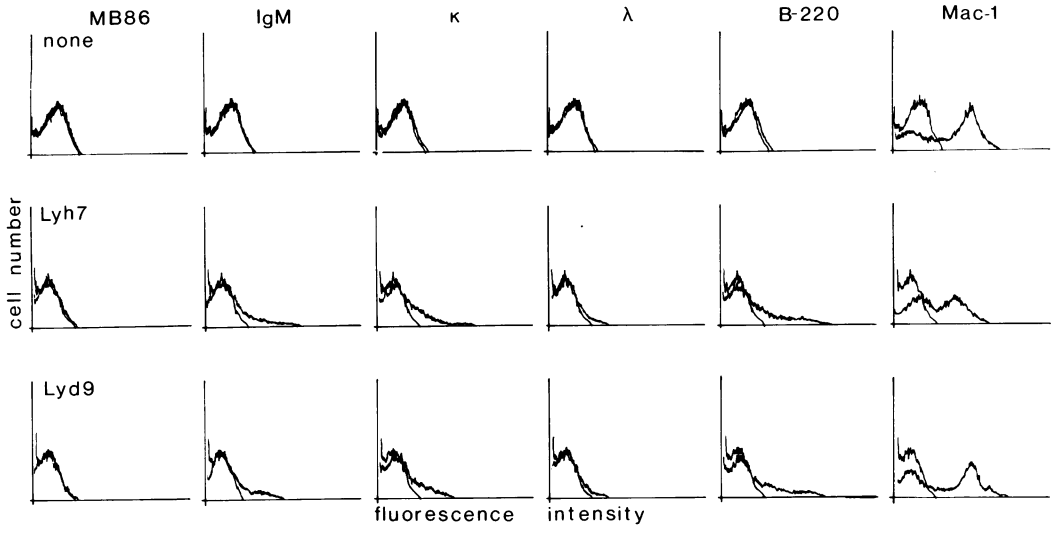

Fig. 5. The Ly clones give rise to IgM$^+$ B lymphocytes *in vivo*. The Ly clones were transferred into sublethally irradiated Scid mice and the presence of spleen cells expressing the surface markers indicated was determined by FACS analysis 4 weeks later. None = control Scid mice that received no Ly clones.

Table I. The Lyd9, LyH7 and Lyb9 clones give rise to B lymphocytes *in vivo*

Ly clone	Scid mice studied	Spleen cells/mouse × 10^6	% positive cells (FACS analysis)										
			μ	κ	λ	MB86	B-220	BP-1	Ly1	Thy1	Lyt2	L3T4	Mac-1
d9	6	19.1–28.0	8.5–14.0	7.5–12.0	1.9–3.0	<1	14.8–18.0	2–4	12–16	5–11	<1	<1	35–61
H7	5	17.4–22.7	9.2–16.5	8.0–15.0	2.0–3.5	<1	13.0–20.0	2–5	11–17	8–13	<1	<1	47–64
b9	4	20.3–31.4	12.3–17.0	10.9–16.1	2.4–3.2	<1	17.0–23.0	3.0–4.5	14.2–19	7–12	<1	<1	32–57
None	5	6.2–13.0	<1–3	<1–3	<1	<1–3	<2	<2	<1	6–10	<1	<1	38–54

The Ly clones were transferred into Scid mice and the presence of spleen cells positive for the surface markers indicated was assessed by FACS analysis 6 weeks later. None = control Scid mice that received no Ly clones. One mouse in the control group had 3% IgM$^+$ MB86$^+$ cells (data included above) and one mouse from the group that received the Lyb9 clone had 4% IgM$^+$ MB86$^+$ (cells of Scid origin); the data from these animals are not included above.

and not by the host. The spleens of two Scid mice, one from the control group (data included in Table I) and one from the group that received the Lyb9 cells (data not included in Table I) had ~3–4% MB86$^+$ IgM$^+$ cells probably reflecting the known leakiness of the Scid mutation (Bosma *et al.*, 1983). The rest of the control Scid mice had no detectable (<1%) mature B cells or B cell precursors in the spleen (Figure 5 and Table I). Interestingly, there were more B-220$^+$ and Ly1$^+$ cells than IgM$^+$ cells in the spleens of Scid mice that received the Ly clones (Table I), indicating the presence not only of mature Ig$^+$ B lymphocytes but also of B cell precursors. It is worth noting that while all Ly clones are BP-1$^+$ before their transfer into Scid mice (Figure 1), up to 5% BP-1$^+$ cells were only detected in the spleens of Scid mice that received the Ly clones 4–6 weeks before analysis (Table I). As the same spleens contained many more B-220$^+$ B cells (13–23%) than BP-1$^+$ cells (Table I), the majority of the Lyd9, LyH7 and Lyb9 cells must have become BP-1$^-$ as they differentiated *in vivo*.

The spleens from Scid mice that received the Ly clones, like those from the control Scid mice, had THY1$^+$ (5–13%) cells but no detectable (<1%) Lyt2$^+$ or L3T4$^+$ T lymphocytes (Table I), indicating that no mature T lymphocytes were generated by the Ly clones.

Growth requirements

We performed experiments aimed to define the growth factors for the continuous growth *in vitro* of the Lyd9, LyH7 and Lyb9 clones. Figure 6 shows the results obtained with the Lyb9 clone

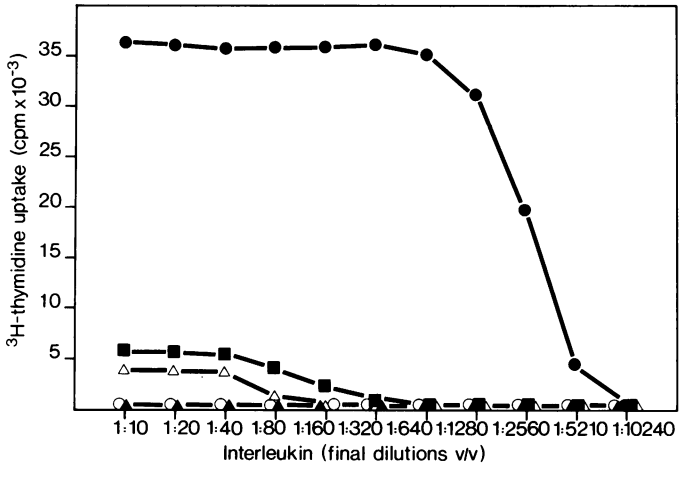

Fig. 6. Growth factors for the Ly clones. Proliferative responses of the Lyd9, LyH7 and Lyb9 clones to rIL1 (○), rIL2 (▲), rIL3 (●), rIL4 (△) and rIL5 (■) was assessed by [^{3}H]thymidine uptake during the last 6 h of a 48-h culture period. The data are expressed as c.p.m. and are the mean of triplicate cultures. (The SE in the experiment shown above was <7.4% of the mean.) Cells cultured in medium only gave 161 c.p.m. The results shown above were obtained with the Lyb9 clone; the same pattern of responses was observed with the Lyd9 and the LyH7 clones.

and illustrates the phenomena observed in the three independent experiments carried out. The Lyb9 cells grow in rIL3 but not in rIL1 or rIL2; they also proliferate in rIL4 and in rIL5, albeit

to a much lesser extent than in rIL3. A similar pattern of responses was exhibited by the Lyd9 and LyH7 clones. All three clones self-renew *in vitro* in rIL3 approximately every 10–12 h and die within 48 h in the absence of exogenous growth factors.

Discussion

The molecular, cellular and functional properties of the three clones described here fulfill the criteria for PRO-B lymphocytes: they contain the Ig genes in the germline configuration, express three B cell lineage characteristic surface markers (B-220, Lyb8 and BP-1) and give rise to IgM$^+$ B lymphocytes both *in vivo* and *in vitro*. The capacity of our Ly clones to differentiate *in vitro* into IgM$^+$ B lymphocytes was also observed by T.Honjo and collaborators (in preparation).

The identification of Ly1$^+$ PRO-B lymphocyte clones and Ly1$^-$ PRO-B lymphocyte clones (Palacios and Steinmetz, 1985; McKearn *et al.*, 1986; Muller-Sieburg *et al.*, 1986), suggests that the Ly1$^+$ and the Ly1$^-$ B cells are distinct lineages that split very early in B cell ontogeny.

The Ly1$^+$ PRO-B lymphocyte clones, unlike Ly1$^-$ PRO-B lymphocytes, were found to be Mac-1$^+$ (Figure 1 and Palacios and Steinmetz, 1985; McKearn *et al.*, 1986; Muller-Sieburg *et al.*, 1986) a marker that is expressed by macrophages and by many Ly1$^+$ B cells (Hayakawa *et al.*, 1983; Davidson *et al.*, 1984; Palacios and Leu, 1986; Holmes and Morse, 1984; Springer *et al.*, 1979). The biological significance of these findings is not understood largely because the function of the Mac-1 molecule either on B cells or on macrophages remains unknown.

The Lyd9, LyH7 and Lyb9 clones express receptors for IL3 and IL2 as assessed by FACS analysis using the CC11 and 7D4 Mabs respectively. They grow in rIL3 but not in rIL1 or rIL2; they also proliferate in rIL4 and rIL5, albeit to a much lesser extent than in rIL3. The finding that the PRO-B lymphocyte clones are sensitive to rIL5 is reminiscent of other haemopoietic growth factors which act both on progenitor cells as well as on mature cells. While rIL3, rIL4 and rIL5 promote proliferation of the clones, none of them—either alone or in combination—seem able to induce the Ly PRO-B lymphocyte clones to differentiate along the B cell pathway *in vitro*.

The rIL2 receptors on the Lyd9, LyH7 and Lyb9 Ly1$^+$ PRO-B lymphocyte clones, like those present on other IL3-sensitive early lymphoid precursors (Sideras and Palacios, 1987) and on IL3-dependent myeloid cell lines (Legros *et al.*, 1985; Koyasu *et al.*, 1986) have no known function. They are probably low-affinity receptors (Koyasu *et al.*, 1986) that do not transmit mitogenic signals (Robb *et al.*, 1984).

We have shown here that Ly1$^+$ PRO-B lymphocyte clones belong to the CC11$^+$, IL3-sensitive population of B cell precursors in the bone marrow (Palacios and Leu, 1986). Interestingly, the other population of B cell precursors in the marrow, which are CC11$^-$ and do not proliferate in IL3, seem to lack precursors for Ly1$^+$ B cells (Palacios and Leu, 1986). The B cell precursors that develop in Whitlock–Witte cultures in the absence of IL3 and IL4 (Whitlock *et al.*, 1987; Hunt *et al.*, 1987), also do not seem to give rise to Ly1$^+$ B lymphocytes (Dasch and Jones, 1986). They resemble our CC11$^-$ B cell precursor population. Thus, the Ly1$^+$ and the Ly1$^-$ B cell precursors appear to be distinct in their requirements for growth *in vitro*. However, more work is clearly necessary to substantiate this view. For instance, it is still possible that the CC11$^+$ Ly1$^+$ PRO-B lymphocytes (e.g. Lyd9, LyH7 and Lyb9 clones), if placed in Whitlock–Witte type of cultures, would switch off the expression of Ly1 and CC11 and that under such a microenvironment they could only generate Ly1$^-$ B lymphocytes. We are currently testing this possibility with the CC11$^+$ Ly1$^+$ PRO-B lymphocyte clones described here.

Another finding of interest in the present study is that the Lyd9, LyH7 and Lyb9 cells give rise *in vitro* as well as *in vivo* to relatively high numbers of $\mu^+\lambda^+$ B lymphocytes (up to 25%). It is known that only ~5% of B cells in the spleen are λ^+ (Takemori and Rajewsky, 1981). Thus it appears that the Lyd9, LyH7 and Lyb9 clones would be prone to generate $\mu^+\lambda^+$ B lymphocytes. This property fits well with previous findings that Ly1$^+$ B lymphocytes often produce λ antibodies (Herzenberg *et al.*, 1986) and that Ly1$^+$ IgM$^+$ B cell lines established in *in vitro* cultures are predominantly λ^+ (Braun *et al.*, 1986). We have isolated the $\mu^+\lambda^+$ B lymphocytes generated *in vitro* by the LyH7 clone and have been able to establish in culture some cell lines. We are in the process of characterizing these B cell lines at the DNA and RNA levels and the results from these studies will be the subject of another communication.

It is worth stressing the finding that 5-Aza appears to facilitate the differentiation *in vitro* of the Lyd9, LyH7 and Lyb9 clones into IgM$^+$ B lymphocytes. This effect of 5-Aza on the Ly clones was also found by other investigators (T.Kinashi *et al.*, in preparation). 5-Aza is a DNA-demethylating agent and the state of DNA methylation often correlates with the expression of several genes including the ones coding for Ig (Storb and Arp, 1983; Jones, 1984). One possible mechanism to account for this is that 5-Aza could induce the Ly clones to rearrange their Ig genes. We did not detect rearrangements of the IgH chain gene, nor cells having cytoplasmic μ chain in 5-Aza-treated Ly cells, but it is impossible to exclude that very few cells had rearranged IgH chain gene which could not be detected with the techniques used. Other possible mechanisms are that 5-Aza might act on the Lyd9, LyH7 and Lyb9 PRO-B lymphocyte clones by rendering their Ig genes 'accessible' to recombinases, or by 'turning on' the recombination system in the cells. This in turn would facilitate/enhance the processes of rearrangement and expression of the Ig genes under the influence of AC and LPS. These possible mechanisms of action of 5-Aza can be tested by transfecting the Ly1$^+$ PRO-B lymphocyte clones treated with 5-Aza and untreated with constructs similar to those employed to demonstrate that a pre-B lymphoma line having an active recombination system could rearrange T cell receptor β chain gene segments (Yancopoulos *et al.*, 1986).

The Ly1$^+$ PRO-B lymphocyte clones described here should prove valuable tools to identify genes responsible for lineage commitment, to study *in vitro* the mechanism(s) of initiation of somatic recombination of Ig genes, to define the molecules involved in the process of differentiation into B lymphocytes and to assess the possible functions of the B-220, Ly1, Lyb8 and BP-1 surface molecules during B cell development.

Materials and methods

Reagents

Iscove's Dulbecco's modified medium (IMDM, GIBCO), fetal calf serum (FCS, Readysysteme AG), *Salmonella abortus* lipopolysaccharide (LPS, Sigma Chemical Co.) 5-Aza (Sigma Chemical Co.), [^{3}H] thymidine (185 MBq, The Radiochemical Centre, Amersham), low-toxicity rabbit complement (Cedarlane Labs), Concanavalin A (ConA, Pharmacia).

Mice

BALB/c, C57BL/6 and CBA/J mice were from our animal facilities. Breeding pairs of C.B.17 Scid mice (Bosma *et al.*, 1983) were donated to our Institute by Dr M.Bosma (Institute for Cancer Research, Philadelphia). Scid mice were bred under pathogen-free conditions in the mouse barrier facility of our Institute and are housed in sterile isolators. Four- to twelve-week-old male and female mice were used.

Antibodies (Mabs)

Mabs against the following cell surface antigens were used: Ly1 (53-73-13), Lyt2 (53-6-72), Thy1.2 (30H12 and γ1γ), B-220 (14.8), Lyb8.2 (Cy341.2), L3T4 (GK1.5), Ia (M5/114 and MKD6), Pgp-1 (I42/5), Mac-1 (M1/70), IL2 receptor (7D4), anti-mouse granulocytes (GM1.2, New England Nuclear), IL3 receptor (CC11), J11d, LFA-1 (FD441.8). The reports describing these antibodies are listed elsewhere (Palacios and Steinmetz, 1985; Palacios and Leu, 1986; Palacios *et al.*, 1987b). The BP-1 Mab specific for cells of the B cell lineage (Cooper *et al.*, 1986), the MB86 anti-IgM allotype antibody (Nishikawa *et al.*, 1986), and the anti-H2 specific Mabs: K^b (20-8-45 and 28-13-35), K^k (16-3-225), D^k (15-5-55) and K^d (31-3-45) (Ozato *et al.*, 1980) were also employed. Mabs Cy341.2, CC11, BP-1 and MB86 were purified from culture supernatants by affinity chromatography using Protein A Sepharose columns (Pharmacia) and conjugated to biotin-*N*-hydroxy succinimide as described by Palacios and Leu (1986). FITC-labeled goat $F(ab')_2$ anti-rat Ig was from Cappel Labs (Cochranville, PA), biotin-conjugated rat anti-mouse Ly1 and FITC- or phycoerythrin-labeled avidin were from Becton and Dickinson (Sunnydale, CA). FITC-conjugated sheep anti-mouse Ig that cross-reacts with rat Ig was a gift of Luciana Forni (Basel Institute for Immunology). FITC-conjugated goat anti-mouse μ, κ or λ chain antibodies were from Southern Biotechnology Associates (Birmingham, AL) and biotin-conjugated goat anti-mouse μ, κ or λ antibodies were from Amersham International (Amersham, UK).

Cytokines

Human rIL1 (Cistrom Technologies), mouse rIL2, rIL3, rIL4 and rIL5 were obtained from X63Ag8 myeloma cells transfected with cDNAs encoding the respective ILs as described in detail elsewhere (H.Karasuyama *et al.*, in preparation). Supernatants from transfected X63Ag8 myeloma cells were tested for biological activity in the following assays: proliferation of CTLL IL2-dependent cell line (Gillis and Smith, 1977), growth of Bc/Bm11 IL3-dependent PRO-B lymphocyte clone (Palacios and Steinmetz, 1985), induction of IgG1 secretion of LPS-activated spleen B cells (Sideras *et al.*, 1985), proliferation of fetal thymocytes (Palacios *et al.*, 1987b) and secretion of IgM by large B cells isolated from spleen. We considered as 1 unit of biological activity the dilution of the cytokine preparation showing 30% of the maximal response. Crude supernatants obtained from: PWM-stimulated human peripheral blood mononuclear cells, ConA-stimulated mouse or rat spleen cells, U-937 human tumor cells, WEHI-3 mouse tumor cells and ConA-stimulated 2.19 T helper cells, were prepared as described previously (Palacios and Steinmetz, 1985; Sideras *et al.*, 1985; Palacios and von Boehmer, 1986; Palacios *et al.*, 1987b).

Preparation of cells

Bone marrow and spleen cell suspensions were prepared as described (Palacios, 1985). Marrow cells from 4-week-old BALB/c mice were treated with the mixture of Mabs GM1.2, M5/144, MKD6 and γ1γ plus complement (Palacios and Leu, 1986) to eliminate myeloid and mature T lymphocytes. Following two cycles of treatment, the marrow cells were washed twice in culture medium [IMDM + FCS (5%) + 2-ME (5×10^{-5} M) + gentamycin (50 μg/ml)] and resuspended in WEHI-CM (culture medium supplemented with supernatants containing IL3 activity produced by WEHI-3 cells) (Palacios, 1985). Optimal concentrations of WEHI-3 supernatants were determined on the Bc/Bm11 PRO-B lymphocyte clone (Palacios and Steinmetz, 1985).

Accessory cells (AC) from spleen of C57BL/6 and CBA/J mice were obtained by centrifugation over BSA gradients (Palacios and Leu, 1985). Spleen cells from Scid mice were checked for the presence of IgM^+, $B\text{-}220^+$, $Thy1^+$, $Mac\text{-}1^+$ and Ia^+ cells by FACS analysis. Spleen Scid cells that had no detectable IgM^+ or $B\text{-}220^+$ were used. AC from C57BL/6 and CBA/J mice and spleen Scid cells were irradiated (2500 rad, X-ray), washed and resuspended in culture medium.

Development of the Lyd9, LyH7 and Lyb9 clones

Marrow cells (from a 4-week-old BALB/c mouse) depleted of myeloid, T lymphocytes and Ia^+ cells were employed for the establishment of 13 cell lines using the same procedure described previously for the establishment of the 86/I $Ly1^+$ B cell precursor line (Palacios and Leu, 1986). One of them, the line called Ly, was chosen for further study because of its phenotype. Clones were derived from this cell line by micromanipulation (Palacios, 1985). Of the 26 clones obtained, 16 were frozen and 10 clones were chosen for further study because they exhibited the highest levels of Ly1 antigen on the cell membrane. These clones have been maintained in culture in WEHI-CM ($2-3 \times 10^5$ cells/ml) up to the present by changing them into fresh WEHI-CM every 2−3 days.

Analysis of the configuration of Ig genes

The state of the Ig genes coding for H and κ light chains in the Ly clones was assessed by Southern blot analysis. DNA from the clones and BALB/c liver was isolated and 10 μg of each was digested with *Bam*HI or with *Eco*RI restriction enzymes (Maniatis *et al.*, 1982). DNA fragments were separated on 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized either with a J_H-specific probe [a mixture of the 2-kb *Eco*RI−*Bam*HI and the 1-kb *Bam*HI fragments which contain the four J_H-gene segments (Steinmetz and Zachau, 1980)] or with a 2.6-kb *Hin*dIII−*Bam*HI fragment containing the c kappa exon (Cory *et al.*, 1980).

Immunofluorescence staining and flow cytometry analysis (FACS)

This was carried out as described in detail before (Palacios *et al.*, 1986) with a FACS I analyzer (Becton-Dickinson, Sunnyvale, CA). Direct- or indirect-immunofluorescence stainings were performed using the following FITC-labeled second step reagents: goat $F(ab')_2$ anti-rat Ig, avidin, and sheep anti-mouse Ig antibody. Negative controls were the cells stained with second step reagent only. Dead cells were excluded from analysis by propidium iodide. Fluorescence emitted by single viable cells was determined using logarithmic amplification and the data collected from 10^4 cells were analyzed with a program in a Consort 30 computer (which displays data in the form of histograms and gives the percentage of positive and negative cells as well as the statistical analysis of the data obtained with the test sample as compared with that from the negative control sample).

Induction of differentiation

Attempts to induce differentiation of the PRO-B cells by using soluble mediators were performed as follows: Ly clones (10^6 cells) were cultured in the presence of saturating concentrations of the following mediators employed either alone or in various combinations: WEHI-3 sup., U-937 sup., PWM-sup., rat or mice spleen ConA-sup. and 2.19 T cell sup. (final concentrations 5−25%), IL1 (100 U/ml), IL2 (500 U/ml), IL3 (100 U/ml), IL4 (500 U/ml), IL5 (500 U/ml), in tissue culture plastic flasks in a final volume of 7 ml of culture medium. After 3−9 days of culture, the cells were washed and assayed for surface IgM, Ia, B-220, BP-1 and Ly1 antigens.

In another set of experiments, the Lyd9, LyH7 and Lyb9 clones suspended in WEHI-CM were exposed to several concentrations of freshly prepared 5-Aza (from 5 to 100 μg/ml). The cultures were monitored for the presence of IgM^+, Ia^+, $Ly1^+$, $B\text{-}220^+$, $BP\text{-}1^+$ and $Lyb8^+$ cells 2, 7, 14, 21 and 40 days after exposure to the drug. The following protocol gave the best and most reproducible results: 5×10^6 cells suspended in 4 ml of WEHI-CM containing 5-Aza 20−10 μg/ml) were incubated at 37°C for 24 h. After this period, 4 ml of fresh WEHI-CM without 5-Aza were added to the cultures and incubated at 37°C. After 3−4 days the cells were harvested, washed twice in culture medium and the viable cells (45−75% of the starting population) were cultured in WEHI-CM without 5-Aza at 37°C for 3 days. Following this culture period the cells were subjected to the entire treatment described above once more. After completion of the second cycle of treatment with 5-Aza, the cells were maintained (or expanded) at $2-3 \times 10^5$ cells/ml of WEHI-CM changing them into fresh WEHI-CM every 2−3 days.

The experiments in which the Ly clones treated with 5-Aza or untreated were stimulated to differentiate by using AC and LPS were performed as follows: Ly cells (2×10^5) were co-cultured with X-ray-irradiated (2500 rad) AC ($0.5-1.0 \times 10^6$ cells from Scid, C57BL/6 or CBA/J mice) and LPS (30 μg/ml) in Linbro macroculture wells in a final volume of 1.5 ml of culture medium (in the absence of exogenous growth factors). The cultures (10−24 wells per Ly clone) were incubated at 37°C for various periods (5, 6, 7, 8, 10, 15 and 20 days); the cultures incubated for a period >8 days were fed at 4- to 5-day intervals by replacing ~0.5 ml of the medium with fresh culture medium. The cells were harvested, washed and assayed for: (i) the expression of several surface markers (see above) by FACS analysis and (ii) the presence of cells secreting IgM and/or IgG by the Protein A plaque assay. The supernatants from the cultures were tested for the presence of Ig by enzyme-linked immuno-assay (ELISA) (Rolink *et al.*, 1987).

Transfer of the Ly clones into Scid mice

Lyd9, LyH7 and Lyb9 clones (5×10^6 in 0.5 ml PBS) were injected intravenously or intraperitoneally into sublethally X-ray-irradiated (250 rad) Scid mice. Control mice were injected with PBS only. All mice were maintained with drinking water containing antibiotics in sterile isolators. The spleens were removed 4 or 6 weeks later and cell suspensions were prepared. The presence of IgM^+, κ^+, λ^+, $BP\text{-}1^+$, $MB86^+$, $B\text{-}220^+$, $Ly1^+$, $THY1.2^+$, $Lyt2^+$, $L3T4^+$ and $Mac\text{-}1^+$ cells was assayed by FACS analysis.

Proliferative cell responses to interleukins

Cells were harvested in the logarithmic phase of growth, washed three times in 50 ml BSS each and resuspended in culture medium at the desired concentrations. Cells (10^4) were incubated in microplate wells in a final volume of 200 μl of culture medium containing the following ILs (final dilutions from 1:10 to 1:10 240): rIL1, rIL2, rIL3, rIL4 or rIL5. The cultures in triplicate were incubated at 37°C for 24 and 48 h. Cell proliferation was measured by [^{3}H]-thymidine uptake (18.5 KBq/well) during the last 5−6 h of the culture periods. The data are expressed as c.p.m. and are the mean of triplicate samples.

Acknowledgements

We are grateful to Dr Werner Haas for critically reading the manuscript; Drs A.Coutinho, P.Kincade, F.Melchers, L.Forni, P.Sideras, E.Shevach,

M.Steinmetz, K.Rajewsky, I.Trowbridge and M.Bosma for providing us with reagents or mice; T.Leu, K.Szabo, A.Peter and G.Dastoornikoo for technical assistance; H.P.Stahlberger for preparation of the illustrations and Ms Catherine Plattner and Ms Carolyn Harley for the excellent preparation of the manuscript. The Basel Institute for Immunology was founded and is supported by F.Hoffmann-La Roche, Ltd. Co., Basel, Switzerland.

References

Alt,F., Blackwell,K., DePinho,R., Reth,M. and Yancopoulos,G. (1986) *Immunol. Rev.*, **89**, 5–27.
Bosma,G., Custer,R. and Bosma,M. (1983) *Nature*, **301**, 527–529.
Braun,J., Citri,Y., Baltimore,D. *et al.* (1986) *Immunol. Rev.*, **93**, 5–21.
Cooper,M.D. (1981) *J. Clin. Immunol.*, **1**, 81–88.
Cooper,M.D., Mulvaney,D., Coutinho,A. and Cazenave,P.A. (1986) *Nature*, **321**, 616–618.
Corbel,C. and Melchers,F. (1984) *Immunol. Rev.*, **78**, 51–72.
Cory,S., Adams,J. and Kemp,D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4943–4947.
Dasch,J. and Jones,P.P. (1986) *J. Exp. Med.*, **163**, 938–946.
Davidson,W., Fredrickson,T., Rudikoff,E. *et al.* (1984) *J. Immunol.*, **133**, 744–753.
Dorskind,K., Schouest,L. and Fletcher,W. (1985) *Cell. Tissue Res.*, **239**, 375–380.
Gillis,S. and Smith,K. (1977) *Nature*, **268**, 154–156.
Hayakawa,K., Hardy,R., Parks,D. and Herzenberg,L.A. (1983) *J. Exp. Med.*, **157**, 202–211.
Hayakawa,K., Hardy,R. and Herzenberg,L.A. (1985) *J. Exp. Med.*, **161**, 1554–1562.
Herzenberg,L.A., Stall,A., Labor,P. *et al.* (1986) *Immunol. Rev.*, **93**, 81–102.
Holmes,K. and Morse,W.C.III (1984) *Curr. Top. Microbiol. Immunol.*, **113**, 224–231.
Hunt,P., Robertson,D., Weiss,D., Rennick,D., Lee,F. and Witte,O.N. (1987) *Cell*, **48**, 997–1007.
Jones,P.A. (1984) In Razín,A., Cedar,H. and Riggs,A.D. (eds), *DNA Methylation: Biochemistry and Biological Significance*. Springer, New York, pp. 165–187.
Koyasu,S., Yodoi,J., Nikaido,T. *et al.* (1986) *J. Immunol.*, **136**, 984–989.
Legros,G., Gillis,S. and Watson,J. (1986) *J. Immunol.*, **135**, 4009–4016.
Maniatis,T., Fritsch,E. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
Manohar,V., Braun,E., Leiserson,W. and Chused,T. (1982) *J. Immunol.*, **129**, 532–537.
McKearn,J., McCubrey,J. and Fagg,B. (1986) *Proc. Natl. Acad. Sci. USA*, **82**, 7414–7420.
Muller-Sieburg,C., Whitlock,C. and Weissman,I. (1986) *Cell*, **44**, 653–658.
Nishikawa,S., Sasaki,Y., Kina,T., Amagai,T. and Katsura,T. (1986) *Immunogenetics*, **23**, 137–141.
Ozato,K., Mayer,N. and Sachs,D.H. (1980) *J. Immunol.*, **214**, 533–539.
Paige,C.J. (1983) *Nature*, **302**, 711–713.
Palacios,R. (1985) *Immunol. Methods*, **III**, 265–278.
Palacios,R. and Leu,T. (1985) *Cell. Immunol.*, **94**, 369–378.
Palacios,R. and Leu,T. (1986) *Immunol. Rev.*, **93**, 125–146.
Palacios,R. and Steinmetz,M. (1985) *Cell*, **41**, 727–734.
Palacios,R. and von Boehmer,H. (1986) *Eur. J. Immunol.*, **12**, 17–23.
Palacios,R., Neri,T. and Brockhaus,M. (1986) *J. Exp. Med.*, **163**, 369–378.
Palacios,R., Kiefer,M., Brockhaus,M., Dembić,Z., Karjalainen,K., Kisielow,P. and von Boehmer,H. (1987a) *J. Exp. Med.*, **166**, 12–33.
Palacios,R., Sideras,P. and von Boehmer,H. (1987b) *EMBO J.*, **6**, 91–95.
Raff,M.C., Szeinberg,M. and Taylor,R.B. (1970) *Nature*, **225**, 553–555.
Raff,M.C., Megson,M., Owen,J.J. and Cooper,M.D. (1976) *Nature*, **259**, 224–226.
Robb,R., Greene,W. and Rusk,C. (1984) *J. Exp. Med.*, **160**, 1126–1132.
Rolink,A.G., Radaszkiewicz,T. and Melchers,F. (1987) *J. Exp. Med.*, **165**, 1675–1687.
Sideras,P. and Palacios,R. (1987) *Eur. J. Immunol.*, **17**, 217–221.
Sideras,P., Bergsted-Lindquist,S., MacDonald,R. and Severinson,E. (1985) *Eur. J. Immunol.*, **15**, 586–592.
Spalding,D. and Griffin,J. (1986) *Cell*, **44**, 507–514.
Springer,T.A., Galfre,G., Secher,D. and Milstein,C. (1979) *Eur. J. Immunol.*, **9**, 301–306.
Steinmetz,M. and Zachau,H.G. (1980) *Nucleic Acids Res.*, **8**, 1693–1707.
Storb,U. and Arp,B. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6642–6647.
Takemori,T. and Rajewsksy,K. (1981) *Eur. J. Immunol.*, **11**, 618–625.
Tonegawa,S. (1983) *Nature*, **302**, 575–581.
Whitlock,C. and Witte,O.N. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3608–3613.
Whitlock,C., Tidmarsh,G., Muller-Sieburg,C. and Weissman,I.L. (1987) *Cell*, **48**, 1009–1021.
Yancopoulos,G., Blackwell,K., Suh,H., Hood,L. and Alt,F.W. (1986) *Cell*, **44**, 251–259.

Received on August 26, 1987; revised on September 16, 1987