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Effects of bestatin on phagocytic cells in cyclophosphamide-treated mice

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Abstract:
The low-molecular weight dipeptide bestatin is a potent inhibitor of aminopeptidase N and has been demonstrated to have antitumor and immunomodulatory effects. The effects of bestatin on interleukin (IL)-1β synthesis and release by peritoneal macrophages stimulated *in vitro* with lipopolysaccharide (LPS) from *E. coli,* the phagocytic and oxidative burst activity from peripheral blood monocytes and granulocytes and the number of blood leukocytes and blood picture in cyclophosphamide-treated mice were tested. Bestatin at doses of 1 and 0.1 mg/kg was injected into cyclophosphamide-treated mice *ip* five times on alternating days or ten times at 24 h intervals. The first dose of bestatin was administered 24 h after a single injection of cyclophosphamide at a dose of 350 mg/kg. It was found that bestatin administered at doses of 1 and 0.1 mg/kg five times on alternating days increased the synthesis and release of IL-1β by resident peritoneal murine macrophages stimulated *in vitro* with LPS in cyclophosphamide-treated mice. The immunocorrecting action of bestatin on the picture of peripheral blood in cyclophosphamide-treated mice was primarily observed with young forms of neutrophilic granulocytes. The changes were observed irrespective of the dosage and the number of subsequent doses applied. Moreover, the administration of bestatin after pharmacological immunosuppression partially prevented the suppressive effects of cyclophosphamide on the oxidative burst activity of peripheral blood monocytes and stimulated the phagocytic activity of granulocytes.

Key words:
bestatin, cyclophosphamide, phagocytosis, oxidative burst, IL-1β, mice

Abbreviations: APN – aminopeptidase N, BE – bestatin, CD – cluster of differentiation, CFU-GM – colony forming unit-granulocyte/macrophage, Con A – concanavalin A, CSF – colony stimulating factor, CY – cyclophosphamide, DCH – delayed-cutaneous hypersensitivity, DTH – delayed-type hypersensitivity, FBS – fetal bovine serum, FMLP – formyl-methionyl-leucyl-phenylalanine, GM-CSF – granulocyte/macrophage colony stimulating factor, HLA-DR – major histocompatibility complex 2, IL – interleukin, LPS – lipopolysaccharide, LTBMC – long-term human bone marrow cultures, MIP-1α – macrophage inflammatory protein, PBS – phosphate buffered saline, PMA – phorbol 12-myristate 13-acetate, TGF-β – transforming growth factor

Introduction

Macrophages, monocytes and neutrophils are phagocytes that are important in the immunological response and immunity of macroorganisms. The cells primarily function in innate immunity because they are not able to recognize the antigens specifically; however, they are also involved in the mechanisms of acquired immunity. Therefore, the ability to modulate the functions of these cells may be therapeutically beneficial for the treatment of many infectious diseases.

Bestatin (ubenimex), a low-molecular weight dipeptide, is known to be a biological response modifier [21]. The drug is also known for its antitumor [7, 10, 11, 22, 24], antibacterial [9], antiviral [4, 30, 31] and antifungal [2] effects as a result of a direct activity in the cells of the immune system. Moreover, this agent stimulates the activity of macrophages [37]. As shown previously, bestatin activates the tumoricidal properties in mouse peritoneal macrophages *in vitro* and tumor cytotoxicity in macrophages of mice treated with the drug [33]. Bestatin also increases the cytotoxic activity of peripheral blood lymphocytes and spleen cells of cancer patients due to the activation of macrophages [15]. In addition, the mitogenic action of this immunomodulator on lymphocytes is also connected with the stimulation of macrophages [14].

Previous studies have shown that bestatin has many diverse effects on the production of cytokines [19, 25, 27, 38]. It stimulates the humoral immune response [17, 31, 39], hematopoiesis [1], augments delayed-type hypersensitivity (DTH) to SRBC and restores the impaired DTH to SRBC and delayed cutaneous hypersensitivity (DCH) to oxazolone [13, 39]. Bestatin inhibits aminopeptidases, especially aminopeptidase N (APN) [3, 18, 36], which is identical to the cell surface molecule CD13 [20]. Membrane-bound APN/CD13 is distributed in hematopoietic cells of myeloid origin and outside of the hematopoietic system in epithelial, endothelial and fibroblast cells. Overexpression of APN/CD13 has been observed in various inflammatory diseases and cancers [3]. As an inhibitor of APN/CD13, bestatin has shown beneficial effects in the treatment of some inflammatory diseases and types of cancer [3, 28, 29]. It is currently used in Japan as an immunomodulator and antitumor drug.

However, there are little data about the influence of bestatin on the immune response impaired by immunosuppressants. Therefore, the purpose of the present study was to determine the effects of bestatin in different dosages and schedules of treatment on the activity of peritoneal macrophages and peripheral blood monocytes and granulocytes as well as on the number of blood leukocytes and blood picture in cyclophosphamide-treated mice. Cyclophosphamide, an alkylating agent used in cancer treatment and autoimmune diseases, is also used in experimental immunopharmacology to induce immunosuppression and estimate the immunocorrecting action of the drugs or substances considered as immunomodulators.

Materials and Methods

Animals

The studies were conducted on female Balb/c mice (8 weeks of age), with each weighing 20 g. The mice were kept under conventional conditions. The animals were fed a commercial, granulated food and water *ad libitum*. The experimental animals were obtained from the Breeding Center of Laboratory Animals at the Institute of Occupational Medicine, Łódź, Poland. The principles of laboratory animal care (NIH publication No. 86–23, revised 1985) as well as the specific national laws on the protection of animals were followed. The study protocol was approved by the Local Ethics Committee in Wrocław, Poland (No. 08/2008).

Drugs and treatment

Pharmacological immunosuppression was induced by a single intraperitoneal (*ip*) injection of cyclophosphamide (Endoxan – Baxter) administered at a dose of 350 mg/kg. Bestatin (in subst., Sigma) at doses of 1 and 0.1 mg/kg was injected into cyclophosphamide-immunosuppressed mice *ip* five times at 48-h intervals or ten times at 24-h intervals. The first dose of bestatin was administered 24 h after the cyclophosphamide injection. Bestatin and cyclophosphamide were dissolved in phosphate buffered saline (PBS, Institute of Immunology and Experimental Therapy, Wrocław, Poland). The trials in the control groups were conducted in parallel. The mice in the control groups received PBS instead of bestatin or cyclophosphamide. The volume of each dose of bestatin, cyclophosphamide or PBS was 0.2 ml per animal. Each experimental group consisted of eight mice.

Measurements

The following indices were measured: (i) the production of interleukin-1β (IL-1β) in the culture supernatants of peritoneal macrophages stimulated *in vitro* with lipopolysaccharide (LPS) from *E. coli* (055:B5, Sigma); (ii) the number of leukocytes in the blood and blood picture; (iii) the phagocytic activity of the blood monocytes and granulocytes; and (iv) the oxidative burst activity of the blood monocytes and granulocytes. The level of IL-1β was determined 24 h after

the last bestatin injection, whereas the number of leukocytes in the blood, the blood picture and the phagocytic and the oxidative burst activity of the blood granulocytes and monocytes were determined 24 and 72 h after the last bestatin administration.

Cytokine assay

The mice were anesthetized with halothane (Narcotan, Zentiva, Prague, Czech Republic) and euthanized by cervical dislocation. Peritoneal exudate macrophages were harvested in sterile, ice-cold PBS with two antibiotics: penicillin 10 U/ml and streptomycin 1 μg/ml (Penicillin-Streptomycin Solution Stabilized, Sigma). The cells were washed and suspended in RPMI-1640 medium (Institute of Immunology and Experimental Therapy, Wrocław, Poland) supplemented with 10% fetal bovine serum (FBS) (Sigma), 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma) and antibiotics (10 U/ml penicillin and 1 μg/ml streptomycin, Sigma), adjusted to a concentration of 1.5 × 10^6 cells/ml, and dispensed in 100 μl volumes in a 96-well flat bottom plate (Sarstedt Inc., Newton, USA). The medium with non-adherent cells was replaced after 3 h incubation at 37°C in normal atmosphere with 5% CO_2. The incubation was continued, and the medium was replaced after 20 h by the medium without FBS but containing LPS from *E. coli*, serotype 055:B5 (Sigma), at a concentration of 2.5 μg/ml. After 24 h of incubation, the supernatants were removed and stored at –70°C [5].

A commercial ELISA kit (Quantikine, R&D Systems, Minneapolis, USA) was used to determine the murine IL-1β (pg/ml) levels in macrophage culture supernatants, according to the manufacturer's instructions. Each sample was tested in duplicate.

Preparation of blood smears

Blood smears were performed on microscopic glass and stained with Giemsa and May-Grunwald reagents. The preparations were analyzed using 1000× magnification in immersion oil. Up to 100 cells were counted on two preparations for each mouse (eight mice per group). The results are presented as the mean values (in total number) for each cell type (lymphocytes, bacilliform neutrophils, segmented neutrophils, basophils, eosinophils and monocytes).

The phagocytic and the oxidative burst activity

The blood samples were collected from retro-ocular arteries of halothane-anesthetized mice in tubes containing a heparin anticoagulant (Equimed, Kraków, Poland). Next, the mice were euthanized by cervical dislocation.

Commercial Phagotest and Bursttest (Phagoburst) kits were used according to the manufacturer's instructions (ORPEGEN Pharma, Heidelberg, Germany).

Fluorescence was analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson Biosciences) and the CellQuest 3.1f. software.

The Phagotest kit contains fluorescein-labeled opsonized *E. coli* (*E. coli*-FITC) and the necessary reagents, which allowed us to measure the percentage of phagocytosing blood granulocytes and monocytes (ingestion of one or more bacteria per cell) and their mean fluorescence intensity (the number of bacteria per cell).

The Bursttest kit contains unlabeled opsonized *E. coli*, phorbol 12-myristate 13-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (FMLP) as stimulants and the necessary reagents. The Bursttest allowed us to determine the leukocyte oxidative burst by measuring the percentage of blood granulocytes and monocytes producing reactive oxygen radicals and their mean fluorescence intensity (enzymatic activity).

Statistical analysis

The differences between the groups were assessed using the one-way analysis of variance (ANOVA) and *post-hoc* analysis using the Scheffé test using STATISTICA 9.1 software. A p value of < 0.05 was considered significant.

Results

The effect of bestatin on the synthesis and release of IL-1β by peritoneal macrophages in cyclophosphamide-treated mice

A single dose of cyclophosphamide (350 mg/kg) did not induce the synthesis and release of IL-1β by murine peritoneal macrophages stimulated *in vitro*

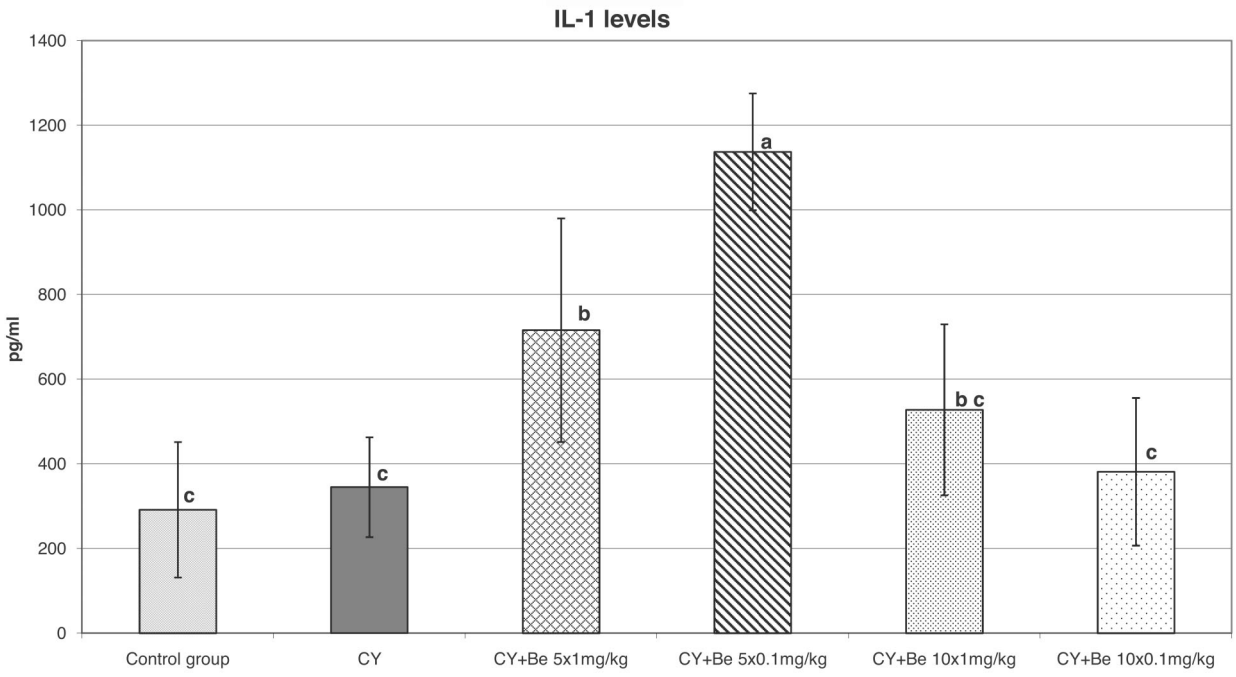

Fig. 1. The effects of bestatin (BE) administered five times on alternating days or ten times at 24-h intervals on IL-1β production by peritoneal macrophages stimulated *in vitro* with LPS in cyclophosphamide (CY)-treated mice; the data represent the mean values (n = 8) and standard deviation. Data with different letters show significant differences ($p < 0.05$)

with LPS from *E. coli* (2.5 μg/ml cell culture) on day 12 after injection of this immunosuppressive drug.

Administration of bestatin after a single injection of a high dose of cyclophosphamide induced the synthesis and release of IL-1β by mouse peritoneal macrophages stimulated *in vitro* with LPS. The effect of bestatin was dependent on the dosage and the number of doses applied. The strongest stimulating effect was observed after five injections of bestatin on alternating days at a dose of 0.1 mg/kg. In this experiment, the concentration of IL-1β increased to greater than three-fold as compared to the control group. In contrast, five injections of bestatin at 48-h intervals at a dose of 1 mg/kg doubled the synthesis and release of IL-1β by mouse peritoneal macrophages stimulated *in vitro* with LPS. No significant differences were observed after ten injections of bestatin (0.1 or 1.0 mg/kg) at 24-h intervals (Fig. 1).

The effects of bestatin on the number of blood leukocytes and the blood picture in cyclophosphamide-treated mice

A single administration of cyclophosphamide at a dose of 350 mg/kg decreased the number of peripheral blood leukocytes and lymphocytes, monocytes, and bacilliform neutrophils on days 12 and 14 following exposure to the drug (Tabs. 1 and 2).

The administration of bestatin after exposure to a high dose of cyclophosphamide partially improved the picture of the peripheral blood in cyclophosphamide-treated mice. However, bestatin administered five or ten times at two subsequent doses did not change the effect of cyclophosphamide on the number of blood leukocytes in the mice. As can be seen in Tables 1 and 2, the exposure to bestatin at doses of 0.1 and 1 mg/kg administered five or ten times completely prevented the suppressive action of cyclophosphamide on the absolute number of bacilliform neutrophils. The strongest stimulating effect was observed after ten injections of bestatin at 24-h intervals at doses of 0.1 mg/kg and 1 mg/kg on day 12 after immunosuppression. In this experiment, the absolute number of bacilliform neutrophils increased by half, as compared to the control group (Tab. 2). The effect of bestatin on the absolute number of lymphocytes was dependent on the dosage, the number of doses applied and the time after exposure to cyclophosphamide. The immunocorrecting effect on the absolute number of lymphocytes was observed on day 14 after

Tab. 1. Changes in the composition of blood cell types in cyclophosphamide (CY)-treated mice after administration of bestatin (BE) five times on alternating days; the data represent the mean values (n = 8) and standard deviation

Number of cells (10^3/µl)	Hours	Group			
		Control	CY 1 350 mg/kg	CY + BE 5 1 mg/kg	CY + BE 5 0.1 mg/kg
Leukocytes	24	7.7 ± 2.3[a]	3.6 ± 0.8[b]	5.0 ± 0.8[b]	4.1 ± 0.9[b]
	72	6.8 ± 1.5[a]	2.9 ± 0.7[b]	4.6 ± 1.1[b]	4.4 ± 1.2[b]
Lymphocytes	24	5.9 ± 1.7[a]	2.4 ± 0.6[b]	3.2 ± 0.6[b]	2.8 ± 0.5[b]
	72	5.2 ± 1.1[a]	1.9 ± 0.5[b]	2.9 ± 0.8[b]	2.8 ± 0.7[b]
Bacilliform neutrophils	24	0.3 ± 0.1[a]	0.03 ± 0.03[b]	0.4 ± 0.06[a]	0.3 ± 0.05[a]
	72	0.3 ± 0.08[a]	0.03 ± 0.03[c]	0.4 ± 0.2[b]	0.3 ± 0.07[ab]
Segmented neutrophils	24	1.2 ± 0.4	1.0 ± 0.2	1.2 ± 0.3	0.9 ± 0.3
	72	1.1 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.4
Basophils	24	0.08 ± 0.08	0.04 ± 0.04	0.03 ± 0.03	0.01 ± 0.02
	72	0.03 ± 0.04	0.02 ± 0.02	0.01 ± 0.02	0.0 ± 0.0
Eosinophils	24	0.04 ± 0.04	0.05 ± 0.03	0.06 ± 0.05	0.05 ± 0.04
	72	0.04 ± 0.04	0.04 ± 0.02	0.05 ± 0.02	0.03 ± 0.03
Monocytes	24	0.1 ± 0.05[a]	0.06 ± 0.03[b]	0.09 ± 0.05[ab]	0.04 ± 0.04[b]
	72	0.1 ± 0.07[a]	0.05 ± 0.02[b]	0.02 ± 0.02[b]	0.02 ± 0.02[b]

Data with different superscript letters show significant differences ($p < 0.05$)

Tab. 2. Changes in the composition of blood cell types in cyclophosphamide (CY)-treated mice after administration of bestatin (BE) ten times at 24 h intervals; the data represent the mean values (n = 8) and standard deviation

Number of cells (10^3/µl)	Hours	Group			
		Control	CY 1 350 mg/kg	CY + BE 10 1 mg/kg	CY + BE 10 0.1 mg/kg
Leukocytes	24	9.2 ± 1.0[a]	5.7 ± 1.0[b]	6.2 ± 1.7[b]	6.1 ± 1.2[b]
	72	4.9 ± 1.0	3.5 ± 1.2	3.9 ±1.0	4.7 ± 0.7
Lymphocytes	24	7.2 ± 1.0[a]	3.8 ± 0.7[b]	3.6 ± 1.0[b]	3.8 ± 0.7[b]
	72	3.8 ± 0.9[a]	2.2 ± 0.6[b]	2.9 ± 0.7[ab]	3.6 ± 0.5[a]
Bacilliform neutrophils	24	0.4 ± 0.2[b]	0.07 ± 0.07[c]	0.6 ± 0.2[a]	0.6 ± 0.09[a]
	72	0.3 ± 0.08[a]	0.06 ± 0.06[b]	0.2 ± 0.07[ab]	0.3 ± 0.09[a]
Segmented neutrophils	24	1.4 ± 0.2	1.6 ± 0.3	1.8 ± 0.5	1.6 ± 0.3
	72	0.6 ± 0.1	0.9 ± 0.3	0.6 ± 0.1	0.7 ± 0.1
Basophils	24	0.05 ± 0.05	0.05 ± 0.05	0.02 ± 0.03	0.02 ± 0.03
	72	0.03 ± 0.03	0.05 ± 0.02	0.04 ± 0.04	0.03 ± 0.02
Eosinophils	24	0.05 ± 0.05	0.08 ± 0.05	0.07 ± 0.06	0.07 ± 0.05
	72	0.04 ± 0.05	0.05 ± 0.03	0.05 ± 0.04	0.03 ± 0.02
Monocytes	24	0.1 ± 0.06[a]	0.09 ± 0.05[ab]	0.04 ± 0.04[b]	0.04 ± 0.03[b]
	72	0.09 ± 0.02	0.1 ± 0.06	0.1 ± 0.05	0.09 ± 0.04

Data with different superscript letters show significant differences ($p < 0.05$)

Tab. 3. The phagocytic activity of peripheral blood granulocytes and monocytes in cyclophosphamide (CY)-treated mice after administration of bestatin (BE) five times on alternating days or ten times at 24 h intervals; the data represent the mean values (n = 8) and standard deviation

Group	% of phagocytosing cells				Mean fluorescence intensity			
	Granulocytes		Monocytes		Granulocytes		Monocytes	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Control group	82.5 ± 4.5^{b}	85.6 ± 6.6	80.2 ± 4.7	77.5 ± 3.8^{a}	83.3 ± 6.6^{ab}	61.0 ± 10.8^{a}	59.0 ± 7.2	44.9 ± 5.0^{a}
CY 1 350 mg/kg	87.3 ± 6.1^{ab}	83.3 ± 6.8	68.5 ± 6.5	62.5 ± 11.9^{ab}	75.3 ± 10.4^{b}	44.8 ± 7.2^{b}	44.9 ± 12.2	33.5 ± 6.6^{b}
CY 1 350 mg/kg + BE 5 1 mg/kg	93.0 ± 3.7^{a}	85.1 ± 3.8	74.6 ± 6.1	62.8 ± 4.7^{ab}	97.0 ± 10.9^{ab}	44.2 ± 7.3^{b}	54.1 ± 15.1	33.0 ± 3.5^{b}
CY 1 350 mg/kg + BE 5 0.1 mg/kg	92.7 ± 2.8^{a}	83.8 ± 6.4	82.1 ± 4.3	59.2 ± 10.1^{b}	98.5 ± 11.0^{a}	43.6 ± 7.7^{b}	64.8 ± 8.5	33.0 ± 6.0^{b}
CY 1 350 mg/kg + BE 10 1 mg/kg	88.9 ± 5.1^{ab}	88.9 ± 4.0	73.8 ± 8.4	71.3 ± 5.0^{ab}	81.4 ± 10.2^{ab}	55.1 ± 7.9^{ab}	54.7 ± 10.7	39.4 ± 5.6^{ab}
CY 1 350 mg/kg + BE 10 0.1 mg/kg	90.3 ± 6.0^{ab}	88.7 ± 3.6	78.3 ± 9.0	65.4 ± 12.2^{ab}	80.0 ± 9.3^{ab}	54.1 ± 6.3^{ab}	48.9 ± 7.7	36.3 ± 7.3^{ab}

Data with different superscript letters show significant differences ($p < 0.05$)

the cyclophosphamide injection in the group administered ten times with 0.1 mg/kg bestatin (Tab. 2). However, bestatin was not able to reduce the suppressive effect of cyclophosphamide on the absolute number of blood lymphocytes in the remaining schedules of treatment (Tabs. 1 and 2). Bestatin did not exert an immunocorrecting effect on the absolute number of monocytes (Tab. 1). The drug administered ten times at all doses under investigation even decreased the absolute number of these cells on day 12 after the cyclophosphamide injection (Tab. 2).

The effects of bestatin on the phagocytic activity of granulocytes and monocytes in cyclophosphamide-treated mice

A single injection of a high dose of cyclophosphamide did not change the percentage of the phagocytosing granulocytes and monocytes. However, cyclophosphamide on day 14 after administration decreased the fluorescence intensity of granulocytes and monocytes, which indicated that the number of ingested bacteria per cell had been reduced (Tab. 3).

Five administrations of bestatin at doses of 0.1 and 1 mg/kg after exposure to a high dose of cyclophosphamide increased the percentage of granulocytes, indicating phagocytosis, as compared to the control group on day 12 after injection of the immunosuppressant. However, the number of ingested bacteria per granulocyte (intensity of fluorescence) increased only in the group administered 0.1 mg/kg of bestatin. In contrast, bestatin administered five times at a dose of 0.1 mg/kg decreased the percentage of monocytes, indicating phagocytosis, on day 14 after cyclophosphamide injection. On day 14 following the exposure to cyclophosphamide, the immunocorrecting action of bestatin on the reduced number of ingested bacteria per one granulocyte or monocyte was not observed (Tab. 3).

The effects of bestatin on oxygen burst activity of granulocytes and monocytes in cyclophosphamide-treated mice

As shown in Table 4, a single injection of cyclophosphamide did not change the percentage of granulocytes producing reactive oxidants and enzyme activity (fluorescence intensity). However, cyclophosphamide decreased the percentage of monocytes producing reactive oxygen metabolites and the enzyme activity of monocytes on day 14 after the injection. Bestatin at a dose of 1 mg/kg administered five times at 48 h intervals increased the percentage of granulocytes producing reactive oxidants on day 12 after cyclophosphamide treatment but decreased the enzyme activity (fluorescence intensity) of these cells on day 14 fol-

Tab. 4. The oxidative burst activity of peripheral blood granulocytes and monocytes in cyclophosphamide (CY)-treated mice after administration of bestatin (BE) five times on alternating days or ten times at 24 h intervals; the data represent the mean values (n = 8) and standard deviation

Group	% of cells producing reactive oxygen radicals				Mean fluorescence intensity			
	Granulocytes		Monocytes		Granulocytes		Monocytes	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Control group	77.7 ± 5.8^{b}	77.8 ± 7.8	25.0 ± 3.8	21.1 ± 5.3^{b}	6.1 ± 0.7	8.8 ± 1.5^{a}	6.9 ± 0.5	8.9 ± 1.9^{a}
CY 1 350 mg/kg	85.7 ± 6.8^{ab}	78.3 ± 5.1	31.0 ± 8.5	13.4 ± 5.1^{c}	7.5 ± 1.5	8.7 ± 1.6^{ab}	5.2 ± 0.6	5.7 ± 0.7^{b}
CY 1 350 mg/kg + BE 5 1 mg/kg	88.4 ± 4.4^{a}	75.2 ± 8.1	34.9 ± 3.0	30.4 ± 6.3^{ab}	7.3 ± 0.7	7.4 ± 0.9^{b}	6.1 ± 0.8	6.3 ± 0.9^{b}
CY 1 350 mg/kg + BE 5 0.1 mg/kg	85.0 ± 4.9^{ab}	83.5 ± 2.8	26.8 ± 3.9	37.1 ± 8.8^{a}	6.8 ± 0.5	8.8 ± 0.9^{ab}	5.9 ± 1.0	6.4 ± 0.8^{b}
CY 1 350 mg/kg + BE 10 1 mg/kg	86.6 ± 5.1^{ab}	85.1 ± 5.7	33.3 ± 9.7	32.2 ± 11.1^{ab}	7.3 ± 1.2	9.1 ± 1.3^{ab}	5.6 ± 0.9	6.4 ± 1.4^{b}
CY 1 350 mg/kg + BE 10 0.1mg/kg	86.3 ± 4.2^{ab}	85.3 ± 4.9	36.8 ± 8.7	35.3 ± 8.5^{ab}	7.6 ± 0.6	8.4 ± 1.1^{ab}	6.2 ± 1.3	5.7 ± 0.4^{b}

Data with different superscript letters show significant differences ($p < 0.05$)

lowing exposure to cyclophosphamide (compared to the control group). Moreover, bestatin, irrespective of the dosage and the number of subsequent doses applied, completely inhibited the suppressive effect of a high dose of cyclophosphamide on the percentage of the monocytes producing reactive oxidants. The strongest immunocorrecting action was observed after five injections of bestatin at a dose of 0.1 mg/kg on day 14 after immunosuppression. However, the drug was not able to restore the impairment by cyclophosphamide enzyme activity of monocytes (Tab. 4).

Discussion

The results obtained in the present study show that bestatin had a modulating effect on the synthesis and release of IL-1β by murine peritoneal macrophages stimulated *in vitro* with LPS from *E. coli*, the phagocytic and oxidative burst activity of peripheral blood monocytes and granulocytes and the blood picture in cyclophosphamide-treated mice.

Bestatin at doses of 1.0 and 0.1 mg/kg administered five times on alternating days increased the production and release of IL-1β by murine peritoneal macrophages in cyclophosphamide-treated mice. However, cyclophosphamide did not affect the release of pro-inflammatory cytokine IL-1β. Although, Bryniarski et al. [5] reported that peritoneal macrophages from cyclophosphamide-treated mice (50 mg/kg) or murine macrophages incubated *in vitro* with alkylating metabolites, acrolein (10^{-7} M) and phosphoramide mustard (10^{-6} M) increased production of pro-inflammatory IL-6 and IL-12, and the simultaneously decreased production of anti-inflammatory IL-10 and TGF-β cytokines. Shibuya et al. [35] also reported a stimulating effect of bestatin at concentrations of 1 and 100 μg/ml on the synthesis of IL-1, but they studied unstimulated murine peritoneal macrophages. The effect was even greater at lower concentrations (0.1, 1 and 10 μg/ml) in the presence of concanavalin A (Con A). Bestatin at a dose of 5 mg/kg administered to mice orally on days 5 and 3 prior to the removal of a sample of murine macrophages also augmented the release of IL-1. In our study, the enhanced synthesis and release of this cytokine was observed 24 h after the last dose of bestatin. However, in our experimental model, the schedule and the method used to measure the concentrations of IL-1β were different. Further, Lkhagvaa et al. [19] observed different effects of bestatin on the synthesis of cytokines by stimulated and unstimulated monocytes and macrophages. The

drug at concentrations of 10 and 50 μg/ml suppressed the synthesis of IL-6 and IL-8 by human monocytes stimulated *in vitro* with LPS. In addition, bestatin used at a higher concentration (50 μg/ml) also inhibited the production of MIP-1α (macrophage inflammatory protein) but increased the synthesis of IL-10. In contrast, bestatin increased the production of cytokines by unstimulated human monocytes. The drug at a concentration of 50 μg/ml increased the synthesis of IL-6 by these cells. Bestatin at a concentration of 50 μg/ml exhibited a suppressive action on the production of cytokines by alveolar macrophages isolated from patients with sarcoidosis. The drug inhibited the overproduction of IL-6 and IL-8 in these cells. The action of bestatin, i.e., inhibition of the synthesis of IL-6, IL-8 and MIP-1α, which are considered as pro-inflammatory cytokines and stimulation of the production of the anti-inflammatory IL-10 cytokine, allows us to propose that bestatin may be effective in the treatment of various inflammatory diseases [19].

IL-1 plays an important role in the development of inflammatory processes. Lkhagvaa et al. [19] reported the suppressive effect of bestatin on the production of pro-inflammatory cytokines (IL-6, IL-8 and MIP-1α). However, the results of our studies showed an increase in the synthesis of IL-1β after bestatin treatment, although there were some differences in the experimental protocol. In our study, bestatin was administered *in vivo* to mice and not to the cells in culture, which might be one of the reasons for the different results. Further studies are needed to explain the action of bestatin on the production of cytokines.

The results of the present study also show that bestatin exerts a partial immunocorrective effect on white blood cells in cyclophosphamide-treated mice. Bestatin increased the absolute count of lymphocytes and neutrophils (decreased by cyclophosphamide), but the effect was dependent on the dosage and the number of doses applied. The immunocorrecting action of bestatin was primarily observed with young forms of neutrophilic granulocytes (bacilliform neutrophils) because an increase in the absolute number of these cells was observed after five and ten exposures to the bestatin doses under investigation on days 12 and 14 after cyclophosphamide injections.

The results of earlier studies conducted *in vitro* [8] showed that bestatin exerted a stimulating effect on hematopoiesis in long-term human bone marrow cultures (LTBMC). Bestatin at concentrations of 0.1 and 1 μg/ml added to the culture at the onset and at each weekly medium change for 5 weeks activated the production of mature cells and myeloid progenitors in LTBMC through the stimulating action on the secretion of IL-6. Bestatin within the concentration ranges of 0.001–1 μg/ml augmented the G-CSF and GM-CSF-induced colony formation of human bone marrow cells [34]. It also enhanced the ability of L920 cell supernatant, which shows a macrophage-colony stimulating activity, to induce colony formation of mouse bone marrow progenitor cells, reaching the maximum effect at a concentration of 0.01 μg/ml [23]. Abe et al. [1] reported that a single intraperitoneal injection of bestatin within a dose range of 2.5–100 mg/kg increased the frequency and absolute number of CFU-GM in the mice. The effect was also observed after intravenous and oral administration. However, the dose of immunosuppressant was much lower (200 mg/kg) than in our study. The immunocorrecting action of bestatin was observed when the administration of bestatin began 2–24 h following the injection of cyclophosphamide but was not observed when bestatin was injected prior to the immunosuppressant. Okamura et al. [26] showed that bestatin at a concentration of 100 μg/ml incubated with human mononuclear cells for 24 h increased granulocyte-macrophage colony stimulating factor activity. Considering the results obtained in our study and reports by other authors, we can assume that bestatin may exert a stimulating action on normal and cyclophosphamide-suppressed hematopoiesis of mice by directly affecting the bone marrow progenitor cells and indirectly affecting the synthesis and release of colony stimulating factors and other cytokines by cells, such as macrophages and monocytes [1].

The purpose of the present study was to also investigate the immunocorrective action of bestatin on the phagocytic and oxidative burst activity of peripheral blood granulocytes and monocytes in cyclophosphamide-treated mice. As we have shown, the effect was dependent primarily on the kind of cells and, to a lesser extent, on the dosage and the number of subsequent doses applied.

The results of earlier studies conducted *in vitro* and *in vivo* [32] show that bestatin increased the phagocytic and oxidative burst activity of human monocytes and mouse macrophages. Bestatin also enhanced the spontaneous migration of human granulocytes and the migration of these cells in the presence of chemotactic stimuli and in the phagocytosis of human granulocytes. However, bestatin did not augment the oxida-

tive burst activity of these cells, as measured by their ability to reduce nitroblue tetrazolium [16].

Ino et al. [12] reported a stimulating effect of bestatin on human monocytes, which also confirms the ability of the drug to enhance the phagocytic and oxidative burst activity of these cells. These findings were also confirmed in our studies and other reports.

Neopterin, a low molecular compound derived from guanosine triphosphate, is released by activated monocytes and macrophages and is considered to be a reliable marker of activation of these cells. In addition, their activation was exhibited by an increased expression of a CD16 molecule, a low affinity receptor for IgG (FcgRIII), and an HLA-DR molecule belonging to the second class of the major histocompatibility complex on monocytes/macrophages.

The increased expression of CD16 and HLA-DR molecules on monocytes and release of neopterin by these cells after bestatin treatment was found in lymphoma patients after autologous bone marrow transplantation. Bestatin administered orally at low doses (10 or 30 mg/day) for 60 days increased the serum neopterin level, whereas high doses (90 or 180 mg/day) increased the expression of CD16 and HLA-DR molecules and the level of the granulocyte/macrophage colony stimulating factor (GM-CSF). However, bestatin did not affect the frequency and the absolute number of monocytes [12].

It is assumed that the activation of monocytes by bestatin stimulates the release of monokines by these cells, including CSF, which can explain the adjuvant action of the drug on the regeneration of the hematopoietic system and its stimulating action on the activity of T and B lymphocytes [12].

In conclusion, it can be stated that bestatin was able to stimulate phagocytic activity of peripheral blood granulocytes and restore the impaired oxidative burst activity of monocytes in cyclophosphamide-treated mice. Further, the drug restored the absolute number of neutrophils reduced by cyclophosphamide in a manner irrespective of the dosage and schedule of the treatment. The drug also increased IL-1β expression by peritoneal murine macrophages. We propose that bestatin, a potent immunocorrecting drug, may be of potential therapeutic value in the restoration of the immune status of patients undergoing chemotherapy.

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References:

1. Abe F, Matsuda A, Schneider M, Talmadge JE: Effects of bestatin on myelopoietic stem cells in normal and cyclophosphamide-treated mice. Cancer Immunol Immunother, 1990, 32, 75–80.
2. Aoyagi K, Itoh N, Abe F, Abe S, Uchida K, Ishizuka M, Takeuchi T, Yamaguchi H: Enhancement by ubenimex (bestatin) of host resistance to *Candida albicans* infection. J. Antibiot (Tokyo), 1992, 45, 1778–1784.
3. Bauvois B, Dauzonne D: Aminopeptidase-N/CD13 (EC 3.4.11.2.) Inhibitors: chemistry, biological evaluations and therapeutic prospects. Med Res Rev, 2006, 26, 88–130.
4. Bourinbaiar AS, Lee-Huang S, Krasinski K, Borkowsky W: Inhibitory effect of the oral immune response modifier, bestatin, on cell-mediated and cell-free HIV infection in vitro. Biomed Pharmacother, 1994, 48, 55–61.
5. Bryniarski K, Szczepanik M, Ptak M, Zemelka M, Ptak W: Influence of cyclophosphamide and its metabolic products on the activity of peritoneal macrophages in mice. Pharmacol Rep, 2009, 61, 550–557.
6. Chełmońska-Soyta A, Miller RB, Ruhnke L, Rosendal S: Activation of murine macrophages and lymphocytes by *Ureaplasma diversum*. Can J Vet Res, 1994, 58, 275–280.
7. Ezawa K, Minato K, Dobashi K: Induction of apoptosis by ubenimex (Bestatin) in human non-small-cell lung cancer cell lines. Biomed Pharmacother, 1996, 50, 283–289.
8. Fujisaki T, Otsuka T, Takamatsu Y, Eto T, Harada M, Niho Y: Effects of bestatin on hematopoiesis in long-term human bone marrow cultures. Biomed Pharmacother, 1995, 49, 69–74.
9. Harada Y, Kajiki A, Higuchi K, Ishibashi T, Takamoto M: The mode of immunopotentiating action of bestatin: enhanced resistance to *Listeria Monocytogenes* infection. J Antibiot (Tokyo), 1983, 36, 1411–1414.
10. Hirayama Y, Sakamaki S, Takayangi N, Tsuji Y, Sagawa T, Chiba H, Matsunaga T, Niitsu Y: Chemotherapy with ubenimex corresponding to patient age and organ disorder for 18 cases of acute myelogeneous leukemia in elderly patients-effects, complications and long-term survival. Gan To Kagaku Ryoho, 2003, 30, 1113–1118.
11. Ichinose Y, Genka K, Koike T, Kato H, Watanabe Y, Mori T, Iioka S et al., NK421 Lung Cancer Surgery Group: Randomized double-blind placebo-controlled trial of bestatin in patients with resected stage I squamous-cell lung carcinoma. J Natl Cancer Inst, 2003, 95, 605–610.
12. Ino K, Bierman PJ, Varney ML, Heimann DG, Kuszynski CA, Walker SA, Talmadge JE: Monocyte activation by an oral immunomodulator ubenimex (bestatin) in lymphoma patients following autologous bone marrow transplantation. Cancer Immunol Immunother, 1996, 43, 206–212.
13. Ishizuka M, Masuda T, Kanbayashi N, Fukasawa S, Takeuchi T, Aoyagi T, Umezawa H: Effect of bestatin on mouse immune system and experimental murine tumors. J Antibiot (Tokyo), 1980, 33, 642–652.
14. Ishizuka M, Sato J, Sugiyama Y, Takeuchi T, Umezawa H: Mitogenic effect of bestatin on lymphocytes. J Antibiot (Tokyo), 1980, 33, 653–662.

15. Iwahashi M, Tanimura H, Yamaue H, Tsunoda T, Tani M, Noguchi K, Mizobata S et al.: In vitro augmentation of cytotoxic activity of peripheral blood lymphocytes and spleen cells of cancer patients by ubenimex. Anticancer Res, 1994, 14, 1557–1562.
16. Jarstrand C, Blomgren H: Bestatin, a new immunomodulator, enhances migration and phagocytosis of human granulocytes in vitro. J Clin Lab Immunol, 1981, 5, 67–69.
17. Knoblich A, Müller WE, Harle-Grupp V, Falke D: Enhancement of antibody formation against herpes simplex virus in mice by the T-cell mitogen bestatin. J Gen Virol, 1984, 65, 1675–1686.
18. Kuramochi H, Motegi A, Iwabuchi M, Takahashi K, Horinishi H, Umezawa H: Action of ubenimex on aminopeptidase activities in spleen cells and peritoneal macrophages from mice. J Antibiot (Tokyo), 1987, 40, 1605–1611.
19. Lkhagvaa B, Tani K, Sato K, Toyoda Y, Suzuka C, Sone S: Bestatin, an inhibitor for aminopeptidases, modulates the production of cytokines and chemokines by activated monocytes and macrophages. Cytokine, 2008, 44, 386–391.
20. Look AT, Ashmun RA, Shapiro LH, Peiper SC: Human myeloid plasma membrane glycoprotein CD 13 (gp150) is identical to aminopeptidase N. J Clin Invest, 1989, 83, 1299–1307.
21. Mathé G: Bestatin, an aminopeptidase inhibitor with a multi-pharmacological function. Biomed Pharmacother, 1991, 45, 49–54.
22. Mishima Y, Terui Y, Sugimura N, Matsumoto-Mishma Y, Rokudai A, Kuniyoshi R, Hatake K: Continuous treatment of bestatin induces anti-angiogenic property in endothelial cells. Cancer Sci, 2007, 98, 364–372.
23. Nemoto K, Abe F, Karakawa K, Horinishi H, Umezawa H: Enhancement of colony formation of mouse bone marrow cells by ubenimex. J Antibiot (Tokyo), 1987, 40, 894–898.
24. Niimoto M, Hattori T: Prospective randomized controlled study on bestatin in resectable gastric cancer. Biomed Pharmacother, 1991, 45, 121–124.
25. Noma T, Klein B, Cupissol D, Yata J, Serrou B: Increased sensitivity of IL2-dependent cultured T cells and enhancement of in vitro IL-2 production by human lymphocytes treated with bestatin. Int J Immunopharmacol, 1984, 6, 87–92.
26. Okamura S, Omori F, Haga K, Baba H, Kawasaki C, Tanaka T, Sugimachi K, Niho Y: Ubenimex stimulates production of colony-stimulating factor from human peripheral mononuclear cells in vitro. Bestatin 16 ICC Proceedings, Jerusalem, Israel, 1989, June 11–16, p. 12.
27. Okura A, Ishizuka M, Takeuchi T: Effect of ubenimex on the production of murine interferon. J Antibiot (Tokyo), 1988, 41, 261–263.
28. Ota K: Review of ubenimex (Bestatin): clinical research. Biomed Pharmacother, 1991, 45, 55–60.
29. Ota K, Uzuka Y: Clinical trials of bestatin for leukemia and solid tumors. Biotherapy, 1992, 4, 205–214.
30. Pulido-Cejudo G, Conway B, Proulx P, Brown R, Izaguirre CA: Bestatin-mediated inhibition of leucine aminopeptidase may hinder HIV infection. Antiviral Res, 1997, 36, 167–177.
31. Sasaki S, Fukushima J, Hamajima K, Ishii N, Tsuji T, Xin K-Q, Mohri H, Okuda K: Adjuvant effect of Ubenimex on a DNA vaccine for HIV-1. Clin Exp Immunol, 1998, 111, 30–35.
32. Schorlemmer HU, Bosslet K, Dickneite G, Lüben G, Sedlacek HH: Studies on the mechanisms of action of the immunomodulator Bestatin in various screening test systems. Behring Inst Mitt, 1984, 74, 157–173.
33. Schorlemmer HU, Bosslet K, Sedlacek HH: Ability of the immunomodulating dipeptide bestatin to activate cytotoxic mononuclear phagocytes. Cancer Res, 1983, 43, 4148–4153.
34. Shibuya K, Chiba S, Hino M, Kitamura T, Miyagawa K, Takaku F, Miyazano K: Enhancing effect of ubenimex (bestatin) on proliferation and differentiation of hematopoietic progenitur cells, and the suppressive effect on proliferation of leukemic cell lines via peptidase regulation. Biomed Pharmacother, 1991, 45, 71–80.
35. Shibuya K, Hayashi E, Abe F, Takahashi K, Horinishi H, Ishizuka M, Takeuchi T, Umezawa H: Enhancement of interleukin 1 and interleukin 2 releases by ubenimex. J Antibiot (Tokyo), 1987, 40, 363–369.
36. Suda H, Aoyagi T, Takeuchi T, Umezawa H: Inhibition of aminopeptidase B and leucine aminopeptidase by bestatin and its stereoisomer. Arch Biochem Biophys, 1976, 177, 196–200.
37. Talmadge JE, Lenz BF, Pennington R, Long C, Phillips H, Schneider M, Tribble H: Immunomodulatory and therapeutic properties of bestatin in mice. Cancer Res, 1986, 46, 4505–4510.
38. Tsunogake S, Furusawa A, Nagashima S, Nakamura Y, Enokihara H, Shishido H: Effect of aminopeptidase inhibitors on the production of various cytokines by peripheral blood mononuclear cells and stromal cells and on stem cell factor gene expression in stromal cells: comparison of ubenimex with its stereoisomers. Int J Immunotherapy, 1994, 10, 41–47.
39. Umezawa H, Ishizuka M, Aoyagi T, Takeuchi T: Enhancement of delayed-type hypersensitivity by bestatin, an inhibitor of aminopeptidase B and leucine aminopeptidase. J Antibiot (Tokyo), 1976, 29, 857–859.

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