Properties and activities of aminopeptidases in normal and mitogen-stimulated human lymphocytes

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(Received 20 June 1984/Accepted 25 October 1984)

Human peripheral lymphocytes were found to contain at least two distinct aminopeptidases, designated cytosol aminopeptidase and microsomal aminopeptidase, which differed from one another with respect to intracellular localization, substrate specificity, metal-ion activation, K_m value and electrophoretic mobility. No change in these aminopeptidase activities was observed in cultured lymphocytes in the absence of mitogen throughout the cultivation period. The addition of phytohaemagglutinin or concanavalin A to the culture medium caused, in dose-dependent manner, a significant increase in cytosol aminopeptidase activity in lymphocytes. On the other hand, no increase in microsomal aminopeptidase activity was observed under the same conditions. The biochemical properties of aminopeptidases in stimulated cultured lymphocytes were identical with those of the enzymes in peripheral lymphocytes and unstimulated cultured lymphocyte. The phytohaemagglutinin dose-response curves for lymphocyte activation as measured by the DNA synthesis rate and for cytosol aminopeptidase activity were observed to be similar. However, when DNA synthesis was temporarily blocked by hydroxyurea, the rate of increase of aminopeptidase activity was unaffected. Pokeweed mitogen only slightly increased the cytosol aminopeptidase activity in cultured lymphocytes, although the lymphocytes were highly activated.

Aminopeptidases that catalyse the hydrolysis of amino acid amides have been demonstrated in a variety of mammalian tissues and body fluids (Behal *et al.*, 1965; Joseph & Sanders, 1966; Tamura *et al.*, 1973, 1975), and have been isolated and purified from several organs (Hanson *et al.*, 1965; Wachsmuth *et al.*, 1966; Maroux *et al.*, 1973; Niinobe & Fujii, 1980; Ledeme *et al.*, 1981). However, in spite of these extensive studies, the intracellular functions of aminopeptidases remain obscure.

It has been reported that some aminopeptidase activity might play an important role in the regulating process of lymphocyte activation (Saito *et al.*, 1978; Ishizuka *et al.*, 1980). Therefore study of aminopeptidases in lymphocytes, especially

under conditions of activation, may contribute to the elucidation of the intracellular functions of aminopeptidases. However, hitherto little information has been reported concerning lymphocyte aminopeptidases.

In the present paper the properties and activities of aminopeptidases in peripheral and in cultured lymphocytes, which were activated by various mitogens, are reported.

Materials and methods

Chemicals and enzymes

Reagents were obtained from the indicated sources: Ficoll-paque (Pharmacia Fine Chemicals, Uppsala, Sweden), phytohaemagglutinin-P (PHA) (Difco Laboratories, Detroit, MI, U.S.A.), pokeweed mitogen (PWM) and foetal-calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.), concanavalin A and silica particles (Japan Immunoresearch Laboratories, Takasaki, Japan), hydroxyurea and enzyme substrates (Sigma

Abbreviations used: C-AP, cytosol aminopeptidase; M-AP, microsomal aminopeptidase; PBS(–), Mg^{2+} and Ca^{2+} -free phosphate-buffered saline (140 mM-NaCl/9 mM-sodium phosphate buffer, pH 7.4); PHA, phytohaemagglutinin; PWM, pokeweed mitogen.

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Chemical Co., St. Louis, MO, U.S.A.), [6-³H]-thymidine (21Ci/mmol) (Amersham International, Amersham, Bucks., U.K.), glutamate dehydrogenase (NADPH-dependent) from *Proteus inconstans* (Ono Pharmaceutical Co., Osaka, Japan), and leucine dehydrogenase from *Bacillus sphaericus* (Toyobo Co., Osaka, Japan).

Lymphocyte preparation

Human peripheral lymphocytes for subcellular fractionation were obtained from the discarded fractions after the preparation of a platelet transfusion. Lymphocytes for culture were obtained from heparinized venous blood freshly taken from healthy individuals. Phagocytic cells were removed in the following manner: 10% (v/v) silica-particle suspension [5% (w/v) suspended in PBS(-)] was added to blood samples, which were then incubated for 60min at 37°C with constant shaking. Cells that had phagocytosed silica particles were removed by centrifugation at 900g for 10min, and the leucocyte-rich fraction (buffy coat) was withdrawn. This fraction was mixed with an equal volume of PBS(-) and then centrifuged in Ficoll-paque at 400g for 20min. Lymphocytes were carefully withdrawn from the interface, and washed three times with PBS(-). Total and viable cell counts were determined by microscopy. Cell viability, as determined by Trypan Blue exclusion, was 98% or greater.

Subcellular fractionation

Cells were suspended in 17mM-Tris/HCl buffer, pH7.6, containing 0.83% NH₄Cl to lyse any contaminating erythrocytes and then washed twice with PBS(-). Packed cells were suspended in 2vol. of 0.25M-sucrose in 3mM-Tris/HCl buffer, pH7.4, containing 2mM-EDTA. Homogenization was performed with a Polytron homogenizer (Silverson Machines) and the completion of homogenization was monitored by using a phase-contrast microscope. The homogenate was fractionated by differential centrifugation. The nuclear fraction (sedimented by 700g for 10min), the light-mitochondrial fraction (sedimented by 8500g for 10min), the heavy-mitochondrial fraction (sedimented by 24000g for 10min), the microsomal fraction (sedimented by 105000g for 60min) and the cytosol fraction (105000g supernatant) were obtained successively. The nuclear fraction was washed once with 1.15% (w/v) KCl. All fractions obtained, except for the cytosol fraction, were suspended in the above buffer. In order to release latent enzyme activity, all fractions were subjected to three cycles of freezing and thawing (-70°C to 37°C) and to sonication for four 10s periods on ice. In preliminary experiments, the recovery of exogenous lymphocyte

aminopeptidases after this releasing procedure was at least 87%.

Cell culture

Cells (1 × 10⁶ cells/ml) were cultured in a culture medium (RPMI 1640; Nissui Seiyaku, Tokyo, Japan) containing 10% (v/v) foetal-calf serum in polystyrene tubes (16mm × 125mm) at 37°C in a humidified atmosphere of CO₂/air (1:19). After preincubation for 24h, reagents were added to the culture medium at zero time and lymphocytes continued to be cultured. Cells were then harvested at each measuring time point by centrifugation at 400g for 10min.

Enzyme preparation

After lysis of any contaminating erythrocytes by the method described above, cells were washed twice with PBS(-) and were resuspended in 0.5ml of PBS(-). This cell suspension was frozen and thawed (-70°C to 37°C) and then centrifuged at 105000g for 40min. The supernatant was analysed for enzyme activities and properties as described below. The resulting pellet was resuspended in 0.5ml of PBS(-) and sonicated for three 10s periods on ice for enzyme assay.

Incorporation of [³H]thymidine label

A 0.2ml sample of the cultured lymphocytes was transferred from a culture tube to a micro-titre plate 6h before harvesting of the cells. [³H]Thymidine (0.3μCi) was added to each culture, and cells were incubated for 6h under the same conditions as described above. Cells were then treated with trichloroacetic acid (final concn. 5%, w/v), washed and collected on a glass-fibre filter. The radioactivity on the filter was counted in a xylene scintillant with a liquid-scintillation counter.

Enzyme assay

On the basis of the theory of coupled enzyme reactions (Takagahara *et al.*, 1983), L-leucine amide-splitting activity was measured by a coupling assay that monitored the rate of NH₃ formation by coupling to the indicator enzyme, glutamate dehydrogenase. The standard reaction mixture contained 23.4mM-leucine amide, 2.4mM-2-oxoglutarate, 0.14mM-NADPH, 2000 units of glutamate dehydrogenase (*K_m* for NH₃ 1.1mM)/l, 80mM-Tris/HCl buffer, pH8.0, and enzyme in a final volume of 0.62ml. Incubation was carried out at 37°C. The reaction was started by addition of the substrate and the enzyme activity was monitored after 3min of lag time by measuring the rate of NADPH oxidation as the change in absorbance at 340nm for 3min. In this method, both theoretically and experimentally, up to 200units (μmol/min) of

enzyme/l provided a linear change in absorbance and 99% of the enzyme activity could be detected.

A sample-blank reaction rate and a reagent-blank reaction rate were determined by substituting buffer solution for substrate and enzyme respectively in the complete assay system. Both blank reaction rates were effectively negligible. In preliminary experiments with lymphocyte homogenate as enzyme, addition of L-aspartic acid or L-alanine to the assay medium had no effect on enzyme assay. Therefore there seemed to be no possibility that transaminases and/or deaminases could contribute to the changes measured.

L-Leucine *p*-nitroanilide-splitting activity was measured as described by Szasz (1967).

Electrophoresis

Electrophoretic analysis was performed at 4°C on a Cellogel membrane (Chemetron Co., Milan, Italy) with 0.05M-sodium barbital/0.01M-barbital buffer, pH 8.6, and an operative current of 1 mA/cm of membrane. Activity staining was performed with L-leucine amide or L-leucine *p*-nitroanilide as substrate at 37°C in an activity stain system containing 23.4 mM-L-leucine amide or 24.8 mM-L-leucine *p*-nitroanilide, 2000 units of leucine dehydrogenase/l, 1.5 mM-NAD⁺, 0.4 mM-Thiazolyl Blue, 0.05 mM-phenazine methosulphate and 80 mM-Tris/HCl buffer, pH 8.0.

Protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Subcellular distribution and properties of human peripheral-lymphocyte aminopeptidases

Fig. 1 shows a zymogram of aminopeptidases in lymphocytes after cellulose acetate-membrane electrophoresis. The aminopeptidases in human peripheral lymphocytes were electrophoretically separated into two aminopeptidases, one fast-moving and the other slow-moving. The slow-moving aminopeptidase was activity-stained with both L-leucine amide and L-leucine *p*-nitroanilide as substrates, whereas fast-moving aminopeptidase was activity-stained only with L-leucine amide.

Table 1 presents the intracellular distribution of aminopeptidase in human peripheral lymphocytes. L-Leucine amide-splitting activities were obtained from two fractions, cytosol fraction and microsomal fraction. Electrophoretic analysis of aminopeptidase in the cytosol fraction revealed only one band, corresponding to the fast-moving aminopeptidase, and electrophoresis of the microsomal fraction also showed only one band, corresponding to the slow-moving aminopeptidase (Fig. 1). The aminopeptidase in the cytosol fraction preferentially hydrolysed L-leucine amide as compared with L-leucine *p*-nitroanilide and was markedly activated by Mn²⁺ (2 mM: activity 247%). On the other hand, the aminopeptidase in the microsomal fraction hydrolysed L-leucine *p*-nitroanilide to a considerable extent, as well as L-leucine amide, and was not activated by Mn²⁺ (activity 98%). Apparent *K_m* values for L-leucine amide were obtained by using the Lineweaver-Burk plot. A *K_m* value of 4.2 mM was calculated for the amino-

Fig. 1. Cellulose acetate membrane electrophoresis of lymphocyte aminopeptidases

The activity staining was performed with L-leucine amide (Leu-NH₂, lanes a-e) or L-leucine *p*-nitroanilide (Leu-*p*-NA, lanes f-h) as substrate. Lanes a and f, whole-homogenate fraction of human peripheral lymphocytes; lanes b and g, cytosol fraction of human peripheral lymphocytes; lanes c and h, microsomal fraction of human peripheral lymphocytes; lane d, supernatant fraction of lymphocytes cultured for 72 h in the absence of PHA; lane e, supernatant fraction of lymphocytes cultured for 72 h in the presence of PHA (5 µg/ml). For full experimental details see the text.

Table 1. Intracellular localization of aminopeptidases in human peripheral lymphocytes

The fractions were prepared as described in the Materials and methods section. Results are presented as percentages recovered of that in the homogenate fraction.

Fraction	Recovery (%)		
	L-Leucine amide-splitting activity	L-Leucine <i>p</i> -nitroanilide-splitting activity	Protein
Homogenate	100	100	100
Nuclear	0.6	1.0	7.1
Heavy mitochondrial	5.6	5.6	25.6
Light mitochondrial	10.3	9.7	11.4
Microsomal	52.3	72.6	12.5
Cytosol	36.0	7.2	31.8
Total	104.7	96.1	88.4

peptidase in the cytosol, and a K_m value of 0.7 mM was obtained for the aminopeptidase in the microsomal fraction.

The above results demonstrated that human peripheral lymphocytes contain at least two distinct aminopeptidases, designated cytosol aminopeptidase (C-AP) and microsomal aminopeptidase (M-AP).

As almost all of C-AP activity was recovered, with little contamination by M-AP, in the supernatant solution of the cell suspension which was frozen and then thawed, this solution was used in subsequent experiments for C-AP assay with L-leucine amide as substrate. M-AP was assayed by using the resulting pellet with L-leucine *p*-nitroanilide as substrate.

Effect of PHA and concanavalin A on aminopeptidase activity in cultured lymphocytes

Fig. 2 shows changes in C-AP and M-AP activities in cultured lymphocytes in response to PHA. No change in these aminopeptidase activities was observed in lymphocytes cultured without mitogen throughout the cultivation period. The addition of PHA to the culture medium caused an increase in C-AP activity in cells in proportion to the mitogen dosage (Fig. 3). The increase in C-AP activity in PHA-induced cells was already evident 24 h after the addition of mitogen, and the highest activity was observed at 72 h after cultivation with PHA.

The same results were obtained with concanavalin A (5 μ g/ml) as mitogen, i.e. the elevation of C-AP activity was already detected at 24 h and the highest activity (117.6 units/g of protein) was observed at 72 h.

With respect to the electrophoretic mobility (Fig. 1), the apparent K_m value for L-leucine amide (4.2 mM) and the metal-ion activation (2 mM- Mn^{2+} : activity 221%), the properties of C-AP in lymphocytes cultured with PHA were identical

Fig. 2. Change in aminopeptidase activity in cultured lymphocytes in the presence and in the absence of PHA. After preincubation for 24 h, PHA (5 μ g/ml) was added to the culture at zero time (indicated by the arrow). For full experimental details see the text. ○, C-AP, and △, M-AP, in lymphocytes cultured in the presence of PHA; ●, C-AP and ▲, M-AP, in lymphocytes cultured in the absence of PHA. Each point represents the mean $\pm$ S.E.M. for four to eight cultures.

with those in lymphocytes cultured without mitogen and those in peripheral lymphocytes.

On the other hand, no elevation of M-AP activity was observed throughout the cultivation period with any dose of PHA, indicating that the increase in C-AP activity was not just the result of a general increase in enzyme content.

Relationship between C-AP activity and [3H]thymidine incorporation in cultured lymphocytes stimulated by PHA

Fig. 3 shows the dose-response relationship for lymphocyte activation as measured by a [3H]-

Fig. 3. Dose-response curve of C-AP activity and $[^3\text{H}]$ -thymidine incorporation in the cultured lymphocytes with various doses of PHA

Lymphocytes were cultured in the presence of various doses of PHA for 72 h. For full experimental details see the text. Each point represents the mean $\pm$ S.E.M. for three to five cultures.

thymidine incorporation and elevation of C-AP activity in cells that were cultured for 72 h. The similarity between the PHA dose-response curves for the $[^3\text{H}]$ thymidine incorporation and for the C-AP activity suggests that there is a close correlation between lymphocyte stimulation and active elevation of C-AP activity in cells. However, even when DNA synthesis as measured by the incorporation of labelled thymidine was temporarily blocked by hydroxyurea, the rate of elevation of C-AP activity was not affected (Fig. 4).

Effect of PWM on aminopeptidase activities in cultured lymphocyte

It is generally recognized that human peripheral lymphocytes can be separated into two subpopula-

Fig. 4. Effect of hydroxyurea on the elevation of C-AP activity in cultured lymphocytes stimulated by PHA. After preincubation for 24 h, both PHA (5 $\mu\text{g/ml}$) and hydroxyurea (1 mM) were added to cultures at zero time (indicated by arrow) and lymphocytes were cultured for 24 h. Cells are then washed with medium and resuspended in fresh medium including PHA, but not including hydroxyurea, for continued culturing. For full experimental details see the text. ○, $[^3\text{H}]$ Thymidine incorporation and C-AP activity in lymphocytes not treated with hydroxyurea; ●, $[^3\text{H}]$ thymidine incorporation and C-AP activity in lymphocytes treated with hydroxyurea. Each point represents the mean $\pm$ S.E.M. for three or four cultures.

tions, T- and B-cells, and that PHA and concanavalin A selectively stimulate human T-cells. Fig. 5 shows the effect of PWM, a T- and B-cell mitogen, on lymphocyte enzymes. Unlike the situation with PHA or concanavalin A, even when lymphocyte activation as measured by $[^3\text{H}]$ thymidine incorporation was highly stimulated by an optimal concentration of PWM, the elevation of C-AP activity was less significant. Little change in M-AP activity was observed in lymphocytes cultured with PWM.

Fig. 5. Change in aminopeptidase activity in cultured lymphocytes stimulated by PWM

After preincubation for 24h, PWM (10 µg/ml) was added to the culture at zero time (indicated by arrow). For full experimental details see the text. ○, C-AP, activity, △, M-AP activity, and □, [³H]thymidine incorporation, in lymphocytes stimulated by PWM. Each point represents the mean ± S.E.M. for three to five cultures.

Discussion

The present study demonstrated for the first time that human peripheral lymphocytes contain at least two distinct aminopeptidases, designated cytosol aminopeptidase (C-AP) and microsomal aminopeptidase (M-AP), which differ from one another in their enzymic and physicochemical properties. C-AP in lymphocytes seems to be a leucine aminopeptidase (EC 3.4.11.1), described first by Linderstrom-Lang (1930) and subsequently by others. This conclusion was based on similarities seen in other tissue enzymes with respect to intracellular localization (Patterson *et al.*, 1963, 1965; Ledeme *et al.*, 1981), little activity for L-leucine *p*-nitroanilide (Patterson *et al.*, 1963; Niinobe & Fujii, 1980; Ledeme *et al.*, 1981) and behaviour with metal ions (Smith & Spackman, 1955; Ledeme *et al.*, 1981). The electrophoretic mobility of C-AP was also identical with that of human liver leucine aminopeptidase partially purified by the method of Niinobe & Fujii (1980) (S. Kanda & H. Kohno, unpublished work). However, further definition of the nature of the lymphocyte aminopeptidases requires the isolation and the characterization of the enzymes.

The studies described in the present paper indicate that an elevation of C-AP activity, which is not commonly considered to be an inducible enzyme, was found in cultured lymphocytes in the presence of PHA or concanavalin A. The increase in C-AP activity was, as with other enzymes that have been demonstrated to increase in specific

activity, undoubtedly associated with lymphocyte activation, because this increase was caused by the addition of mitogen in a dose-dependent manner and because the PHA dose-response curve for the C-AP activity was identical with that for lymphocyte activation. Furthermore, the elevation of C-AP activity was already observed at 24h after the addition of mitogen, and was not affected even when DNA replication was blocked. Since the increase in cell size was still not significant at 24h and the increase in mitosis was not significant for 48h in culture, this change in the enzyme activity was not due to the general increase in cell size as described by Harrison *et al.* (1981), and not due to the appearance of a new population of cells, but presumably due to gene expression. In addition, C-AP showed similar profiles in normal and activated cells, suggesting that the same structural gene was expressed.

On the other hand, unlike PHA or concanavalin A, little increase in C-AP activity was observed in lymphocytes stimulated by PWM, even when the incorporation of labelled thymidine was strongly stimulated. Interestingly, the increase in production of β_2 -microglobulin, a protein closely associated with the control of lymphocyte activation (Ostberg *et al.*, 1976), was observed in lymphocytes stimulated by PHA, but not by PWM (Fanger & Bernier, 1973). In addition, it was demonstrated that a bestatin, a potent inhibitor of leucine aminopeptidase, strongly affected blastogenesis of lymphocytes, suggesting that leucine aminopeptidase may play an important role in the control of lymphocyte activation (Saito *et al.*, 1978; Ishizuka *et al.*, 1980).

This study was supported in part by a grant (no. 57570834) from the Japan Ministry of Education.

References

- Behal, F. J., Asserson, B., Dawson, F. & Hardman, J. (1965) *Arch. Biochem. Biophys.* **111**, 335–344
- Fanger, M. W. & Bernier, G. M. (1973) *J. Immunol.* **111**, 609–617
- Hanson, H., Glasser, D. & Kirshke, H. (1965) *Hoppe-Seyler's Z. Physiol. Chem.* **340**, 107–125
- Harrison, E. H., Zbuzek, V., Labadie, J. H. & Bowers, W. E. (1981) *Biochim. Biophys. Acta* **676**, 321–328
- Ishizuka, M., Sato, J., Sugiyama, Y., Takeuchi, T. & Umezawa, H. (1980) *J. Antibiot.* **33**, 653–662
- Joseph, R. L. & Sanders, W. J. (1966) *Biochem. J.* **100**, 827–832
- Ledeme, N., Hennon, G., Vincent-Fiquet, O. & Plaquet, R. (1981) *Biochim. Biophys. Acta* **660**, 262–270
- Linderstrom-Lang, K. (1930) *Hoppe-Seyler's Z. Physiol. Chem.* **188**, 48–68
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- Maroux, S., Louvard, D. & Baratti, J. (1973) *Biochim. Biophys. Acta* **321**, 282-295
- Niinobe, M. & Fujii, S. (1980) *J. Biochem. (Tokyo)* **87**, 195-203
- Ostberg, L., Lindblom, J. B. & Peterson, R. A. (1976) *Eur. J. Immunol.* **6**, 108-113
- Patterson, E. K., Hsiano, S. & Keppel, A. (1963) *J. Biol. Chem.* **238**, 3611-3620
- Patterson, E. K., Hsiano, S., Keppel, A. & Sorof, S. (1965) *J. Biol. Chem.* **240**, 710-716
- Saito, M., Takegoshi, K., Aoyagi, T., Umezawa, H. & Nagai, Y. (1978) *Cell Immunol.* **40**, 247-262
- Smith, E. L. & Spackman, D. H. (1955) *J. Biol. Chem.* **212**, 271-299
- Szasz, G. (1967) *Am. J. Clin. Pathol.* **47**, 607-610
- Takagahara, I., Yamauti, J., Fujii, K., Yamashita, J. & Horio, T. (1983) *J. Biochem. (Tokyo)* **93**, 1145-1157
- Tamura, Y., Niinobe, M., Arima, T., Okuda, H. & Fujii, S. (1973) *Biochim. Biophys. Acta* **327**, 437-445
- Tamura, Y., Niinobe, M., Arima, T., Okuda, H. & Fujii, S. (1975) *Cancer Res.* **35**, 1030-1034
- Wachsmuth, E. D., Fritze, I. & Pfeleiderer, G. (1966) *Biochemistry* **5**, 169-182