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Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin

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During B cell differentiation, at least three stages can be defined in terms of their growth signal requirement by using two different growth signals, which are recombinant interleukin 7 (IL-7) and a stromal cell clone PA6 which does not produce IL-7; first a PA6 dependent stage, second a PA6 + IL-7 dependent stage and third an IL-7 dependent stage. In order to test the possibility that this differentiation of growth signal requirement is controlled by the expression of functional immunoglobulin molecules, we have investigated the frequencies of PA6 + IL-7 dependent and IL-7 dependent cells which are present in the bone marrow of either μ-chain or κ-chain gene transgenic mice. In a μ-chain gene transgenic mouse, the frequency of PA6 + IL-7 dependent cells is selectively reduced, while that of IL-7 dependent cells is selectively reduced in a κ-chain gene transgenic mouse. This result suggests that expression of a functional μ-chain gene drives PA6 + IL-7 dependent cells to differentiate into the subsequent IL-7 dependent stage. Likewise, when μ-chain positive IL-7 dependent cells express a functional light-chain gene, their growth signal requirement changes into an IL-7 unreactive stage.

Key words: B cell development/Ig gene rearrangement/interleukin-7/stromal cell/transgenic mouse

Introduction

During the postnatal life of the mouse, pluripotent hemopoietic stem cells continue to give rise to early B lineage progenies which undergo rearrangement of variable(V)-gene segments of immunoglobulin (Ig) heavy(H)-chain gene and subsequent light(L)-chain gene, eventually differentiating into mature B cells which express complete IgM molecules on their surface (for review see Kincade, 1987). Although the site of V-gene recombination is precisely determined by a set of signal sequences adjacent to each V-gene segment (Early *et al.*, 1980; Sakano *et al.*, 1980), this process always creates both deletion and insertion of nucleotides (Sakano *et al.*, 1979, 1980; Early *et al.*, 1980; Alt and Baltimore, 1982). Such an error prone feature of V-gene recombination has obvious positive aspects such as (i) the generation of enormous diversity at the recombination site and (ii) the avoidance of the expression of two functional antibodies on a single B cell (allelic exclusion: Coleclough *et al.*, 1981). However, it also has a negative aspect in generating non-functional B cells which have failed to form productive V-genes. Previous studies on the Ig gene configuration of pre-B cell leukemia and that of pre-B cells in long-term bone marrow culture suggest that the frequency of non-functional B cells would be higher than functional ones (Korsmeyer *et al.*, 1981; Alt and Baltimore, 1982; Whitlock *et al.*, 1983). In contrast, in the peripheral lymphoid organs, almost all B lineage cells are functional ones bearing complete Ig molecules on their surface. Thus, B cell differentiation is a unique system where both functional and non-functional cells are generated but only functional ones are selected and allowed to leave the bone marrow into the periphery.

How is this selection of functional B cells achieved during intramarrow B cell development? To answer this question, we need to understand how the proliferation of B cell precursors is regulated in the bone marrow. Recently, we have demonstrated that stromal cell signals required for cell proliferation during the entire process of B cell differentiation can be divided into IL-7 (Namen *et al.*, 1988) and other molecules, all of which are expressed in an IL-7 defective stromal cell clone PA6 (Sudo *et al.*, 1989). Using recombinant IL-7 and PA6 stromal cell clone separately, we have investigated the growth signal requirements of intramarrow B cell precursors and found that B cell differentiation proceeds through the following three steps with different growth signal requirements. The cells at the earliest stage require PA6 but not IL-7 for proliferation. When commitment into B lineage is determined and Ig-gene rearrangement starts, proliferation of the cells requires both IL-7 and PA6. Along with the extensive proliferation at this stage, some but not all cells acquire the ability to proliferate in response to IL-7 alone. Finally, upon maturation into surface IgM-bearing B cells, they eventually lose the reactivity to any of the stromal cell signals (Nishikawa *et al.*, 1988; Suda *et al.*, 1989; Sudo *et al.*, 1989; Hayashi *et al.*, 1990). Interestingly, we demonstrated that differentiation from the second stage into the third stage is completely arrested in the mouse with severe combined immune deficiency (*scid*: Hayashi *et al.*, 1990). This suggests strongly that expression of a functional H-chain gene is a prerequisite for inducing the change of growth signal requirement, because recent studies unequivocally indicated that no other processes apart from antigen receptor gene rearrangement are directly affected by the *scid* mutation (Schuller *et al.*, 1986; Malynn *et al.*, 1988; Hendrickson *et al.*, 1988; Lieber *et al.*, 1988; Okazaki *et al.*, 1988). Based on these observations, we previously proposed a model of B cell differentiation whereby cell proliferation is controlled by stromal cell molecules, while differentiation is regulated by intracellular events triggered by the expression of Ig molecules (Hayashi *et al.*, 1990).

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The present study attempts to test this model by using transgenic mice harboring functional μ-chain or κ-chain genes. If expression of a functional Ig gene is the signal inducing the change in growth signal requirement of B cell precursors, we would expect that in such transgenic mice, all B lineage cells are driven to the next stage by the functional Ig molecules derived from transgenes, which will eventually cause a loss of B cell precursors with particular growth signal requirements. Our present results clearly demonstrate that this is indeed the case.

Results

Experimental design based on our model of B cell differentiation

Figure 1 depicts our model of intramarrow B cell differentiation. In this model, the definition of the differentiation stage is made solely on the basis of its growth signal requirement, irrespective of its Ig-gene configuration or expression of surface antigens. Thus, in order to avoid confusion with previous staging using other parameters, we used our own designation. Growth signals used for defining these stages were recombinant IL-7 (Namen *et al.*, 1988; Sudo *et al.*, 1989) and a stromal cell clone PA6 which does not express a detectable amount of IL-7 (Kodama *et al.*, 1982; Nishikawa *et al.*, 1988; Sudo *et al.*, 1989). B-pro-I is the earliest stage in which the cell requires only PA6 for proliferation. B-pro-II is defined as a stage where cell proliferation requires both PA6 and IL-7, and CFU-IL7 is the stage where cell proliferation requires IL-7 alone. In this model, it is further assumed that although cell proliferation is supported by external growth signals derived from stromal cells, cell differentiation is directed by the expression of functional Ig molecule. B-pro-II is a specific stage for the cells which are on the way to complete H-chain gene rearrangement, and only those which succeed in expressing a functional μ-chain gene can enter the next, CFU-IL7, stage.

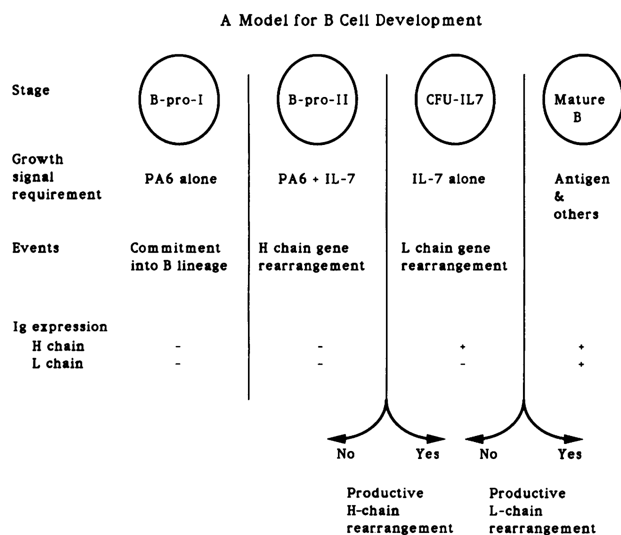

Fig. 1. B cell differentiation supported by IL-7 and other stromal cell molecules and directed by the expression of Ig molecules: a model. Staging of the differentiation was made on the basis of the growth requirements of B lineage cells, according to the results in our previous paper (Hayashi *et al.*, 1990). Events correlated with each stage are our hypothesis which will be tested in this article using Ig-gene transgenic mice.

Likewise, the CFU-IL7 stage is the stage for L-chain rearrangement, and those cells which undergo a productive L-chain rearrangement, consequently sμ^+ cells, will become IL-7 unreactive (Lee *et al.*, 1989; Suda *et al.*, 1989). This model, therefore, predicts that in μ-chain transgenic mice, where almost all B cell precursors can express a functional μ-chain gene, the cells at B-pro-II stage are driven to enter CFU-IL7 stage, which eventually results in the selective decrease of the cells in this stage. Likewise, in κ-chain transgenic mice, selective loss of the cells in CFU-IL7 stage is expected, because expression of a functional κ-chain transgene drives the cell from this stage into mature B cells which have no IL-7 reactivity. In this study, these predictions were tested by assessing the frequencies of the cells at B-pro-II and CFU-IL7 stages in the bone marrow of transgenic mice harboring either μ-chain gene or κ-chain gene. Although the culture conditions used for assaying B-pro-II also allow CFU-IL7 to proliferate, our limiting dilution assay is nearly specific to B-pro-II, because the proliferative ability of CFU-IL7 is less extensive than B-pro-II (Nishikawa *et al.*, 1989) and CFU-IL7 does not usually continue to proliferate for more than 7 days (Lee *et al.*, 1989; Suda *et al.*, 1989; Sherwood and Weissman, 1990; our unpublished observation). In the present assay, the frequency analysis of B-pro-II was carried out 12 days after the initiation of assay culture.

H-chain transgenic mice

pSV-Vμ1 containing a functionally rearranged V_H genomic gene from a hybridoma, B1-8.δ (Neuberger and Rajewsky, 1981), and Cμ exons from BALB/c mice were provided by Dr M.Neuberger (Neuberger, 1983) and introduced into C57BL/6 mice. A transgenic line which carries $\sim 10-15$ copies of this construct was selected and used for this study. In this particular line, the number of mature B cells in the spleen was maintained at $\sim 70\%$ of the normal level, and 90% of sμ^+ cells expressed the allotype from the transgene (data not shown). Thus, in this line, the efflux of mature B cells from the bone marrow into periphery is maintained at $\sim 70\%$ of the normal level and a majority of B cells express the transgene as expected. Next, bone marrow cells were harvested from this line, and subjected to frequency analysis for B-pro-II which was measured by limiting dilution assay in culture containing the PA6 cell layer and 20 U/ml recombinant IL-7, and CFU-IL7 which was measured by colony forming cell assay in medium containing methylcellulose and IL-7 (Table I). Colony forming cells reactive to recombinant IL-3 were also measured as a control. Figure 2 summarizes the relative pool sizes of B-pro-II, CFU-IL7 and sμ^+ cells in bone marrow and spleen of this line in

Table I. Frequencies of B-pro-II and CFU-IL7 in the μ-chain gene transgenic mouse

Mice	B-pro-II	CFU-IL7	CFU-IL3
Littermate	1/242 ± 62	1/238 ± 26	1/193 ± 22
μ-chain transgenic	1/2689 ± 285	1/289 ± 65	1/179 ± 21

The frequency of B-pro-II was carried out in 96-well cluster dishes containing 200, 400, 600 or 800 bone marrow cells per well. Ninety-six wells were used for each cell dilution. Colony assay for the cells reactive to IL-7 or IL-3 was carried out in medium containing methylcellulose and either recombinant IL-7 or IL-3. Each figure represents the mean and standard deviation of triplicate cultures.

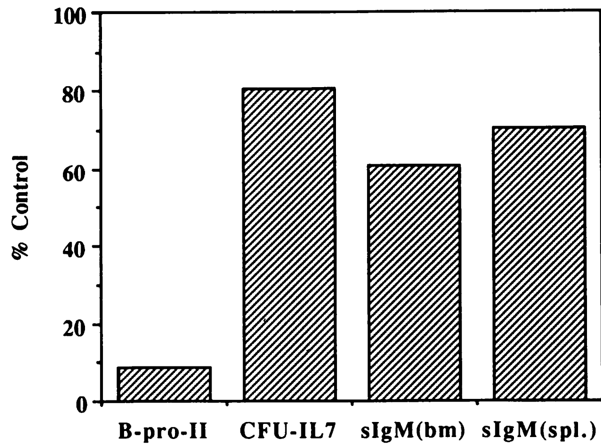

Fig. 2. Relative pool sizes of B-pro-II, CFU-IL7, sμ^+ in the bone marrow and spleen of μ-chain gene transgenic mice. Frequencies of B-pro-II and CFU-IL7 in the bone marrow of either μ-chain gene transgenic mice or transgene negative littermates were measured by the limiting dilution assay in a 96-well cluster dish containing the PA6 monolayer and recombinant IL-7 (20 U/ml) and colony assay in media containing methylcellulose and IL-7 respectively. Frequencies of surface μ^+ cells in the bone marrow and spleen were determined by flow fluorocytometry. The frequency of cells at each stage in the μ-chain gene transgenic mouse was divided by that of the littermates and expressed as a percentage of normal.

comparison with those in a littermate. As predicted, B-pro-II was selectively lost, while in later stages, both CFU-IL7 and sμ^+ cells were kept at a constant level (70–80% of normal). Interestingly, however, the frequency of B-pro-II never became zero in repeated experiments (data not shown). In order to see whether these proliferating cells are B precursors which have lost transgene expression, the cells in 11 independent positive wells were separately stained by anti-allotype antibodies. In contrast to our expectation, all the growth positive wells contained a significant number of cells which expressed IgM from the transgene. On the other hand, only few cells, if any, which express IgM from endogenous genes were detected in the same wells (Figure 3). This result indicates that a small fraction of B cell precursors, even after the expression of a functional μ-chain gene, can continue to proliferate under these assay conditions.

L-chain transgenic mice

We used a C57BL/6 transgenic mouse carrying a functional genomic κ-gene from a hybridoma, B13H4C8, which is generated from spleen cells of NZB mice and secretes a monoclonal antibody against mouse erythrocytes (Ozaki *et al.*, 1984). Due to lack of an appropriate marker to identify the expression of the transgene, we used an RNase protection

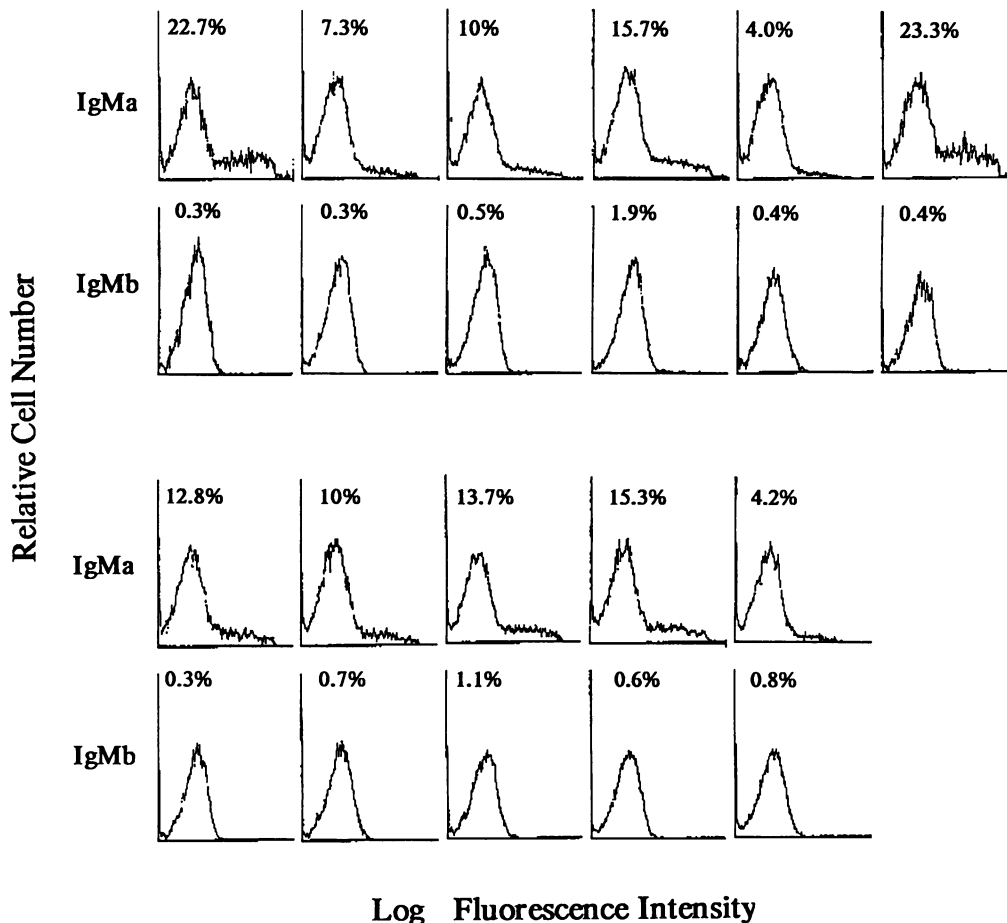

Fig. 3. Surface allotype of the cells proliferating in the limiting dilution wells. For measuring the frequency of B-pro-II, a limiting dilution assay in 96-well cluster dishes containing the PA6 monolayer and IL-7 was used. Although the frequency was low, B lineage cells did generate in several wells of the assay. Eleven wells which contained >10 000 B220$^+$ cells were selected, and stained with either anti-IgM^b or anti-IgM^a monoclonal antibodies. The result was from two separate assays.

assay to detect transgene expression. RNA from spleen cells of the transgenic mouse as well as from control hybridoma cells could protect the probe, generating a 247 bp protected band, while no band was detected using RNA from a littermate spleen (Okamoto, Murakami, and Honjo, unpublished observation). In this transgenic line, however, the number of sμ^+ cells was reduced to half the normal level. Thus, in this mouse strain, the efflux of mature B cells from bone marrow into the periphery is maintained at half the normal level. Next, we measured the frequencies of B-pro-II and CFU-IL7 in the bone marrow of the κ-chain gene transgenic mouse (Table II). The relative pool sizes of B-pro-II, CFU-IL7 and sμ^+ cells in the bone marrow and spleen of this transgenic line are presented in Figure 4. As we expected, the frequency of CFU-IL7 was reduced dramatically to 1/20 of the normal level. In contrast, the frequency of B-pro-II was approximately half of the normal level, which corresponds to the magnitude of reduction in mature B cells in bone marrow and spleen.

Table II. Frequencies of B-pro-II and CFU-IL7 in κ-chain gene transgenic mice

Mice	B-pro-II	CFU-IL7	CFU-IL3
Littermate	1/797 ± 157	1/1104 ± 232	1/353 ± 58
κ-chain transgenic	1/1812 ± 214	1/23 333 ± 15 275	1/381 ± 59

See footnote to Table I.

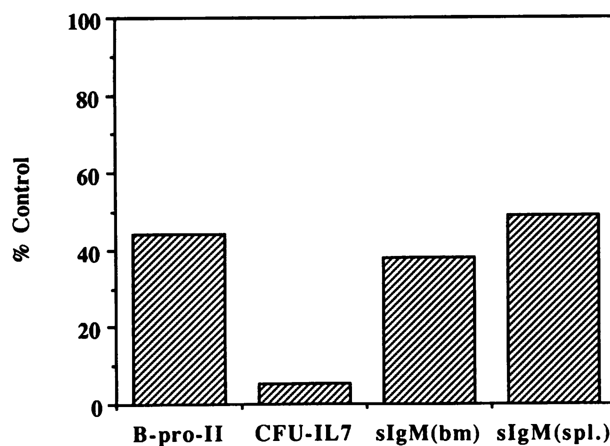

Fig. 4. Relative pool sizes of B-pro-II, CFU-IL7 and sμ^+ cells in the bone marrow and spleen of the κ-chain gene transgenic mouse. The experimental protocol and data presentation are the same as in Figure 2.

Again contrasting with our prediction, the number of CFU-IL7 stage cells in the κ-chain gene transgenic mice was not zero in repeated CFU-IL7 assays. In order to investigate whether these colony forming cells have lost the transgene, we picked up and pooled the colonies from either transgenic mice or normal mice and stained them with anti-κ antibody (Figure 5). Consistent with our previous report (Suda *et al.*, 1989), CFU-IL7 from control mice contained ~30% of sκ^+ cells. In contrast, almost all CFU-IL7 from the κ-transgenic mice were sκ^+, suggesting that these cells proliferated despite the expression of a complete IgM molecule. Moreover, the fact that all the CFU-IL7 in the κ-transgenic mice are sκ^+ supports our notion that CFU-IL7 is the cell which has undergone productive H-chain rearrangement.

Discussion

B cell differentiation directed by the expression of Ig molecules

In our previous studies, using recombinant IL-7 and an IL-7 defective stromal cell clone PA6 as separate growth signals, we investigated the growth signal requirement of intramarrow B cell precursors, and demonstrated that B cell differentiation from hemopoietic stem cells must pass two subsequent stages, B-pro-II whose proliferation requires both IL-7 and PA6 and CFU-IL7 which proliferate in response to IL-7 alone (Suda *et al.*, 1989; Sudo *et al.*, 1989; Hayashi *et al.*, 1990). The question then addressed is how this differentiation of growth signal requirement is regulated. Our

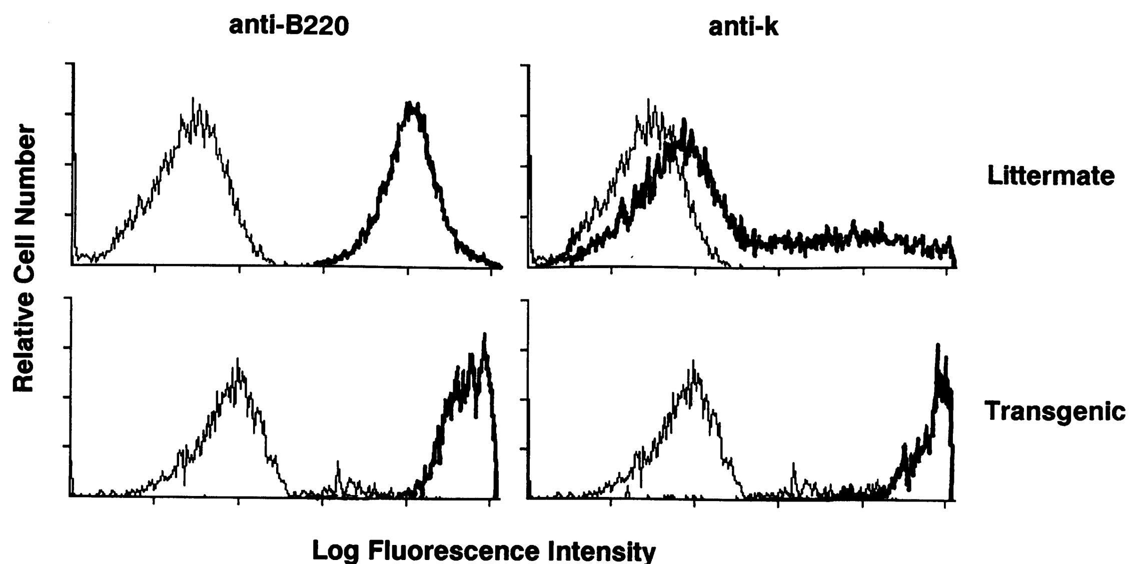

Fig. 5. Surface staining of cells in the colonies generated in response to IL-7. All large colonies generated from the bone marrow cells from κ-chain gene transgenic mice containing >500 cells were picked up and pooled. Thirty colonies from the CFU-IL7 assay of the littermates were also picked up, pooled and used as a control. The harvested cells were washed and stained with either anti-κ monoclonal antibody or anti-B220 antibody followed by FITC conjugated anti-rat-κ antibody (thick line). The thin line indicates control staining by FITC-conjugated anti-rat-κ antibody alone.

present results together with previous observations suggest strongly that it is neither regulated by external signals derived from the microenvironment, nor by a cell-autonomous program encoded in the genome. Instead, functional molecules expressed by B lineage cells, namely various forms of Ig molecules, may be the primary signal which drives the cells to change their growth signal requirement. First, we have demonstrated that differentiation from B-pro-II into CFU-IL7 is arrested in the *scid* mouse, indicating that expression of a functional μ-chain gene is a prerequisite for this differentiation step (Nishikawa *et al.*, 1989; Hayashi *et al.*, 1990). The recent study of Fried and Bosma confirmed this notion by showing that the introduction of a functional μ-chain gene into the *scid* mouse results in the appearance of pre-B cells which can proliferate in response to IL-7 alone (M.Fried and M.Bosma, personal communication). These experiments suggest that the CFU-IL7 stage following that of B-pro-II is reached only after productive H-chain gene rearrangement. In the present study, we assessed the effect of a μ-chain transgene on the frequency of B-pro-II itself. We found that in the μ-chain gene transgenic mouse where almost all B lineage cells express the μ-chain transgene, the frequency of B-pro-II is dramatically decreased. This is consistent with a logical prediction from our hypothesis, namely that B-pro-II, upon expression of a functional μ-chain gene, is driven to enter the CFU-IL7 stage. Likewise, the decrease of CFU-IL7 in the κ-chain gene transgenic mouse indicates that expression of a functional κ-chain gene is the signal to drive CFU-IL7 into mature B cells in which IL-7 reactivity is lost (Lee *et al.*, 1989; Suda *et al.*, 1989).

In earlier studies, it was shown that the size of the B lineage pool was reduced in μ-chain transgenic mice to varying extents (Herzenberg *et al.*, 1987; Müller *et al.*, 1989; also reviewed in Storb, 1989). Our present study provides an interpretation of this puzzling phenomenon. No time would be required for H-chain gene rearrangement in μ-chain gene transgenic mice, therefore, by definition, B lineage cells could proceed immediately from B-pro-II to CFU-IL7. As a consequence, the size of the intramarrow B cell pool, which may depend strongly on proliferation at the B-pro-II stage, may also be reduced. The same explanation might also be applicable to the observation of Nussenzweig *et al.* (1988b) that the incidence of lymphoma in c-*myc* transgenic mice decreased in a double transgenic mouse carrying both c-*myc* and human μ-chain transgenes, because introduction of a μ-chain transgene would cause a stage-specific reduction of cell proliferation, while this particular proliferation might contribute to the efficiency of tumorigenesis induced by the c-*myc* transgene.

Taken together, we believe that our present and previous studies along with studies of other groups allow us to conclude that, although cell proliferation during B cell development is controlled by stromal cell molecules including IL-7, the growth signal requirements of B cell precursors are determined by the form of Ig molecule expressed at the various stages of differentiation.

Exception to the rule and future direction

Although our present results fit in general with our model described above, they also demonstrate that there exist B precursors which behave in a conflicting manner to this model, namely actively proliferating B-pro-II in the μ-chain gene transgenic mouse, and CFU-IL7 in the κ-chain gene transgenic mouse. Because most of B-pro-II from the μ-chain transgenic mouse and CFU-IL7 from the κ-chain gene transgenic mouse were demonstrated to express transgenes rather than endogenous Ig genes, these cells must be exceptions to the rule rather than cells which have failed to express the transgenes. Moreover, the rate of appearance of these exceptional cells, which should be zero if our model is correct, appeared to be too high to be neglected. The same seems to be the case also for the allelic exclusion rule, because it has been shown that a considerable number of B cells in the transgenic mouse start rearrangement of endogenous Ig genes and sometimes co-express them (Weaver *et al.*, 1985; Nussenzweig *et al.*, 1988a; Stall *et al.*, 1988; Rath *et al.*, 1989). Indeed, in the spleen of the H-chain transgenic mice used in this study, $\sim 10\%$ of all mature B cells express an endogenous μ-chain gene. These results suggest that the signal provided by the expression of a transgene is not always effective for inducing the subsequent differentiation processes, although the reason for this remains for future study. Nevertheless, it is now possible to obtain a sufficient number of such exceptional cells by culturing in the presence of appropriate growth signals. Such cell lines would be helpful to elucidate why the expression of a transgene was not effective in these particular cells.

Another observation inconsistent with our model is that the expression of the transgene also affects earlier stages of B lineage differentiation than the stage predicted by our model. Because of our inability to control the timing of transgene expression, the introduced transgenes might be expressed from the earliest phase of differentiation. However, assuming that our model is correct, we would have thought that premature expression of a transgene may have little, if any, effect on the growth of an earlier stage. In contrast, the frequency of B-pro-II was reduced to $<50\%$ of the normal level in the κ-chain gene transgenic mouse. Again, the reason for this remains to be elucidated.

What kind of molecules can convert the expression of Ig gene into the signal inducing B cell differentiation? For this, the B lineage cell should be equipped with molecules which can detect the expression of a functional Ig gene and transduce its signals. Recent studies indicate that there are in fact such candidate molecules expressed at the earliest phase of B lineage differentiation, namely μ-chain associative proteins. Among those which have already been identified, mb-1 was shown to be a part of signal transducer complex associated with μ-chain (Hombach *et al.*, 1988; Sakaguchi *et al.*, 1988; N.Sakaguchi, personal communication). On the other hand, lambda 5 and $V_{pre\text{-}B}$ which are supposed to bind the N-terminal half of the μ-chain could play a role as sensors or intracellular ligands which may be able to cross-link μ-chain (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987; Karasuyama *et al.*, 1990; Tsubata and Reth, 1990; reviewed in Melchers, 1989). Therefore, we speculate that the earliest B cells are the cells equipped with all necessary molecules which can utilize the expressed functional μ-chain molecules as a component of the transmembrane signal apparatus. Although this is a highly speculative picture, we believe that understanding the functional role of μ-associative molecules in early B cell development will provide a clear insight into the molecular mechanisms which support Ig directed B cell differentiation.

Materials and methods

Transgenic mice

Transgenic mice were produced as described previously (Yamamura *et al.*, 1984; Araki *et al.*, 1989). The μ-chain gene introduced was pSV-Vμ1 (Neuberger, 1983) which was digested with *Xho*I. The κ-chain gene introduced was a cloned *Bam*HI fragment of κ-gene from the hybridoma B13H4C8, thus without 3′ κ-enhancer. Transgenic mouse lines were selected by expression of the transgenes in the spleen. μ-chain transgenic mouse lines which carry 10–15 copies of the transgene, and κ-chain transgenic mice which carry 30–40 copies of transgenes were used in this study.

Frequency analysis of B cell precursors and cell staining

Frequency assays for B-pro-II, CFU-IL7 and CFU-IL3 were carried out as described in our recent papers (Suda *et al.*, 1989; Hayashi *et al.*, 1990). For indirect staining of the cells, we used culture supernatants of hybridomas, RA3-6B2 (anti-B220; Coffman, 1983), or 187.1 (anti-mouse-κ; Yelton *et al.*, 1981) followed by fluorescein isothiocyanate (FITC) labeled anti-rat-κ monoclonal antibody. FITC labeled monoclonal anti-IgM allotype antibodies, MB86 for IgM^b (Nishikawa *et al.*, 1986) and DS-1 for IgM^a (Stall *et al.*, 1988) were used for allotype discrimination, and FITC-labeled goat anti-mouse-IgM antibody was used for measuring the frequency of total sμ^+ cells.

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