ACTION OF UBENIMEX† ON AMINOPEPTIDASE ACTIVITIES IN SPLEEN CELLS AND PERITONEAL MACROPHAGES FROM MICE

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(Received for publication March 10, 1987)

The action of ubenimex on aminopeptidase (APase) activity was studied in intact spleen cells and peritoneal macrophages from mice. Ubenimex strongly inhibited hydrolyzing activities against arginine-β-naphtylamide (Arg-NA), Lys-NA and Pro-NA in both cells. Inhibition of hydrolysis of Leu-NA, Met-NA and Ala-NA was relatively small or not observed. When both cells were incubated in HANKS' solution, hydrolyzing activities against Arg-NA, Lys-NA and Pro-NA were released to the medium, while Leu-NA and Met-NA-hydrolyzing activities were mostly bound. In addition, the Leu-NA-hydrolyzing activity in the spleen cells was kinetically studied. The Arg-NA and Leu-NA-hydrolyzing activities in four fractions prepared from the homogenate of spleen cells were also studied kinetically. From these studies it was suggested that ubenimex inhibits aminopeptidase B and a Pro-NA-hydrolyzing enzyme more effectively than Leu-APase in intact spleen cells and peritoneal macrophages from mice.

Ubenimex, a dipeptide found in the culture filtrate of *Streptomyces olivoreticuli*, has aminopeptidase (APase)-inhibitory[1~3], immunomodulatory[4~8] and antitumor activities[4,9,10]. The antitumor activity has been shown to be exerted *via* the immunomodulatory activity *in vivo*[4,9~11]. The APase-inhibitory activity has been studied mostly *in vitro* and largely independently of the immunomodulatory and antitumor activities. APases have been shown to participate in pathoimmunological processes. For instance, APases were suggested to be involved in inflammatory processes[12~14] where immunomodulation was thought to play a role. APase activities increased in association with the activation and differentiation of macrophages *in vivo*[15,16]. Additionally, Leu-APase and APase B-inhibitory activities of ubenimex and its analogs in a cell-free system were shown to correlate with enhancing effects in delayed-type hypersensitivity *in vivo*[5]. These results encouraged us to study further the action of ubenimex on APase activities in intact immunocompetent cells to understand the *in vivo* action of ubenimex.

Materials and Methods

Mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals. Ubenimex was prepared at Nippon Kayaku Co., Ltd.[17]. Arginine-β-naphtylamide (Arg-NA), Leu-NA, Ala-NA, Met-NA, Asp-NA and Pro-NA were purchased from Sigma Chemical Co. and His-NA and Lys-NA from Koch-Light Laboratories Ltd. Ficoll-sodium metrizoate and DEAE-cellulose

† Hereafter, by recommendation of WHO, the name of ubenimex is used for bestatin.
††† Deceased.

(DE-52) were obtained from Japan Immunoresearch Laboratories Co., Ltd. and Whatman Ltd., respectively.

Intact spleen cells and macrophages were prepared from nine to ten-week-old female BALB/c mice as described below. For preparation of spleen cells, the removed spleens were swollen by injecting HANKS' solution, cut at one end to make a small outlet and the inner cells were gently squeezed out with the aid of forceps. The cells were suspended in HANKS' solution and centrifuged at $400 \times g$ for 20 minutes through a Ficoll-sodium metrizoate layer to separate the lymphocyte fraction. The lymphocyte fraction was washed three times with HANKS' solution and suspended in fresh HANKS' solution. The suspension was immediately used as the intact spleen cells. For preparation of macrophages, the peritoneal cavities were washed twice with 3 to 5 ml of HANKS' solution and the cells in the washes were harvested by centrifugation, suspended in serum-free RPMI 1640 at 2×10^5 cells/ml, poured into Falcon 3046 plates and incubated for 90 minutes in a 5%-CO_2 incubator at 37°C. The monolayered cells were washed three times with warm HANKS' solution to exclude nonadherent cells. The washed cells were covered with fresh HANKS' solution and immediately used as the intact macrophages. The number of viable cells was determined by the trypan blue method.

APase activities were assayed by measuring β-naphthylamine liberated from amino acid-NA after incubation. A HANKS' solution containing amino acid-NA and an enzyme source as detailed in each figure legend was incubated for 30 minutes at 37°C and cooled to 0°C to terminate the reaction. The liquid phase of the mixture was separated by centrifugation, heated for 5 minutes on boiling water and subjected to fluorometric determination of β-naphthylamine with a Hitachi 650-10S fluorescence spectrophotometer. APase activity was calculated from the amount of β-naphthylamine formed due to the added enzyme source. *Km* values were determined according to LINEWEAVER and BURK plot. *Ki* values in inhibition of Leu-NA-hydrolyzing activity of intact spleen cells were estimated from DIXON plot. Other *Ki* values were estimated by the method of LINEWEAVER and BURK.

Results

Intact spleen cells were first examined for APase activities. The results are shown in Table 1. Amino acid-NA-hydrolyzing activities varied depending on the substrates. Among them, Ala-NA,

Table 1. APase activities of intact spleen cells and inhibition of the activities by ubenimex.

The incubation mixture for assay of the activities contained 1.5×10^6 intact spleen cells and 0.2 mM amino acid-NA in a total volume of 0.75 ml. Ubenimex was added at 10 μg/ml to examine the inhibitory effect. The number of intact spleen cells scarcely varied during the incubation in both assays.

Substrate	Activity (nmol/minute/ 2×10^6 cells)	Inhibition (%)
Arg-NA	0.10	95
Lys-NA	0.23	59
Leu-NA	0.46	31
Met-NA	0.65	19
Ala-NA	1.07	37
His-NA	0.16	13
Pro-NA	0.05	91
Asp-NA	0.01	NT

NT: Not tested.

Table 2. APase activities of intact macrophages and inhibition of the activities by ubenimex.

The incubation mixture for assay of the activities contained 2×10^5 macrophages in a total volume of 1.0 ml. After the incubation the medium was separated by decantation and directly subjected to heating on boiling water. The other manipulations and conditions were the same as those described in the legend of Table 1. The number of macrophages scarcely changed during incubation in both assays.

Substrate	Activity (nmol/minute/ 2×10^6 cells)	Inhibition (%)
Arg-NA	0.51	91
Lys-NA	0.44	60
Leu-NA	0.55	0
Met-NA	0.82	0
Ala-NA	0.95	48
His-NA	0.12	9
Pro-NA	0.11	96
Asp-NA	0.01	NT

NT: Not tested.

Met-NA and Leu-NA-hydrolyzing activities were highest, whereas Asp-NA-hydrolyzing activity was extremely low. Ubenimex inhibited all the activities. The strongest inhibition was seen on Arg-NA-hydrolyzing activity and a similarly strong inhibition on Pro-NA-hydrolyzing activity. Inhibitions of His-NA, Met-NA and Leu-NA-hydrolyzing activities were smallest. With intact macrophages, APase activities similar to those found with intact spleen cells were observed except that Arg-NA-hydrolyzing activity was about 5 times higher (Table 2). Inhibitory effects of ubenimex were practically the same as those obtained with the spleen cells or lower. Particularly, ubenimex did not inhibit Leu-NA and Met-NA-hydrolyzing activities.

Since both immunocompetent cells were similar to each other in APase activities as well as inhibition by ubenimex, only the spleen cell activities were further studied unless otherwise indicated. APases might be released from intact spleen cells during the incubation period for the activity assays described above. This possibility was next examined and the results are shown in Fig. 1. After incubation for 1 hour, the release of Arg-NA-hydrolyzing activity into the medium was the highest. Arg-NA-hydrolyzing activity released into the medium required 0.1 M NaCl for maximum activity. Arg-NA-hydrolyzing activity increased with time up to 240 minutes, whereas increase of activity in pellet was much

Fig. 1. Release of APase activities from intact spleen cells.

For the release 2×10^6 intact spleen cells were incubated in 1.0 ml of HANKS' solution at 37°C for 1 hour unless otherwise indicated. After the incubation the medium was separated from the cell pellet by centrifugation for 5 minutes at $450\times g$ and both medium and pellet were subjected to the assay of APase activities. The pellet was washed with an excess amount of HANKS' solution before the assay. Assay of APase activities was carried out as described in the legend of Table 1 using Arg-NA (a), Pro-NA (b), Lys-NA (c), Ala-NA (d), Leu-NA (e) and His-NA (f) as the substrate except that the total volume of the incubation mixture was increased to 1.0 ml. Released activities are shown in the figure as the percentage of each medium activity to the sum of the medium and pellet activities. The inset shows a time dependency of the release of Arg-NA-hydrolyzing activity. Closed and open circles indicate medium and pellet activities, respectively. All the activities shown in the figure are mean of triplicate data.

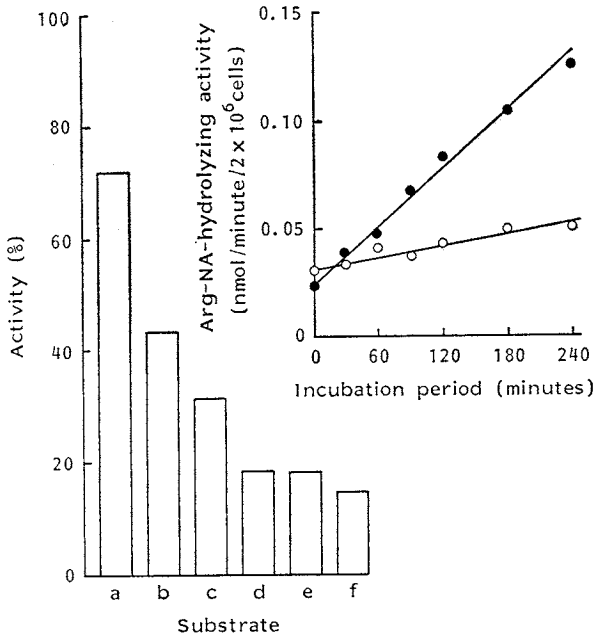

Fig. 2. DIXON plot on inhibition of the Leu-NA-hydrolyzing activity of intact spleen cells by ubenimex.

The APase activity was assayed as in the legend of Table 1 except that Leu-NA was added at 0.04 mM (●) and 0.01 mM (○) and that the total volume was doubled. DIXON plots in the inset were prepared on first phases in the main figure using activities obtained by subtracting the activities estimated on the extrapolated lines from the corresponding observed activities.

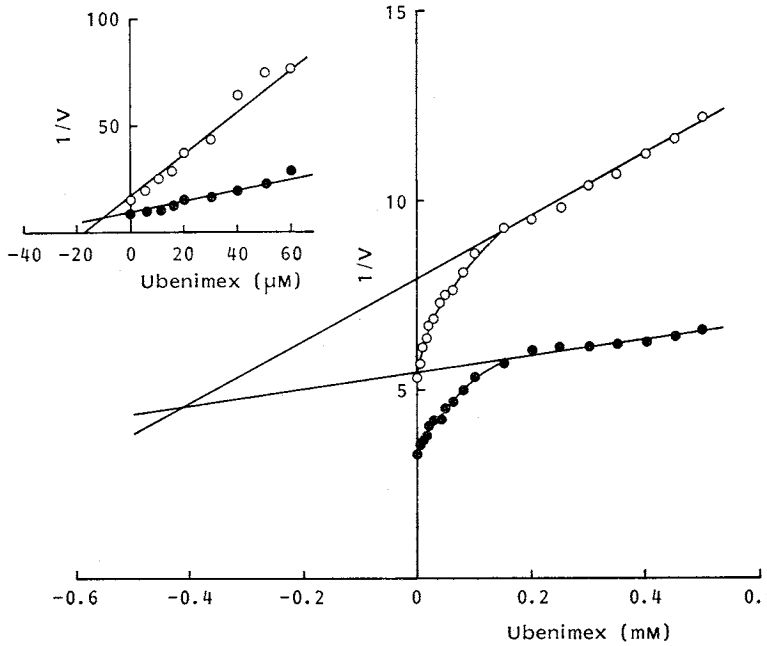

Table 3. Kinetic parameters obtained for Arg-NA and Leu-NA-hydrolyzing activities in 105,000×*g* precipitate and DEAE-fractions.

The fractions were prepared at 5°C as described below. Intact spleen cells were frozen, thawed and homogenized in HANKS' solution. The homogenate was centrifuged at 105,000×*g* for 1 hour. The supernate was applied to a 0.8×14-cm DEAE-cellulose column previously equilibrated with 0.01 M phosphate-KOH, pH 7.2. APase activities were eluted at a flow rate of 0.4 ml/minute by application of a linear gradient from 0.01 to 0.5 M phosphate-KOH, pH 7.2. Total volume of the eluting buffer was 200 ml and 2-ml-fractions were collected. When the fractions were subjected to the assay of Arg-NA and Leu-NA-hydrolyzing activities, three separate peaks, that is, DEAE I, II and III were found on the chromatogram in the increasing order of phosphate concentration. DEAE I and II exclusively contained Leu-NA and Arg-NA-hydrolyzing activities, respectively. DEAE III inseparably contained both activities. Arg-NA and Leu-NA-hydrolyzing activities were assayed under the same conditions as those in the legend of Table 1 except that the HANKS' solution was replaced with 0.05 M phosphate-KOH, pH 7.2.

Fraction	Substrate	*Km* (M)	*Ki* (M)
105,000×*g* Precipitate	Leu-NA	1.5×10^{-5}	2.6×10^{-5}
DEAE I	Leu-NA	2.3×10^{-4}	1.2×10^{-4}
DEAE II	Arg-NA	5.9×10^{-5}	4.4×10^{-8}
DEAE III	Leu-NA,	1.3×10^{-5},	3.2×10^{-7},
	Arg-NA	1.6×10^{-5}	4.7×10^{-7}

less, as shown in the inset of Fig. 1. Pro-NA and Lys-NA-hydrolyzing activities were also released but the percentages were less (Fig. 1). Leu-NA-hydrolyzing activity was largely found associated with the spleen cells. With macrophages, similar release was observed and Arg-NA-hydrolyzing activity was again most efficiently released (data not shown).

Table 3 shows kinetic parameters for Arg-NA and Leu-NA-hydrolyzing activities in four fractions prepared after homogenation of intact spleen cells. Most of the Arg-NA-hydrolyzing activity was found in $105,000 \times g$ supernate after the homogenation. The supernate activity was further separated into two peaks, that is, DEAE II and III on a DEAE-cellulose chromatogram. Ubenimex strongly and competitively inhibited both Arg-NA-hydrolyzing activities. Leu-NA-hydrolyzing activity was found in three fractions ($105,000 \times g$ precipitate, DEAE I and III) and ubenimex strongly inhibited the activity of DEAE III. In contrast, inhibition of the activity of $105,000 \times g$ precipitate was much less and that of the activity of DEAE I was still less, although competitive inhibition was seen with both. *Km*/*Ki* ratio obtained from the DEAE I activity was practically the same as that obtained for the precipitate activity. Therefore, we consider that the DEAE I activity is due to a soluble Leu-NA-hydrolyzing APase formed from the bound type at the time of homogenation. Kinetic study on the inhibition of Leu-NA-hydrolyzing activity was additionally done with intact spleen cells since the intact activity was largely bound. As shown in Fig. 2, DIXON plots were biphasic. Both inhibitions were competitive with the substrate, relatively small and the *Ki* values were 4.15×10^{-4} and 1.01×10^{-5} M.

Discussion

Ubenimex inhibited Leu-NA-hydrolyzing activity with intact spleen cells as LEYHAUSEN *et al.* previously found for intact T-lymphocytes, B-lymphocytes and thioglycollate-elicited macrophages from mice[3]. However, the inhibition was rather small as compared with those of the other APase activities. In addition, inhibitions of Met-NA and Ala-NA-hydrolyzing activities of both immunocompetent cells were similarly small or not detected. In contrast to these results, ubenimex strongly inhibited Arg-NA, Lys-NA and Pro-NA-hydrolyzing activities of both types of intact cells.

Even though incubation for the release of APase activities was performed two times longer than that for the assay of the activities, Arg-NA, Lys-NA and Pro-NA-hydrolyzing activities were seen to be largely released from intact spleen cells and macrophages. Therefore, the activities seen with intact cells in Tables 1 and 2 are due to both the bound and the released forms of APases. The released Arg-NA-hydrolyzing activity required 0.1 M NaCl for maximum activity as APase B from rat liver did[18]. Thus, this activity is considered to be mostly exerted by an APase B-like enzyme. The released Arg-NA-hydrolyzing activity was found as a large separate peak on a DEAE-chromatogram obtained under the same conditions as those in the present study and the peak was located similarly to the DEAE II (data not shown). The DEAE II fraction hydrolyzed Arg-NA exclusively from Leu-NA and had *Km* and *Ki* values similar to those for the released Arg-NA-hydrolyzing activity (data not shown). Therefore, the DEAE II activity is also considered to be due to an APase B-like enzyme. The bound Arg-NA-hydrolyzing activity found in the study on the release of APase activities may be due to an APase B-like enzyme and that may be weakly bound to the surface of both intact immunocompetent cells, since most of the Arg-NA-hydrolyzing activity of intact spleen cells was found in the $105,000 \times g$ supernate after homogenation. The weakly bound Arg-NA-hydrolyzing activity in intact spleen cells may be recovered as the DEAE II peak after the DEAE-fractionation. The DEAE III enzyme may be the so called nonspecific APase[19], since it inseparably contained Arg-NA and Leu-NA-hydrolyzing activities.

The *Ki* values of 4.15×10^{-4} and 1.01×10^{-5} M obtained for the Leu-NA-hydrolyzing activity of intact spleen cells further indicate that the inhibitory activity of ubenimex against the intact cell activity is relatively small. This activity must be due to a Leu-APase-like enzyme since it was largely found in the $105,000 \times g$ precipitate after homogenation and only the precipitate activity gave *Km* and *Ki* values similar to those for the intact activity.

Leu-APase from various sources seems to most effectively hydrolyze Leu-NA and Ala-NA and less effectively Met-NA and Phe-NA[19]. APase B effectively and specifically hydrolyzes Arg-NA and Lys-NA[18]. In addition, APases similar to the Leu-APase and APase B occur in mouse spleen cells and macrophages[3,16]. Taking these into consideration, we conclude here that ubenimex inhibits APase B

and Pro-NA-hydrolyzing activities more effectively than Leu-APase with intact spleen cells and resident peritoneal macrophages from mice. Ubenimex has been suggested to bind to Leu-APase on the cell surface of immunocompetent cells at the starting of the immunomodulatory action[3,5,20,21]. Ubenimex may also exert immunomodulatory activity *via* binding to APase B and Pro-NA-hydrolyzing APase released from and bound to immunocompetent cells. Further studies on the action of ubenimex on both APases should be done in relation to its immunomodulatory activity.

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