IL-2 Enhancing Factor(s) in B Cell Supernatants from Patients with Rheumatoid Arthritis or Systemic Lupus Erythematosus

KOHTARO TOMURA, HOIL KANG, KOH MITAMURA, MASAMI TAKEI, MIKI KARASAKI, SHIGEO KOYASU* and SHIGEMASA SAWADA

The Division of Clinical Immunology, the First Department of Internal Medicine, Nihon University School of Medicine, Tokyo 173, and *Department of Cell Biology, the Tokyo Metropolitan Institute of Science, Tokyo 113

TOMURA, K., KANG, H., MITAMURA, K., TAKEI, M., KARASAKI, M., KOYASU, S. and SAWADA, S. IL-2 Enhancing Factor(s) in B Cell Supernatants from Patients with Rheumatoid Arthritis or Systemic Lupus Erythematosus. Tohoku J. Exp. Med., 1989, 159 (3), 171–183 — Culture supernatants of B cells from patients with rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) in the active stage enhanced interleukin 2 (IL-2) dependent proliferation of CTLL A/J cells. This activity, designated B cell-derived growth-enhancing factor-2 (BGEF-2), was recovered by gel filtration of a molecular weight between 15,000 and 20,000. BGEF-2 itself did not show IL-2 activity nor IL-1 activity, and BGEF-2 activity was not detected in the following cytokines: Interferon-α (IFN-α), interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 6 (IL-6). Furthermore, BGEF-2 was distinguishable from B cell-derived growth-enhancing factor described in a previous paper [Kang et al. (1987) J. Immunol., 139, 1154-1160]. BGEF-2 was produced by B cells from patients with RA or SLE only when the patients were in the active stage. BGEF-2 enhanced IL-2-dependent growth of peripheral blood T cells from patients with active RA, but did not enhance the growth of T cells from healthy volunteers. These results suggest that BGEF-2 is a B cell-derived lymphokine which plays an important role in the pathogenesis of RA and SLE. ——— RA ; SLE ; IL-2 ; BGEF-2 ; B-cell

Immunologic abnormalities such as hypergammaglobulinemia and the production of autoantibodies accompany rheumatic diseases (Decker et al. 1979; Delfaissy et al. 1980). It is known that in rheumatoid arthritis (RA) the synovial
cells produce interleukin 1 (IL-1), which may be involved in the pathogenesis of bone cartilage destruction (Floyd et al. 1982; Fontana et al. 1982). A decrease in the ability of T cells to produce interleukin 2 (IL-2), a deficiency in the response of T cells to IL-2 and polyclonal B cell activation have been reported in patients with RA and systemic lupus erythematosus (SLE) (Abdou et al. 1976; Linker-Israeli et al. 1983; Miyasaka et al. 1984; Comb et al. 1985).

Several reports have suggested the possibility that cytokines are involved in the pathogenesis of rheumatic diseases (Morgan and Weigle 1980; Jurgensen et al. 1986; Muraguchi et al. 1986; Romangnani et al. 1986; Dobashi et al. 1987). We recently have reported a new lymphokine, B cell-derived growth-enhancing factor (BGEF) produced by a B cell line established from peripheral blood lymphocytes of a patient with RA (Kang et al. 1987). BGEF enhances IL-1 activity in several respects, such as IL-1-induced proliferation of murine thymocytes and IL-1-induced production of IL-2.

Here we report that the supernatants from B cells of patients in the active stage of RA or SLE enhanced IL-2-induced proliferation of a murine-cloned T cell line, CTLL A/J. We also describe the properties of this new factor, designated B cell-derived growth-enhancing factor-2 (BGEF-2), which differs from other cytokines such as IL-1, IL-2, interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interferon (IFN)-α, IFN-γ, tumor necrosis factor (TNF) and BGEF.

PATIENTS AND METHODS

Patients

The group of RA patients consisted of 30 individuals, 21 women and 9 men, age 27 to 56, all of whom fulfilled the criteria of the American Rheumatism Association (ARA) (Ropes et al. 1958) and included 25 patients in the active stage. All patients with RA were taking nonsteroidal anti-inflammatory drugs or low doses of prednisolone (2.5 to 10 mg/day).

The group with SLE consisted of 21 individuals, 16 women and 5 men, aged 14 to 60, all of whom fulfilled the ARA revised criteria for the classification of SLE (Tan et al. 1982) and included 5 patients in the active stage.

All patients with inactive SLE were taking low doses of prednisolone (2.5 to 10 mg/day). The activity of RA was assessed according to the ARA classification, including the duration of morning stiffness, grasping power, evaluation of the articular index and erythrocyte sedimentation rate. Active SLE was evaluated by the criteria of Budman et al. (1977) as the presence of clinically identifiable active disease (convulsion, serositis, nephritis, arthritis) in at least one organ system, not including the skin. All patients were graded as having active or inactive SLE by their attending physicians.

The control group consisted of 15 healthy aged-matched individuals.

Cell preparation

Heparinized peripheral blood (about 60 ml) was treated with silica suspension (KAC-2; Ohtsuka assay, Tokyo), incubated at 37°C for 1 hr, and then centrifuged through a lymphocyte-separating medium (LSM; Litton Bionetics, Kensington, UK) at 1,500 rpm for 30 min. The intermediate layer containing lymphocytes was collected, and the cells were washed three times with RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA) medium. These lymphocytes were passed through a G-10 (Pharmacia Fine Chemicals A B, Uppsala,
B Cell Derived IL-2 Enhancing Factor of RA or SLE Patients

Sweden) column to remove residual monocytes. Finally these cells were passed through a nylon wool column to obtain B cells and T cells by a slight modification of the method of Julius et al. (1973).

In brief, 0.5 g of nylon wool (Wako Pure Chemical Industries, Osaka), and the column were equilibrated with RPMI 1640 medium containing 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, MA, USA). Four milliliters of cell suspension (1-5 x 10^6 cells) was applied to column, and the column was incubated in air with 5% CO_2 at 37°C for 1 hr and then washed with approximately 60 ml of RPMI 1640 medium. The passed cells were collected and used as the T cell-enriched fraction.

A fraction enriched with B lymphocytes was recovered from the column by mechanical agitation. The nylon wool was removed from the syringe and compressed vigorously in RPMI 1640 medium. T cells remaining (about 30%) in the B cells-enriched fraction were removed by cytotoxic treatment with monoclonal anti-leu-4 antibody (Becton Dickinson, Mountain View, CA, USA) plus rabbit complement, according to the method of Falkoff et al. (1987).

After the removal of T cells by cytotoxic treatment, the B cell fraction contained more than 94% B cells, as determined by flow cytometry using a FACS analyzer (Becton Dickinson, Sunnyvale, CA, USA) with FITC-conjugated goat F (ab')^2 anti-human immunoglobulin (Tago Inc., Burlingame, CA, USA).

T cells prepared by passage through the nylon wool column twice contained more than 85% T cells, as determined by staining with monoclonal anti-leu-4 antibody.

Furthermore, the purity of these cells was established by the proliferative response to mitogens. The fractions of monocytes in both fractions were less than 2%, as determined by nonspecific esterase staining or staining with anti-leu-M3 antibody (Becton Dickinson).

B cell and T cells were cultured in microtiter plates at a cell density of 1 x 10^6 cells/ml in RPMI 1640 containing 10% FCS in air with 5% CO_2 at 37°C for 72 hr. RPMI 1640 medium was generally supplemented with 10 mM HEPES, 50 U/ml penicillin G, 50 μg/ml streptomycin and 0.2% sodium bicarbonate. Each supernatant was obtained as a B cell supernatant or T cell supernatant.

Phytohemagglutinin (PHA) was obtained from Difco Laboratories (Detroit, MI, USA).

Gel filtration

Gel filtration was performed using a calibrated Sephacryl S-200 column (1.3 x 45 cm, 15 ml/hr, Pharmacia Fine Chemical A B, Uppsara, Sweden) equilibrated with phosphate buffered saline. Marker proteins used were bovine serum albumin (BSA; m.w. 67,000), ovalbumin (m.w. 45,000) and soybean trypsin inhibitor (m.w. 22,000).

Biological assay of IL-1

Murine (C3H/HeN mice; Charles River, Tokyo) thymocytes were separated into peanut agglutinin (PNA; E, Y. Laboratories Inc., San Mateo, CA, USA) agglutinated and nonagglutinated cells (PNA^- thymocytes) according to the method of Reisner et al. (1976). As indicator cells, 5 x 10^5 cells in 100 μl of PNA^- thymocytes were incubated in microtiterplates with 50 μl of sample solution with or without other stimuli, at 37°C for 55 hr, followed by 16 hr pulse labeling with 0.5 μCi [methyl^3H] thymidine/well (Amersham International, Amersham, Tokyo). For the determination of [methyl^3H] thymidine incorporation, cells were harvested onto a labomesh LM-101 glass filter, and the acid insoluble radioactivity was determined by a liquid scintillation spectrophotometer. IL-1 (Genzyme, Boston, MA, USA) activity was also examined by fibroblasts. Cells from the fibroblast cell line CCD-1SK (Dainippon Pharmaceutical Co., Tokyo) were washed and suspended in Dulbecco's MEM (Nissui Seiyaku Co., Tokyo) at a cell density of 1 x 10^6 cell/ml. A 100 μl portion of these cells was incubated in microtiterplates at 37°C in air with 5% CO_2 for 16
The cells were then cultured in medium containing IL-1 with or without B cell supernatant at 37°C in air with 5% CO₂ for 48 hr, followed by 16 hr pulse labeling with 0.5 μCi [methyl³H] thymidine/well (Schmidt et al. 1982).

**IL-2 assay.** IL-2 (Fuji Rebio, Tokyo) was determined by DNA synthesis of the indicator T cell clone, CTLL A/J, according to the method described previously (Koyasu et al. 1983). Samples serially diluted twofold with RPMI 1640 containing 5% FCS to 100 μl/well. Indicator cells (2 × 10⁴ cells in 100 μl of the same medium) were added subsequently and incubated at 37°C for 20 hr, followed by 4 hr pulse labeling with 0.5 μCi [methyl³H] thymidine/well.

Percent effect was calculated by the following equation:

\[
\text{Percent effect} = \left( \frac{(\text{IL-2} (0.5 \text{ U/ml}) + \text{B cell supernatant (50 μl/well)}) \text{ cpm} - \text{IL-2} (0.5 \text{ U/ml}) \text{ cpm}}{\text{IL-2} (0.5 \text{ U/ml}) \text{ cpm}} \right) \times 100
\]

One unit/ml of IL-2 induces 50% of maximum DNA synthesis of 5 × 10⁴/ml CTLL A/J cells.

**IL-4 assay.** IL-4 was determined by DNA synthesis of IL-4 dependent cell clone, Sez 627, according to the method of Maeda (Maeda et al. 1988). Indicator cells (2 × 10⁴ cells in 100 μl of medium) were incubated with samples at 37°C for 68 hr, followed by 4 hr pulse labeling with 0.5 μCi [methyl³H] thymidine/well.

**IL-5 assay.** IL-5 was determined by using BCLI tumor assay, according to the method of Kinashi et al. (1986). BCLI spleen cells were cultured for 2 days with the indicated additions and the medium was assayed for IgM by radioimmunoassay.

**IL-6.** IL-6 was determined by DNA synthesis of the IL-6 dependent cell line, B45.3, according to Dr. H. Karasuyama (personal communication; Department of Immunology, Tokyo University School of Medicine). Indicator cells (5 × 10⁴ cells in 100 μl of RPMI medium containing 5% FCS) were incubated with samples at 37°C for 42 hr, followed by 6 hr pulse labeling with 0.5 μCi [methyl³H] thymidine/well.

**Interferon (IFN) assay.** IFN activity was determined by the reduction of Sindbis virus growth on FL cells as previously described (Virelizier et al. 1977).

**TNF assay.** The cytolytic activity is based on the ability of TNF to lyse A673 cells in the presence of cyclohexide, as measured by crystal violet dye uptake of residual viable cells (Yonehara et al. 1989).

### Results

**Culture supernatants of B cells from patients with RA and SLE enhanced IL-2-induced proliferation of T cell clone and thymocytes**

When CTLL A/J cells were cultured with a suboptimal concentration of IL-2 in the presence of culture supernatants of B cells from patients with active RA or SLE, these supernatants enhanced IL-2-induced proliferation of CTLL A/J cells (Fig. 1). IL-2-induced proliferation of PNA-thymocytes was also enhanced by the supernatant (data not shown). In contrast, culture supernatant of B cells from patients with inactive or healthy individuals did not show this enhancing activity. In addition, T cells from patients with RA or SLE or healthy individuals did not show this enhancing activity.

When the B cell supernatants were subjected to gel filtration through Sephacryl S-200, enhanced IL-2-induced cell proliferation of PNA-thymocytes activity was recovered in compounds with a molecular weight ranging between 15,000 and
20,000 (Fig. 2A, B). These fractions enhanced the IL-2-induced proliferation of CTLL A/J cells in a dose-dependent manner, although they did not induce the proliferation of CTLL A/J cells in the absence of IL-2 (Fig. 3) nor enhance the maximum level of DNA synthesis induced by IL-2. It was reported that BGEF did not possess this IL-2-enhancing activity on CTLL A/J cells (Kang et al. 1987). So we have concerned this IL-2-enhancing activity is differed from BGEF previously described. The test of the following experiments we used the peak fractions (tube Nos. 38-40 in Fig. 2) designated BGEF-2. The peak fractions were obtained from B cell supernatants of all patients with RA and SLE in the active stage. BGEF-2 was not obtained from T cell supernatants from these patients. On several patients with RA and SLE in the active stages, diminution of BGEF-2 activity was found when they were in remission stage after treatments (data not shown). In all subjections to gel chromatography, IL-1 enhancing activity named as BGEF, a protein with a m.w. 60-65 kD, was not observed in any of these supernatants.

IL-1 activity measured by thymocyte proliferation was not detected in any fractions. In addition, BGEF-2 did not induce the proliferation of fibroblasts nor enhanced in IL-1-induced proliferation of fibroblasts, indicating that BGEF-2 is different from IL-1 (data not shown).
Effect of BGEF-2 on CTLL A/J cells

Kinetics of the proliferation of CTLL A/J cells induced by a combination of IL-2 and BGEF-2 were studied (Fig. 4A and B). Significant enhancing activity of BGEF-2 on IL-2-induced proliferation of CTLL A/J cells was observed during cultivation up to 36 h as measured by both thymidine incorporation and cell counts.

When CTLL A/J cells which had been exposed to BGEF-2 were washed and cultured further with IL-2 alone, enhancing activity of BGEF-2 on IL-2-induced proliferation of CTLL A/J cells was not observed (data not shown).
Comparison between BGEF-2 and other cytokines

We examined whether other known cytokines exhibit the BGEF-2 activity. IFN-α and γ were used at the concentration of 100-10,000 U/ml and IL-4 and IL-5 were used at 1-100 U/ml. IL-6 and TNF-α were used at the concentrations of 0.05-5 ng/ml and 0.05-10 ng/ml, respectively. No cytokine had any IL-2 enhancing activity on CTLL A/J cell proliferation at any concentrations (Fig. 5).

In reverse, these cytokines activities of BGEF-2 were examined. BGEF-2 did not have activities of IL-4, and IL-6. (Table 1) TNF activity of BGEF-2 (25% v/v) was not detected (<100 pg/ml). INF activity of BGEF-2 (25% v/v) was not detected (<0.3 IU). IL-5 activity of BGEF-2 (25% v/v) was not detected (personal communication from Dr. T. Honjo, Kyoto University, Japan).

Effect of BGEF-2 on the proliferation of peripheral blood T cells

Several reports on deficient reaction of T cells from patients with RA and SLE to IL-2 have been reported (Linker-Israeli et al. 1983; Miyasaka et al. 1984; Comb et al. 1985).

Effect of BGEF-2 on the proliferation of peripheral blood T cells was
Fig. 4. Effect of BGEF-2 on the proliferation of CTLL A/J cells by IL-2. A and B, CTLL A/J cells were cultured for 12 hr, 24 hr and 36 hr with IL-2 in the absence (○—○) or presence (●—●) of BGEF-2 (25% v/v), and DNA synthesis was determined by pulse labeling of the cells with (³H) thymidine (A) or cell count (B). BGEF-2 alone (△—△) had no effect on cell proliferation. The results are expressed as the mean cpm (A) or cell count/well (B) of triplicate cultures.

Fig. 5. BGEF-2 activity (i.e., IL-2 enhancement activity in CTLL A/J cells) of other cytokines. No cytokine had any IL-2 enhancement effect on CTLL A/J cell proliferation at any concentrations. In this figure each concentration as followed: INFα and INFγ were used at 10,000 μg/ml. IL-4 and IL-5 were used at 100 μg/ml. IL-6 and TNF at the concentration of 100 μg/ml. Open circles and bars refer to the mean cpm and s.d. of triplicate cultures.
B Cell Derived IL-2 Enhancing Factor of RA or SLE Patients

Table 1. Lymphokine dependent cell proliferation

<table>
<thead>
<tr>
<th></th>
<th>IL-6 dependent cell 1)</th>
<th>IL-4 dependent cell 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(B 45.3)</td>
<td>(Sez627)</td>
</tr>
<tr>
<td>Medium</td>
<td>4140 cpm</td>
<td>3737 cpm</td>
</tr>
<tr>
<td>Human rIL-6</td>
<td>100 u/ml</td>
<td>18876</td>
</tr>
<tr>
<td></td>
<td>1 u/ml</td>
<td>15015</td>
</tr>
<tr>
<td>Human rIL-4</td>
<td>10 u/ml</td>
<td>n.t.</td>
</tr>
<tr>
<td></td>
<td>1 u/ml</td>
<td>25319</td>
</tr>
<tr>
<td>BGEF-2</td>
<td>25% v/v</td>
<td>12601</td>
</tr>
<tr>
<td></td>
<td>4586</td>
<td>3937</td>
</tr>
</tbody>
</table>

n.t., not tested.

1) B 45.3 cells were cultured with IL-6 (100 u/ml, 1 u/ml) or BGEF-2 (25% v/v). B 45.3 cells (5 × 10⁴ cells in 100 µl of RPMI 1640 containing 5% FCS) were incubated with IL-6 or BGEF-2 at 37°C for 42 hr, followed by 6 hr pulse labeling with 0.5 µCi [methyl ³H] thymidine/well. B 45.3 cells were not proliferative by BGEF-2.

2) Sez627 cells were cultured with IL-4 (10 u/ml, 1 u/ml) or BGEF-2 (25% v/v). Sez627 cells (2 × 10⁴ cells in 100 µl of RPMI 1640 containing 5% FCS) were incubated with IL-4 or BGEF-2 (25% v/v) at 37°C for 68 hr, followed by 4 hr pulse labeling with 0.5 µCi [methyl ³H] thymidine/well. Sez627 cells were not proliferative by BGEF-2.

examined. Neither IL-2 nor BGEF-2 induced any significant proliferation of peripheral blood T cells without stimulation by PHA (Fig. 6).

In the presence of 5 µg/ml of PHA, 0.5 U/ml of IL-2 induced the proliferation of T cells from healthy individuals to the maximum level and was not

![Fig. 6. Effect of BGEF-2 on IL-2 dependent proliferation of human peripheral blood T cells. The T cells (1 × 10⁶/ml) were prepared as described in Patients and Methods. T cells from patients with RA or from healthy individuals were cultured for 48 hr with or without suboptimal doses of PHA (5 µg/ml). The results are expressed as the mean cpm of triplicate cultures. Their mean values and s.d. are given by open circles and bars.](image-url)
enhanced by BGEF-2. In contrast, the same concentration of IL-2 did not induce the maximum level of DNA synthesis in T cells from the patients with active RA. However the maximum level of DNA synthesis in these T cells from patients with active RA was observed when BGEF-2 was added to the culture.

**DISCUSSION**

In the present study, we found that B cells from patients with active RA or SLE produced a factor which did not show IL-1 nor IL-2 activity but which enhanced IL-2-induced proliferation of T cells. This factor, designated BGEF-2, has a molecular weight between 15,000 and 20,000. BGEF-2 was not produced by B cells from healthy individuals or T cells from patients with active disease, suggesting that BGEF-2 is produced only by B cells in patients with active RA or SLE and may play a crucial role in the pathogenesis of RA and SLE.

In a previous paper (Kang et al. 1987), we reported the discovery of a factor produced by a B cell line established from the peripheral blood lymphocytes of a patient with RA. The cell line, TKS-1, produced a factor designated BGEF which enhanced IL-1- and IL-2-induced proliferation of murine thymocytes (Kang et al. 1987). We also have reported that B cell supernatants from patients with RA possess activity which enhances IL-1-induced proliferation of murine thymocytes (Kang et al. 1987). However, in several respects, BGEF-2 as described in this paper, is distinguishable from IL-1 enhancing factor named BGEF as described in the previous paper. BGEF has a molecular weight in the range of 60,000 to 65,000, whereas BGEF-2 has a molecular weight between 15,000 and 20,000.

BGEF enhanced IL-1 activity as measured by the induction of the proliferation of murine thymocytes and the production of IL-2 from T cells, but did not enhance IL-2-induced proliferation of an IL-2-dependent T cell line. In contrast, BGEF-2 enhanced IL-2-induced proliferation of the IL-2-dependent T cell line, murine thymocytes and human peripheral blood T cells, but did not enhance the IL-1-induced proliferation of fibroblasts. Neither factor showed IL-1 or IL-2 activity. From these results, we concluded that BGEF-2 was distinct from BGEF. The factor produced by TKS-1 cells, and designated BGEF, should be named BFEF-1, hereafter.

Recent studies have suggested that several cytokines are involved in the pathogenesis of rheumatic diseases. Several reports have show that IFN-α and IFN-γ possess B cell growth activity and are able to act synergistically with IL-2 on B cell proliferation (Morgan and Weigle 1980; Romangnani et al. 1986). IL-4, IL-5 and IL-6 have been implicated in polyclonal B cell activation in rheumatic diseases (Jurgensen et al. 1986; Muraguchi et al. 1986; Dobashi et al. 1987). Thus, we attempted to establish the identity of BGEF-2 by comparing its activity to those of other cytokines (Fig. 5). The results clearly indicated that BGEF-2 in different form those cytokines at any concentrations, and that
BGEF-2 is a unique cytokine produced by B cells from patients with active RA and SLE.

A deficiency in the reaction of T cells to IL-2 has been reported in patients with RA or SLE (Linker-Israeli et al. 1983; Abdou et al. 1984; Miyasaka et al. 1984; Comb et al. 1985). In the presence of BGEF-2, peripheral blood T cells from patients with RA showed maximal proliferation, comparable to that of healthy individuals (Fig. 6). This suggests that BGEF-2 can restore the ability of T cells to react to IL-2 in patients with RA during the active stage of the disease, when B cells are producing BGEF-2.

Recent studies reported that deficient IL-2 production of the lymphocytes from the patients with RA and SLE was restored by several stimulations. Elevated soluble IL-2 receptor levels in the sera and synovial fluids of the patients with RA was reported (Keystone et al. 1988). So the system of IL-2 and IL-2 receptors is thought to be very important in explaining about chronic immunemediated inflammation of RA and SLE.

Moreover, Everson et al. (1988) reported IL-2 enhancing factor(s) derived from dendric cells from synovial effusions of the patients with RA. It is unknown whether it is identical to our BGEF-2 or not, but our paper strongly supported their findings.

BGEF-2 activity was produced by B cells harvested from patients with active RA or SLE. The mechanisms triggering the production of BGEF-2 by B cells and the involvement of BGEF-2 in the pathogenesis of rheumatic diseases are extremely interesting and should be investigated further in other studies.

Acknowledgments

We are grateful to Dr. T. Honjo (Kyoto University, Japan) for providing valuable materials (IL-4, IL-5) and critical discussion for his examination of IL-4 and IL-5 activities about BGEF-2. We thank Dr. T. Hirano (Osaka University, Japan) for providing human IL-6 and for his examination of IL-6 activity about BGEF-2.

We also thank Dr. I. Yahara for his valuable discussion and encouragement.

References


129-134.


