

## Bradykinin Induces Interleukin-6 and Synergizes with Interleukin-1

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### ABSTRACT

Bradykinin was found to induce production of IL-6 in human diploid fibroblasts, as well as in a hepatoma-derived cell line, but not in a human melanoma or an osteosarcoma cell line. With the exception of the melanoma cell line, these cells were also found to be responsive to IL-1 $\beta$ . The response to bradykinin was faster but less high than that induced by IL-1. Experiments in which IL-1 ( $-\alpha$  or  $-\beta$ ) and bradykinin were applied simultaneously revealed a synergistic interaction. Of the other cytokines tested, TNF- $\alpha$  and IFN- $\gamma$  weakly induced IL-6. Neither IL-2, IFN- $\alpha$ , nor IFN- $\beta$  was able to induce IL-6, either in the absence or the presence of bradykinin. These observations constitute further evidence for the existence of interactions between cytokine and noncytokine peptides, thus linking the neuroendocrine and immune systems.

### INTRODUCTION

Bradykinin (BK)<sup>1</sup> is a nonapeptide generated from plasma or tissue kininogen. Its biological activities include the ability to induce vasodilation and pain, to enhance vascular permeability, and to stimulate arachidonic acid metabolism. The molecule is involved as a regulator in various physiological and pathological processes (for review see ref. 1), e.g., wound healing, local inflammation due to infection or autoimmune disease, and septic shock (2).

It has been suggested that cells in inflamed tissues are more responsive to bradykinin than those in normal tissues (3,4), and that this increased sensitivity to bradykinin is due to the effect of certain inflammation-associated cytokines. Along this line, it has been shown that bradykinin-induced PGE<sub>2</sub> production by synovial cells, fibroblasts, and colonic tissue can be enhanced by preincubation of these cells or tissues with interleukin-1 (IL-1) or tumor necrosis factor (TNF) (5-7).

Here we present evidence for the existence of additional interactions between the cytokine network and the kinin system. We show that bradykinin has the ability to induce a cytokine (IL-6) and that, in doing so, it synergizes with certain other cytokines.

### MATERIALS AND METHODS

#### Reagents

The following materials were prepared in our laboratory: human natural interleukin-1 $\beta$  (IL-1 $\beta$ ) (8), purified human natural interferon- $\alpha$  (IFN- $\alpha$ ), IFN- $\beta$  (9), and goat anti-IL-1 $\beta$ -antibodies. The following materials were purchased: human recombinant IL-1 $\alpha$  and human recombinant IL-2 (Janssen Pharmaceutica, Beerse, Belgium), human recombinant TNF- $\alpha$  (Genzyme Corporation, Boston, MA), human recombinant IFN- $\gamma$  (Roche, Gent, Belgium), poly(rI) · poly(rC) (P-L Biochemicals, Inc., Milwaukee, WI), bradykinin-acetate and (Thi<sup>5,8</sup>, D-Phe<sup>7</sup>)-bradykinin-acetate (Sigma Chemical Co., St. Louis, MO). Eagle's minimum essential medium (EMEM) with Earle's salts, and fetal calf serum (FCS) (Gibco, Paisley, Scotland).

#### Cell cultures

E<sub>1</sub>SM and E<sub>6</sub>SM (different strains of human diploid fibroblasts), Malavu (human hepatoma-derived), Bowes (human melanoma), and MG-63 (human osteosarcoma) cells were plated in 24-well polystyrene culture dishes (24 × 1.9 cm<sup>2</sup>,

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<sup>1</sup>Abbreviations used: BK, bradykinin; poly(rI) · poly(rC), polyriboinosinic · polyribocytidylic acid.

Nunc, Roskilde, Denmark) and allowed to grow to confluency or saturation density ( $\pm 1$  week) in EMEM with 10% FCS. For induction, medium was removed, cells were washed three times with serum-free EMEM, and 1 ml of EMEM containing IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , poly(rI) · poly(rC), alone or in combination with bradykinin or vehicle [equivalent volume of phosphate-buffered saline (PBS)] was added.

The inducing effect of IL-1 $\beta$  was neutralized by polyclonal anti-IL-1 $\beta$  antibodies. For this purpose a 1/300 dilution of the antibody was preincubated for 2 h at 37°C with IL-1 $\beta$  concentrations of 1 and 10 U/ml before application to the cell culture. The bradykinin analogue [(Thi<sup>5,8</sup>,D-Phe<sup>7</sup>)-bradykinin-acetate] at a final concentration of  $10^{-4}$  M was added to the cells 30 min before addition of bradykinin at concentrations of  $10^{-6}$  and  $10^{-4}$  M.

Experiments were in general conducted using a 24-h incubation period, with concentrations ranging from 0 to  $10^{-4}$  M for bradykinin and 0 to 100 U/ml for IL-1 $\beta$ . At the end of the treatment period, the culture medium was collected and stored at  $-20^{\circ}\text{C}$  until it was assayed for IL-6.

### IL-6 assay

Hybridoma growth factor (HGF) activity for IL-6 was determined on the factor-dependent 7TD1 mouse-mouse hybridoma as described (10). Briefly, cells were grown in microtiter cultures (2000 cells/200  $\mu\text{l}$ ) in the presence of serial ( $0.5 \log_{10}$ ) dilutions of supernatants from stimulated cells or an internal standard (10,000 U/ml; 1 U/ml is equivalent to 1 pg/ml) of pure natural HGF/IL-6 (11,12). The potency of the samples was evaluated from the cell densities (colorimetric determination of

hexosaminidase levels) after 4 days culture, one unit of HGF corresponding to half-maximal growth of the hybridoma cells.

To exclude the possibility that IL-6 activity measured in cell supernatants after addition of bradykinin or cytokines, was due to direct effects of these agents in the IL-6 assay, bradykinin ( $10^{-10}$ ,  $10^{-8}$ ,  $10^{-6}$ , and  $10^{-4}$  M), IL-1 $\beta$  [10 U/ml; 1 U/ml is equivalent to 10 pg/ml (12)], IFN- $\gamma$  (100 U/ml), poly(rI) · poly(rC) (50  $\mu\text{g}/\text{ml}$ ), and combinations were added directly to the 7TD1 hybridoma test cells. No toxicity or stimulating effects were observed.

### Endotoxin assay

To exclude the possibility that IL-6 induction was caused by contamination by endotoxin, all solutions containing the inducers at the highest concentrations used were tested for the presence of endotoxin. The presence of endotoxin was quantitated using the *Limulus* amoebocyte lysate assay as previously described (13). Standard curves were made with *Escherichia coli* 055:B5 endotoxin. The method has a detection limit of 20 pg lipopolysaccharide per ml. The IL-1 $\beta$  (2000 U/ml) and bradykinin ( $10^{-2}$  M) solutions contained 75 and 70 pg/ml of endotoxin, respectively. Although endotoxin is a potent inducer of IL-6 in certain cell types such as monocytes, in concentrations as high as 50,000 pg/ml it failed to induce significant amounts of IL-6 on fibroblasts (12). The measured concentrations thus are low enough to be neglected, as is also confirmed by the IL-1 and bradykinin neutralization experiments.

## RESULTS

The dose relationship for IL-6-induction by IL-1 $\beta$  or bradykinin was analyzed in the human diploid fibroblast strain E<sub>1</sub>SM and the human Malavu cell-line. IL-1 $\beta$  was applied at doses of 0.1 to 100 U/ml, bradykinin at doses ranging from  $10^{-8}$  to  $10^{-4}$  M, and IL-6 levels were determined after 24 h incubation. Figure 1A and B shows that both substances were able to induce IL-6. As expected, a dose of 1 unit/ml of IL-1 was sufficient to induce significant production of IL-6, and maximal stimulation was achieved with 10 to 100 units/ml. Bradykinin also induced production of IL-6, although it was less potent than IL-1 $\beta$ . Stimulation with doses equivalent to those active in other bioassays used for bradykinin ( $10^{-7}$  M) was poor. Higher doses induced significant increases of IL-6 levels in a dose-dependent fashion.

Specificity of induction was assessed by the use of an IL-1 $\beta$ -specific polyclonal antibody and by an analogue of bradykinin. The IL-6 production by IL-1 in the presence or absence of antibodies, respectively, was 6 and 500 units of IL-6/ml for 1 unit of IL-1/ml (= 99% reduction), and 10 and 800 units of IL-6 for 10 units of IL-1/ml (= 99% reduction). Since the basal level of IL-6 production equals 10 U/ml, this means that the IL-6 inducing effect of IL-1 could be completely neutralized by the antibodies. The IL-6 production for bradykinin induction in the presence or absence of analogue, respectively, are 250 and 800 units of IL-6/ml for  $10^{-6}$  M BK and 320 and 1000 units IL-6/ml for  $10^{-4}$  M BK. Since the basal level of IL-6 production equals 64 U/ml and the level of production in the

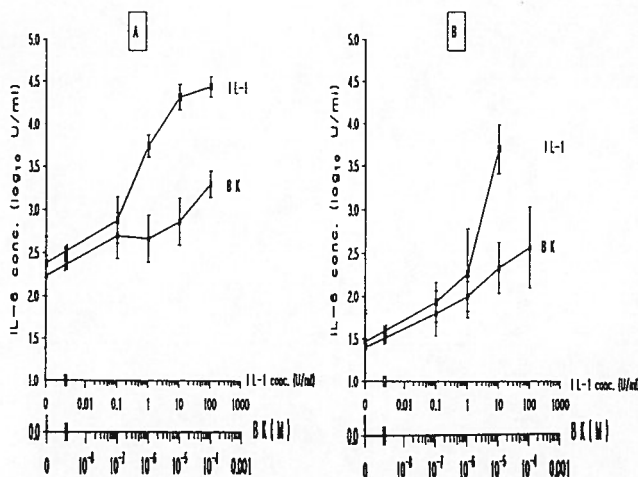


FIG. 1. Dose-response curve of IL-6 production by diploid fibroblasts (A) and Malavu cells (B) stimulated with IL-1 or bradykinin. Confluent cultures of E<sub>1</sub>SM fibroblasts (A) and Malavu cells (B) were treated with different concentrations of IL-1 $\beta$  or bradykinin. After 24 h incubation, the culture fluids were assayed for IL-6 production. Titers of IL-6 are expressed as log<sub>10</sub> U/ml as determined by hybridoma cell proliferation. Means and standard errors for five (A) and three (B) experiments are shown.

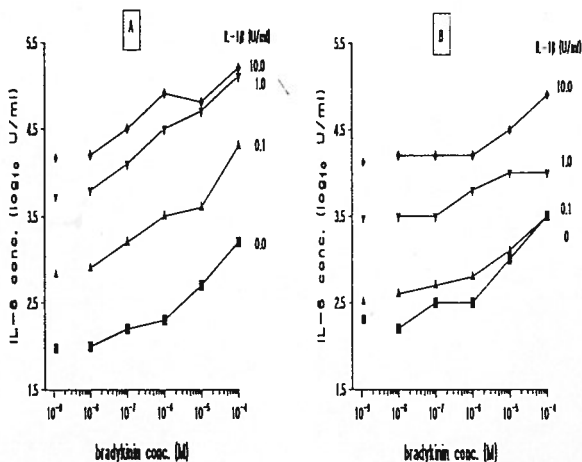


FIG. 2. Synergy between IL-1 and bradykinin for IL-6 production in diploid fibroblasts (A) and Malavu cells (B). Confluent cultures of  $E_1SM$  fibroblasts (A) and the Malavu cell-line (B) were treated simultaneously with different concentrations of bradykinin and IL-1 $\beta$  for 24 h.

presence of analogue alone equals 320 U/ml, this means that preexposure of the cells to the analogue reduced IL-6 titers after induction by bradykinin to levels approximately one-third of those obtained without antagonization. These results are in agreement with the known intrinsic agonistic activity of this bradykinin-receptor antagonist.

To explore the possibility that IL-1 $\beta$  and bradykinin synergize in inducing IL-6, diploid fibroblasts and the Malavu cell line were treated for 24 h with different combinations of both agents and the IL-6 titers were determined in the culture fluids. Figure 2A and B illustrates the presence of synergy in that the IL-6 responses (in  $\log_{10}$  units/ml) to bradykinin increased when IL-1 was added, and that the magnitude of the increments due to addition of bradykinin was independent of the IL-1 dose applied.

Synergy between IL-1 and bradykinin was confirmed by additional experiments of similar design done on a different diploid cell strain ( $E_6SM$ ) as well as on several tumor cell lines. It can be seen from Table 1 that the response of the  $E_6SM$  cells to IL-1 and to bradykinin was less pronounced than that of the  $E_1SM$  cell strain. Synergy is evident from the comparison between IL-6 yields measured in cultures treated with IL-1/bradykinin combinations and those calculated by summation of the yields (units/ml) observed in cultures treated with each inducer only.

In the next experiment on  $E_1SM$  and Malavu cells we analyzed the time kinetics of IL-6 production following a dose of 10 U/ml IL-1 $\beta$  or  $10^{-4}$  M bradykinin, or a combination of both (Fig. 3A and B). After 8 h, bradykinin-induced IL-6 reached a maximum titer. IL-6 production in cultures treated with IL-1 $\beta$  alone or in combination with bradykinin continued to increase over the entire 48-h observation period. Again synergy between the two agents is apparent from comparison of the IL-6 yields. Bradykinin when applied alone seems to accelerate rather than to increase the production of IL-6.

Finally, it was assessed whether synergism between bradykinin and cytokines is restricted to IL-1 $\beta$ . Therefore  $E_6SM$  cells were treated with IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , or poly(I:C) in the absence and presence of bradykinin. As shown in Table 2, significant IL-6 induction was seen only in cells treated with IL-1 $\alpha$ , IL-1 $\beta$ , or dsRNA. Weak induction of IL-6 was observed with TNF- $\alpha$  and IFN- $\gamma$ , while the other cytokines did not significantly augment baseline production. Addition of bradykinin, while synergistic with IL-1 and IFN- $\gamma$ , acted all but additively with the other stimulants tested.

## DISCUSSION

Bradykinin and IL-1 were found to induce production of IL-6 in human diploid fibroblasts, as well as in a hepatoma-derived cell line (Malavu). The bradykinin-induced response was faster but less pronounced than that induced by IL-1. Experiments in

TABLE 1. SYNERGISTIC INDUCTION OF IL-6 BY BRADYKININ AND IL-1 $\beta$  IN DIFFERENT CELL LINES

Treatment <sup>a</sup>		IL-6 yield ( $\log_{10}$ units/ml) in cultures of				
BK ( $10^{-4}$ M)	IL-1 $\beta$ (10 U/ml)	$E_1SM$	$E_6SM$	Malavu	Bowes	MG-63
—	—	2.0	0.8	1.1	1.7	1.7
+	—	3.2	1.0	2.4	1.7	1.2
—	+	4.2	2.8	3.7	1.8	2.6
+	+	5.2	3.7	4.6	1.8	2.5
		(4.2) <sup>b</sup>	(2.8)	(3.7)	(2.0)	(2.6)

<sup>a</sup>IL-6 samples harvested after 24 h incubation with indicated stimulants.

<sup>b</sup>Expected yield without synergy, as calculated by summation of IL-6 yields (in units/ml) obtained after stimulation with bradykinin or IL-1 $\beta$  only.

TABLE 2. SYNERGISTIC INDUCTION OF IL-6 BY BRADYKININ AND IL-1 IN DIPLOID FIBROBLASTS: COMPARISON WITH OTHER CYTOKINES OR POLY(rI) · POLY(rC)

Cytokine	Dose	Bradykinin	
		Absent	Present
—		0.8 <sup>a</sup>	1.0
IL-1 $\alpha$	2.5	2.3	3.1 (2.3) <sup>b</sup>
(U/ml)	25	2.9	3.7 (2.9)
IL-1 $\beta$	1	2.5	3.3 (2.5)
(U/ml)	10	2.8	3.7 (2.8)
IL-2	0.1	0.7	1.0 (1.2)
(U/ml)	1	0.7	0.7 (1.2)
	10	1.2	1.2 (1.4)
IFN- $\alpha$	5	0.7	0.8 (1.2)
(U/ml)	50	0.8	0.9 (1.2)
	500	1.0	1.2 (1.3)
IFN- $\beta$	5	0.5	0.7 (1.1)
(U/ml)	50	0.7	0.8 (1.2)
	500	1.0	1.2 (1.3)
IFN- $\gamma$	0.35	1.1	2.0 (1.4)
(ng/ml)	3.5	1.3	2.1 (1.5)
	35	1.6	2.0 (1.7)
TNF- $\alpha$	0.5	1.0	1.2 (1.3)
(ng/ml)	5	1.2	1.6 (1.4)
	50	1.4	1.8 (1.5)
poly-IC	5	2.4	2.1 (2.4)
( $\mu$ g/ml)	50	3.0	3.2 (3.0)

<sup>a</sup>IL-6 yield (log U/ml) after incubation of E<sub>6</sub>SM cultures with indicated combination of cytokine and bradykinin ( $10^{-4}$  M).

<sup>b</sup>In parentheses: expected yield without synergy, as calculated by summation of IL-6 yields (in units/ml) obtained after stimulation with bradykinin or the cytokine only.

which both inducers were applied simultaneously revealed a synergistic interaction.

Synergy of bradykinin with IL-1 has been described for the production of PGE<sub>2</sub> in synovial cells, fibroblasts or colonic tissue (5–7,14). Therefore, our results extend the spectrum of this synergy to the case of IL-6-production by different cell types, including diploid human fibroblasts. Although others have reported that TNF- $\alpha$  is a good inducer of IL-6, our data indicate that TNF- $\alpha$  is a poor inducer of IL-6 in fibroblasts as compared to IL-1. This observation confirms previous reports (15,16). Further, no synergy between TNF- $\alpha$  and bradykinin could be observed for the induction of IL-6 from diploid fibroblasts.

Bradykinin is known to stimulate release of IL-1 and TNF from macrophages (14). Hence bradykinin may be involved in a positive feedback loop in which it first activates macrophages to release monokines, which in turn enhance responsiveness of

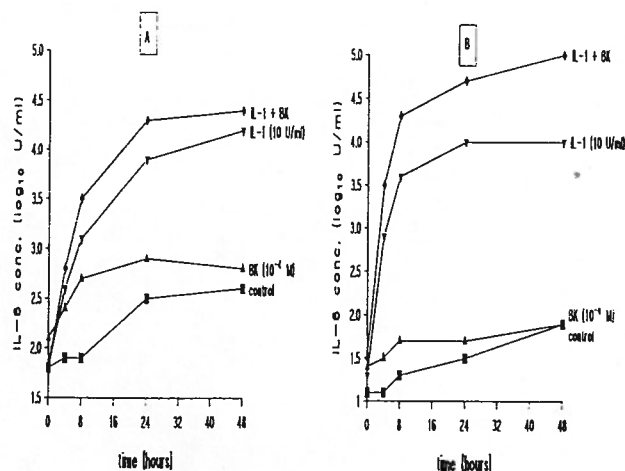


FIG. 3. Time course curve of IL-6-production by diploid fibroblasts (A) and Malavu cells (B) stimulated with IL-1 $\beta$  and bradykinin. Confluent cultures of E<sub>1</sub>SM fibroblasts (A) and the Malavu cell line (B) were treated with bradykinin ( $10^{-4}$  M), IL-1 $\beta$  (10 U/ml), a combination of both, or were left untreated. Culture fluids were assayed for IL-6-production after different time periods.

bradykinin-sensitive tissues. This mechanism may contribute to the further regulation of the inflammatory response by enhancing the production of secondary mediators (PGE<sub>2</sub>, IL-6, and possibly others). Furthermore, interactions between cytokines and noncytokine peptides are not limited to bradykinin. Substance P, neurokinins A and B, bombesin, and thrombin too have been shown to interact with the regulatory functions of IL-1 (14,17). In addition, somatostatin, substance P, neurokinins A and B, and vasoactive intestinal polypeptide have been described to enhance the production of IL-1, TNF, and interferons (18–22).

The studies on PGE<sub>2</sub> induction in synovial cells have shown that bradykinin alone is relatively ineffective, but does give rise to a dose-dependent induction when its application is preceded by treatment with IL-1 or TNF (5,18). Our findings suggest that bradykinin may act as an inflammatory mediator in both normal and already inflamed tissue, but that its efficiency increases when it acts in cooperation with cytokines, as is the case in inflammation.

Bradykinin exerts many inflammatory activities: it enhances vascular permeability, stimulates arachidonic acid metabolism, induces pain, and affects vascular tone (23). Thus, it has been implicated in rhinitis (24), septic shock (2), and chronic inflammatory diseases (18) such as arthritis (5,6). The induction of IL-6 by bradykinin has not been reported previously, and its biological significance is not clear. IL-6 may exert antiinflammatory effects by suppressing the production of IL-1 and TNF (25,26) and by inducing the production of acute phase proteins (27). This suggests that IL-6 induction by bradykinin, in the absence or presence of other inducers such as IL-1, serves the purpose of moderating the proinflammatory effects of these substances.

Due to regulatory effects on smooth muscle contraction, bradykinin is involved in cardiac, vascular, pulmonary, uterine, and gastrointestinal physiology (23). It is possible therefore that synergy between cytokines and bradykinin may be of physiological and pathological importance in certain noninflammatory processes. This has been described for vascular smooth muscle (28-30), and may in part explain the therapy-resistant hypotension in septic shock.

In conclusion, our study documents the existence of synergy between members of the kinin and cytokine families, thus illustrating cross-influences between the neurohumoral and the immune system.

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