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Inhibitory mechanism of the proliferative responses of resting B cells: feedback regulation by a lymphokine (suppressive B-cell factor) produced by Fc receptor-stimulated B cells

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SUMMARY

We investigated the effect of a lymphokine termed 'suppressive B-cell factor' (SBF), which is produced by FcRγ (Fc receptor for IgG)-stimulated B cells or hybridoma TS4.44, and is known to suppress B-cell responses *in vivo* and *in vitro* by inhibiting their proliferation. Small B cells, fractionated by Percoll density gradient, from athymic nude mice (BALB/c) secreted SBF after binding EA (sheep erythrocytes sensitized with IgG mouse anti-sheep erythrocyte antibody), and the proliferation of small but not large B cells was preferentially suppressed by SBF in response to LPS *in vitro*. Proliferation of purified B cells from BALB/c nu/nu mice, induced by a synergistic interaction between $F(ab')_2$ fragment of goat anti-mouse IgM antibody and B-cell stimulating factor (BSF_1), was almost completely abrogated by the co-existence of SBF during the 72-hr culture period. However, the co-culture with SBF for the last 24 or 48 hr, as well as of B cells pretreated with SBF for 1 hr at 37°, partially inhibited the growth response. These findings suggest that SBF operates on resting B cells and holds them in a resting state. This notion would be further supported by the fact that SBF inhibited G_0-G_1 transition. Taken together, we conclude that SBF acts on the early step of B-cell activation, thereby inhibiting B-cell growth. Arrest of resting B cells in the G_0 phase and failure of an increase in functional receptors for BSF_1 seem to be responsible for the suppression of B-cell responses.

INTRODUCTION

Various lymphokines stimulate B cells to proliferate and differentiate into antibody-forming cells in mice and humans (Swain, Wetzel & Dutton, 1985). It is demonstrated that at least two successive signals are required to activate murine resting B cells to proliferate (Howard *et al.*, 1982, 1983). The first is provided by the anti-μ antibody, possibly by a given antigen (Parker, 1982). The second is delivered by a lymphokine prepared from the culture supernatant of thymoma cells (EL-4) that are stimulated with phorbol myristate acetate (PMA). This factor, designated the B-cell growth factor (BCGF) (Swain *et al.*, 1983), and recently B-cell stimulating factor 1 (BSF_1), is physicochemically different from interleukin-2 (IL-2) (Farrar *et al.*, 1983) and interferon-γ (IFN-γ), which are also known to cause B-cell growth and differentiation (Zubler *et al.*, 1984; Nakagawa *et al.*, 1985).

The immune responses induced by antigen and various mediators undergo feedback regulation, which results in the suppression of the B-cell response. Antigen/mitogen-activated T cells act as suppressors by producing suppressive lymphokines (Tada & Okumura, 1979; Schimpl & Wecker, 1972). Besides the direct induction by antigen/mitogen stimulation, we showed that antibody molecules stimulate B cells via their Fc receptor (FcR) after forming immune complexes, thereby blocking B-cell response (Masuda *et al.*, 1978; Miyama, Yamada & Masuda, 1979; Miyama-Inaba *et al.*, 1982). The Fc receptor-dependent B-cell suppression observed is mediated in an antigen non-specific manner by a lymphokine produced by B cells themselves, the growth of which was blocked after binding immune complexes. This factor, the molecular size of which is approximately 43,000, was named the suppressive B-cell factor (SBF)

Abbreviations: BCGF, B-cell growth factor; BSF_1, B-cell stimulating factor 1; CSF, colony-stimulating factor; EA, sheep erythrocytes sensitized with IgG fraction of mouse anti-sheep erythrocyte antibody; EDTA, ethylenediamine tetraacetate; FcRγ, Fc receptor for IgG; FCS, fetal calf serum; HBSS, Hanks' balanced solution; [^{3}H]TdR, [methyl-^{3}H]thymidine; IBF, immunoglobulin binding factor; IFN-γ, interferon-γ; IL-2, interleukin-2; LPS, lipopolysaccharide; LT, lymphotoxin; PMA, phorbol 12-myristate 13-acetate; SBF, suppressive B-cell factor; sIg, surface immunoglobulin; SRBC, sheep red blood cell; TPA, 12-0-tetradecanoyl phorbol-13-acetate.

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because of its suppressive effect on only B cells. SBF is different from other known suppressive lymphokines, including lymphotoxin (LT) and immunoglobulin binding factors (IBF), in its biological and physiochemical properties (Miyama *et al.*, 1979; Suzuki *et al.*, 1983a, b, 1985). Our observation has, thus, suggested the existence of a feedback system that is mediated by SBF in consequence of a B–B interaction.

Therefore, in this study we investigated how SBF interferes with the activation and proliferation of B cells induced by the anti-μ antibody in synergy with BSF_1, the function of which is counteractive to SBF.

MATERIALS AND METHODS

Mice

Nu/nu and nu/+ BALB/c mice of both sexes were maintained in the Institute of Experimental Animals, Faculty of Medicine, Kyoto University, under specific pathogen-free conditions and were used at 8–10 weeks of age.

Culture medium

The culture media used throughout were Hanks' balanced salt solution (HBSS) and RPMI-1640 (Nissui Seiyaku Co. Ltd, Osaka, Japan). The latter was usually supplemented with 7·5% fetal calf serum (Flow Laboratories, North Ryde, NSW, Australia), 200 U/ml of penicillin G, 100 μg/ml of streptomycin and 5×10^{-5} M of 2-mercaptoethanol.

Antibodies

Monoclonal anti-Thy-1.2 antibody (Clone F7D5, Olac, Bicester Oxon, U.K.) was used for the depletion of T cells. The $F(ab')_2$ fraction of the affinity-purified IgG of goat anti-mouse μ-chain (Cappel, Cooper Biochem. Inc., Malvern, PA) was used at a concentration of 10 μg/ml together with BSF_1. The IgG fraction of rabbit anti-sheep red blood cell (SRBC) antisera was used for elaborating B cells bearing Fc receptors for IgG (FcRγ) after coupling with SRBC (EAγ).

Conventional SBF (cSBF)

Nu/nu spleen cells were fractionated by zonal gradient centrifugation into three populations using Percoll (Pharmacia, Uppsala, Sweden) according to a modified method described by DeFranco *et al.* (1982) after passage through a Sephadex G-10 column to deplete the adherent cells. Approximately 40–50% of loaded cells sedimented between 60% and 80% Percoll in our hand. Since the profile of red fluorescence of these cells, when stained with acridine orange and analysed on a flow cytofluorometer (see below), showed a sharper and more symmetrical peak than that of whole spleen B cells, these small cells were used for the present studies as resting B cells. Fifty million /ml of these cells were mixed with an equal volume of 10% SRBC coupled with a subagglutinating dilution of IgG antibody (EA), agitated gently at 37° for 15 min, then centrifuged at 100 *g* for 5 min, and settled at 4° for 30 min. Approximately 40% of the cells bound EA. FcRγ^+ cells were separated from EAγ non-rosette-forming cells by the Ficoll-Isopaque density gradient centrifugation. The pellet (FcRγ^+ cells) was re-rosetted and re-centrifuged by the same procedure. Twenty-four hour culture supernatants of 1×10^7/ml FcRγ^+ B cells in RPMI-1640 were designated as cSBF and used for experiments without further purification.

Monoclonal SBF produced by hybridoma cells (mSBF)

Details were as described previously (Suzuki *et al.*, 1983). In brief, BALB/c nu/nu FcRγ^+ B cells prepared as described above were fused with HAT-sensitive 3T3-4E cells using 50% polyethylene glycol 1000. The culture supernatants of the established SBF-producing hybridoma, named TS-4.44, were collected and concentrated 20-fold using an Amicon YM-10 membrane. As reported, the supernatant of TS-4.44 showed almost identical characteristics with conventional SBF in its molecular size (43,000 as determined by gel filtration and SDS-PAGE) and biological activities. This was used for experiments without further purification.

LPS response for SBF assay

Two-hundred thousand nu/nu spleen cells in 0·1 ml of culture medium mixed with 0·1 ml of either RPMI-1640 or serially two-fold diluted test samples in 96-well flat-bottomed microculture plates (Coaster, Cambridge, MA) in the presence or absence of 50 μg/ml lipopolysaccharide (LPS, *E. coli*, 026:B6, Difco, Detroit, MI). Cultures were performed in triplicate at 37° in a humidified atmosphere of air containing 5% CO_2. After 56 hr, cultures were pulsed with 0·5 μCi [methyl-^{3}H]thymidine ([^{3}H]TdR, 20 Ci/mM, Amersham International, Amersham, Bucks, U.K.) and harvested 16 hr later to count the [^{3}H]TdR incorporation on a Packard liquid scintillation counter. Suppressive effect of SBFs on the LPS responses of B cells was expressed as percentage suppression as follows:

$$\% \text{ suppression} = \left(1 - \frac{\text{c.p.m. LPS response with SBF} - \text{c.p.m. background with SBF}}{\text{c.p.m. LPS response without SBF} - \text{c.p.m. background without SBF}}\right) \times 100.$$

SBF activity was expressed as described elsewhere (Gillis, Ferm & Smith, 1978): plots of percentage suppression versus two-fold dilution were constructed by least-squares fitting, from which the dilution of SBF samples yielding 50% of maximal percentage suppression was derived, and units (U) per millilitre SBF in the experimental samples were calculated by:

$$\text{U/ml SBF} = \frac{\text{dilution of experimental sample for 50\% of maximal \% suppression}}{\text{dilution of standard SBF for 50\% of maximal \% suppression}}.$$

All the procedures were calculated automatically using a computer program. Standard SBF obtained from nu/nu BALB/c mouse as described above was assigned a value of 1 U/ml.

Partially purified BSF_1

BSF_1 was purified from the culture supernatant of EL-4 thymoma cells stimulated with 12-0-tetradecanoyl phorbol 13-acetate (TPA) in RPMI-1640 medium containing 1% FCS (Farrar *et al.*, 1983). In brief, the 24-hr culture supernatant was concentrated on an Amicon YM-5 membrane and fractionated on a Sephacryl S-200 column. S-200 filtrated fractions containing BSF_1 activity were applied to a HPLC MonoQ column, and developed with a NaCl linear gradient. The active eluates were further applied to a phenyl sepharose (CL-4B) hydrophobic column equilibrated with 0·02 M PBS (pH 7·2). The column was washed with the starting PBS and then developed with a

gradient of 1:1 starting buffer to 60% ethandiol in 0·02 M PBS. The BSF_1 fractions thus obtained contained neither IL-2 nor CSF activity as shown previously (Hilfiker, Moore & Farrar, 1981). Fifty percent of the maximum proliferative activity was designated as 1 U. BSF_1 activity was expressed as described above using c.p.m. instead of percentage suppression.

Assays for B-cell proliferation

Spleen cells from either nu/+ or nu/nu BALB/c mice were treated with an optimum dose of anti-Thy-1.2 monoclonal antibody at 4° for 30 min and then guinea-pig complement at 37° for 45 min. Cells depleted of T cells were passed through a Sephadex G-10 column. B cells thus prepared did not respond to Con. A. Cells were cultured in quadruplicate at a concentration of 1×10^5/well in 96-well flat-bottomed microculture plates. For anti-μ co-stimulated B-cell proliferation, 10 μg/ml of the $F(ab')_2$ fraction of affinity-purified IgG of goat anti-mouse μ-chain were added to B cells. As 5 U/ml of BSF_1 and 10 μg/ml of anti-μ antibody usually induced the maximal synergistic responses of B cells, these amounts of reagents were used unless otherwise stated. In order to investigate the effect of SBF on the proliferative responses of B cells, either cSBF or mSBF was added to the wells at a concentration of 4 U/ml. These critical amounts were determined by preliminary tests as optimum for the suppression of B-cell proliferation. The total volume of the culture was adjusted to 0·2 ml per well. Cultures were performed as described above. After 56 hr of culture, samples were pulsed with 0·5 μCi of [^{3}H]TdR and harvested 16 hr later. [^{3}H]TdR incorporation was counted on a Packard liquid scintillation counter. The suppressive effect of SBFs on B-cell proliferation in this assay was expressed as percentage suppression, according to the same formula as used for the suppressive effect on the LPS response.

Pretreatment of B cells by SBF

B cells (1×10^7) were suspended in 4 U of cSBF in 1 ml, incubated at 37° for the indicated periods, washed three times with HBSS, and then re-suspended in the culture medium. These B cells were applied to the proliferation assay as described above.

Absorption of BSF_1

B cells (1×10^6/ml) were preactivated by 10 μg/ml of anti-μ with or without SBF for 16 hr. These B cells were resuspended in 50 U of BSF_1 to a cell density of 3×10^7/ml after washing three times with HBSS, and incubated at 37° for 45 min followed by further incubation at 4° for 1 hr. Then, supernatants were collected and [^{3}H]TdR incorporation was determined from a two-fold dilution series of these samples to estimate the residual BSF_1 activity as described above.

Cell-cycle determination

The cell-cycle distribution of individual B cells was measured by flow cytometry after differential staining of cellular DNA and RNA with the metachromatic dye acridine orange (Darzykiewicz *et al.*, 1976). B cells were stimulated with 10 μg of anti-μ and/or 5 U/ml of BSF_1 in the presence or absence of 4 U/ml of SBFs for 16 hr. Approximately 5×10^5 cells thus cultured were suspended in 0·2 ml of PBS and mixed with 0·5 ml of the solution containing 0·1% (v/v) of Triton X-100 (Sigma Chemical Co., St Louis, MO), 0·2 M sucrose, 10^{-4} M EDTA, and 2×10^{-2} M citrated phosphate buffer at pH 3·0. The cells were stained 1 min later by the addition of 1 ml of the solution containing 0·002% acridine orange (Polysciences Inc., Warrington, PA), 0·1 M NaCl, and 10^{-2} M citrated phosphate buffer at pH 3·8. After 5 min of equilibration at room temperature, the fluorescence intensity of individual cells was measured on a flow cytofluorometer (Ortho Spectrum III, Ortho Diagnostic Systems Inc., Westwood, MA).

RESULTS

First, we ascertained that approximately 40–50% of the small B cells separated from nu/nu spleens byPercoll gradient centrifugation formed EA-rosettes and produced SBF by 24 hr culture (data not shown). This is consistent with our previous finding obtained using Thy-1.2-negative spleen cells passed through a Sephadex G-10 column (Miyama *et al.*, 1979). Moreover, the LPS response of small B cells was more markedly inhibited by co-culture with SBF than that of large B cells (Table 1). These observations suggest that there is a regulatory circuit mediated by SBF in the small B cells, probably in the G_0 phase. Using these small B cells, we next confirmed that the synergy between anti-μ antibody and BSF_1 was dose dependent, and that 10 μg/ml of anti-μ and 5 U of BSF_1 for 1×10^5 purified B cells showed the maximal synergism (data not shown).

Figure 1 demonstrated that the background responses to the critical amount of each of these reagents alone were significantly low, and that marked proliferation of B cells occurred in synergy with these two stimulants. Addition of cSBF at a concentration of 4 U/ml into the cultures, as expected, resulted in a remarkable decrease in the proliferation response. The suppression caused by cSBF was also dose-dependent (data not shown), and this amount of cSBF was generally used for the subsequent experiments.

For successful proliferation, B cells in a resting state are unresponsive to BSF_1, and co-stimulation with anti-μ is required as also shown in Fig. 1. This raises a question which is susceptible for SBF, resting, anti-μ-triggered, or both cells. As shown in Fig. 2, the suppressive effect of cSBF on B-cell proliferation caused by a synergistic action between anti-μ and BSF_1 decreased in a time-dependent manner: approximately

Table 1. Suppressive effect of cSBF on proliferative response of small and large B cells to LPS

B cells*	LPS	cSBF	c.p.m. (mean ± SD)	% suppression
Large	−	−	1344 ± 708	
	+	−	59,361 ± 4154	
	−	+	1596 ± 306	
	+	+	33,212 ± 1944	45·5
Small	−	−	944 ± 238	
	+	−	45,016 ± 945	
	−	+	889 ± 142	
	+	+	11,288 ± 1365	76·4

B cells were layered onto Percoll step gradients composed of 50%, 60%, 70% and 80% Percoll layers. Cells forming a band between 50% and 60%, and bands between 60% and 80%, were designated here as large and small B cells, respectively.

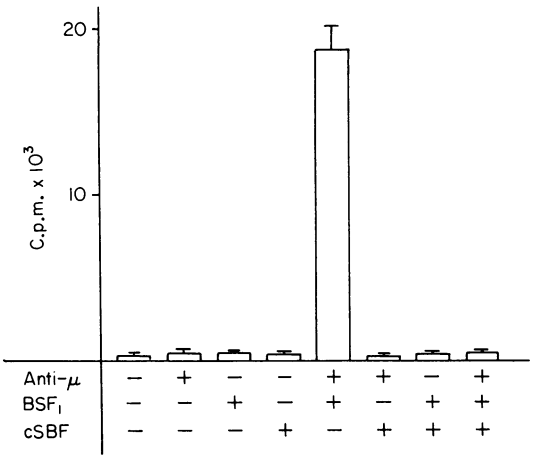

Figure 1. The suppressive effect of cSBF on B-cell proliferation by anti-μ and BSF_1. BALB/c spleen B cells, prepared as described in the Materials and Methods, were cultured in quadruplicate at a concentration of 10^5 cells/0·2 ml/well in the presence (+) or absence (−) of 5 U/ml BSF_1, 10 μg/ml anti-μ antibody, and/or 4 U/ml cSBF, for 3 days. Each well was pulsed with 0·5 μCi of [^{3}H]TdR for 16 hr before the termination of culture. The results are shown as mean ± SD (c.p.m.). Marked synergism was observed between anti-μ and BSF_1 (18,788 ± 1496), in contrast to weak proliferative response to anti-μ (394 ± 64) or BSF_1 (508 ± 82) alone. Background was 371 ± 105. The addition of cSBF into the culture led to a severe decrease in the synergistic proliferative response (670 ± 99). Percentage suppression was 99·6.

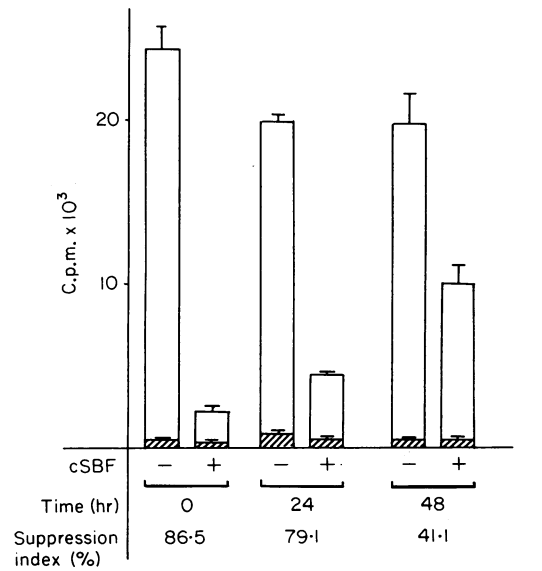

Figure 2. The suppressive effect of cSBF on B-cell proliferation by anti-μ and BSF_1 with time. BALB/c splenic B cells, prepared as described in the Materials and Methods, were cultured in quadruplicate at a concentration of 10^5 cells/0·2 ml/well in the presence of 5 U/ml BSF_1 and 10 μg/ml anti-μ. Medium or cSBF (4 U/ml) was added to wells 0, 24 and 48 hr after initiation of the culture. Each well was pulsed with 0·5 μCi of [^{3}H]TdR for 16 hr before the termination of the 3-days culture. The results are shown as mean ± SD (c.p.m.). Open bars represent proliferative responses caused by anti-μ and BSF_1 with or without cSBF. Hatched bars indicate backgrounds with or without cSBF.

86% at 0 hr and 41% at 48 hr. These findings suggest that SBF acts on the resting B cells, while the cells that had already received an on-going signal from anti-μ stimulation and entered into the cell cycle were not affected by SBF.

If so, a 3-day culture in the presence of cSBF during the experimental period would cause marked suppression of proliferation by providing a continuous action of SBF, which restores B cells to the resting state. In fact, the suppression was significant but partial (approximately 60% suppression) when the whole B cells, which probably contain at least two cell populations—one in the resting state and one in the activated state—were pretreated with cSBF at 37° for 1 hr (Table 2a) or 16 hr (Table 2b), suggesting that SBF causes the resting B cells to adapt to their present state. In contrast, B cells that are in cell cycle, including the G_1/S phase, might not be affected by SBF.

Anti-μ antibody acts on resting B cells, causing entry into G_1 phase (De Franco *et al.*, 1982). BSF_1 also activates resting B cells, preparing entry into S phase more promptly when cultured with low concentrations of anti-μ antibody plus BSF_1 (Rabin, Ohara & Paul, 1985). In our synergistic B-cell proliferation assay system between anti-μ antibody and BSF_1, a critical dose of anti-μ antibody is supposed to induce receptors for BSF_1 on resting B cells. If SBF acts preferentially on resting B cells, the possible induction of receptors for BSF_1 by anti-μ stimulation may be inhibited by SBF, thereby preventing the cells from receiving a signal from BSF_1 for proliferation. As shown in Table 3, this appears to be the case. Thus, 50 U of BSF_1 were mixed and incubated with B cells that had been precultured with the anti-μ antibody alone, SBF alone, or the two together for 16 hr. When the standard BSF_1 assay was carried out using untreated B cells and these absorbed samples, more than 90% reduction of the BSF_1 activity was attained by absorption with anti-μ antibody-stimulated B cells. On the other hand, B cells precultured with SBF, despite the presence of the anti-μ antibody, absorbed approximately 30% of the BSF_1 activity, and similar findings were obtained for the cells precultured with either SBF or medium alone, implying that, although resting B

Table 2. Hyporesponsiveness of SBF-treated B cells to BSF_1

		Medium-treated B cells	SBF-treated B cells	% suppression
(a)*				
Exp. 1	Background	371 ± 105†	325 ± 53	
	BSF_1	508 ± 82	436 ± 114	
	Anti-μ + BSF_1	18,788 ± 1496	7723 ± 1216	59·3 ($P < 0·001$)
Exp. 2	Background	2872 ± 431	2265 ± 134	
	BSF_1	5080 ± 1253	4109 ± 649	
	Anti-μ + BSF_1	20,175 ± 510	9711 ± 575	55·2 ($P < 0·001$)
(b)				
Exp. 3	Background	1516 ± 193	1426 ± 306	
	BSF_1	2873 ± 294	3363 ± 351	
	Anti-μ + BSF_1	44,194 ± 4454	14,726 ± 947	68·8 ($P < 0·001$)

*B cells were treated by SBF for 1 hr in (a) and for 16 hr in (b).
†Mean ± SD (c.p.m.).

Table 3. Absorption of BSF_1 activity by B cells after various pretreatments*

	Residual BSF_1 activity (U/ml)	% absorption
BSF_1 unabsorbed	50	—
BSF_1 absorbed with B cells precultured with medium	34·3 ± 4·4	31·4
BSF_1 absorbed with B cells precultured with anti-μ	3·8 ± 0·9	92·4
BSF_1 absorbed with B cells precultured with anti-μ and SBF	33·9 ± 6·3	32·2
BSF_1 absorbed with B cells precultured with SBF	34·7 ± 5·7	30·6

*B cells (1×10^6/ml) were preactivated with 10 μg/ml of anti-μ in the presence or absence of 4 U/ml SBF for 16 hr. These B cells were resuspended in 50 U of BSF_1 to a cell density of 3×10^7/ml, and incubated at 37° for 45 min followed by further incubation at 4° for 1 hr. Then, the supernatant was collected and the [^{3}H]TdR incorporation was calculated from two-fold dilution series of these samples to estimate residual BSF_1 activity as in Fig. 1. The results were expressed as units measured by probit analysis (mean ± SD).

cells possess substantial amount of BSF_1 receptors, anti-μ antibody induces a high amount of the receptors on them.

The results shown in Table 3 suggest two possibilities: SBF inhibits either the induction of BSF_1 receptors on the membrane of B cells that entered the G_1 phase in response to anti-μ stimulation, or G_0-G_1 transition itself. The next experiments demonstrated that the second possibility is the most likely.

As depicted in Fig. 3 and summarized in Table 4, G_0–G_1 transition of B cells, 95·1% of which were in the G_0 stage (Fig. 3a), was induced by co-cultivation with the anti-μ antibody (Fig. 3b) and further enhanced in synergy with BSF_1 (Fig. 3d) as measured by an increase in red fluorescence indicating RNA, from 1·7% to 16·0% and 51·6%, respectively (Table 3). However, cSBF drastically inhibited the transition of B cells thus activated (Fig. 3c and e), from 16·0% to 2·5% and 51·6% to 2·1% respectively. Table 4 shows that the mSBF-induced suppression of the G_0–G_1 transition is similar to that induced by cSBF. Hence, these findings suggest that SBF prevents resting B cells from entering the cell cycle in an early stage of their activation and that, once B cells are activated, they lose sensitivity to SBF.

DISCUSSION

We demonstrated that SBF, which is an immunosuppressive lymphokine produced by murine Fc receptor-bearing B cells, presumably in the resting state after binding immune complexes, abrogated murine B-cell activation by anti-μ antibody, thereby inhibiting B-cell growth by BSF_1.

The observation that cSBF, as well as mSBF, inhibits G_0–G_1 transition by anti-μ triggering can be explained by the current understanding of B-cell activation (Parker, 1980; Cambier & Monroe, 1984; Swain *et al.*, 1985). Anti-μ antibody causes cross-linkage of sIg, leading to RNA and DNA synthesis and expression of functional surface receptors for lymphokines inducing B-cell growth or differentiation (Parker, 1982). One such factor, BSF_1, used in this study, is a T-cell factor produced by TPA-stimulated EL-4 cells (Farrar *et al.*, 1983). In agreement with other recent studies (Cambier & Monroe, 1984), we also observed that aproximately 16% of the resting B cells enter the G_1 phase in response to anti-μ and 52% in synergy with BSF_1 during the 16-hr culture period. This suggests that SBF interrupts the transmission of an activation signal from the anti-μ antibody, resulting in the arrest of resting B cells in the G_0 phase and a concomitant block of an increase in functional receptors for BSF_1. In fact, more than 90% of BSF_1 activity was absorbed by anti-μ stimulated B cells, compared to 30% by normal resting B cells (Table 3). This suggests that resting B cells express substantial receptors for BSF_1. These data are not inconsistent with the current understanding about BSF_1 as an activation factor. BSF_1 increases the expression of Ia antigen on

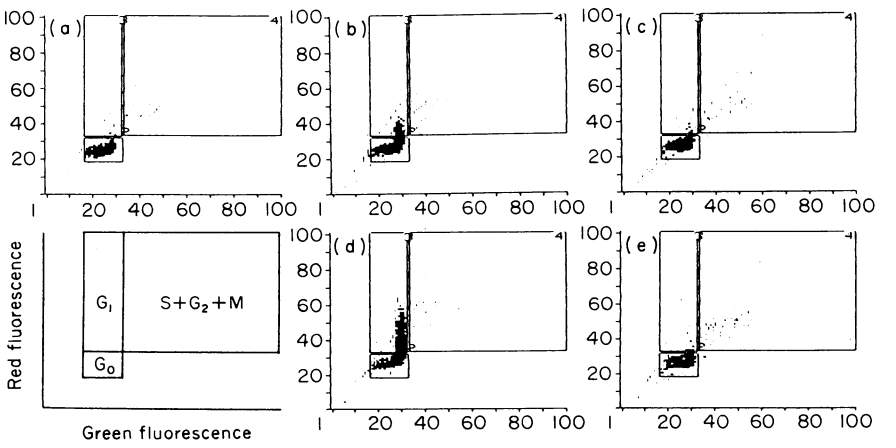

Figure 3. Inhibitory effect of SBF on G_0–G_1 transition of B cells by anti-μ. 1×10^5/ml of B cells were stimulated with 10 μg/ml anti-μ with or without 5 U/ml BSF_1, in the presence or absence of 4 U /ml SBF for 16 hr. The cell-cycle distribution of individual B cells was measured by flow cytometry after differential staining of cellular RNA and DNA with acridine orange as described in the Materials and Methods. (a) B cells cultured in medium only; (b) B cells stimulated with anti-μ; (c) B cells stimulated by anti-μ in the presence of SBF; (d) B cells stimulated by anti-μ and BSF_1; (e) B cells stimulated by anti-μ and BSF_1 in the presence of SBF.

Table 4. Inhibitory effect of SBF on G_0–G_1 transition of B cells by anti-IgM

	−	−	−	+	+	+	+	+	+	Anti-IgM
	−	−	−	−	−	−	+	+	+	BSF_1
	−	+	−	−	+	−	−	+	−	cSBF
Cell cycle	−	−	+	−	−	+	−	−	+	TS4.44
G_0	95·1*	92·4	92·1	80·0	92·7	93·4	45·5	92·3	92·4	
G_1	1·7	3·0	2·8	16·0	2·5	2·5	51·6	2·1	3·1	
$S+G_2+M$	2·4	3·8	4·3	2·3	3·5	3·2	2·0	4·2	3·4	

* 5×10^3 B cells were measured by flow cytometry after differential staining of cellular DNA and RNA with acridine orange and the cell frequency distribution in each cell cycle was expressed as a percentage.

resting B cells (Noelle *et al.*, 1984) and acts to prepare them to respond to anti-μ antibody and BSF_1 (Rabin *et al.*, 1985). Thus, both anti-μ antibody and BSF_1 are able to activate resting B cells. However, it has been demonstrated that the activating mechanisms involved in both cases are quite different (Mizuguchi *et al.*, 1986). We have found that SBF inhibits phosphatidylinositol turnover, thereby suppressing B-cell activation by anti-μ antibody, but not by BSF_1 (manuscript in preparation).

There is a clear distinction between small and large B cells partitioned by Percoll in terms of responsiveness to SBF. Thus, the LPS response of small but not large (blastic) cells is markedly inhibited by co-culture with SBF. Interestingly, the suppressive effect of SBF on B-cell responses to anti-μ antibody and BSF_1 in the 72-hr culture decreased in a time-dependent way when SBF was added to the culture on Day 0, 1 or 2. These findings suggest that SBF acts on resting B cells, but not on cells that have already received an on-going signal by anti-μ stimulation and have entered the cell cycle. The finding that the proliferative response of SBF-pretreated whole B cells to anti-μ and BSF_1 after removal of SBF is partially inhibited further supports this concept. Thus, SBF receptors seem to be constitutively expressed on resting, but not on activated, B cells.

The immune regulation mediated by FcR of either T or B cells appears to be dependent on the antibody molecules produced in response to an antigenic stimulation. The lymphokines, including SBF, examined here, and immunoglobulin binding factor (IBF) produced by T cells (Gisler & Fridman, 1975), are responsible for the suppression of B-cell responses. Thus, antibody molecules themselves or immune complexes formed in the course of immune responses seem to benefit the host under physiological conditions. We know that SBF activity is absorbed by splenic B, but not by T cells or macrophages (Masuda *et al.*, 1978) and that not only $FcR\gamma^+$ B, but also $FcR\gamma^-$ B cells are suppressively effected by SBF when examined by either LPS or antibody responses (Miyama *et al.*, 1978), suggesting that most, but not all (Park *et al.*, 1986), resting B cells may be susceptible for SBF. Moreover, we have observed that immune complexes inhibit the growth response of resting B cells to anti-μ antibody and BSF_1 when binding to FcR, and that this step seems to be necessary for causing the subsequent production of SBF (M. Inaba, T. Masuda, T. Ohno and K. Ajisaka, submitted for publication). The implication is that SBF-mediated immune suppression, which seems to be advantageous for maintaining immune homeostasis, involves an autocrine- and paracrine-like regulatory mechanism in B cells in the G_0 phase.

Several studies on the biochemical nature of SBF indicate that SBF is a glycoprotein of 43,000 molecular weight synthesized and secreted by FcRγ-stimulated B, but not EA non-rosetting B cells or other types of cells (Masuda *et al.*, 1978; Miyama *et al.*, 1979; Suzuki *et al.*, 1983a b). However, one may persist in a possibility that SBF may be a T-cell product, such as IFNγ, which has been reported to inhibit B-cell responses to anti-μ antibody and BSF_1 (Mond *et al.*, 1985), as in the case of SBF. The SBF used in the present study was prepared by small B-cell fraction of athymic nude mice to rule out this possibility as far as possible. Of course, we are still unable to discount a possible involvement of residual T or NK cells in nude mice in the production of SBF. However, no IFN activity was detected in our SBF samples when assayed by the reduction of viral cytopathic effect using L cells and vesicular stomatitis virus. Moreover, SBF scarcely retained to the affinity column coupled with IgG fraction of rabbit anti-IFNγ antibody but was recovered in the effluent fraction (manuscript in preparation). In contrast to INFγ, SBF molecules possess Ia antigen and are acid resistant, since the activity was well preserved in the acid (pH 2·2) eluate from SBF-loaded affinity columns coupled with anti-H-2 antibody (Miyama *et al.*, 1979). The fact that INFγ does not inhibit the induction of resting B cells from G_0 to early G_1 phase (Mond *et al.*, 1985) while SBF does, and that, in contrast to this, IFNγ is reported to promote the proliferation of anti-μ-activated human B lymphocytes (Romagnani *et al.*, 1986), also indicated that SBF molecules are distinct from IFNγ. Taken together, it is unlikely that SBF is a T-cell product such as INFγ.

It is interesting to note that the mode of action of SBF is in striking contrast to that of BSF_1, which causes the proliferation, but not the suppression, of activated but not resting B cells. Although BSF_1 is a T-cell factor, production of a B-cell growth factor by human activated B cells has recently been observed (Jurgensen, Ambrus & Fauci, 1986; Muraguchi *et al.*, 1986). This also suggests the presence of an autocrine or paracrine pathway in B cells for their proliferation and differentiation as in the case of T cells, the proliferation of which is supported by a T-cell product, IL-2 (Smith & Cantrell, 1985).

We are currently involved in the determination of the SBF gene, biological analysis of SBF receptors, and investigation of the molecular mechanism of the suppressing effect of SBF on B-cell activation.

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REFERENCES

CAMBIER J.C. & MONROE J.G. (1984) B cell activation. V. Differential signalling of B cell membrane depolarization, increased Ia expression, G_0 to G_1 transition, and thymidine uptake by anti-IgM and anti-IgD antibodies. *J. Immunol.* **133,** 576.

DARZYNKIEWICZ S., TRAGANOS F., SHARPLESS T. & MELAMED M.R. (1976) Lymphocyte stimulation: a rapid multiparameter analysis. *Proc. natl. Acad. Sci. U.S.A.* **73,** 2881.

DEFRANCO A.L., RAVECHE E.S., ASOFSKY R. & PAUL W.E. (1982) Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. exp. Med.* **55,** 1523.

FARRAR J.J., HOWARD M., FILLER-FARRAR J. & PAUL W.E. (1983) Biochemical and physicochemical characterization of murine B cell growth factor: a lymphokine distinct from interleukin 2. *J. Immunol.* **131,** 1838.

GILLIS S., FERM M.M., OU W. & SMITH K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GISLER R.H. & FRIDMAN W.H. (1975) Suppression of *in vitro* antibody synthesis by immunoglobulin-binding factor. *J. exp. Med.* **142,** 507.

HILFIKER M.L., MOORE R.N. & FARRAR J.J. (1981) Biologic properties of chromatographically separated murine thymoma-derived interleukin 2 and colony-stimulating factor. *J. Immunol.* **127,** 1983.

HOWARD M., FARRAR J., HILFIKER M., JOHNSON B., TAKATSU K., HAMAOKA T. & PAUL W.E. (1982) Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. exp. Med.* **155,** 914.

HOWARD E., MIZEL S.B., LACHMAN L., ANSEL J., JOHNSON B. & PAUL W.E. (1983) Role of interleukin 1 in anti-immunoglobulin-induced B cell proliferation. *J. exp. Med.* **157,** 1529.

JURGENSEN C.H., AMBRUS J.L. & FAUCI A.S. (1986) Production of B cell growth factor by normal human B cells. *J. Immunol.* **136,** 4542.

MASUDA T., MIYAMA M., KURIBAYASHI K., YODOI J., TAKABAYASHI A. & KYOIZUMI S. (1978) Immunological properties of Fc receptor on lymphocytes. V. Suppressive regulation of humoral immune response by Fc receptor bearing B lymphocytes. *Cell. Immunol.* **39,** 238.

MIYAMA M., KURIBAYASHI K., YODOI J., TAKABAYASHI A. & MASUDA T. (1978) Immunological properties of Fc receptor on lymphocytes. I. Functional differences between Fc receptor positive and negative lymphocytes in humoral immune repsonses. *Cell. Immunol.* **35,** 253.

MIYAMA M., YAMADA J. & MASUDA T. (1979) Immunological properties of Fc receptor on lymphocytes. VI. Characterization of suppressive B-cell factor (SBF) released from Fc receptor-bearing B cells. *Cell. Immunol.* **44,** 51.

MIYAMA-INABA M., SUZUKI T., PAKU Y. & MASUDA T. (1982) Feedback regulation of immune responses by immune complexes: possible involvement of a suppressive lymphokine by FcR-bearing B cells. *J. Immunol.* **128,** 882.

MIZUGUCHI J., BEAVEN M.A., OHARA J. & PAUL W.E. (1986) BSF_1 action on resting B cells does not require elevation of inositol phospholipid metabolism or increased $[Ca^{2+}]i$. *J. Immunol.* **137,** 2215.

MOND J.J., FINKELMAN F.D., SARMA C., OHARA J. & SERRATE S. (1985) Recombinant interferon-γ inhibits the B cell proliferative response stimulated by soluble but not by Sepharose-bound anti-immunoglobulin antibody. *J. Immunol.* **135,** 2513.

MURAGUCHI A., NISHIMOTO H., KAWAMURA N., HORI A. & KISHIMOTO T. (1986) B cell-derived BCGF functions as autocrine growth factor(s) in normal and transformed B lymphocytes. *J. Immunol.* **137,** 179.

NAKAGAWA T., HIRANO T., NAKAGAWA N., YOSHISAKI K. & KISHIMOTO T. (1985) Effect of recombinant IL 2 and γ-IFN on proliferation and differentiation of human B cells. *J. Immunol.* **134,** 959.

NOELLE R., KRAMMER P.H., OHARA J., UHR J.W. & VITETTA E.S. (1984) Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. *Proc. natl. Acad. Sci. U.S.A.* **81,** 6149.

PARK Y.-H., SUZUKI T., MIYAMA-INABA M., MASUDA T., YOSHIDA Y. & UCHINO H. (1986) Feedback regulation of antibody formation by suppressive B cell factor: preferential suppression of high-affinity antibody production by memory B lymphocytes. *Int. Arch. All. appl. Immunol.* **81,** 156.

PARKER D.C. (1980) Induction and suppression of polyclonal antibody responses by anti-Ig reagents and antigen-nonspecific helper factors. *Immunol. Rev.* **52,** 115.

PARKER D.C. (1982) Separable helper factors support B cell proliferation and maturation to Ig secretion. *J. Immunol.* **129,** 469.

RABIN E.M., OHARA J. & PAUL W.E. (1985) B-cell stimulatory factor 1 activates resting B cells. *Proc. natl. Acad. Sci. U.S.A.* **82,** 2935.

ROMAGNANI S., GIUDIZI M.G., BIAGIOTTI R., ALMERIGOGNA F., MINGARI C., MAGGI E., LIANG C.-M. & MORETTA L. (1986) B cell growth factor activity of interferon-γ. Recombinant human interferon-γ promotes proliferation of anti-μ-activated human B lymphocytes. *J. Immunol.* **136,** 3513.

SCHIMPL A. & WECKER E. (1972) Replacement of T-cell function by a T-cell product. *Nature (Lond.)*, **237,** 15.

SMITH K.A. & CANTRELL D.A. (1985) Interleukin 2 regulates its own receptors. *Proc. natl. Acad. Sci. U.S.A.* **82,** 864.

SUZUKI T., MIYAMA-INABA M., MASUDA T., UCHINO H. & ISHII K. (1983a) Monoclonal SBF produced by hybridoma: *in vitro* and *in vivo* suppression of B tumour-cell proliferation. *Immunology* **50,** 595.

SUZUKI T., MIYAMA-INABA M., MASUDA T., UCHINO H., MURAKAMI A. & TANAKA H. (1983b) Suppressive B cell factor (SBF) produced by FcR-bearing B cells: suppression of B but not non-B cell proliferation. *Immunology*, **50,** 149.

SUZUKI T., PAKU Y.H., MIYAMA-INABA M., MASUDA T., SAJI H., NAGATA I. & UCHINO H. (1985) A novel lymphokine derived from human IgG Fc receptor-bearing B cells: its suppressive effect on activated T and B lymphocytes including tumour cells *in vitro*. *Immunology*, **55,** 427.

SWAIN S.L., HOWARD M., KAPPLER J., MARRACK P., WATSON J., BOOTH R., WETZEL G.D. & DUTTON R.W. (1983) Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. *J. exp. Med.* **158,** 822.

SWAIN S.L., WETZEL G.D. & DUTTON R.W. (1985) B cell growth and differentiation factors. In: *Lymphokines 10. Growth and Differentiation of B Cells* (eds M. H. Schreier and K. A. Smith), p. 1. Academic Press, New York.

TADA T. & OKUMURA K. (1979) The role of antigen-specific T cell factors in the immune response. *Adv. Immunol.* **28,** 1.

ZUBLER R.H., LOWENTHAL J.W., ERARD F., HASHIMOTO N., DEVOS R. & MACDONARD H.R. (1984) Activated B cells express receptors for, and proliferate in response to, pure interleukin 2. *J. exp. Med.* **160,** 1170.