

# TERMINAL MATURATION OF RESTING B CELLS BY PROLIFERATION-INDEPENDENT B CELL DIFFERENTIATION FACTORS

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The pathway of normal B cell activation is a network of complex regulatory events (1-3). Although the goal of a directed immune response is to generate antigen-specific antibodies, a large component of this response is actually polyclonal and nonspecific. Examples of this phenomenon are seen regularly with antigen-specific immunization, as there is a concomitant rise in antibody titers directed against noncrossreacting antigens (4). This nonspecificity appears to be mediated, in large part, by soluble factors that stimulate either growth or differentiation of local B cell clones (1-3, 5, 6). Current models of lymphokine regulation of B cell maturation suggest that resting,  $G_0$  B cells are preactivated by antigen *in vivo*, or anti-IgM antibodies *in vitro*, to become responsive to B cell-stimulatory factor (BSF-1, reference 1).<sup>1</sup> After exposure to growth factors, B cells proliferate and generate receptors for factors regulating terminal maturation, antibody production, and secretion (B cell differentiation factors [BCDF], 1-3). Recently, it has become evident that such a model is not sufficient to explain the heterogeneity of B cell growth (7) and differentiation (8) factors. Furthermore, we and others (8-10) have now described nonspecific proliferation-inducing factors (B cell proliferation factor [BCPF] and BSF-1) that act on resting,  $G_0$  B cells without preactivation, a finding that suggests that, even at this early stage, B cells have receptors for growth factors.

An analogous situation may hold true for differentiation factors. In fact, there has been some evidence for the dichotomy of proliferation and differentiation. Previous studies (11) using monoclonal leukemic B cell populations showed that these cells could undergo terminal maturation without evidence of cell division. Furthermore, induction of proliferation with anti- $\mu$  or anti- $\gamma$  antibodies successfully blocked PWM-induced B cell maturation (12), although it is unclear whether this was related to active proliferation or the presence of anti-Ig in culture. In the murine system, B cell maturation factors (13-14) that appear to act on resting splenic B cells have been reported. In normal B cell preparations, we have observed that actively proliferating B cells in culture do not undergo terminal maturation consistently; rather, there appears to be an inverse correlation between proliferation and differentiation. In this paper we extend these observations to show that active proliferation actually blocks B cell responses to B cell

<sup>1</sup> *Abbreviations used in this paper:* BCDF, B cell differentiation factor; BCGF, B cell growth factor; BCPF, B cell proliferation factor; BSF-1, B cell-stimulatory factor 1, BCAF-1; CM, culture medium; HU, hydroxyurea; SAC, *Staphylococcus aureus* Cowan I strain organisms; SPA, Staph protein A.

differentiation signals and that terminal maturation to plasma cells can occur, without any division, in resting B cells. These findings may help to explain much of the nonspecificity inherent in the immune response and may serve to change our conceptual model of normal B cell maturation.

### Materials and Methods

*Cell Separation and Culture Conditions.* PBMC from normal controls were obtained after Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation. T/B cell separation was performed as previously described using neuraminidase treated SRBC (15). All cultures were performed in RPMI 1640 (KC Biologic, Lenexa, KY), 1% penicillin/streptomycin (Gibco Laboratories, Grand Island, NY), 2 mM glutamine (Gibco Laboratories) and 10% FCS (Hazelton-Dutchland, Denver, PA), henceforth termed culture medium (CM). In some experiments, formalin-fixed *Staphylococcus aureus* Cowan I strain organisms (SAC) (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) were added to culture.

*Lymphokine Sources.* Human B cell differentiation factors MOW 10, MOP1L, and MTP7 were derived from cloned T cell hybridomas from three different fusions (16). These factors were devoid of any IL-2, IFN- $\gamma$ , B cell growth factor (BCGF), or BCPF activity (16). BCDF-RAC was obtained as supernatant from an IL-2-dependent T cell clone (9), 48–72 h after restimulation with IL-2. BCPF was obtained from the T cell hybrid MOW 1 (9, 16). Unless otherwise indicated, all supernatants were used from standard stocks at 10% vol/vol.

*Counterflow Elutriation.* We performed elutriation with a JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA), using a modification of the method of Wahl et al. (17). The chamber was equilibrated with PBS/1% BSA at a constant speed (2,000 rpm) at 18°C in a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA).  $1-3 \times 10^6$  non-T cells were slowly injected into the chamber with an initial flow rate of 6 ml/min. Eight 150-ml fractions were collected by stepwise increases in flow rate while maintaining a constant rotor speed. Fractions were pelleted, washed in PBS, resuspended, and sized and plotted by a Channelyzer (Coulter Electronics Inc., Hialeah, FL). A standard curve for cell size was generated with calibrated beads.

*Assay for B Cell Proliferation.* Pansorbin (0.1–0.001% vol/vol) or BCPF (10–0.01%) was added to  $10^6$  non-T cells in CM on day 0. At 24, 48, 72, 96, and 110 h, triplicate 100- $\mu$ l aliquots were removed from these cultures and transferred to microwell cultures with the addition of 2  $\mu$ Ci of [ $^3$ H]methyl thymidine (Schwarz/Mann Div., Orangeberg, NY; sp act, 1.9 mCi). 18 h after the addition of the isotope, the wells were harvested and counted, as previously described (16). Data for day 3, usually the highest response date, are depicted in the results section. Hydroxyurea (HU) (Sigma Chemical Co.) was added to some cultures at  $10^{-3}$ – $10^{-7}$  M, concentrations documented to suppress T and B cell mitogen responses.

*Assay for Antibody Secretion.* A reverse hemolytic plaque assay was performed on day 6, as previously described (16), using Staph protein A (SPA)-coated SRBC and goat anti-human Ig as developing antiserum (Cappel Laboratories, Cochranville, PA).

*Immunofluorescence Staining.* Isolated fractions were stained with OK3, T4, T8, and M1 from Ortho Diagnostic Systems Inc. (Raritan, NJ), VG 2 (framework anti-Ia monoclonal kindly provided by Dr. Shu Man Fu, Oklahoma Medical Research Foundation, Oklahoma City, OK), goat anti-human Ig (Cappel Laboratories), EL2 (mAb analogous to 4F2/B cell activation marker) (18), anti-Tac (kindly provided by Dr. Thomas Waldmann, National Cancer Institute, Bethesda, MD), and B1 (Coulter Electronics Inc.). Staining and analyses were performed as previously described (15).

### Results

*Proliferation Is a Negative Signal for B Cell Differentiation.* In contrast to some proposed models of B cell maturation, we have noted, empirically, that cultured

TABLE I  
Proliferation Negatively Impacts B Cell Differentiation

SAC (vol/vol)	[ <sup>3</sup> H]thymidine incorporation (cpm)*	PFC per well <sup>†</sup>				
		0	MOW10	MOPIL	MTP7	RAC
0	225	90	3,120	6,480	1,920	2,150
0.1	33,126	0	10	40	90	70
0.01	21,465	10	40	40	120	30
0.001	9,123	10	2,520	3,680	920	2,260
BCPF(%)						
10	7,125	80	110	20	180	150
1	4,122	80	140	690	210	600
0.1	578	60	4,230	3,240	1,560	1,910

\*10<sup>5</sup> B cells cultured in triplicate in the presence of SAC or BCPF for 72 h. [<sup>3</sup>H]methyl thymidine is added for the final 18 h of culture with results expressed as mean cpm of triplicate cultures. The data presented are representative of at least five experiments.

<sup>†</sup>10<sup>6</sup> B cells are cultured for 6 d in the presence of one of four different BCDF preparations ± SAC or BCPF. A reverse plaque assay for total Ig is performed on day 6. The data are representative of at least five experiments.

B cells undergoing active proliferation do not result in terminal maturation to Ig-secreting cells. To address this observation more directly, we sought to determine the effects of factors inducing proliferation on the B cell response to BCDF(s). Either high-dose SAC (0.1–0.01% vol/vol) or BCPF (10–1%) were added on day 0 to cultures of isolated B cells with one of four different BCDF preparations. As can be seen in Table I, in each case where active proliferation is noted, the response to BCDF(s) was blunted or absent, compared with cultures without any proliferation signal. This inhibition was reversed as the degree of proliferation decreased, with lower concentrations of SAC or BCPF. This inhibition of maturation was not due to a change in the kinetics of the BCDF response (Fig. 1), as no PFC response was demonstrable up to day 12 when BCPF was added on day 0. Furthermore, even when BCDF was added from 24–96 h after SAC or BCPF, no terminal differentiation was noted, suggesting that these proliferative signals were not delaying expression of receptors for BCDF. Supernatants of proliferating B cells (induced by SAC) did not, in themselves, induce inhibition of BCDF response, showing that proliferating B cells were not secreting any inhibitory factors (data not shown). In addition, the inhibition could not be reversed by replacing cultures with fresh medium nor by changes in cell concentrations, suggesting that nutrient depletion and crowding did not appear to play a role (data not shown).

*Differentiation Occurs in the Absence of Proliferation.* To further characterize the role, if any, of proliferation in normal B cell maturation, we added varying concentrations of HU (10<sup>-3</sup>–10<sup>-7</sup> M) to cultures of B cells and any one of four different BCDF preparations. These concentrations of HU were shown, in separate experiments, to block both T and B cell responses to mitogens and antigens (Table II and data not shown). As noted in Table II, HU appears to inhibit the PFC response to the conventional B cell maturation signal, T cells and PWM, as well as to BCDF(s), MTP7, and RAC without affecting cell recovery

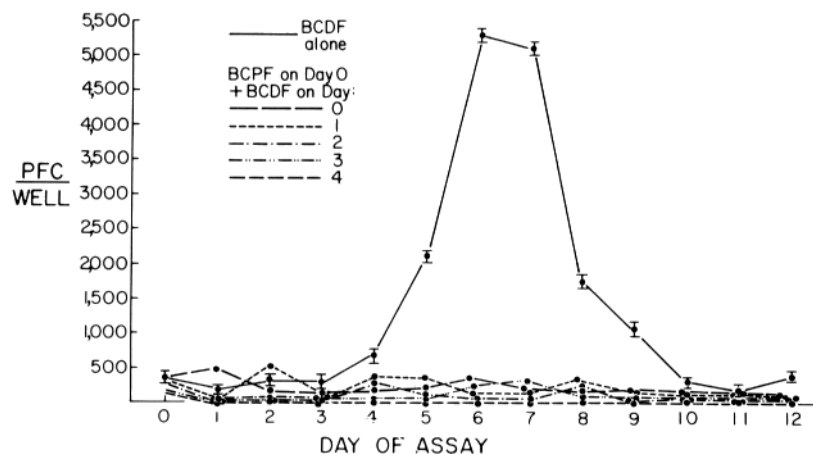


FIGURE 1. Kinetics of BCDF response after the addition of MOW 1/BCPF (10% vol/vol) to  $10^6$  B cells on day 0. MOPIL/BCDF (10% vol/vol) is added to these cultures on either day 0 or after 24, 48, 72, or 96 h. A reverse hemolytic plaque assay is performed every day up to day 12, as described in Materials and Methods. Response to MOPIL/BCDF alone is depicted by the solid line graph (—).

TABLE II  
*Proliferation-dependent and -independent BCDFs*

Hydroxyurea* (concentration)	PFC per well <sup>‡</sup>					T cells + PWM
	0	MOW10	MOPIL	MTP7	RAC	
0	20	5,470	2,210	1,630	1,010	7,130
$10^{-3}$ M§	0	0	10	0	0	0
$10^{-4}$ M	0	110	610	0	10	0
$10^{-5}$ M	20	6,120	2,700	90	0	10
$10^{-6}$ M	90	9,390	2,560	1,200	630	3,170
$10^{-7}$ M	50	9,920	3,160	1,410	1,110	5,160

\*Varying concentrations of HU ( $10^{-3}$ – $10^{-7}$  M) are added to cultured B cells  $\pm$  BCDF on day 0. Dose range was chosen for ability to inhibit both T (PHA) and B (SAC) cell mitogen responses; at  $10^{-3}$  M, 100% inhibition for T and B;  $10^{-4}$ , 90% T/93% B;  $10^{-5}$ , 91% T/95% B;  $10^{-6}$ , 82% T/91% B;  $10^{-7}$ , 61% T/53% B.

<sup>‡</sup>As per Table I.

<sup>§</sup>Viability and recovery of  $10^6$  B cells in culture on day 6  $\pm$  HU as follows: no HU, 71%;  $10^{-3}$  M, 23%;  $10^{-4}$  M, 48%;  $10^{-5}$  M, 76%;  $10^{-6}$  M, 73%;  $10^{-7}$  M, 70%.

and viability. In contrast, both MOPIL and MOW 10 induce PFC responses comparable to control cultures in the presence of  $10^{-5}$  and  $10^{-6}$  M HU. At higher concentrations ( $10^{-3}$  and  $10^{-4}$  M) both cell recovery and viability were reduced, accounting for the marked inhibition of all responses (Table II); however, at lower concentrations ( $10^{-5}$ – $10^{-7}$  M) cell recovery and viability were unaffected. Similar data were obtained using thymidine blockade (data not shown). These findings suggest that there may be two types of BCDF(s): A proliferation-dependent variety, analogous to the conventional T cell and PWM signal; and a proliferation-independent type that can induce terminal maturation without expansion of the B cell clone. Furthermore, this provides additional

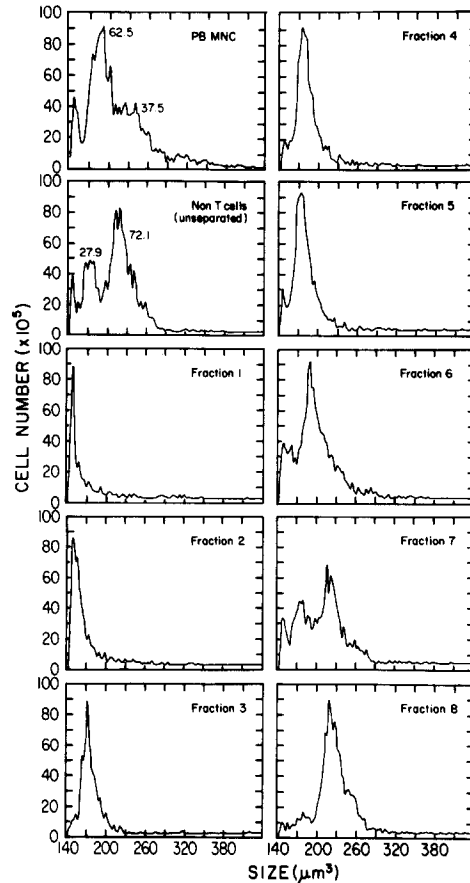


FIGURE 2. Fractions of non-T cells are collected by counterflow elutriation and analyzed for size by a Channelyzer (Coulter Electronics, see Materials and Methods). Unseparated mononuclear cells and isolated non-T cells are depicted for comparison. Cell size was extrapolated from standardized calibrated beads (expressed as  $\mu\text{m}^3$ ).

evidence for the heterogeneity of B cell differentiation factors, as previously proposed (8, 9).

*Proliferation-independent BCDFs Induce Terminal Maturation of Dense Resting B Cells.* An alternative explanation for the findings noted above is that both MOP1L and MOW 10 are acting on B cells that have either already undergone proliferation or have been activated *in vivo*. To isolate purer populations of resting and activated cells, B cells were separated by counterflow elutriation. In Fig. 2 profiles of unseparated non-T cells, as well as eight isolated fractions, are depicted by size and cell volume (range, 140–215  $\mu\text{m}^3$ ). The staining characteristics of each fraction are noted in Table III. The early fractions (1–3) are remarkable for the absence of any activation marker (4F2, Tac). The 4F2<sup>+</sup> cells increase thereafter to a maximum in fraction 8 (macrophages). Tac was present in fractions 5–7, with peak staining in fraction 6.

Each fraction was analyzed for response to three different B cell maturation signals, MOW 10 (our proliferation-independent BCDF), SAC and IL-2, a more

TABLE III  
Staining Characteristics of Elutriated Non-T Cells

Non-T fraction	Positive staining (%)				
	sIg	4F2	TAC	OKT3	OKM1
0*	24.6	49.6	1.4	1.3	53.2
1	3.2	<0.1	<0.1	<0.1	<0.1
2	4.2	<0.1	<0.1	<0.5	<0.1
3	14.6	<0.1	<0.1	0.8	<0.1
4	31.6	0.4	<0.1	<0.5	<0.1
5	27.6	3.2	0.5	<0.5	3.7
6	37.9	6.7	31.9	<0.5	3.1
7	19.6	21.9	3.7	<0.5	16.7
8	11.4	79.3	0.7	<0.5	62.1

Aliquots of each elutriated fraction were stained with specific mAbs as described in Materials and Methods. Data are representative of at least six elutriations.

\*Unseparated.

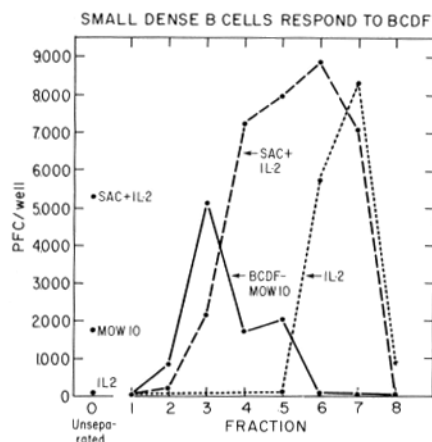


FIGURE 3. Response to Bcdf/MOW 10 (—), IL-2 (---), or IL-2 and SAC 0.001% (- - -) by elutriated fractions, measured on day 6 by a reverse hemolytic plaque assay. Fraction 0 is nonelutriated non-T cells showing baseline responses.

conventional pure B cell signal (19) and IL-2, recently shown to induce differentiation of Tac<sup>+</sup> B cells (20–23). Fig. 3 depicts a representative experiment. The response to MOW 10 appears to reside in the early B cell fractions, corresponding with the dense resting B cell. In contrast, IL-2 only affects terminal maturation of Tac<sup>+</sup>-activated B cells, confirming data reported by others (24). The combined signal of SAC and IL-2 is effective in virtually all fractions and is probably related to the ability of SAC to induce Tac positivity on B cells (25).

Thus it appears that MOW 10 is acting on dense resting 4F2<sup>-</sup> B cells, not in vivo-activated cells, and suggests that even resting B cells have a full complement of receptors for both growth and differentiation factors.

### Discussion

Some previous models of B cell maturation have suggested that B cell proliferation is an integral and necessary prerequisite for terminal maturation and antibody secretion. The data presented in this study directly conflict with such a schema, and extend previous observations of malignant B cell clones (11) and of the murine system (13–14). Using response to T cell hybridoma-derived BCDFs as an assay for terminal maturation, we have shown that active proliferation of B cells actually inhibits B cell differentiation, as measured by antibody secretion. These data were reproducible using two different B cell proliferation signals (high-dose SAC or BCPF) and four distinct BCDF preparations, so that this effect did not appear to be due to an unusual combination of B cell signals. The marked inhibition of B cell maturation was not due to a change in the kinetics of BCDF response as no PFC were detectable even when cultures were assayed up to day 12. Furthermore, addition of BCDF to cultures up to 96 h after stimulation with BCPF did not result in any PFC response, suggesting that proliferation signals were not acting to induce receptors for BCDF. Other considerations such as depletion of nutrients in the media, cell crowding, and factors secreted from proliferating B cells were found not to play a significant role in the phenomenon described (data not shown).

The finding that active proliferation inhibited response to BCDF led us to question whether such responses required any proliferation of B cells. Previous studies using leukemic B cells have suggested that terminal maturation can occur without proliferation (11), but these cells may have already been beyond the proliferative stage. In our system, responses to BCDFs MOW 10 and MOPIL appeared to remain intact in the presence of concentrations of HU or thymidine known to inhibit T and B cell responses to mitogen. In contrast, similar concentrations of HU or thymidine abrogated response to two other BCDF preparations, MTP7 and RAC, as well as the more conventional differentiation signal, T cells, and PWM. Thus it appears that there are proliferation-independent as well as -dependent BCDFs, providing further evidence for the heterogeneity of BCDFs, and suggesting that some BCDFs might act at earlier stages of B cell maturation. However, since these BCDFs may be acting on already *in vivo*-activated B cells that had undergone proliferation, it was necessary to isolate resting  $G_0$  B cells characterized by small cell volumes and the absence of surface activation markers 4F2 and Tac (26, 27); this was accomplished by counterflow elutriation. Addition of BCDF/MOW 10 to isolated resting cells did result in maturation to antibody-secreting cells. In fact, as might have been predicted from the earlier data, more activated B cells (with high spontaneous proliferation) did not respond to this BCDF. Rather, as has been shown by others (23), these activated Tac<sup>+</sup> B cells differentiated in the presence of IL-2. The combination signal of SAC (0.001%) and IL-2 induced differentiation at virtually all stages of B cell activation, a finding that can be explained by the ability of SAC to activate B cells to become Tac<sup>+</sup> (25). We and others (8–10) have previously shown that resting B cells can respond to B cell proliferation signals. Recently other groups (28–30) have provided evidence that BSF1 is a differentiation factor for resting cells as well. However, in this context, differentiation was defined as the ability of this factor to induce receptors for proliferation signals or increased expression

of class II molecules and not terminal maturation, as we report here. Thus, in conjunction with the data presented in this study, and previous studies using malignant B cells or murine splenocytes (11–14), one can state that the resting B cell has a full complement of receptors for both growth and differentiation factors. The rationale for such a finding is less clear. Although the major Ig response with antigen-specific stimulation is directed against the specific antigen, there is a great deal of nonspecificity inherent in the immune response. Part of this nonspecificity may be due to the action of polyclonal BCDFs on local B cell clones, specific and nonspecific. If antigen-specific signals are weak, nonspecific signals may serve to boost the response dramatically. In the presence of antigen locally, antigen-specific B cells are more abundant and predominate in the response. The capability of resting cells to respond to these factors allows for a more rapid and dramatic response. The fine tuning of a specific immune response might come only after several antigenic challenges.

### Summary

Using response to four different BCDF preparations as a model of B cell maturation, we have shown that induction of B cell proliferation abrogates terminal maturation of these cells. In fact, response to some BCDFs can occur in the presence of inhibitors of DNA replication, suggesting that there are proliferation-independent as well as proliferation-dependent BCDFs. These findings cannot be explained by changes in the kinetics of the BCDF response, nor can they be reversed by repletion of media or changing cell densities. Proliferation-independent BCDFs appear to exert their effects on dense, resting 4F2<sup>-</sup> B cells rather than more activated B cells. This is in contrast to B cell differentiation signals of IL-2 alone or SAC and IL-2 in concert. These data suggest that the current models of B cell activation and maturation may require some reorganization, relegating the proliferative phase of B cell maturation to a lesser role. In addition, evidence is provided for the fact that the resting B cell may have the full complement of receptors for BCDF as well as BCGF and BCPF and may help account for the inherent nonspecificity of the immune response.

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