

GROWTH-SUPPORTING ACTIVITY OF FRAGMENT Ba OF
THE HUMAN ALTERNATIVE COMPLEMENT PATHWAY
FOR ACTIVATED MURINE B LYMPHOCYTES

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Factor B, a 92,000 dalton, single chain glycoprotein, is the major serine protease of the alternative pathway (AP) of the C system (1); it is encoded by a gene located in the MHC (2). During activation of the AP, factor B binds to C3b in the presence of magnesium and is then cleaved by factor D, releasing the 33,000 dalton N-terminal fragment Ba. The 60,000 dalton C-terminal fragment, Bb, which carries the enzymatic site, is rapidly released upon decay-dissociation of the C3-convertase C3bBb (1, 3). Guinea pig Ba has been reported to be chemotactic, in vitro, for guinea pig polymorphonuclear leukocytes (4).

Several observations led us to hypothesize that factor B, and more likely one of its activation fragments, could play a role in the control of growth and maturation of B lymphocytes: (a) factor B is a major constitutive protein synthesized and secreted by macrophages (reviewed in reference 5), as well as by some B lymphocytes (6). Treatment of macrophages with bacterial LPS increases the production of factor B (7); (b) the requirement for macrophages in the polyclonal activation of murine B lymphocytes by LPS is still controversial (8). However, it is widely accepted that accessory cells and/or their secreted factors help for an optimal response of B lymphocytes to LPS (9); (c) finally, LPS, as well as some other B cell mitogens, may activate the AP (10), thus allowing the cleavage of factor B by factor D, in the presence of C3b, thereby releasing its activation fragments Bb and Ba in the microenvironment of the cells.

In the present study we have investigated the action of human factor B and its activating enzyme factor D, as well as the activation fragments Bb and Ba on the growth of activated mouse spleen B lymphocytes. Indeed, human and mouse factor B display very high homology in amino acid sequence (11); moreover, murine factor B can be cleaved by human factor D. We show that purified fragment Ba is strongly mitogenic for LPS-preactivated B cell blasts, whereas it has no effect on the growth rates of Con A-activated T cell blasts or CTL. Furthermore, fragment Ba was also shown to be a potent growth factor for in vivo activated B cell blasts isolated from unprimed mice of the LPS nonresponder C3H/HeJ strain.

We propose that fragment Ba should be regarded as a B cell stimulatory factor

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(BSF); up to now, this is the only biological activity that has been reported for human Ba.

Materials and Methods

Purified Components. Factor B (1), factor D (12), and C3 (13) were purified from human plasma according to the standard procedures, with minor modifications. Factor B (20 mg) was cleaved into its fragments Bb and Ba by a 90 min incubation at 37°C with C3 (200 µg) and factor D (20 µg), in veronal-buffered saline containing 0.012 M MgSO₄. Factor B, Ba, and Bb were purified by gel filtration on an Ultrogel AcA54 (IBF, Villeneuve la Garenne, France), followed by Fast-protein liquid chromatography- (FPLC) anion exchange chromatography on a Mono-Q column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 20 mM phosphate buffer, pH 7.2.

Mice. C57Bl/6, B10BR, and C3H/HeJ mice of both sexes were bred in our own facilities, and used at 8–12 wk of age.

Assays. The assay for B cell stimulatory factor (BSF) was carried out on: (a) LPS-preactivated B cell blasts; anti-Thy-1.2 (HO.13-4-9, a kind gift of Dr. Gottlieb, Sloan-Kettering Institute for Cancer Research, New York) and C treated mouse spleen cells were cultivated with LPS (50 µg/ml; Difco Laboratories Inc., Detroit, MI), in RPMI-1640 medium (Flow Laboratories Ltd., Irvine, Scotland), containing 10% heat-inactivated FCS (Flow Laboratories Ltd.), antibiotics, 2-ME (50 µM, L-glutamine (2 mM), and sodium pyruvate (0.11 mg/ml), as described previously (14). After 18–20 h, living cells, consisting of >90% surface Ig-positive cells, were harvested by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Cells were extensively washed and subcultured in flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA), at 10⁵ cells/ml, in RPMI-1640 serum-free medium supplemented with antibiotics, human albumin (0.4 mg/ml), transferrin (0.75 µg/ml), insulin (2 µg/ml), 2-ME, L-glutamine, and sodium pyruvate. Medium and LPS restimulation provided the negative and positive controls, respectively; (b) in vivo activated B cell blasts from unprimed mice of the LPS-non responder C3H/HeJ strain; spleen cells were treated with anti-Thy-1.2 and complement. Cells were then separated on a discontinuous Percoll gradient (Pharmacia Fine Chemicals), from which only the cell fraction of density between 1,059 and 1,064 was collected. Finally, low density cells were extensively washed and subcultured in round-bottomed microtiter plates (Falcon Labware), at 3 × 10⁵ cells/ml, in RPMI-1640 serum-free medium supplemented as described above.

T cell growth factor (TCGF) assays were performed on T cell blasts (10⁵ cells/ml) obtained from Con A-stimulated cultures, and on a TCGF-dependent cytotoxic T cell line, as described (14). Medium and a standard preparation of TCGF provided the negative and positive controls, respectively.

In all assays, complement peptides were titrated in a twofold serial dilution into the assay wells; proliferation was assayed on day 2 after subculture, cells were pulsed with [³H]TdR (1 µCi/culture) for 6 h, harvested, and counted; results are expressed as the cpm per culture.

Results

Effects of Cleavage of Factor B by Factor D on Proliferation of LPS-preactivated B Cell Blasts. Factor B was added at a final concentration of 25 µg/ml, to serum-free cultures, in which factor D was titrated in a twofold serial dilution starting from 16 µg/ml. LPS-preactivated B cell blasts did not maintain growth when recultured in serum-free medium alone (660 ± 110 cpm), but they did so when restimulated by LPS (15,328 ± 2,101 cpm).

As shown in Fig. 1, the addition of factor D to cultures containing factor B strikingly enhanced [³H]thymidine uptake. A plateau level of maximum [³H]-thymidine uptake was seen for concentrations of factor D ranging from 16 to 4 µg/ml (30,971 ± 2,370 cpm). Background level was reached for concentrations

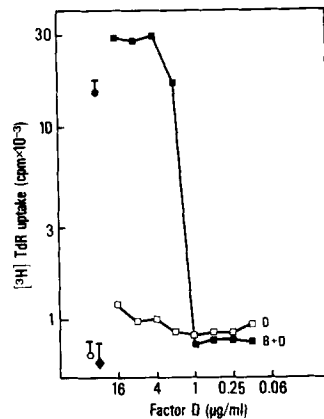


FIGURE 1. Effects of cleavage of factor B by factor D on proliferation of LPS-preactivated B cell blasts. Factor D was tested either alone (□) or in the presence of 25 µg/ml of factor B (■), in a 1:2 serial dilution starting at 16 µg/ml. Control responses to LPS (●), factor B alone (◆), or medium (○) were 15,328 ± 2,101 cpm, 660 ± 110 cpm, and 594 ± 163 cpm, respectively.

lower than 1 µg/ml. By contrast, factor D alone, even at high concentrations, did not significantly enhance [³H]thymidine uptake by LPS-preactivated B cell blasts; proliferation obtained in presence of factor B alone (594 ± 163 cpm) was also comparable to the medium control.

Effects of Fragment Ba on Proliferation of Activated B Cells. Since factor B had to be cleaved by factor D to exhibit growth-promoting activity, we have investigated the effects of its activation fragments Ba and Bb on proliferation of activated B cells. Serial dilutions of factor B, Bb, and Ba were added to serum-free cultures of LPS-preactivated B cell blasts. Complement peptides concentrations are expressed as molarity (M), allowing direct comparison of the responses to B, Bb, and Ba; 2 µM of factor B, Bb, and Ba corresponding to 180, 120, and 60 µg/ml, respectively.

As shown in Fig. 2a, the addition of 60 µg/ml (2 µM) of highly purified Ba to serum-free cultures of LPS-preactivated B cell blasts increased [³H]thymidine uptake up to nine times the background; a linear dose-response was obtained for Ba concentrations ranging from 3 to 60 µg/ml. By contrast, neither factor B, nor its fragment Bb, show any growth-promoting activity (Fig. 2a). The response to Ba reaches maximum levels for concentrations above 120 µg/ml (4 µM, Fig. 2b).

In order to determine if the growth-supporting activity of Ba was restricted to B cells preactivated by LPS, or if it might as well act on other activated B cells, we have investigated its effect on *in vivo*, activated B cell blasts obtained from unprimed mice of the LPS-nonresponder C3H/HeJ strain. Fig. 2c shows that Ba has the ability to maintain growth of low density B cells of unprimed C3H/HeJ mice; moreover, the magnitude and the dose-dependency of the response are comparable to those obtained with the LPS-preactivated B cell blasts.

Fragment Ba Has No TCGF-like Activity. The cellular specificity of the growth-supporting activity of Ba was further probed by investigating its effect on the growth of T lymphocytes. We used both TCGF-dependent CTL cells and Con

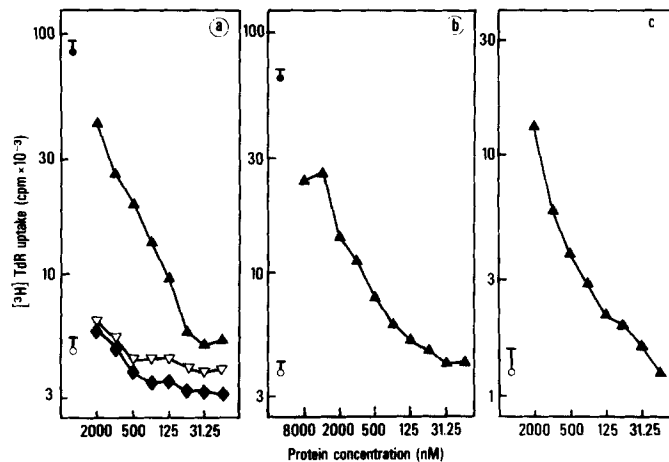


FIGURE 2. Effects of fragment Ba on B cell blast proliferation. Purified factor B (\blacklozenge), Bb (∇), and Ba (\blacktriangle) were added to LPS-preactivated B cell blast cultures in a 1:2 serial dilution starting at 2 μM (a) or 8 μM (b), with LPS restimulation (\bullet) and medium (\circ) as controls. (c) Ba was added to cultures of in vivo, activated B cell blasts from unprimed C3H/HeJ mice; Ba 1:2 serial dilution started at 2 μM . Note the change of scale in c.

TABLE I
Fragment Ba Has No TCGF-like Activity

Cells cultured	$[^3\text{H}]$ Thymidine incorporation (cpm \pm SD)		
	Medium	TCGF-CM	Ba (60 $\mu\text{g}/\text{ml}$)
CTL cells	4,034 \pm 340	352,860 \pm 35,611	4,345 \pm 936
Con A-activated T cells	945 \pm 130	125,267 \pm 9,580	1,011 \pm 224

Con A-activated T cells and TCGF-dependent CTL cells were cultivated at 10^5 cells/ml in the presence or absence of 60 $\mu\text{g}/\text{ml}$ of purified Ba, with TCGF-CM as positive control. Results are expressed as the average cpm of six replicate cultures \pm SD.

A-activated T cells as responder cells. Highly purified Ba was added to the cultures at a final concentration of 60 $\mu\text{g}/\text{ml}$, which gave substantial proliferative responses with activated B cell blasts.

As shown in Table I, Ba failed to maintain proliferation of TCGF-reactive T cells; $[^3\text{H}]$ thymidine incorporation was identical whether or not Ba was added to the medium, whereas the addition of conditioned medium containing TCGF activity supported the growth of both CTL cells and Con A-activated T cells very efficiently.

Discussion

The results presented here describe the first biological activity of fragment Ba of the human alternative complement pathway as a B cell stimulatory factor. We show that highly purified fragment Ba supports growth of in vitro, LPS-preactivated B cell blasts; moreover, Ba also maintains proliferation of the in vivo, activated B cell blasts from unprimed mice of the LPS-nonresponder C3H/HeJ strain, showing that the growth-supporting activity of Ba is not restricted to LPS-preactivated B cell blasts. By contrast, Ba has no effect on proliferation of TCGF-reactive T cells.

Miyama et al. reported that treatment of macrophages with LPS considerably increases synthesis and secretion of factor B (7). Furthermore, since both factor D and C3 are synthesized by macrophages (reviewed in reference 5), AP activation by LPS may occur in the microenvironment of the LPS-activated cells. In fact, we could show that the addition of factor B and factor D to serum-free cultures containing macrophages supported growth of the LPS-preactivated B cell blasts very efficiently. Since neither factor B alone nor factor D alone had any significant effect, we conclude that the growth-supporting activity is not due to a possible contamination of the protein preparations by LPS or other mitogens, but is more likely mediated by one of the activation fragments released during the cleavage of factor D. We have established that fragment Ba has the ability to support growth of the LPS-preactivated B cell blasts, it is therefore tempting to speculate that Ba is a physiological mediator for B cell activation by LPS.

It has been reported that fragment Ba generated during AP activation is a short-lived product (14), in that the C-terminal arginine (Arg) and lysine (Lys) residues are rapidly released by a serum carboxypeptidase B. Similarly, both the anaphylatoxins C3a and C5a have a C-terminal Arg that modulates the expression of their biological activities. The role of the two C-terminal basic residues of Ba in the control of the B cell growth has not been formally investigated. Since cleavage of factor B has been done in the absence of carboxypeptidase B inhibitor, it is most likely that purified fragment Ba preparations contain a mixture of its native form and its des_{Arg} and $\text{des}_{\text{Arg-Lys}}$ fragments. In fact, it was necessary to add 120 $\mu\text{g/ml}$ of purified Ba to LPS-preactivated B cell blasts to reach the plateau level of maximal proliferative response, whereas cleavage of 25 $\mu\text{g/ml}$ of factor B by high concentrations of factor D was enough. Under such experimental conditions, extensive cleavage of factor B would yield a maximal concentration of 8 $\mu\text{g/ml}$ of Ba; this indicates that Ba generated through cleavage of factor B in the cultures is more efficient than purified Ba itself, suggesting that the native form of Ba, which is progressively released in the microenvironment of the cells, is more active than fragments that have lost Lys and/or Arg.

Summary

We have investigated the effects of cleavage of factor B by its activating enzyme, factor D, as well as its activation fragments Bb and Ba, on the growth of mouse spleen B lymphocytes preactivated by LPS. Neither factor B nor factor D show any growth-supporting activity when tested alone. The coaddition of factor B and factor D to serum-free cultures of LPS-preactivated B cell blasts increased the proliferation of the responding cells up to the level obtained by restimulation with LPS. Such growth-supporting activity was shown to be mediated by Ba, whereas Bb did not show any significant effect. Furthermore, this effect was not restricted to the LPS-preactivated B cell blasts; in fact, Ba also supported the growth of *in vivo*, activated B cell blasts of unprimed mice of the LPS-nonresponder C3H/HeJ strain. In contrast, Ba did not maintain growth of Con A-activated T cells or TCGF-dependent CTL cells.

Taken together, these results describe the first biological activity of human Ba as a B cell stimulatory factor.

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