MITOGENIC POTENTIALS OF BESTATIN, AMASTATIN, ARPHAMENINES A AND B, FK-156 AND FK-565 ON SPLEEN LYMPHOCYTES†

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The following aminopeptidase (AP) activities were found to be associated with the surface of mouse spleen cells: Leu-AP (138 pmol/10^5 cells×minute) and AP-B (16 pmol/10^5 cells× minute with Lys-β-naphthylamide as substrate and 21 pmol/10^5 cells×minute with Arg-β-naphthylamide substrate); AP-A activity was not detected by the assay system applied. The immunoactive peptide bestatin inhibited the Leu-AP, while AP-B activity decreased in the presence of both arphamenines A and B and bestatin. No effects on these enzymes were caused by amastatin (an AP-A inhibitor), FK-156, FK-565 and Bu-2743E; the latter peptide turned out to be not an inhibitor of cell surface associated microsomal Leu-AP but an inhibitor of cytosolic Leu-AP. The immunoactive peptides bestatin, arphamenines A and B, and amastatin increased [^{3}H]thymidine incorporation into spleen cells containing lymphocytes and macrophages. These mitogenic actions were not observed when macrophages were removed from the cultures or the cells had been stimulated with ConA or LPS. The lactoyl- and heptanoyl peptides FK-156 and FK-565 caused a mitogenic action on lymphocytes independently of the presence of macrophages. The inhibitor of cytosolic Leu-AP did not change the incorporation into lymphocytes.

In a fundamental study UMEZAWA *et al.*[1] found that aminopeptidases which hydrolyze *N*-terminal peptide bonds are located on surfaces of mammalian cells. These enzymes are not released extracellularly. Searching for inhibitors of these aminopeptidases, UMEZAWA *et al.* discoverd; bestatin[2], inhibiting microsomal leucine aminopeptidase (EC 3.4.11.2) (Leu-APm), cytosolic leucine aminopeptidase (EC 3.4.11.1) (Leu-APc) and aminopeptidase B (EC 3.4.11.6) (AP-B)[3,4], amastatin[2], inhibiting aminopeptidase A (EC 3.4.11.7) (AP-A) and Leu-APc[5], and arphamenines A and B[6], inhibiting specifically AP-B[6]. Of pharmacological interest was the finding that the inhibitors, bestatin[7~10], amastatin[3] and arphamenines A and B[3] enhance immune responses.

For bestatin, which was shown to be a promising antitumor agent (see ref 3), both enzymatic[4], immunochemical data[11,12] and binding studies[13] revealed, Leu-APm to be the target molecule on the cell surface. However, recent data indicate that bestatin exhibits a broader inhibition specificity on membrane-bound aminopeptidases[14]. Therefore, we determined in a comparative study the effect of bestatin and other aminopeptidase inhibitors, *e.g.* the leucine aminopeptidase inhibitor Bu-2743[15], on [^{3}H]thymidine incorporation of murine lymphocytes. In this study we have included FK-156 and FK-565, two additional immunoactive peptides[16]. Moreover, we tried to correlate inhibition of aminopeptidase(s), localized on the surface of murine spleen cells, with mitogenic response caused by these

† This contribution is dedicated to Dr. MILDRED SCHEEL.

inhibitors.

Materials and Methods

Compounds

Concanavalin A (ConA) (No. C 7275), lipopolysaccharide (LPS) (No. L 4130), amino acid-β-naphthylamides (Leu-NA, Arg-NA, Lys-NA and Glu-NA) and Leu-APm (from swine kidney; 25 units/mg) were obtained from Sigma, St. Louis, MO (U.S.A.); [*methyl*-^{3}H]thymidine (specific activity 78 Ci/mmol) from The Radiochemical Centre, Amersham (England); Leu-APc (from swine kidney; 100 units/mg) from Boehringer, Mannheim (Germany).

Microbial Peptides

Bestatin, amastatin, arphamenines A and B were gifts of Nippon Kayaku Co. Ltd., Tokyo; FK-156 and FK-565 were obtained from Fujisawa Pharmaceutical Co. Ltd., Osaka and Bu-2743E from Bristol-Myers Research Institute, Tokyo.

Preparation of Lymphocytes

Spleen cells were prepared from 5~6 weeks old male outbread NMRI mice. The cells were dissociated mechanically by squeezing the tissue through an 80-mesh steel screen. Erythrocytes were removed from the single cell suspension by NH_4Cl-treatment. The spleen cells were suspended in RPMI 1640 medium.

Two populations of spleen cells were tested; macrophage-containing cell populations and macrophage-depleted cultures. They were prepared as described[9].

Aminopeptidase Assays

Cell surface-associated AP activities were determined as described earlier[1,4]. For determination of AP-A, Glu-NA was used as substrate; for AP-B activity determination, Arg-NA and Lys-NA were added; and for Leu-AP determination, Leu-NA was included. $3\sim6\times10^5$ cells (macrophages-containing lymphocytes) were incubated (37°C, 30 minutes) in the presence of 0.5 mM substrate. The reaction product was quantified photometrically.

The activities of soluble Leu-APc and Leu-APm were determined essentially as described[4]; 0.18 μg of Leu-APm or 2.5 μg of Leu-APc were added to an 1-ml assay.

Determination of Mitogenic Effects

Spleen cells were suspended in medium, supplemented with 20% fetal calf serum, at a density of 1.5×10^7 cells/ml[9]. 5×10^5 cells each were placed into a final volume of 200 μl in cups of sterile flat-bottomed microtitration plates (Costar No. 3596). The assays were cultured in a fully humidified atmosphere of 5% CO_2 and air at 37°C. Where indicated 2 μg/ml of ConA or 20 μg/ml of LPS were added to the cultures. The lymphocytes were cultivated for 72 hours; 18 hours before the end of the culture 0.2 μCi of [^{3}H]thymidine was added to each cup. Incorporated thymidine was determined, after freezing the cells for 2 hours, on Whatman GF/A filters and rinsing them with a "Titertek-Cell Harvester" (Flow) apparatus. The filters were then dried (60°C; 40 minutes) and counted with 2 ml of scintillation liquid.

Each experiment was done in quadriplicate.

Statistical Evaluation

T-Test to determine the significance of the [^{3}H]thymidine incorporation effects on lymphocytes at different inhibitor concentrations was performed according to Student[17].

Results

Cell Surface Enzyme-inhibition Studies

According to AOYAGI *et al.*[1] we determined Leu-AP, AP-A and AP-B activities on the surface of murine spleen cells by using different amino acid-naphthylamides as substrates as described in detail

Table 1. Effect of various peptides on leucine aminopeptidase (Leu-AP) and aminopeptidase B (AP-B) activities, associated with the cell surface of spleen lymphocytes (macrophages-containing cultures). The determinations were performed as described under "Methods" using Leu-NA, Arg-NA or Lys-NA as substrates. The values represent the means of five samples each.

Peptides added (3 μg/ml)	AP-activities of lymphocytes (in %)		
	Leu-AP (Leu-NA)	AP-B	
		(Arg-NA)	(Lys-NA)
Control	100±8	100±9	100±9
Bestatin	59±4	48±4	53±4
Bu-2743E	102±8	98±9	105±9
Arphamenine A	104±9	37±3	46±4
Arphamenine B	99±8	45±4	51±5
Amastatin	94±8	99±9	103±9
FK-156	103±8	106±10	99±9
FK-565	106±8	105±9	101±9

under "Methods". The highest activity was determined for Leu-AP with 138±7 pmol/10^5 cells×minute (with Leu-NA as substrate) while the activity of AP-B was lower and amounted to 16±1 (Lys-NA) or 21±1 pmol/10^5 cells×minute (Arg-NA). No AP-A activity (substrate: Gul-NA) could be detected under the assay conditions used.

Inhibition studies with the different peptides revealed (Table 1) that cell surface-associated Leu-AP was only inhibited by bestatin, while AP-B was inhibited both by bestatin and arphamenines A and B. In completion of an earlier study[15] we determined the specificity of Bu-2743E and found in *in vitro* tests, that this peptide preferentially inhibited Leu-APc and not Leu-APm (Table 2). Because only the latter enzyme is cell surface-associated[4] the observed insensitivity of that Leu-AP activity which is associated with lymphocyte cell surfaces towards Bu-2743E becomes plausible.

Table 2. Inhibition of soluble cytosolic and microsomal leucine aminopeptidase activity by Bu-2743E. The activity is given as per cent of the control. The values represent the means of five samples each.

Bu-2743E concentration (μg/ml)	Enzyme	Enzyme activity (%)
0	Leu-APc	100±7
0.03	〃	98±7
0.1	〃	82±6
0.3	〃	43±3
1.0	〃	29±2
3.0	〃	17±1
10.0	〃	14±1
0	Leu-APm	100±7
0.1	〃	98±8
1.0	〃	104±7
10.0	〃	89±6

Mitogenic Effect on Spleen Lymphocytes

The leucine aminopeptidase inhibitors bestatin and Bu-2743E differentially influenced [^{3}H]thymidine incorporation into DNA of murine spleen cells (Table 3). Bestatin was determined to stimulate significantly incorporation into splenic lymphocytes if macrophages are present in the cultures. Highest stimulation (to 140%) was measured at a concentration of 1 μg bestatin/ml (*P* value *versus* control: <0.001). No significant effect was measured in macrophage-free cultures or in macrophage-containing cultures which were treated with the mitogens ConA or LPS. On the other side, Bu-2743E did not change at all significantly the incorporation at all combinations summarized in Table 3.

Like bestatin, both arphamenines A and B only stimulated significantly those cultures which firstly contained macrophages and secondly were not stimulated with mitogens (Table 4). The strongest

Table 3. [^{3}H]Thymidine incorporation by murine spleen lymphocytes incubated with different concentrations of the leucine aminopeptidase inhibitors bestatin and Bu-2743E.

Inhibitor pretreatment (μg/ml)		Mitogens added to cultures; incorporation (cpm/culture)					
		Macrophages-containing cultures			Macrophages-depleted cultures		
		None	ConA	LPS	None	ConA	LPS
None		40,014	129,027	134,551	50,151	136,874	128,304
Bestatin	0.001	39,450	127,514	132,015	52,998	141,296	131,280
	0.01	39,312	125,779	129,927	53,509	132,946	134,407
	0.1	48,222	136,411	135,139	48,107	136,688	132,219
	1.0	56,160	130,612	130,343	50,735	135,603	129,811
	10.0	41,364	124,804	128,762	48,136	133,552	129,743
Bu-2743E	0.01	39,472	128,976	129,385	52,558	134,390	127,290
	0.1	36,410	129,584	132,602	48,696	135,029	129,754
	1	41,887	131,113	135,153	49,282	139,632	129,419
	10	40,657	127,041	133,846	53,351	135,749	132,372

Table 4. Effects of the aminopeptidase B inhibitors arphamenines A and B on [^{3}H]thymidine incorporation into lymphocytes.

Inhibitor pretreatment (μg/ml)		Mitogens added to cultures; incorporation (cpm/culture)					
		Macrophages-containing cultures			Macrophages-depleted cultures		
		None	ConA	LPS	None	ConA	LPS
None		41,284	126,498	129,593	46,530	128,200	124,096
Arphamenine A	0.01	40,176	130,679	127,712	45,609	126,231	125,790
	0.1	46,915	127,857	126,153	46,242	127,261	124,752
	1.0	49,443	128,028	129,305	46,267	128,659	124,430
	3.0	47,841	129,126	126,114	44,606	125,907	121,975
	10.0	39,124	126,773	130,578	47,662	127,033	125,520
	20.0	37,391	124,428	127,822	43,187	121,459	118,941
Arphamenine B	0.01	41,888	127,829	128,454	45,736	127,660	125,425
	0.1	43,104	129,330	127,477	46,541	127,518	127,527
	1.0	46,086	128,969	130,039	46,323	129,881	126,914
	3.0	51,368	124,634	131,748	43,457	125,701	125,416
	10.0	44,927	127,577	127,650	45,172	128,162	127,823
	20.0	38,655	121,334	119,668	42,931	123,530	121,291

increase was observed with 1 μg arphamenine A/ml (to 120%; $P<0.005$) or with 3 μg arphamenine B/ml (to 124%; $P<0.005$).

Amastatin was determined to stimulate [^{3}H]thymidine incorporation into lymphocytes/macrophages even stronger than was found in the experiments with bestatin. The optimal stimulatory concentration was around 0.1 μg/ml (increase to 145%; $P<0.001$). In macrophage-free lymphocyte cultures no effect of amastatin was measured. Mitogen-stimulated cultures were not affected by amastatin (Table 5).

The lactoyl- and heptanoyl-peptides FK-156 and FK-565 significantly stimulated [^{3}H]thymidine incorporation into macrophages/lymphocytes at concentrations higher than 1 μg/ml (Table 6). The optimal doses were determined to be 3 μg FK-156/ml (increase to 149%; $P<0.001$) or 1 μg FK-565/ml (to 158%; $P<0.001$) respectively. Unlike the hitherto mentioned peptides, the FK-compounds also stimulated the incorporation into lymphocytes in the absence of macrophages. At a

Table 5. Incorporation of [^{3}H]thymidine into lymphocytes pretreated with amastatin, an inhibitor of aminopeptidase A.

Inhibitor pretreatment (μg/ml)		Mitogens added to cultures; incorporation (cpm/culture)					
		Macrophages-containing cultures			Macrophages-depleted cultures		
		None	ConA	LPS	None	ConA	LPS
None		42,816	131,574	127,954	53,404	134,166	128,428
Amastatin	0.01	53,587	133,848	126,804	49,723	136,302	126,930
	0.1	61,979	129,523	130,179	53,276	133,938	126,365
	1.0	59,112	128,295	128,641	55,608	130,625	130,397
	10.0	40,676	130,301	125,874	48,059	129,783	131,512

Table 6. Influence of the peptides FK-156 and FK-565 on [^{3}H]thymidine incorporation into lymphocytes.

Peptide pretreatment (μg/ml)		Mitogens added to cultures; incorporation (cpm/culture)					
		Macrophages-containing cultures			Macrophages-depleted cultures		
		None	ConA	LPS	None	ConA	LPS
None		39,160	131,280	126,447	49,897	129,306	124,928
FK-156	0.3	44,655	129,264	127,736	48,632	127,820	124,761
	1	49,581	126,020	129,186	51,390	132,144	121,255
	3	58,157	128,965	127,446	53,789	127,798	126,870
	10	56,577	126,875	125,364	54,721	130,258	125,704
	30	54,589	123,644	122,611	42,208	129,297	124,395
FK-565	0.3	39,644	129,013	123,325	51,981	131,902	123,014
	1	62,032	127,676	126,323	58,928	129,615	124,239
	3	58,420	130,631	125,721	63,583	130,915	124,518
	10	58,448	125,639	122,470	61,520	133,132	126,814
	30	57,313	125,147	119,492	59,226	129,963	119,503

concentration of 3~10 μg/ml both compounds caused an increase of [^{3}H]thymidine incorporation by more than 10% ($P<0.01$). Mitogen-treated cultures were not affected both by FK-156 and by FK-565.

Discussion

UMEZAWA's concept to modulate cell functions *via* an inhibition of cell surface-bound aminopeptidases[1] proved to be a realistic approach. The first experimental support came from studies with the aminopeptidase inhibitor bestatin[1,18]. This dipeptide affects both the Leu-APm[4] and perhaps also further aminopeptidases[14] which occur on the cell surface of mammalian cells and causes — *via* their inhibition — a stimulation of metabolism of those cells *in vivo*, which are involved in immune system[10,18]. This finding was also confirmed *in vitro* by the demonstration that bestatin displays a mitogenic effect on spleen cells which include lymphocytes and macrophages (ref 9; and this report).

Recently a further leucine aminopeptidase inhibitor, Bu-2743E, was described[15], whose biological activity remains to be determined. In the present study it is shown that this peptide inhibited the soluble Leu-APc and caused no effect on the overall leucine aminopeptidase activities, present on the surface of spleen cells. Mitogenicity tests with spleen cells showed no influence of Bu-2743E on these cells. These results are in perfect agreement with earlier studies which failed to demonstrate the existence of Leu-APc on the cell surface of mammalian cells (see ref 11 and 19). Obviously spleen cell surfaces do not seem to be provided with target molecules for Bu-2743E.

Arphamenines A and B are two aminopeptidase B inhibitors if tested both in *in vitro* assays[6] and in systems containing intact spleen cells (this contribution) if Arg-NA and Lys-NA were used as substrates. Earlier studies demonstrated the property of these inhibitors to augment delayed type hy-

persensitivity to red blood cells *in vivo*[6]. In the present contribution, we succeeded in proving the mitogenic effect of the arphamenines on lymphocytes also *in vitro*. Future studies must clarify whether the arphamenines indeed interact on the cell surface with AP-B, because this enzyme is thought to be localized in the cytosol[18].

The finding that the AP-A inhibitor amastatin causes a mitogenic effect on lymphocytes was not unexpected. From earlier histochemical studies[19] and more recent enzymic determinations[1], evidence was presented that AP-A is also localized on the cell membrane. However, the activity of this enzyme on spleen cells is very low and could not be detected by the exopeptidase assay with Glu-NA as substrate, applied by us.

The inhibitors mentioned above, stimulate mitogenic activity of splenic lymphocytes (determined by [^{3}H]thymidine incorporation measurements) only under conditions at which macrophages are present in the cultures. These results suggest that these potential immunomodifiers function through an activation of macrophages. Another class of chemically well defined immunoactive peptides was discovered[20], FK-156 and FK-565, which exhibited mitogenic activity of lymphocytes, irrespectively of the presence of macrophages, as shown in the present study. Under the conditions used these peptides stimulated [^{3}H]thymidine incorporation by up to 60%. The target molecule(s) for the FK-compounds are not known; the presented data exclude the possibility that they interact with cell surface-associated Leu-AP or AP-B.

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