MITOGENIC EFFECT OF BESTATIN ON LYMPHOCYTES

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The incorporation of ^{3}H-thymidine into the acid insoluble fraction of spleen cells was increased by intraperitoneal injection of 10 μg bestatin/mouse. Bestatin (0.01, 0.1, 1.0 μg/ml) which was added to mouse spleen cell culture increased ^{3}H-thymidine incorporation into lymphocytes, but 10 μg/ml did not. This mitogenic action of bestatin was not observed when adherent cells were removed or T cells were destroyed, suggesting that bestatin causes the proliferate T cells probably through the activation of macrophages. Bestatin given to mice did not modulate mitogenicity of lectins. Bestatin treatment of human peripheral buffy coat cells increased ^{3}H-thymidine incorporation into lymphocytes. The addition of bestatin at a high concentration such as 100 μg/ml to mouse spleen cell cultures exhibited a high mitogenic effect on B cells in preference to T cells but this effect was not seen with bestatin at 50 μg/ml. In this case, the mitogenicity of Con A or LPS was expanded and antibody formation to SRBC in spleen cell cultures were also stimulated.

As reported in previous papers, bestatin[1] produced by streptomyces inhibits aminopeptidases which are membrane-associated enzymes[2], binds to cells and enhances immune responses[4,5]. Bestatin 0.1, 1, 10 or 100 μg/mouse enhanced delayed-type hypersensitivity to sheep red blood cells but 1,000 μg/mouse did not have this effect[4,5]. As reported in the preceding paper[6], bestatin restored delayed-type hypersensitivity reduced by intravenous injection of cyclophosphamide or by intraperitoneal inoculation of EHRLICH carcinoma cells and low doses, 1, 10 or 100 μg/mouse, suppressed the growth of slowly growing subcutaneous tumors of GARDNER lymphosarcoma and IMC carcinoma.

In this report, we will report the mitogenic effect of bestatin.

Materials and Methods

Mice

Specific pathogen-free CDF_1 mice (Balb/c $\times$ DBA/2, female, 8$\sim$12 weeks old) were obtained from Institute of Medical Science, University of Tokyo and were used throughout the experiments unless otherwise noticed.

Bestatin

Bestatin (Lot 751158TS, NK421, Lot 2) was dissolved in distilled water at 4 mg or 1 mg/ml for animal experiments or in RPMI 1640 (Nissui Seiyaku Co., Ltd. Tokyo) at 200 μg/ml for cell culture experiments. These solutions were diluted to appropriate concentration with distilled water or RPMI 1640 medium. Bestatin in 0.25 ml was given to each mouse. Bestatin in 0.05 ml was added to spleen cell culture.

Mitogens

Concanavalin A (Con A, Pharmacia Fine Chemicals AB. Uppsala, Sweden) and phytohaemagglutinin P (PHA-P, Difco Laboratories, Detroit, Mich.) were employed as T cell mitogens and Pokeweed mitogen (PWM, Grand Island Biological Co., N.Y.) and lipopolysaccharide (LPS, *Escherichia coli* 0111, Difco Laboratories, Detroit, Mich.) as B cell mitogens.

Mitogenic Action of Bestatin *in vivo*

Bestatin in 0.25 ml, 0.1, 1, 10, 100, or 1,000 μg/mouse, was given to mice intraperitoneally, 18 hours thereafter, spleen was taken from each mouse and teased to obtain spleen cells. Spleen cells were suspended in serum-free RPMI 1640 and washed once by $800 \times g$ centrifugation, and suspended in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS, Lot 90380, Microbiological Associates, Bethesda, Md., or Lot 527A Colorado Serum Co. Col.) at 1.5×10^6 cells/ml. Three ml of spleen cell suspension was placed in each glass tube (11×100 mm) with a Morton cap and was incubated at 37°C for 3 days in a fully humidified atmosphere of 5% CO_2 and air. Eighteen hours before assay, to each tube was added 1 μCi/tube of ^{3}H-thymidine (^{3}H-TdR, 6 ^{3}H-Thymidine NET-355, New England Nuclear, Boston, Mass.). Thereafter (18 hours after the addition), the incorporation of ^{3}H-TdR into DNA of cultured cells was determined. Cells from each tube were washed with 5 ml of a cold saline thoroughly and suspended in 0.1 ml of saline. The cell suspension was collected with a pipetman (Gilson France S. A., Villier-le-Bel, France) and was distributed on a glass filter (Whatman, 3MM. Whatman Inc., Clifton, N. J.). The filters were soaked in 5% trichloroacetic acid for 60 minutes, twice and were dried. The amount of ^{3}H-TdR incorporated into acid insoluble fraction was determined by liquid scintillation spectrometry and expressed as total count per minute (cpm) per tube.

Mitogenic Effect of Bestatin on Murine Lymphocytes *in vitro*

Spleen cells prepared from normal mice were suspended in RPMI 1640 medium supplemented with 20% heat-inactivated FCS at 15×10^6 cells/ml. This medium was used for cell culture throughout the experiments otherwise stated. Three ml aliquots of this cell suspension were distributed into Petri dish (Falcon 3002, Div. Becton, Dickinson, and Co. Oxnard, Calif.) and bestatin in 0.15 ml RPMI 1640 was added. They were cultured in a fully humidified atmosphere of 5% CO_2 and air at 37°C for 18 hours on a rocking platform (6~8 cycle/min, Bellco Glass Inc., Vineland, N. J.).

Thereafter, non-adherent cells in each culture were collected by centrifugation, and resuspended in 4 ml of medium. The four ml aliquots were placed on 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and centrifuged at $400 \times g$ for 40 minutes at 20°C. After centrifugation, lymphocytes layer was collected and washed with serum-free RPMI 1640 three times. Lymphocytes were suspended in the medium at 1.5×10^6 cells/ml, and 3 ml in a glass tube or 0.2 ml in a well of micro test plate (Falcon 3042) were incubated at 37°C for 3 days in a fully humidified atmosphere of 5% CO_2 and air. Triplicate cultures were performed for each condition. Eighteen hours before assay, ^{3}H-TdR 1 μCi/tube or 0.1 μCi/well was added to each culture and the incorporation of ^{3}H-TdR into cells was determined. The procedure to measure ^{3}H-TdR into cells placed in a glass tube was described above. The incorporation of ^{3}H-TdR into cultures in a microplate was measured by using a cell harvester (Labo Mash, Labo Science Co., Ltd., Tokyo). The amount of ^{3}H-TdR into cells was determined by a liquid scintillation spectrometry. In the case spleen cells were divided into two populations, T cell-depleted cell population and macrophage-depleted cell population. T cell-depleted cell population was prepared as follows: spleen cells were incubated with 0.2 ml of anti-Thy 1, 2 serum (Searle Diagnostic. High Wycombe, England) in 10.8 ml of serum-free RPMI 1640 at 5×10^7 cells/ml at 37°C for 60 minutes washed twice with serum-free medium and resuspended in 10.8 ml of serum-free medium containing 1 ml of mouse red cell-absorbed complement; one hour thereafter, cells were washed thoroughly and were adjusted to 1.5×10^6 cells/ml; each culture in a glass tube was contained 3 ml of cell suspension and incubated for 3 days; triplicate cultures were performed; the determination of ^{3}H-TdR incorporation was described already.

In a group of experiments, the bestatin-treated spleen cells were treated with anti-Thy 1, 2 serum before sedimentation by Ficoll-Paque. The procedure was the same as described above.

Macrophage-depleted cell population was prepared by methods reported by MOSIER[7] or ISHIZUKA *et al.*[8].

The mitogenic action of bestatin at a high concentration on spleen cell culture was tested. Spleen cells were suspended in RPMI 1640 supplemented with 10% FCS at 1.5×10^6 cells/ml and bestatin dissolved in 0.05 ml was added. In some groups, Con A or LPS was added at 0.5 μg/ml. Triplicate cultures were performed in 0.2 ml in sterile Micro test II plates (Falcon 3042). Plates were incubated for

3 days at 37°C in a humidified atmosphere of 5% CO_2 and air. The incorporation of ^{3}H-TdR was determined as described above.

Antibody Formation *in vitro*

Antibody formation to SRBC in dissociated spleen cell culture was tested by the method described by MISHELL and DUTTON[9] and CLICK *et al.*[10].

Mitogenicity of Bestatin on Human Peripheral Blood Lymphocytes *in vitro*

Human buffy coat cells, which included lymphocytes and monocytes were obtained from 20 ml of fresh venous blood in heparin by gravity sedimentation and were suspended in RPMI 1640 supplemented with 20% FCS at concentration of 4×10^6 cells/ml. Three ml aliquots were placed in a Petri dish (Falcon 3002) and added 0.2 μg/ml of bestatin in 0.05 ml. Buffy coat cells with bestatin were incubated at 37°C for 18 hours in a fully humidified atmosphere of 5% CO_2 and air on a rocking platform. Then, cells were washed and lymphocytes were collected by Ficoll-Paque sedimentation. The following procedures were described as above (Fig. 1).

Results

Mitogenicity of Bestatin in Mice

As shown in Fig. 2, when spleen cells were taken from mice 18 hours after intraperitoneal injection of mice and ^{3}H-TdR incorporation into DNA was examined. Bestatin 10 μg/mouse or more increased the incorporation of ^{3}H-TdR.

Fig. 2. The incorporation rate of ^{3}H-TdR into DNA of spleen cells of mice given bestatin 24 hours before culture.
The standard deviation did not exceed 8%.

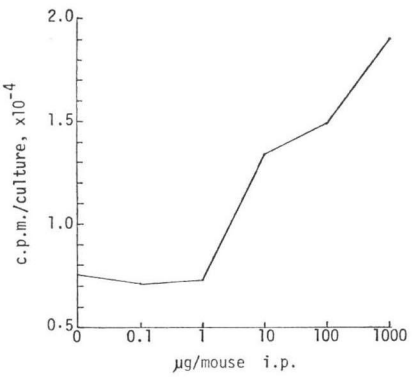


Fig. 1. Procedure for determination of mitogenicity of bestatin in low concentrations *in vitro*.

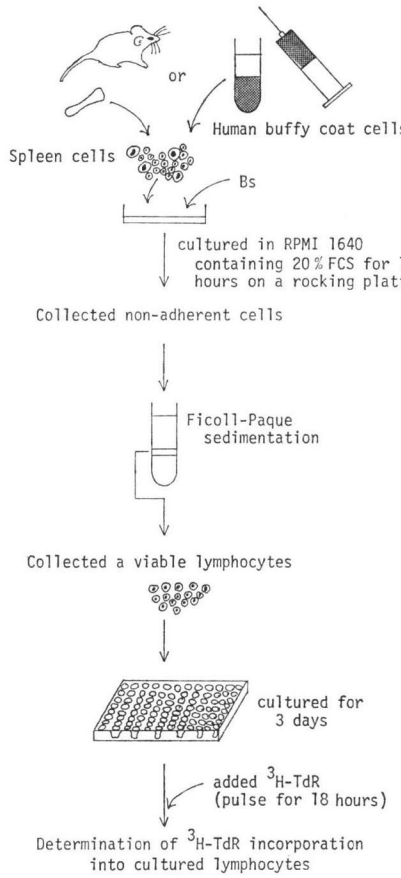


Table 1. Incorporation of ^{3}H-TdR into spleen cells taken from mice treated with bestatin in the presence and absence of mitogens.

Treatment of mice	Mitogens added to the culture c.p.m./culture		
	none	PHA (0.3%)	PWM (3.0%)
None	7,575	20,335	33,948
Bestatin 1,000 μg	19,015	24,822	42,533
〃 100 μg	14,794	20,321	32,889
〃 10 μg	13,415	22,796	33,748
〃 1 μg	7,279	18,713	29,575

Bestatin was given to mice and 24 hours thereafter, spleen cells were collected and 3 ml aliquots (4.5×10^6 cells) were cultured with or without mitogens in a glass tube for 3 days in 5% CO_2 and air. ^{3}H-TdR (1 μCi/culture) was added 18 hours before the harvest of cultured cells. Triplicate cultures were performed in each group. The standard deviation did not exceed 5%.

The rate of incorporation increased parallel to the increase of bestatin dose up to 1 mg/mouse. At this dose the incorporation reached the plateau. This effect of bestatin increasing ^{3}H-TdR incorporation can reflect its mitogenic effect on lymphocytes.

To determine whether bestatin augmented the mitogenic effect of mitogens on splenic lymphocytes, spleen cells taken from mice to which bestatin 10 μg or 1 mg was given were cultured with PHA or with PWM and the incorporation rate of ^{3}H-TdR into DNA of cells was measured. As shown in Table 1, the effect of mitogens on spleen cells taken from mice to which 1 mg of bestatin had been given was slightly enhanced. Bestatin 10 μg or 100 μg/mouse did not show any effect on mitogen-stimulated blastogenesis.

Mitogenic Effect of Bestatin on Murine Splenic Lymphocytes *In Vitro*

The mitogenic effect of bestatin at concentrations lower than 10 μg/ml *in vitro* was examined. Spleen cells which contained whole cell populations were treated with bestatin for 18 hours. After bestatin treatment, non-adherent cells were collected and washed thoroughly out to remove bestatin completely. The non-adherent cells thus obtained were sedimented in Ficoll-Paque and lymphocytes were collected. The lymphocytes thus obtained were cultured for 3 days and the incorporation of ^{3}H-TdR into cells were determined. As shown in Table 2, treatment with bestatin in lower doses enhanced incorporation of ^{3}H-TdR into splenic lymphocytes by 50% to 100%, although with a high dose, 10 μg/

Table 2. Incorporation of ^{3}H-TdR into lymphocytes separated from spleen cells pretreated with bestatin *in vitro*.

Bestatin pretreatment μg/ml	Mitogens added to cultures			
	Exp. I			Exp. II
	None (BS[1]/none)	c.p.m./culture		None (BS/none)
		Con A	LPS	
None	5,748 (1.00)	18,956	24,669	2,644 (1.00)
Bestatin 10	3,672 (0.63)	17,060	16,570	— —
〃 1	9,185 (1.60)	15,068	20,823	6,039 (2.28)
〃 0.1	8,831 (1.54)	24,667	22,966	5,055 (1.91)
〃 0.01	9,037 (1.57)	17,100	22,289	5,565 (2.10)
〃 0.001	6,788 (1.18)	17,523	20,962	— —

1) Bestatin
The standard deviation did not exceed 8%.

Table 3. Influence of fetal calf serum (FCS) on mitogenic action of bestatin (BS) to splenic lymphocytes *in vitro*.

Pretreatment with		Lymphocytes cultures			
BS	FCS	without FCS		with FCS	
		c.p.m./culture	BS/none	c.p.m./culture	BS/none
None	none	154	1.00	1,722	1.00
Bestatin	none	338	2.19	2,251	1.30
None	FCS	334	1.00	4,385	1.00
Bestatin	FCS	764	2.28	9,737	2.22

Spleen cells or lymphocytes were cultured with or without 20% FCS. Bestatin was added to spleen cell cultures at 1 μg/ml. The standard deviation did not exceed 8%.

Table 4. Correlation between cell cluster formation and incorporation of ^{3}H-TdR in lymphocyte cultures treated with or without bestatin.

Bestatin pretreatment	Lymphocyte cultures			
	No. of cluster/culture	BS[1]/none	c.p.m./culture	BS/none
None	112.3±12.6	1.00	5,964±257	1.00
Bestatin, 0.01 μg/ml	210.0± 5.1	1.87	13,270±433	2.22

1) Bestatin

Clusters were enumerated by microscopy (×200) 3 days after the start of the lymphocyte culture.

Table 5. Incorporation of ^{3}H-TdR to lymphocytes separated from whole spleen cells, T-depleted cells, and macrophage-depleted cells treated with bestatin.

Treatment of spleen cells	Bestatin treatment	Lymphocyte cultures	
		c.p.m./culture[1]	BS/none
None	None	17,979	1.54
	BS[2]	27,620	
T Cell-depletion	None	19,457	0.93
	BS	18,122	
Macrophage-depletion	None	15,208	0.99
	BS	15,136	

1) mean c.p.m. of triplicate cultures. The standard deviation did not exceed 5%.

2) Bestatin, 1 μg/15×10^{6} cells/ml

Table 6. Elimination of bestatin (BS) mitogenic action to lymphocytes by treatment with anti-Thy 1, 2 serum and complement.

T Cell-depletion after bestatin treatment	Treatment of whole spleen cell cultures c.p.m./culture		
	without BS (a)	with BS (b)	b/a
None	15,132	29,370	1.94
T Cell-depleted	14,715	12,534	0.85

Spleen cells were treated with 1 μg/ml of bestatin for 18 hours and were treated with anti-Thy 1, 2 serum and complement, then, the cells were sedimented in Ficoll-Paque. Lymphocytes were collected and cultured. The standard deviation did not exceed 5%.

ml, the effect was not observed. Mitogenicity of Con A or LPS on lymphocytes was not stimulated by the pretreatment with bestatin.

To determine whether FCS in medium influences the effect of bestatin, spleen cells were cultured with bestatin in medium with or without FCS and after sedimentation, lymphocytes from each culture were cultured in medium with or without FCS. As shown in Table 3, in all cases bestatin pretreatment stimulated the incorporation of ^{3}H-TdR. FCS addition to the medium, especially the addition to the lymphocyte culture increased ^{3}H-TdR incorporation.

The mitogenicity of bestatin on lymphocytes were accompanied with the formation of cell cluster in cultures. The cell clusters were consisted of a mass of cells, more than 100 cells, and were broken easily to single cells by dispersion using a pasteur pipette. Although clusters were also observed in lymphocyte cultures prepared from non-treated spleen cells, the number of clusters was significantly smaller than that of bestatin-treated group. The number of clusters in cultures was counted by a microscope at ×200. As shown in Table 4, bestatin pretreatment of spleen cells increased the cell clusters in lymphocyte cultures and this increase was in a good agreement with the incorporation of ^{3}H-TdR into lymphocytes.

In order to determine which lymphocytes in spleen cells are activated by pretreatment with bestatin to increase incorporation of ^{3}H-TdR, spleen cells taken from non-treated normal mice were divided into three cell populations: non-treated whole spleen cell population, T-depleted spleen cell population (Spleen cells were treated with anti-Thy 1, 2 serum and complement) and macrophage-depleted spleen cells (Adherent cells were removed). Thereafter, each cell population of these divided cells was cultured

in RPMI 1640 containing 20% FCS and 1.0 μg of bestatin on a rocking platform for 18 hours at 37°C in an atmosphere of 5% CO_2 and air. After 18 hours bestatin treatment, non-adherent cells were sedimented in Ficoll-Paque to remove dead non-adherent granulocytes. Thereafter, lymphocytes in each group were washed thoroughly, cultured for 3 days and the incorporation of ^{3}H-TdR into each culture was determined.

As shown in Table 5, lymphocytes separated from whole spleen cell culture treated with bestatin, resulted in increased incorporation of ^{3}H-TdR into lymphocytes by about 50%. On the contrary, bestatin treatment of T-depleted or macrophage-depleted spleen cell population did not increase the incorporation of ^{3}H-TdR. These results indicate that macrophages are required for the mitogenic action of bestatin to T cells.

In order to determine whether bestatin treatment causes the proliferation of T cells, whole spleen cells were cultured with bestatin for 18 hours and bestatin-stimulated lymphocytes were collected. Thereafter, this lymphocyte population was treated with anti-Thy 1, 2 serum and complement. After Ficoll-Paque sedimentation, lymphocytes were collected and cultured for 3 days and the incorporation of ^{3}H-TdR into lymphocytes was measured. As shown in Table 6, the incorporation into lymphocytes from bestatin-treated spleen cells was increased about 2 times, but by treating with anti-Thy 1, 2 serum and complement, this increase was eliminated. These results suggest that bestatin causes the proliferation of T cells probably through the activation of macrophages.

Mitogenicity of Bestatin on Human Peripheral Blood Lymphocytes *In Vitro*

Human buffy coat cells containing lymphocytes and monocytes were prepared from each 20 ml of venous blood of two normal healthy donors and cultured bestatin with 0.2 μg/ml. Eighteen hours thereafter, lymphocytes were collected and cultured for 3 days. As shown in Table 7, bestatin exhibited its mitogenic action on human peripheral lymphocytes. In two cases shown in Table 7, bestatin treatment of buffy coat cells caused to increase the incorporation of ^{3}H-TdR into lymphocytes about 2 or 3 times.

Table 7. ^{3}H-TdR incorporation to human lymphocytes separated from buffy coat cells treated with bestatin.

Buffy coat cells from	Bestatin treatment	Lymphocytes culture	
		c.p.m/ culture	Bestatin/ none
H. I.	None	2,060	1.00
	Bestatin	4,802	2.33
T. S.	None	990	1.00
	Bestatin	2,905	2.93

The standard deviation did not exceed 5%.

Mitogenicity of Bestatin Added to Spleen Cell Cultures and its Effect on Antibody Formation *In Vitro*

The effect of addition of high concentrations of bestatin to mouse spleen cell cultures was examined. Spleen cell cultures were added bestatin at 0.01, 0.1, 100 μg/ml and cultured for 3 days at 37°C in 5% CO_2 and air. The effect was examined by measuring the incorporation of ^{3}H-TdR into cells. As shown in Table 8, the incorporation of ^{3}H-TdR into spleen cells was increased only in the case of addition of bestatin 100 μg/ml and compared with the control, the incorporation was increased by about 5 times. The addition of 100 μg/ml was also stimulated mitogenic action of Con A and LPS on spleen cells. Bestatin and each mitogen were added to spleen cell cultures simultaneously. Bestatin less than 50 μg/ml showed no effect.

To determine which cell population in spleen is stimulated by addition of bestatin 100 μg/ml,

spleen cells were divided into three cell populations: whole spleen cells, macrophage-depleted and T cell-depleted spleen cells. These cell populations were cultured with bestatin 100 μg/ml for 3 days. The incorporation of ^{3}H-TdR was determined and stimulatory effect of bestatin was estimated by mitogenic index in each cell population. As shown in Table 9, addition of each bestatin dose, Con A or LPS alone increased the incorporation of ^{3}H-TdR into whole spleen cells. The addition of bestatin stimulated the incorporation 2.6 times. Bestatin at 100 μg/ml also increased the incorporation of ^{3}H-TdR into T cell-depleted spleen cells as well as non-treated whole spleen cells. Bestatin of macrophage-depleted spleen cells was observed with a reduction of about 50%. These results indicate that the high concentration of bestatin stimulates B cells, partly through activation of macrophages. The effect of Con A or LPS on macrophage-depleted spleen cells was reduced in both cases, particularly the mitogenic index of Con A or LPS was reduced by 86% or 63% compared with non-treated spleen cells. On T cell-depleted spleen cells, the mitogenic index of Con A was reduced markedly and the effect of LPS was affected slightly.

In combination with bestatin, although bestatin stimulated the mitogenic index of Con A on spleen cells by 40%, that of LPS was stimulated by more than 100%. The stimulatory effect on LPS was observed in parallel with the mitogenic index of bestatin alone, and on macrophage- or T cell-depleted spleen cells, the addition of bestatin also stimulated the mitogenicity of Con A or LPS in parallel with the index of bestatin alone. Results indicate that in combination with Con A and LPS, bestatin can enhance the mitogenicity of Con A and LPS.

These results suggest that the addition of bestatin at 100 μg/ml to spleen cells stimulate B cell and

Table 8. The mitogenic action of bestatin (BS) added to spleen cell cultures

Bestatin μg/ml	Addition to spleen cell cultures					
	none		Con A		LPS	
	c.p.m./culture	BS/none	c.p.m./culture	BS/none	c.p.m./culture	BS/none
100	3,678	4.99	26,631	1.55	12,844	2.92
1	699	0.95	18,843	1.09	4,304	0.98
0.01	777	1.05	15,842	0.92	4,321	0.98
0	737	1.00	17,232	1.00	4,388	1.00

Spleen cells (1.5×10^6 cells/ml) were cultured with or without bestatin and 0.5 μg/ml of Con A or LPS for 3 days. The cultures were performed in triplicate and the standard deviation did not exceed 5%.

Table 9. Mitogenic activity of bestatin added to macrophage- and T cell-depleted spleen cell cultures

Addition to cultures	Mitogenic index[1] of spleen cells					
	Non-treated		Macrophage-depleted		T Cell-depleted	
	None	Bestatin	None	Bestatin	None	Bestatin
None	1.0[a]	2.6	1.0[b]	1.5	1.0[c]	2.1
Con A	14.2 (1.0)[2]	19.9 (1.4)	2.3 (1.0)	3.6 (1.6)	1.8 (1.0)	3.9 (2.2)
LPS	8.7 (1.0)	18.5 (2.1)	3.2 (1.0)	4.5 (1.4)	6.1 (1.0)	11.6 (1.9)

a) 512±8.3 c.p.m./culture b) 4,739±112.3 c.p.m./culture c) 590±17.5 c.p.m./culture

1) Mitogenic index (M. I.) = $\frac{\text{c.p.m. of each cell culture added mitogens or/and bestatin}}{\text{c.p.m. of each cell culture alone}}$

2) A parenthesis: Ratio in cultures added mitogens = $\frac{\text{M. I. of each cell culture with bestatin}}{\text{M. I. of each cell culture without bestatin}}$

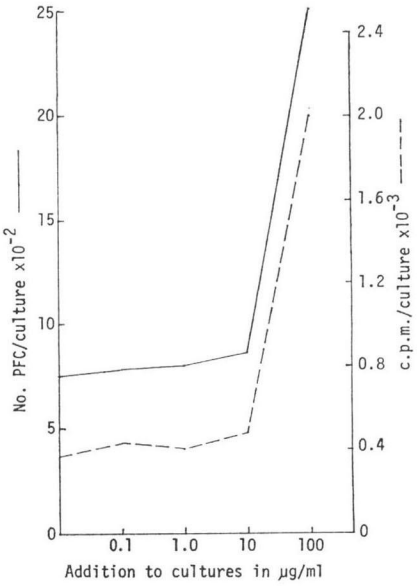
enhance to proliferate T cells slightly, since the addition could stimulate T cell-depleted spleen cells in almost parallel index that of non-treated spleen cells, and the stimulation may partly be mediated by macrophages. It is also shown that bestatin can enhance the mitogenicity of T and B cell mitogens.

The effect of bestatin on antibody formation was tested *in vitro*. Spleen cell cultures were added 1 μg to 100 μg/ml of bestatin and were cultured with SRBC as antigen for 4 days. The number of antibody-forming cells was enumerated in terms of plaque forming cells. As shown in Fig. 3, antibody-forming cells were increased about 3.4 folds in addition of bestatin 100μg/ml. The stimulatory effect was also observed in parallel with the incorporation rate of ^{3}H-TdR into spleen cells.

These effects of bestatin on spleen cell cultures were not observed in the case of its lower concentration than 10 μg/ml.

Fig. 3. Correlation between antibody formation and incorporation rate of ^{3}H-TdR in spleen cell cultures added bestatin.

The standard deviation did not exceed 10% in No. of PFC and 5% in c.p.m.



Discussion

As described in preceding reports[4,6], bestatin was effective in augmenting cell-mediated immunity in lower doses and retarded syngeneic solid tumors depending on timing of administration in mice. These effects of bestatin were not observed at higher doses, more than 1 mg/mouse. Therefore, the action of bestatin was studied in low doses or low concentrations, less than 100 μg/mouse or 10 μg/ml, on lymphoid cells. The administration of bestatin to mice stimulated the incorporation rate of ^{3}H-TdR into DNA of spleen cells in doses of more than 10 μg/mouse.

It was felt that the effect of bestatin reflects its mitogenic activity on murine lymphocytes and we attempted to determine its effect in spleen cell cultures. The addition of less than 10 μg/ml bestatin to the cell culture system did not show any effects. Therefore, spleen cells which include lymphocytes and macrophage were pretreated with bestatin. After pretreatment, lymphocytes were collected and incorporation rate of ^{3}H-TdR into lymphocyte was tested. Mitogenicity was observed in lower concentrations, 1 μg to 0.01 μg/ml. The effect was not observed at concentration of 10 μg/ml.

The effect of bestatin was only observed in lymphocytes from bestatin-treated spleen cells which included T cells and adherent cells but not in lymphocytes from bestatin-treated spleen cells from which T cells and/or adherent cells were removed. Results shown in Tables 6 and 7 indicate that bestatin activated macrophages and/or T cells and the activation provides proliferation of T cells. Hence, we have two possible explanation concerning the mitogenicity of bestatin: (1) Bestatin primarily activates T cells, which produce lymphokines for activation of macrophages and macrophage activation stimulates T cell proliferation. (2) Bestatin primarily activates macrophages and macrophage activation provides to stimulate proliferation of T cells. In both cases, however, bestatin stimulates proliferation of T cells through activation of macrophages[11,12].

Recently, MÜLLER *et al.*[13] showed that administration of bestatin provide to increase incorporation of ^{3}H-TdR only into T cells but not into B cells. The results in this report confirm this observation.

As reported previously high concentration, 50 μg/ml of bestatin also stimulates mitogenicity of Con A or PHA-P on small lymphocytes of guinea pig[14,15]. In this case, the lymphocytes might be not included sufficient macrophages to be activated by bestatin. As shown in Table 3, mitogenicity of Con A or LPS was not enhanced on lymphocytes stimulated by low concentration of bestatin. The difference can be explained as a dose-dependent effect of this substance.

The addition of 100 μg/ml of bestatin to cultures exhibited a high mitogenic response on spleen cells. Bestatin also enhanced the mitogenicity of Con A or LPS. The experiment shown in Table 9 indicates that the addition of high concentration of bestatin is mitogenic on B cells for the most part of its effect and on T cells, partly through activation of macrophages. The high concentration was also effective in enhancing antibody formation against SRBC *in vitro*. The stimulatory effect on antibody formation was shown to be parallel with the incorporation rate of ^{3}H-TdR. It can be noticed that bestatin did not stimulate the generation of suppressor cells on antibody formation to SRBC *in vitro*.

Bestatin is mitogenic on splenic lymphocytes, in low concentration, less than 1 μg/ml. The mitogenicity on T cells can be observed by pretreatment of macrophage and T cells with it and in high concentration, 100 μg/ml, the mitogenicity mainly on B cells, can be observed by addition to spleen cell cultures. The mechanisms of mitogenicity of the high and low concentrations remain to be clarified. The mitogenicity in low concentration may reflect action of bestatin in enhancement of DTH or in retardation of tumor growth in animals. Therefore, bestatin enhancing immune responses in animal through activation of macrophages and T cells, but in high dose, bestatin may activate B cells.

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