BESTATIN, A NEW SPECIFIC INHIBITOR OF AMINOPEPTIDASES, ENHANCES ACTIVATION OF SMALL LYMPHOCYTES BY CONCANAVALIN A

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SUMMARY

Bestatin, a new competitive aminopeptidase-inhibitor of dipeptide nature, was shown to enhance markedly activation of peripheral blood lymphocytes by concanavalin A (Con A). More than 40 percent stimulation over the control (the culture with Con A only) was observed at 50 μg/ml of bestatin, and this stimulatory effect was most predominant at an early stage of lymphocyte blastogenesis by Con A : bestatin had most effect when added to the culture simultaneously with Con A and no appreciable effect when added 44 h after Con A. The effect of bestatin on T lymphocyte activation *in vitro* is discussed in relation to its *in vivo* enhancing effect on cell-mediated immunity and a role in lymphocyte blastogenesis of some proteolytic activities possibly located at the cell surface is emphasized.

Small lymphocytes in culture are stimulated to enlarge, synthesize DNA and divide by various stimulants (1). Since the process may reflect some behavior of lymphocytes *in vivo* during the immune responses (1-4), it has recently aroused much interest; in fact, speculations on its significance have greatly contributed to current ideas on the immune process and on regulation of growth and cell differentiation in general (1,5,6). The mechanism of activation is unknown, but an intriguing suggestion is that its initiation may depend on some proteolytic activities possibly located at the cell surface (7-10). In this laboratory, early events in cellular activation by lectins and other polyclonal lymphocyte stimulants have been studied using various protease-

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inhibitors; the inhibitors used were small molecular peptides isolated from the culture-filtrate of *Actinomycetes* and they were characterized by Umezawa *et al.*(11,12). We previously reported that leupeptin (acetyl- or propionyl-L-leucyl-L-leucyl-L-arginal), a competitive inhibitor of neutral serine-proteases, such as plasmin, trypsin and papain, markedly inhibited blastoid transformation of lymphocytes by phytohaemagglutinin (PHA) under conditions that did not affect cell viability (8,9). This paper shows that a specific aminopeptidase-inhibitor of peptide nature, bestatin ((2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl)-(S)-leucine) (13), modulates and enhances activation of small lymphocytes by Con A.

MATERIALS AND METHODS

Lymphocytes were prepared aseptically from the peripheral blood of individual outbred albino guinea pig of mixed origin, weighing 350-500 g, by sedimentation in 3 % gelatin-Hanks solution, and then centrifugation of the leukocyte-rich plasma on a gradient of ficoll-sodium metrizoate (Lymphoprep, Nyegaard & Co. A/S, Oslo) at 400 x g for 30 min. The lymphocytes were cultured essentially as described previously (8,9). RPMI-1640 medium (Rosewell Park Memorial Institute, Buffalo) was supplemented with 10 % heat-inactivated (56°C for 30 min) fetal calf serum and antibiotics (streptomycin, 100 µg/ml, and penicillin, 100 U/ml). Con A (Sigma Chemical Co., St.Louis) and, or the competitive aminopeptidase-inhibitor, bestatin (Institute of Microbial Chemistry, Tokyo), at an appropriate concentration was added to the cells after various periods of incubation. Cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. DNA synthesis was measured as ^{3}H-thymidine incorporation into acid-insoluble materials, as described previously (8,9).

RESULTS AND DISCUSSION

First we examined the effects of bestatin on DNA synthesis in normal resting small lymphocytes and those activated by Con A. Con A was added at a concentration of 5 µg/ml, because, as seen from the dose-response curve in Fig. 1, this was optimal for activating lymphocytes of guinea pig peripheral blood. The results in Table 1 show that an appropriate amount of bestatin greatly increased activation of DNA synthesis by this concentration of Con A, but slightly though significantly inhibited ^{3}H-thymidine incorporation into normal resting lymphocytes. Its effect on

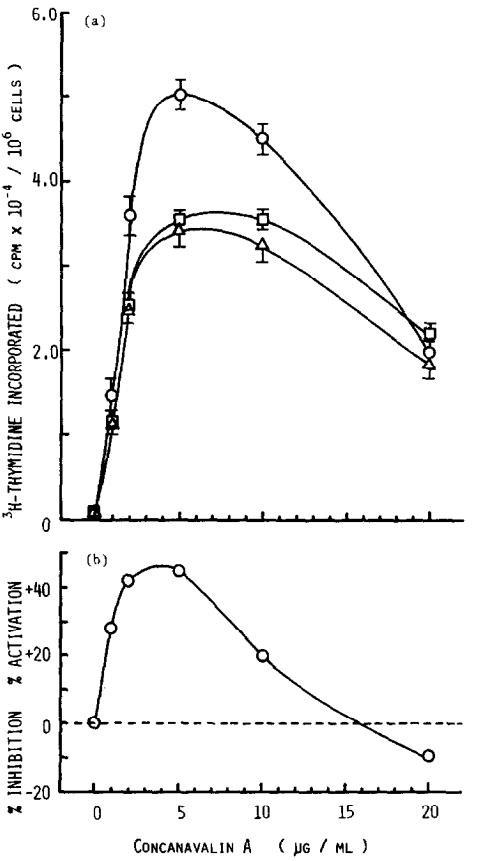

Fig. 1. Dose-response curves for activation of DNA synthesis by Con A in the presence and absence of Bestatin. Small lymphocytes (1 x 10^6 viable cells/ml) were isolated and cultured essentially as described for Table 1. (a) (□), Con A (5 µg) alone added to cultures at zero time; (○), Con A (5 µg) and Bestatin (50 µg) added to cultures together at zero time; (△), Con A (5 µg) added at zero time and Bestatin (50 µg) added at 44 h. DNA synthesis was determined by pulse-labeling with ^{3}H-thymidine from 48-64 h as described for Table 1. Points are means of values in triplicate cultures, and vertical bars show ± s.e.m. (b) Percentage activation of blastogenesis by Bestatin added at zero time as a function of the Con A concentration, calculated from the results in Fig. 1(a).

resting lymphocytes seemed to depend upon its concentration, but this effect was not a cytotoxic effect, because no cytotoxicity was observed on addition of about 200 µg/ml of bestatin to lymphocyte cultures. It seems possible that bestatin may inhibit DNA repair in resting cells. On the other hand, 50 µg/ml of bestatin

Table 1

Effect of Bestatin on ^{3}H-thymidine incorporation into normal resting lymphocytes and those activated by Con A

Addition to Culture			^{3}H-Thymidine Incorporation (cpm/culture ± s.e.m.)	Relative Incorporation(%)
None			915 ± 65	
Bestatin	10 µg		900 ± 110	
	50 µg		804 ± 81	
	100 µg		714 ± 94	
Con A	5 µg		39,010 ± 847	100*
Con A(5µg) plus Bestatin		10 µg	44,471 ± 1,422	114
		50 µg	56,565 ± 1,784	145
		100 µg	39,400 ± 1,021	101

Lymphocytes from the peripheral blood of normal guinea pigs were purified by sedimentation in 3 % gelatin-Hanks solution, and then centrifugation as described in the text. Lymphocytes were cultured in triplicate in small flat-bottomed test-tubes (13 x 75 mm) at concentrations of 1 x 10^6 viable cells per ml in RPMI-1640 medium containing 10 % fetal calf serum. Con A and/or various amounts of bestatin were added at zero time, and cultures were pulse-labeled with 0.5 µCi of ^{3}H-thymidine (5.0 Ci/mmol, Amersham, England) from 48-64 h. Cells were collected on glass-fiber filters, and washed with Hanks solution, and then 0.9 % NaCl, 5 % trichloracetic acid and methanol as described previously (8,9). Radioactivity was determined in a liquid scintillation counter and is expressed as cpm ± s.e.m.

* ^{3}H-Thymidine incorporation in the presence of Con A alone is taken as 100 %.

enhanced lectin-activated DNA synthesis (blastogenesis) induced by Con A more than 40 % (over the control with Con A only). More than 120 µg/ml of bestatin was rather inhibitory to both lectin-activated DNA synthesis and DNA synthesis of non-activated cells.

We next studied the influence of bestatin on the unimodal dose-response to Con A. As shown in Fig. 1, dose-response curve for activation of guinea pig lymphocytes by Con A showed first

Table 2

Effect of the time of addition of Bestatin relative to Con A on lymphocyte blastoid transformation

Period between additions of Con A and Bestatin (hours)	^{3}H-Thymidine Incorporation Bestatin (-) (cpm/culture ± s.e.m.)	Bestatin (+)	Percentage Activation* (%)
0	38,124 ± 952	55,204 ± 2,922	44.8
14		53,450 ± 4,229	40.2
24		54,250 ± 3,282	42.3
36		41,784 ± 2,422	9.6
44		38,581 ± 2,284	0.2

Small lymphocytes (1 x 10^6 viable cells/ml) were prepared and activated with Con A (5 µg) *in vitro* as described in Table 1. Bestatin (50 µg) was added to the cultures with or at various times after, Con A. DNA synthesis was determined as described in Table 1.

* Percentage activation represents:

$$\frac{^3\text{H-thymidine uptake with both Con A and Bestatin}}{^3\text{H-thymidine uptake with Con A alone}} \times 100 - 100$$

increase (stimulation) and then decrease (inhibition) with a maximum at a concentration of 5-10 µg/ml. This curve was very similar to those for lymphocytes from other species (14-16). On addition of 50 µg/ml of bestatin simultaneouly with Con A, lectin-activated DNA synthesis was again enhanced over a wide range of Con A concentrations, and the optimum concentration was slightly changed to a lower values (2-5 µg/ml) (Fig. 1b). The percentage stimulation by bestatin was roughly proportional to the extent of activation by Con A: it increased both stimulation and inhibition by Con A. In contrast, on addition 44 h after Con A, bestatin caused no stimulation but slight inhibition of Con A-activated

DNA synthesis. Thus, bestatin only seems to have a stimulatory effect at a certain stage of blastogenesis by Con A. Therefore, we next examined the most effective time for addition of bestatin relative to Con A. As shown in Table 2, bestatin had most effect when added with Con A , much less effect when added 36 h after Con A, and no appreciable effect when added 44 h after Con A.

Autoradiographic analysis and other studies in the lines of this experiment showed that: (a) to be effective, bestatin must come in contact with the lymphocytes before they are fully activated by Con A. Full activation of guinea pig peripheral blood lymphocytes by Con A takes about 24 h (unpublished data), and similar values have been reported by others (5,14-16), (b) bestatin must remain in the culture with Con A for at least 24-30 h to exert an effect (unpublished data), and (c) bestatin seems to increase the number of lymphocytes that respond to Con A activation.

Bestatin, which was isolated from the culture filtrate of *Streptomyces olivoreticuli*, has been characterized as a specific competitive inhibitor of aminopeptidase B and leucine aminopeptidase (17). It enhanced cell-mediated immunity, i.e. delayed-type hypersensitivity, and immune resistance to cancer (18,19), suggesting that it may enhance the functions of T lymphocytes. In support of these findings on its relation to immune phenomena *in vivo*, we found in this work that it greatly enhanced activation of T-cells *in vitro* by the polyclonal stimulant, Con A, and that it exerted this effect in a limited early stage of blastogenesis.

Many recent results have indicated the importance of proteases in control of cell proliferation. Since malignantly transformed cells resemble protease-treated normal cells in many ways, proteolytic activities on the cell membrane have been thought to be responsible for autocatalytic stimulation of cell division

(20-25). Further, there is suggestive evidence that some proteases, such as aminopeptidases (19), neutral serine-proteases (9,10,24), thiol-proteases and cathepsins (10), and plasminogen-activator (23, 25-27), are bound to the surface of mammalian cells, including normal fibroblasts, malignantly transformed cells, and lymphocytes. It seems possible that in small lymphocytes these surface membrane-bound proteases might be important in altering the cell surface membrane, resulting in discharge of a specific signal initiating a sequence of complicated metabolic alterations inducing change from the resting to the proliferative stage.

However, the effect of bestatin may not be due to specific inhibition of aminopeptidases, but to stimulation of some other biological activities in the lymphocytes. It is also unknown whether bestatin has a direct action on small lymphocytes or whether it acts on some intermediate cells, such as macrophages. Studies on the effects of bestatin on other types of cells, including B lymphocytes and leukaemic lymphoblasts will be reported soon.

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