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B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac

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A stromal cell clone, ST2, which can support both myelopoiesis and B lymphopoiesis of adult bone marrow was used as an *in vitro* microenvironment for investigating the ontogeny of the B cell progenitor in murine embryos. The B cell progenitor clonable on an ST2 layer first become detectable in the embryonal body rather than in the yolk sac around day 9.5 of gestation. As soon as it develops in the embryo, it enters the blood circulation and becomes detectable both in the developing fetal liver and the yolk sac of the 10 day embryo. On the other hand, mast cell and polymorphonuclear cell progenitors, which are also generated on the ST2 layer, develop first in the yolk sac and migrate to the fetal liver around day 10−11 of gestation. At the late stage of embryonal development, day 15−16 of gestation, the B cell progenitor enters the femur as vascularization of the femur starts. These results suggest that the localization of the committed stem cells for various hemopoietic cell lineages differs in the early embryo, although the localization of the pluripotent stem cells is yet to be determined.

Key words: B cell ontogeny/stromal cell clone/B cell progenitor/hemopoietic stem cell

Introduction

It has been established that B lymphocytes, like other cells of hemopoietic tissue, are generated from pluripotent hemopoietic stem cells (Abramson *et al.*, 1977; Williams *et al.*, 1984; Dick *et al.*,1985; Keller *et al.*, 1985). Previous studies demonstrated that hemopoietic stem cells are first detected in the yolk sac of 7−9 day embryos, and on day 10−11 of gestation migrate to the developing fetal liver (Moore and Metcalf, 1970). Although yolk sac origin of hemopoietic stem cells is the most plausible interpretation of the available data, several opposing results have also been reported. First, Tyan and Herzenberg (1967) showed that the T cell progenitor was more abundant in the embryonal body than in the yolk sac of 9 day embryos. In the same study, they also showed that the cell reconstituting B cell lineage was detected both in the yolk sac and embryonal body of 9 day embryos. Furthermore, Harrison *et al.* (1979) and Sonoda *et al.* (1983) have demonstrated the inability of yolk sac cells to cure the anemia of W/W^v mice. Thus, whether the stem cell in the yolk sac is pluripotent, as are those in fetal liver and adult bone marrow, and where the commitment of stem cell to lymphoid cell lineage occurs are questions which still need to be investigated. Since these early studies used experimental systems which are essentially the same as those being utilized now, namely spleen colony assay (Till and McCulloch, 1961), *in vitro* colony assay (Metcalf *et al.*, 1967), or adoptive transfer assay (Tyan, 1968), it has been difficult to design a new approach to these questions. Since Dexter *et al.* (1977) first described conditions to maintain bone marrow hemopoiesis *in vitro*, however, a new option for investigating hemopoiesis, long term bone marrow culture, has been developed. Furthermore, several stromal cell clones which are able to support the growth and differentiation of hemopoietic cell lineages, including the B cell lineage, have been established (Collins and Dorshkind, 1987; Hunt *et al.*, 1987; Whitlock *et al.*, 1987). These stromal cell clones support B lymphopoiesis under the conditions described by Whitlock and Witte (1982), while they also support myelopoiesis under the culture conditions described by Dexter *et al.* (1977). These studies clearly indicate that such stromal cell clones can serve as an *in vitro* microenvironment for hemopoiesis and might become a powerful tool for studying the ontogeny of the hemopoietic system.

In the present study, we used a stromal cell clone, ST2, which was established in our laboratory, and addressed ourselves to the question of when and where the B cell progenitor clonable on ST2 layer develops. The result showed that various hemopoietic cells, including $B220^+$ lymphoid cells and mast cells, are generated on an ST2 layer from embryonal tissues and the B cell progenitor becomes detectable around day 9−10 of gestation, first in the embryonal body rather than in the yolk sac, whereas mast cell and myeloid cell progenitors are detected first in the yolk sac.

Results

Generation of B cells from fetal liver cells on the monolayer of ST2

It is well established that fetal liver is the major site of B lymphopoiesis during embryonal life (Melchers *et al.*, 1975; Raff *et al.*, 1976). Thus far, using the most sensitive assay for B cell progenitors, the liver of the 12 day embryo is the earliest source from which mature B cells are generated *in vitro* (Paige, 1983; Paige *et al.*, 1984). Thus, in order to test the ability of ST2 to support the differentiation of B cells, we cultured the fetal liver cells from 11 or 12 day embryos on an ST2 monolayer. Two weeks after the initiation of the culture, the cells were harvested by gentle pipetting, and analysed cytologically with May-Gruenwald−Giemsa staining and flow-cytometry after staining with anti-μ or anti-B220 antibodies (Table I and Figure 1). Upon co-culture with ST2, the fetal liver cells grew vigorously, and the cell recovery from each culture was ~40-fold more than the number inoculated at the beginning. Lymphoid cells were the major cell type present in the culture of all groups. Besides the lymphoid cells, under our culture conditions, polymorpho-

Table I. Cells generated on an ST2 layer from fetal liver of 11 and 12 day embryos[a]

Cultured cells	Cell recovery per flask	Differential Counts (%)[b]			
		Ly ($B220^+$,$s\text{-}\mu^+$)[c]	PMN	mast	other MNC
11 day fetal liver (1×10^5)	5.4×10^6	72 (60, 13)	2	12	14
12 day fetal liver (1×10^5)	4.3×10^6	68 (43, 18)	10	11	11

[a]1×10^5 fetal liver cells were cultured on an ST2 layer for 2 weeks, and the cultured cells were harvested by gentle pipetting. The cells from duplicate cultures of each group were pooled and analysed. The differential cell counts were performed after staining with May-Gruenwald–Giemsa solution.
[b]Ly; lymphoid cells, PMN; polymorphonuclear cell, MNC; mononuclear cell.
[c]% positive cells in the lymphocyte gate. This value is based on the flow-cytometry in Figure 1.

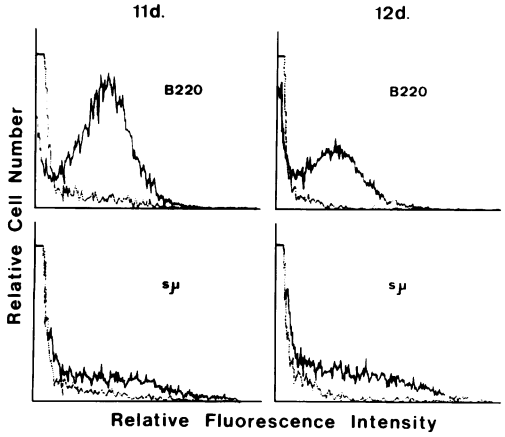

Fig. 1. Flow cytometric analysis of the cells generated on the ST2 layer from 11 and 12 days fetal liver cells. The same cells shown in Table I were stained either with RA3-6B2 (aB220) and subsequent FITC conjugated MARK 1 (anti-rat-k), or with FITC conjugated goat anti-mouse-μ antibody. The cells within the lymphocyte gate were analysed by an Epics V flow-cytometer. Broken lines represent the control profile of the cells stained with FITC conjugated MARK 1 alone.

nuclear cells (from metamyelocyte to mature neutrophil), connective tissue type mast cells with basophilic granules, and other mononuclear cells including mature monocytes, promyelocytes with Azur granules and erythroblasts were maintained. Approximately 50% of these lymphoid cells were $B220^+$, and a significant number of $sIgM^+$ cells were also present (Figure 1). Furthermore, Southern blot analysis of the DNA samples from the same cell populations for JH gene rearrangement demonstrated that there were indeed cells with a rearranged JH gene configuration (analysis on 11 day fetal liver cultures is shown in Figure 5). These results clearly indicate that ST2 supports the growth and differentiation of the B cell progenitor in the 11 day fetal liver and therefore that our culture system is a far more powerful tool for investigating B cell ontogeny *in vitro* than those reported previously (Metcalf, 1976; Melchers, 1977; Paige, 1983; Paige *et al.*, 1984).

B cell progenitor in the earlier embryo

To investigate the ability of ST2 to detect the B cell progenitor, we attempted to induce B cells on ST2 from hemopoietic tissues of the earlier embryo. We cultured the yolk sac cells or the total cells of embryonal body from 9 day embryos, or the yolk sac cells or the fetal liver cells from 10 day embryos. Two weeks after the initiation of the cultures, the cultured cells were harvested, counted and analysed. The cell recovery from the cultures of 9 day embryos was far lower than those from 10 day embryos. Lymphoid cells were present both in the yolk sac and fetal liver cultures of 10 day embryos, whereas no lymphoid cells were detectedin any cultures of 9 day embryos (Table II). In order to examine if the lymphoid cells in the cultures of 10 day embryos include B lineage cells, flow-cytometric analysis was performed on the cells from the fetal liver and yolk sac cultures of 10 day embryos. Figure 2 shows that a significant number of $B220^+$ cells were generated both in the yolk sac and fetal liver cultures of 10 day embryos, though only a few cells were $sIgM^+$. Southern blot analysis of the DNA preparation of the same cell populations with the JH probe were also performed and the result clearly shows that a significant number of the cells in these cultures had rearranged JH genes (Figure 3). These results indicate that the lymphoid cells generated in the cultures of 10 day embryos are indeed B lineage cells.

Interestingly, a considerable number of mast cells and polymorphonuclear cells were generated only in the yolk sac cultures of 9 and 10 day embryos. Thus, as previously shown by Moore and Metcalf (1970) or Sonoda *et al.* (1983), the precursor cells for these cell lineages seem to develop first in the yolk sac and migrate to the fetal liver on day 10–11 of gestation.

B cell progenitor develops first in the embryonal body rather than in the yolk sac

The results in the previous section suggest strongly that the B cell progenitor clonable on ST2 first develops at a certain point between day 9 and 10 of gestation. To determine where and when the B cell progenitor appears, we sacrificed the pregnant mice on day 9.0 (9 a.m.) and 9.6 (11 p.m.) of gestation, prepared the yolk sac and total embryonal body cell suspensions separately, and cultured on ST2. After 2 weeks, the cultured cells were harvested and analysed. Again, mast cells and polymorphonuclear cells were detected only in the yolk sac cultures. The same was also true for the promyelocytes with Azur granules. On the other hand, a significant number of lymphoid cells were detected only in the cultures of the embryonal body of 9.6 day embryos. This is clearly seen in the light scatter analysis comparing the cells in the yolk sac and the embryonal body cultures of 9.6 day embryos (Figure 4). Only a few cells in the yolk sac cultures are distributed in the lymphocyte area, while many cells in the cultures of the embryonal body fell within

Table II. Cells generated on an ST2 layer from fetal liver and yolk sacs of 9 and 10 day embryos[a]

Cultured cells	Cell recovery per flask	Differential counts (%)			
		Ly	PMN	mast	other MNC
10 day yolk sac	38.0×10^5	50	5	16	29
10 day fetal liver	31.0×10^5	73	0	0.2	26
9 day yolk sac	2.7×10^5	0	8	50	42
9 day embryonal body	4.3×10^5	0	0	0	100

[a] 1×10^5 cells were cultured on an ST2 layer for 2 weeks, and the cultured cells were harvested by gentle pipetting. The cells from duplicate cultures were pooled and analysed.

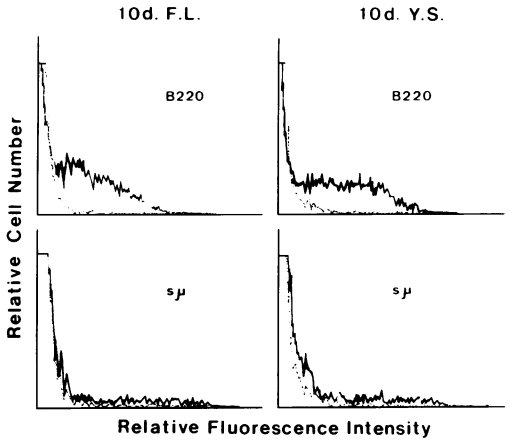

Fig. 2. Flow-cytometric analysis of the cells generated on an ST2 layer from the yolk sac cells and the fetal liver cells of 10 day embryos. The same cells shown in Table II were analysed as in Figure 1.

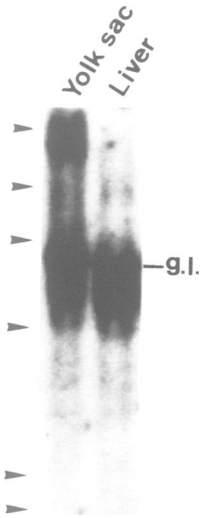

Fig. 3. Southern hybridization of the DNA prepared from the same cells analysed in Figure 2. DNA was prepared from the total cells remaining after the analysis shown in Table II and Figure 2. All DNA was digested with *Eco*RI to completion, and the amount of DNA was estimated by fluorescence spectrophotometry after staining with DAPI. DNA (3.0 and 5.7 μg) was recovered from the cultured cells of the fetal liver and the yolk sac of 10 day embryos, respectively, and all recovered DNA was separated on a 0.8% agarose gel. The DNA was blotted to a nitrocellulose filter and hybridized with the JH probe. The size of the germ line JH band was indicated as g.l.

this area. Flow-cytometric analysis was performed on these cells within the lymphoid cell gate and given in parenthesis in Table III. Indeed, a significant number of $B220^+$ cells were present in these cultures, although no $sIgM^+$ cells were detected in the same cell populations (data not shown). In order to further confirm that the cells present are B lineage cells, we prepared the DNA from the cells in the same cultures after one more week incubation, and analysed for the JH gene rearrangement. As a control, DNA samples from 11 day fetal liver cultures were also analysed (Figure 5). Rearranged JH bands are present on the lane of the embryonal body culture while only the germ line JH band was visible on the lane of the yolk sac culture. Interestingly, the pattern of the distribution of the rearranged JH bands was almost identical between the 9.6 day embryonal body cultures and the 11 day fetal liver cultures, suggesting that a regular process of heavy chain gene rearrangement is taking place on ST2. Although our calculation of embryo age is not absolute because of the inevitable intra- and inter-litter developmental variation, the present results indicate clearly that the B cell progenitor clonable on ST2 develops first in the embryonal body rather than in the yolk sac.

Entry of the B cell progenitor into bone marrow occurs on day 15–16 of gestation

During embryonal development, hemopoietic stem cells migrate from fetal liver to bone marrow. In this final section, we attempt to determine the time of entry of the B cell progenitors into bone marrow. From day 14 of gestation, when chondrification of femur begins, femora become distinguished from the surrounding tissues. We took femora from 14, 15 or 16 day embryos. All femora from a litter were washed extensively with several changes of medium to remove contamination by peripheral blood cells, crushed with forceps and cultured on the ST2 layer. Two weeks after the culture, the floating cells were harvested by gentle pipetting, counted and analysed. Since virtually no floating cells were recovered from the cultures of femora of 14 or 15 day embryos, $<2 \times 10^3$ cells per flask, no cytological analysis was able to be performed on these cultures. In contrast, 5.7×10^6 cells were recovered from the culture of day 16 femora, and 96% of the cells were lymphoid cells. Flow-cytometric analysis demonstrated that 72% of the cells were $B220^+$ and 19% of the cells were $sIgM^+$ (Figure 6). This result indicates that the entry of B progenitors into bone marrow commences on day 15–16 of gestation, as soon as vascularization of the femur starts.

Discussion

In the present study, we used a stromal cell clone (ST2) as an *in vitro* microenviroment for investigating B cell ontogeny

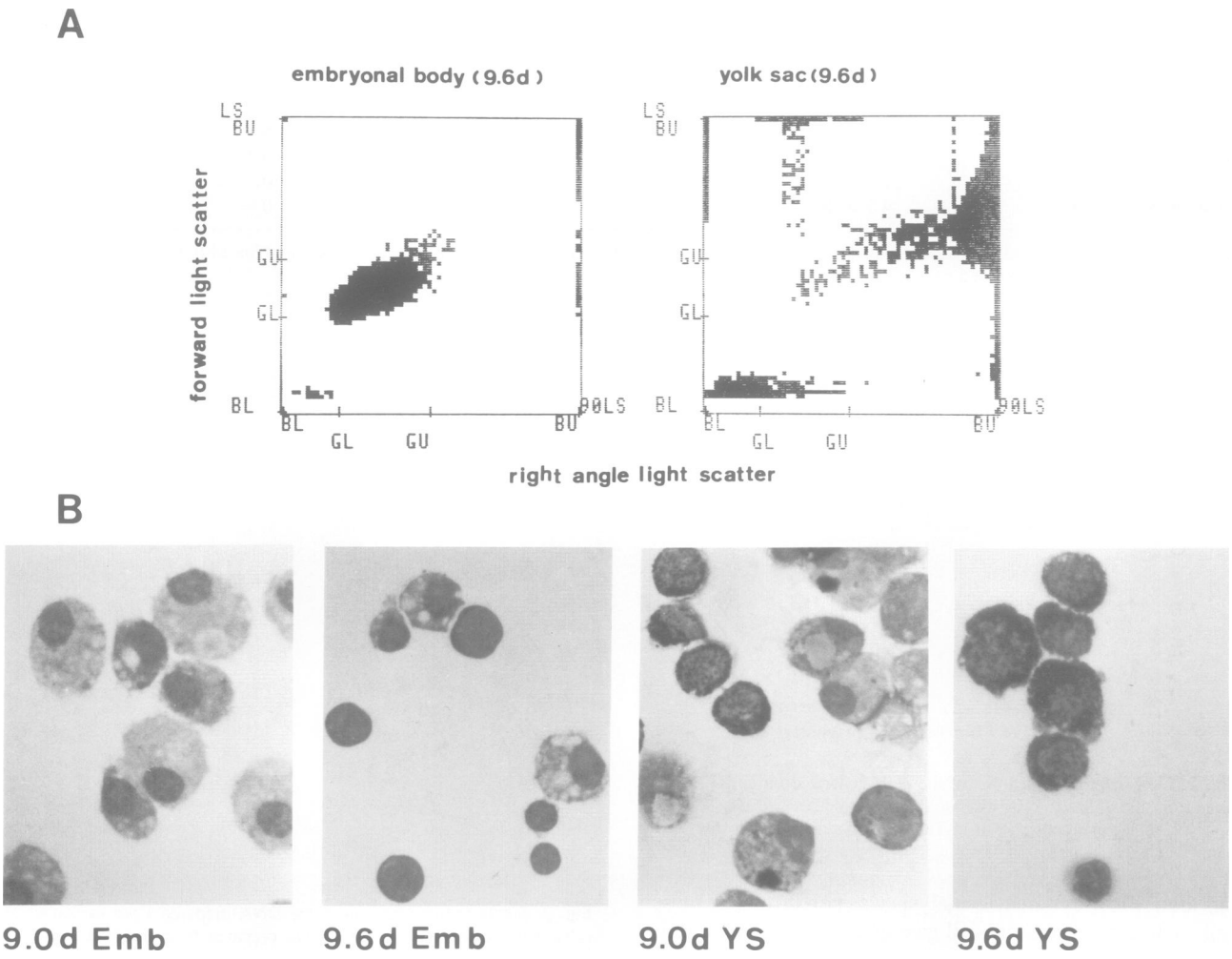

Fig. 4. Morphology of the cells in the cultures of the yolk sac and the embryonal body of 9.0 and 9.6 day embryos. (**A**) Light scatter analysis of the cells in the yolk sac and the embryonal body cultures of Experiment 2 in Table III was performed by Epics V. The gate set for a stromal cell dependent B cell clones was indicated by G.U. and G.L. (**B**) The pictures of the cells from Experiment 2 in Table III. 9.0d Emb; macrophages are dominant, no lymphoid cells, 9.6d Emb; lymphoid cells of various sizes and macrophages, 9.0d YS; mast cells with basophilic granules, macrophages and promyelocytes with Azur granules, 9.6d YS; connective tissue type mast cells of various sizes and mature neutrophils.

Table III. Cells generated on an ST2 layer from yolk sacs and embryonal bodies of 9.0–9.6 day embryos[a]

Experiment	Cultured cells	Cell recovery per flask	Differential counts (%)			
			Ly(%B220$^+$)[b]	PMN	mast	other MNC
1.	9.0d yolk sac	3.6×10^5	0(ND[c])	5.8	42.9	51.3
	9.0d embryonal body	2.0×10^5	0.4(ND)	0	0	99.6
	9.6d yolk sac	2.3×10^5	1.0(7)	0.8	6.9	91.3
	9.6d embryonal body	7.4×10^5	16.3(47)	0	0	83.7
2.	9.0d yolk sac	6.2×10^5	0(0)	10.1	56.2	33.7
	9.0d embryonal body	1.8×10^5	0.2(0)	0.7	0.5	98.6
	9.6d yolk sac	2.3×10^5	2.2(2)	4.6	76.7	16.5
	9.6d embryonal body	17.0×10^5	62.1(8)	0	0	37.9

[a]1×10^5 cells were cultured on an ST2 layer for 2 weeks and the cells were harvested by gentle pipetting. The cells from duplicate cultures were pooled and analysed.
[b]The harvested cells were stained with RA3-6B2 and the cells within the lymphocyte gate were analysed.
[c]Not determined.

during early embryonal development. In the previous studies, 12 day fetal liver has been the earliest cell source from which mature B cells are able to be generated *in vitro* (Paige *et al.*, 1984). On the other hand, we were able to detect B cell progenitors in day 9–10 embryos, where no cell bearing known B cell markers had been detected (Velardi and Cooper, 1984). Therefore, we think that culture with the monolayer of the ST2 clone, or with other stromal cell clones described recently (Collins and Dorshkind, 1987; Hunt *et al.*, 1987; Whitlock *et al.*, 1987), may be the most powerful tool for investigating B cell ontogeny *in vitro*. Like other stromal cell clones capable of supporting both myelopoiesis

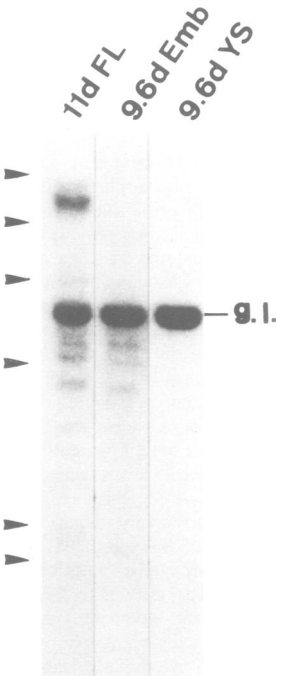

Fig. 5. Southern hybridization of the DNA from cells in cultures of yolk sac and embryonal body of 9.6 day embryos. The cells were harvested from the same cultures shown in Figure 4(A) at the third week of culture. The DNA was prepared from the cells, digested with *Eco*RI and the DNA content was estimated by fluorescence spectrophotometry after staining with DAPI. As a positive control for JH gene rearrangement, the DNA was prepared from the cells of 11 day fetal liver cultures maintained for 4 weeks, and analysed. Digested DNA (5 μg) was separated on a 0.8% agarose gel, blotted to a nitrocellulose filter and hyridized with the JH probe. Arrows represent the sizes of the markers, 23, 9.3, 6.5, 4.3, 2.3 and 2.0 kb from the top, respectively.

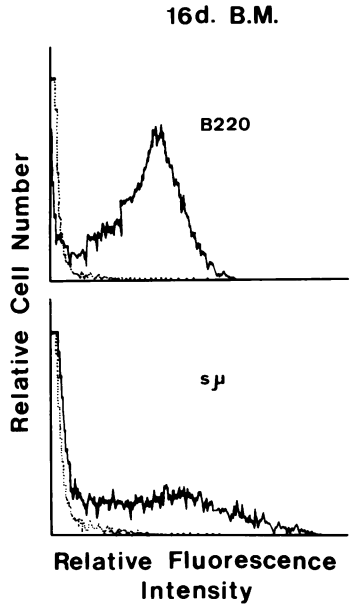

Fig. 6. Flow-cytometric analysis of the cells generated in the 16 day fetal bone marrow culture. Total femur cells from a litter of embryos were cultured on an ST2 layer. Two weeks later, the cells were harvested by gentle pipetting, stained either with anti-B220 and subsequent FITC-anti-rat k or FITC-anti-μ, and analysed by Epics V. The broken lines in the figure represent the control profiles stained with FITC conjugated anti-rat-k alone.

and B lymphopoiesis, ST2 also supports the long term production of various hemopoietic cells. Obviously, even under Whitlock–Witte type culture conditions which preferentially support the growth of the B cell lineage (Whitlock and Witte, 1982), other hemopoietic cells like mast cells, macrophages and polymorphonuclear cells of various differentiation stages are generated and maintained in the culture of the 9 day yolk sac on ST2. Thus, ST2 can detect very early progentiors for various hemopoietic cell lineages, as soon as they develop in embryo. It is important to note, however, that it is still obscure whether ST2 detects the pluripotent stem cells or the stem cells committed to various lineages, and further lineage analysis is required. Thus, at the moment, we designate the cells detected in this study as ST2 clonable hemopoietic progenitor.

The present results clearly demonstrate that the B cell progenitor clonable on ST2 develops on day 9.5–10 of gestation, somewhere in the embryonal body but not in the yolk sac. On the other hand, on day 10 of gestation, B cell progenitors were detectable both in the fetal liver and the yolk sac (Table II), and also in the peripheral blood (unpublished observation). Therefore, as soon as the B cell progenitor develops in the embryonal body, it migrates to the yolk sac by blood circulation. In contrast, as demonstrated by Moore and Metcalf (1970) using *in vitro* and *in vivo* colony assay or Sonoda *et al.* (1983) using intradermal injection assay, the precursors for mast cells and myeloid cells were detected only in the yolk sac of 9–10 day embryos by our culture system. Our results, particularly on the development of the B cell progenitor, contrast with previous studies showing that lymphoid cell progenitor as well as other hemopoietic cell lineages develops first in the yolk sac. Most of these experiments, however, used the 10–11 day yolk sac for reconstituting irradiated mice (Moore and Metcalf, 1970; Paige *et al.*, 1979). Obviously this is not enough to conclude yolk sac origin of lymphoid cell progenitor since according to the results presented here the B cell progenitor was detected both in the yolk sac and embryonal body as early as day 10. On the other hand, Weissman *et al.* (1976) showed that T cell precursors are present in the yolk sac of the 8 day embryo, suggesting strongly that the stem cells in the yolk sac can give rise to lymphoid cells. Due to the complexity of their experimental system, however, we like to think that their result should be interpreted with critical reservation. Our result is more consistent with the result of Tyan and Herzenberg (1968) showing that the B cell progenitors which reconsitute the irradiated host were present both in the embryonal body and the yolk sac of a 9 day embryo, and the T cell progenitors in the same embryo were more abundant in the embryonal body than in the yolk sac, and also with the result in the avian embryo suggesting the embryonal body as origin of the B cell progenitor (Dieterlen-Lievre, 1975). One plausible interpretation of all these contradictory results may be that the pluripotent stem cell arises in the yolk sac but the commitment to B cell lineage occurs in the embryonal body and the variance in the results comes from the difference in the stage of differentiation of the cells detected by *in vivo* experimental systems and by our *in vitro* system, namely, *in vivo* systems detect the pluripotent stem cell while ours detects only the committed progenitor cells. It is, however, of importance to note that previous attempts

to cure the anemia of W/W^v mice by transferring yolk sac cells has not been successful (Harrison *et al.*, 1979; Sonoda *et al.*, 1983). Therefore, it could also be that the stem cells in the yolk sac, although they may be pluripotent, have only limited self-renewal capacity and a further maturation process in the embryonal body is needed to acquire this capacity. The results of Paige *et al.*(1979) also suggest this possibility. Taken together, we think that the yolk sac origin of hemopoietic stem cells is not formally proven yet.

Finally, our result that the progneitor for connective tissue type mast cell appears first in the yolk sac corroborate the previous results of Sonoda *et al.* (1983). Our preliminary result on the frequency of the mast cell progenitors assayed by limiting dilution on ST2 indicated that the frequency of the mast cell progenitors in 9 day yolk sac is ~1/2000, 100-fold more than their estimation. It is of interest to note that although other cell lineages do grow on ST2, connective tissue type mast cells dominate the culture if no B cell progenitor is present. This phenomenon is particularly interesting in view of the previous studies showing the importance of fibroblasts for the growth and differentiation of connective tissue type mast cells (Ginsberg *et al.*, 1982; Levi-Schaffer *et al.*, 1986). Unlike the result of Levi-Schaffer *et al.* (1986) that both interleukin (IL)3 and fibroblasts are required for the development of connective tissue type mast cells, our present results indicate that IL3 is not a requisite for growth of connective tissue type mast cells because no IL3 mRNA is detectable in ST2 cells (paper in preparation). The recent result of Fujita *et al.* (1987) that NIH-3T3 was sufficient for providing the growth signal to connective tissue type mast cells is consistent with our results. Thus, it would be interesting to test the ability of the fibroblast cell lines, which do not support the growth of B lineage cells, for induction of mast cells from fetal tissues. In any case, our culture system is a sensitive *in vitro* tool for investigating mast cell ontogeny.

As mentioned above, the interpretation of our present results is absolutely dependent on the stage of the B cell progenitors clonable on ST2. Therefore, lineage analysis by tagging the proliferating cells on ST2 or colony formation on an ST2 layer overlaid with semi-solid culture medium is currently under way to answer this question. In any case, we would like to emphasize again that the culture system with the stromal cell clone capable of supporting the growth and differentiation of lympho-hemophoietic cell lineages will provide a new quantitative method for investigating the ontogeny of hemopoietic tissue and will reveal many facts which have been left undetected with the technologies hitherto available in experimental hematology.

Materials and methods

Mice

BALB/c mice were purchased from Shizuoka Agricultural Co-operation for Experimental Animal (Shizuoka, Japan). BC8 mice were kindly provided by Dr S.Migita at Kanazawa University, Japan.

Stromal cell line.

ST2 stromal cell clone was established from Whitlock–Witte type long term bone marrow culture of BC8 mice according to the method described by Whitlock *et al.* (1987). Cloning of ST2 was carried out by limiting dilution, seeding the cells in wells of the 96 well cluster dish (Coster, Cambridge, MA) at 1 cell/well dilution. The ST2 clone can grow from a single cell and is contact-inhibited after reaching confluence. Confluent ST2 cells in a T25 flask (Corning 25100, Iwaki Glass Co. Ltd, Japan) were subcultured every 4–5 days into four flasks. For harvesting the cells, we used trypsin–EDTA solution (Gibco Laboratories, NY).

Culture medium

The medium described by Whitlock and Witte (1982) was used throughout this study, and the preparation of this medium was as described previously (Hirayoshi *et al.*, 1987).

Preparation of cells

Female BALB/c mice were mated with males at around 6 p.m. At 9 a.m. the next morning, mice with a vaginal plug were separated and this point was designated day 0.0. The pregnant mice were killed 9–16 days later by cervical dislocation. The uteri were removed, transferred to the dish containing the culture medium and rinsed with the culture medium to remove maternal blood. The embryos with a yolk sac were removed by cutting the uterine membrane, and surround tissues dissected away by fine forceps, and transferred to another dish with the culture medium. The stage of the embryo was further checked at this point using the following criteria; 9 day, a well developed yolk sac; and 10 day, developing liver pigementation. The embryos were washed again by changing the culture medium. For 9.0–9.6 day embryos, the yolk sacs were separated from the embryonal bodies, and the cell suspensions were prepared from both parts by gently drawing tissue through a 27G needle several times. For embryos older than day 10 of gestation, first the yolk sac and then the developing fetal liver along with a small piece of surrounding tissue, were separated from the embryo and the cell suspension was prepared as above. From 14 day embryos, formation of the femur can be identified. Femora were removed from the embryos by fine forceps, crushed by forceps into fine pieces and all the cells, along with the bone, were cultured. All preparations of the tissues were carried out under a dissecting microscope. Cell clumps were removed by passage through a nylon mesh, and cells were washed once with the culture medium.

Cell culture for hemopoiesis

ST2 stromal cells were grown to confluence in a T25 flask. Cells (1×10^5) from various tissues were cultured on this stromal cell layer with 6 ml of culture medium. For the femur cells of 14–16 day embryos, all femur cells from one litter were cultured in a flask without adjusting the cell number. The protocol of feeding and medium exchange for maintaining the culture was as described (Whitlock and Witte, 1982; Hirayoshi *et al.*, 1987). After 2 weeks, the cells were harvested by gentle pipetting, counted and subjected to cytological analysis.

Flow-cytometry

The presence of B cells was determined essentially by a combination of flow-cytometry after staining with anti-B220 antibody and morphological examination with May-Gruenwald–Giemsa staining. The procedures for fluorescence staining with RA3-6B2 (aB220; Coffman, 1983) and anti-μ antibodies were as described previously (Katsura *et al.*, 1985; Hirayoshi *et al.*, 1987). For analysis, Epics V was used and only the cells within the lymphocyte gate were counted. The lymphocyte gate was set each time using ST2 dependent null-pre-B or pre-B/B cell lines (M.Ogawa *et al.*, submitted) as standards.

Differential cell counts

The cytocentrifuge preparation of the cultured cells was stained with May-Gruenwald solution (Merck, Darmstadt, FRG) and then with 2% Giemsa solution (Merck). 500 cells were counted and classified into the folowing four groups; lymphoid cells, mast cells, polymorphonuclear cells (from metamyelocytes to mature neutrophils), and other mononuclear cells including macrophages, erythroblasts, promyelocytes, and a small number of exfoliated ST2 cells. Depending on the age of embryo, the cells of non-hemopoietic tissues (e.g. hepatocytes from 11 day fetal livers) were grown on ST2 layers. Thus, the last group also includes such non-hemopoietic cells.

DNA analysis for immunoglobulin gene rearrangement

Preparation of high mol. wt DNA, Southern blot and hybridization with the JH probe were as described previously (Hirayoshi *et al.*). The amount of DNA applied to the gel was estimated by the method described by Kapuscinski and Skoczylas (1977). Briefly, after digestion with restriction enzymes, an aliquot of the sample was mixed with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer Manheim GmbH, Penzberg, FRG) and analysed by fluorescence spectrophotometry.

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