ENHANCEMENT OF INTERLEUKIN 1 AND INTERLEUKIN 2 RELEASES BY UBENIMEX†

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The effect of ubenimex on the release of interleukin 1 (IL-1) and interleukin 2 (IL-2) from immuno-competent cells was studied. Ubenimex enhanced release of IL-1 from mouse peritoneal macrophages at 1.0 and 100 μg/ml *in vitro* and the release at 1.0 μg/ml was larger. When ubenimex was administered to mice IL-1-releasing activity of the peritoneal macrophages was enhanced 3 and 5 days after the administration but not enhanced 1 day after the administration.

Ubenimex also enhanced IL-2 release from rat spleen cells at 0.1 and 10 μg/ml, when concanavalin A (Con A) was added in the IL-2-releasing system. The enhancement was still observed with mouse spleen cells, when serum was further added. Moreover, thymocyte-proliferating activity was attained in the broths which rat spleen cells were incubated with ubenimex from 0.1 to 10 μg/ml in the absence and presence of Con A.

Ubenimex, an inhibitor of leucine aminopeptidase and aminopeptidase B[1], has been demonstrated to have antitumor activities[2~7] and antibacterial activities[8,9]. Ubenimex has also been found to affect immunological functions. For instances, ubenimex increased antibody-dependent cell-mediated cytotoxicity[6,10], natural killer activity[6,7,10~12] of lymphocytes and cytotoxic activity of peritoneal macrophages against tumor cells[3,13]. In addition, it boosted antibody formation[2,3,14] and enhanced delayed type hypersensitivity[2~4,15], blastogenesis[3,14,16] of immuno-competent cells and colony formation of bone marrow progenitor cells[5].

BLOMGREN[17] suggested that ubenimex enhanced IL-2 release from human peripheral lymphocytes, and NOMA *et al.*[18] more directly showed this. We examined the effect of ubenimex on the release of interleukin 1 (IL-1) and interleukin 2 (IL-2) from murine peritoneal macrophages and splenocytes. The results are described below.

Materials and Methods

Inbred BALB/c, C57BL/6, CD(BALB/c×DBA/2)F_1, outbred ICR mice and inbred Fischer 344 rats were obtained from Charles River Japan Inc. The mice and rats were used at the ages of 6 to 12 weeks and 21 to 26 weeks, respectively. Ubenimex was synthesized at Nippon Kayaku Co., Ltd. by the method of NISHIZAWA *et al.*[19]. Ubenimex was dissolved in sterile saline or RPMI 1640 medium and sterilized by filtration through a Millipore filter with a pore size of 0.22 μm before use. [^{3}H]-thymidine ([^{3}H]TdR) was purchased from Amersham International plc. The specific activity was 40 Ci/mmol. Phytohemagglutinin-P (PHA) and concanavalin A (Con A) were purchased from Difco Laboratory and Pharmacia Fine Chemicals Inc., respectively. Both mitogens were dissolved in RPMI

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

1640 medium in advance and stored in a freezer. α-Methyl-D-mannoside (αMM) was obtained from Sigma Chemical Company. A purified rat T-cell growth factor, which was used here as a standard IL-2, was purchased from Collaborative Research Inc.

Resident macrophages were collected from mouse peritoneal cavities by lavage with RPMI 1640 medium containing 50 μM 2-mercaptoethanol (2-ME), 20 mM HEPES, 100 units/ml penicillin (PC), 100 μg/ml streptomycin (SM) and 5% fetal calf serum (FCS). The macrophages were washed twice with RPMI 1640 medium containing 100 units/ml PC, 100 μg/ml SM and 5% FCS, and suspended in the same medium at 2×10^6 viable cells/ml. One ml of the cell suspension was plated on a Falcon 3001 petri dish and incubated for 3 hours at 37°C under a humidified air containing 5% CO_2. The monolayered cells were washed 3 times with RPMI 1640 medium to remove nonadherent cells. More than 96% and 99.9% of the monolayered cells were identified as macrophages by latex phagocytosis[20] and by non-specific esterase stain[21], respectively. Spleen cells were prepared from mouse and rat as described by NARIUCHI and MATUHASI.[22].

Release of IL-1 was carried out according to TENU *et al.*[23] except that the antibiotics were excluded from the incubation mixture and that the incubation period was reduced to 24 hours because the maximum level of IL-1 activity was attained at 24 hours in this medium. The broth obtained after IL-1 release was sterilized by filtration through Millipore filter and stored at −20°C until IL-1 assay. When ubenimex was added to the IL-1-releasing mixture, the broth was dialyzed twice against RPMI 1640 medium before the sterilization. For IL-2 release 2.5 to 5.0×10^7 spleen cells were incubated for 24 to 48 hours at 37°C in 5 ml of RPMI 1640 medium containing 50 μM 2-ME, 100 units/ml PC and 100 μg/ml SM. FCS and Con A were added where indicated. The broth was similarly dialyzed, sterilized and stored frozen until IL-2 assay.

IL-2 for maintenance of CTLL-2 cells was prepared by incubating Fischer 344 rat spleen cells at 5×10^6 cells/ml for 48 hours at 37°C in RPMI 1640 medium containing 10% FCS and 5 μg/ml Con A. A Costar tissue culture flask was used for the incubation. After the incubation the broth was taken by decantation and centrifuged at 2,500 rpm. αMM was then dissolved in the supernate at 20 mg/ml. The IL-2 preparation was passed through Millipore filter and stored at −20°C until used.

Table 1. The effect of ubenimex on *in vitro* release of IL-1 from macrophages.

Ubenimex (μg/ml)	[^{3}H]TdR incorporation (cpm±SD)	
	Assayed in the absence of mitogen	Assayed in the presence of Con A
0	21,813±2,953	26,833±1,952
0.01	19,421±2,675	26,372±3,408
0.1	22,413±4,697	90,273±17,269**
1.0	42,266±1,217**	111,720±46,539*
10	28,325±8,554	47,186±15,297*
100	31,337±3,846**	39,297±20,641

Release of IL-1 was done as described in Materials and Methods adding indicated amounts of ubenimex. IL-1 was assayed using thymocytes as the target cells. The thymocytes were included at 7.5×10^6 cells/ml in each assay mixture. Con A was included at 5 μg/ml. The incubation was carried out for 72 hours and [^{3}H]TdR was added 5 hours before the termination. Each incorporation is shown in mean±standard deviation (SD) of triplicate or quadruplicate assays. *P* values were determined by t-test in comparison with the incorporation obtained without addition of ubenimex.

* $P<0.05$. ** $P<0.005$.

IL-1 was assayed by measuring [^{3}H]TdR incorporation in thymocytes from 6 week-old BALB/c mice as described by MIZEL *et al.*[24] and OPPENHEIM *et al.*[25]. IL-2 in a single sample was assayed by similarly measuring [^{3}H]TdR incorporation in two different target cells, that is, mouse thymocytes[26] and IL-2-dependent CTLL-2 cells[27,28]. In the former assay one volume of a sample broth was mixed with the same volume of target cell suspension. In the latter one volume of the broth was mixed with 19 volumes of the target cell suspension. In both IL-1 and IL-2 assays incubation mixtures were pulsed by adding 1 μl of 1 μCi [^{3}H]TdR to each well carrying 0.2 ml of the mixtures.

Results

Firstly, ICR, CDF_1 and BALB/c mice were compared with one another in *in vitro* release of IL-1 from each resident macrophage (data not shown). The largest IL-1 release was observed for the macrophages from ICR mice. IL-1

Table 2. The effect of administration of ubenimex on the *in vitro* IL-1-releasing activity of macrophages.

Ubenimex administration schedule (days before mice were sacrificed)	[^{3}H]TdR incorporation (cpm±SD)	
	Assayed in the absence of mitogen	Assayed in the presence of Con A
NA	2,786±391	6,955±1,507
5	4,190±355**	8,837±2,469
3	6,618±1,998*	11,501±1,789**
1	3,808±614*	7,215±2,033

Ubenimex was po administered to ICR mice at the indicated days. The dose was 5 mg/kg. One group consisted of 10 mice. After sacrifice of the mice the macrophages were collected and subjected to *in vitro* IL-1 release. Assay of released IL-1 was carried out as described in the legend of Table 1.

* $P<0.05$. ** $P<0.005$. NA: Not administered.

Table 3. The effect of ubenimex on *in vitro* release of IL-2 from rat spleen cells.

Ubenimex (μg/ml)	[^{3}H]TdR incorporation (cpm±SD)	
	Assayed with thymocytes in the presence of Con A	Assayed with IL-2-dependent CTLL-2 cells
0	1,570±570	2,036±403
0.01	1,295±319	NT
0.1	1,580±270	1,810±708
1.0	4,622±1,449*	2,007±468
10	3,057±1,026*	1,897±516
100	1,574±195	1,370±387
Standard rat IL-2 (U/ml)		
0	1,027±371	1,045±545
5	4,816±795	85,697±4,005
10	8,048±2,444	NT

Spleen cells prepared from Fischer 344 rats were used. The spleen cells were incubated for 48 hours at 5×10^6 cells/ml for IL-2 release and the released IL-2 was assayed using thymocytes and IL-2-dependent CTLL-2 cells as the target cells. The assay incubation mixture using thymocytes was RPMI 1640 medium containing 10% FCS, 1 μg/ml of Con A and that using CTLL-2 cells was RPMI 1640 medium containing 10% FCS, 300 μg/ml glutamine and 25 mM HEPES. The thymocytes were added at 5×10^4 cells in 0.2 ml of the incubation mixture and the CTLL-2 cells were added at 1×10^4. Each assay mixture was incubated for 72 hours or 24 hours, respectively. [^{3}H]TdR was added 4 hours before the termination of the incubation. *P* values were determined by t-test in comparison with the incorporation obtained without addition of ubenimex.

* $P<0.05$. NT: Not tested.

Table 4. Enhancement of IL-2 release from rat spleen cells in the presence of Con A by ubenimex.

Ubenimex (μg/ml)	[^{3}H]TdR incorporation (cpm±SD)	
	Assayed with thymocytes in the presence of Con A	Assayed with IL-2-dependent CTLL-2 cells
0	1,263±302	2,139±1,622
0.01	1,976±616*	NT
0.1	3,449±514**	5,151±279*
1.0	2,340±474**	5,636±3,434
10	1,645±605	5,325±1,675*
100	1,482±534	3,336±2,044
Standard rat IL-2 (U/ml)		
0	1,027±371	429±59
5	4,816±795	42,040±6,365
10	8,046±2,444	43,728±6,126

Spleen cells were prepared from Fischer 344 rats. The spleen cells were incubated for 48 hours at 5×10^6 cells/ml with 5 μg/ml of Con A for IL-2 release. Assay for IL-2 were carried out as described in the legend of Table 3.

* $P<0.05$. ** $P<0.005$. NT: Not tested.

release was less with the CDF_1 macrophages and the least with the BALB/c macrophages. Thus, the macrophages from ICR mice were used in the study of IL-1 release. Table 1 shows the effect of the addition of ubenimex on the IL-1 release. Ubenimex enhanced [^{3}H]TdR incorporation into thymocytes at 1.0 and 100 μg/ml giving a larger incorporation at 1.0 μg/ml. The larger incorporation was about two times more than the incorporations obtained without addition of ubenimex. Essentially similar degrees of enhancement of IL-1 release were observed with the same broths when IL-1 was assayed in the presence of PHA although the incorporations thus obtained were about 1.5 times more (data not shown). Further increased incorporation was ob-

served when assayed in the presence of Con A in place of PHA (Table 1) and the maximum incorporation was still observed at 1.0 μg/ml ubenimex. The increased incorporation in the presence of Con A and PHA may be due to their T cell-differentiating action[29].

The effect of administration of ubenimex on the *in vitro* IL-1-releasing activity of macrophages was examined in the next experiments. Ubenimex was administered as indicated in Table 2. The results are shown in the table. Ubenimex administration significantly increased [^{3}H]TdR incorporation when administered on the 5th day and the 3rd day before sacrifice of mice, but did not when administered on the first day before the sacrifice. These data were obtained by assaying IL-1 without addition of mitogen. When IL-1 was assayed in the presence of PHA, a similar effect of the administered ubenimex was observed (data not shown). When IL-1 was assayed in the presence of Con A in place of PHA larger incorporations were obtained but the effect of ubenimex was again essentially the same as that observed when IL-1 was assayed without mitogen (Table 2). These results indicate that the effect of the administered ubenimex is schedule-dependent.

The effect of ubenimex on *in vitro* IL-2 release in the absence and presence of mitogens was investigated. Since it was known that IL-2-releasing activity of rat spleen cells was higher than that of mouse spleen cells[30], rat spleen cells were mainly used in the study of IL-2 release. After incubation for IL-2 release the broths were extensively dialyzed before IL-2 assay, because even a low level of ubenimex might interfere the assay. The dialysis reduced ubenimex concentration to less than one thousandth. For instance, ubenimex at 100 μg/ml in the incubation mixture was at 0.026 μg/ml after the dialysis.

Ubenimex effect on IL-2 release from spleen cells from Fischer 344 rats was investigated without extraneous addition of mitogen and serum. After incubation of the spleen cells in IL-2-releasing system with and without addition of ubenimex, IL-2 in the broth was assayed using thymocytes and IL-2-dependent CTLL-2 cells as the target cells. The results are shown in Table 3. Ubenimex was seen to enhance IL-2 release from rat spleen cells when IL-2 was assayed using thymocytes. A larger [^{3}H]TdR incorporation into the thymocytes was given at 1.0 μg/ml ubenimex and it was about three times more than the incorporations obtained without addition of ubenimex. The enhancing effect was not observed when IL-2 was assayed using IL-2-dependent CTLL-2 cells as the target cells. Even when 10 times the volume of the broths were subjected to the assay, the effect could not be detected (data not shown). This may be due to a lower sensitivity of the IL-2-dependent CTLL-2 assay to low levels of IL-2 although this assay is considered to be more specific for IL-2 determination[28] than the assay using thymocytes.

Ubenimex effect on IL-2 release was further studied adding Con A to the IL-2-releasing system. The results are shown in Table 4. Even here ubenimex was shown to enhance IL-2 release when IL-2 was assayed using thymocytes. Ubenimex-enhancing effect was more demonstrative when IL-2 was assayed with IL-2-dependent CTLL-2 cells. That is to say, increase of [^{3}H]TdR incorporation into the CTLL-2 cells was observed with the broths which were gotten after incubation for IL-2 release with addition of ubenimex in concentration from 0.1 to 10 μg/ml. A larger incorporation was given at 1.0 μg/ml ubenimex and it was about 2.5 times more than the incorporations obtained without addition of ubenimex. However, absolute value of IL-2 that induced from rat spleen cells under such conditions might be low compared to standard rat IL-2 value.

Effect of ubenimex on IL-2 release was additionally investigated with Fischer 344 rat spleen cells in the presence of serum in addition to Con A. The largest incorporations among the incorporations

Table 5. The effect of ubenimex on IL-2 release from rat and mouse spleen cells in the presence of serum in addition to Con A.

Animal	Ubenimex (μg/ml)	[^{3}H]TdR incorporation (cpm±SD)	
		Assayed with thymocytes in the presence of Con A	Assayed with IL-2-dependent CTLL-2 cells
Rat	0	2,641±704	56,444±5,977
	0.1	4,166±981*	56,148±2,780
	1.0	5,373±943**	60,454±4,369
	10	4,754±648**	52,204±14,059
Mouse	0	1,934±202	20,967±2,058
	0.1	3,285±551**	31,538±4,493**
	1.0	3,195±453**	32,171±3,770**
	10	3,285±844*	32,755±4,035*
Standard rat IL-2 (U/ml)			
	0	589±320	429±59
	5	1,912±347	42,040±6,365
	10	5,970±990	43,728±6,126

Spleen cells were prepared from Fischer 344 rats and CDF_1 mice. When Fischer 344 rat spleen cells were used as IL-2-releasing cells, an indicated amount of ubenimex, 5% FCS, 5 μg/ml Con A and 5×10^6/ml spleen cells were added to the IL-2-releasing mixture and the mixture was incubated for 48 hours. When CDF_1 mouse spleen cells were used, ubenimex, 5% FCS, 2 μg/ml Con A and 1×10^7/ml spleen cells were added to the IL-2-releasing mixture and the mixture was incubated for 24 hours. The assay for IL-2 was carried out as described in the legend of Table 3 and Materials and Methods except that one volume of a sample broth was mixed with the same volume of CTLL-2 cell suspension.

* $P<0.05$. ** $P<0.005$.

obtained in the present study of IL-2 were observed, as shown in Table 5. Even under such conditions ubenimex still enhanced IL-2 release from 0.1 to 10 μg/ml giving a larger release at 1.0 μg/ml, when IL-2 was assayed using thymocytes. Ubenimex-enhancing effect could not be seen with CTLL-2 cells. Even when 0.1 time the volume of the broths were subjected to the CTLL-2 assay, their [^{3}H]-TdR incorporations were the same as those shown in Table 5. This may be due to a narrow range of detectable IL-2 by CTLL-2 cells and/or to a maximum IL-2 releasing level that ubenimex could not stimulate more. Effect of ubenimex was further studied using CDF_1 mouse spleen cells under the same conditions. Ubenimex also enhanced IL-2 release from 0.1 to 10 μg/ml in both assay using thymocytes and CTLL-2 cells, as shown in Table 5. Prior to this investigation mouse spleen cells were tentatively examined for ubenimex-enhanced effect in IL-2 release using the two preceding conditions but no or slight effect was observed in a wide range of concentrations from 0.01 to 100 μg/ml whatever the target cells were (data not shown).

Discussion

In the present study we found that ubenimex enhanced IL-1 release from mouse resident macrophages. This is the first discovery of ubenimex-enhanced effect in IL-1 release as far as we know. BLOMGREN[17] found that ubenimex enhanced release of mitogenic factors from PHA-stimulated human peripheral lymphocytes. IL-1 might have been included in these mitogenic factors. However, the amount must be undetectably small if at all, judging from his mitogenic factor-releasing systems using lymphocytes as the releasing cells and E-rosette-forming cells as the target cells. The present results shown in Tables 1 and 2 more directly indicated that ubenimex enhanced IL-1 release from mouse macrophages. While there was a difference of [^{3}H]TdR incorporations into thymocytes between Tables 1 and 2, this might be dependent upon whether dialysis was done or not after IL-1 induction.

That is, when ubenimex was added in IL-1-releasing medium, as shown in Table 1, the broths were dialyzed against IL-1-releasing medium. When ubenimex was administered to mice in Table 2, the broths were not dialyzed. The dialysis might exclude some immuno-suppressive low-weight molecules from the broths. Therefore, the dialyzed broths more increased [^{3}H]TdR incorporations into thymocytes than the broths which were not dialyzed. Because mouse macrophages released prostaglandins and these releases were regulated by ubenimex[18].

BLOMGREN[17] suggested the enhancement of IL-2 release by ubenimex from the results described above. Recently, NOMA *et al.*[18] more directly showed the effect of ubenimex in enhancing IL-2 release from PHA-stimulated human peripheral lymphocytes using cultured T cells as the target cells in IL-2 assay. DUNLAP *et al.*[31] also reported that ubenimex increased IL-2 release from Con A-stimulated mouse spleen cells using thymocytes as the target cells in IL-2 assay. In order to demonstrate the effect of ubenimex in enhancing IL-2 release we used IL-2-dependent CTLL-2 cells in IL-2 assay. The assay with IL-2-dependent CTLL-2 cells is currently considered to be the best for IL-2 determination in specificity[28]. As detailed above we clearly demonstrated the IL-2 release-enhancement in the presence of Con A with rat spleen cells and in the presence of serum in addition to Con A with mouse spleen cells. The enhancement using CTLL-2 cells was supported from the results that obtained with thymocytes in IL-2 assay. However, it cannot be denied that the assay using thymocytes may determine other factors which induce thymocyte proliferation, because there is not a perfect correlation between the two IL-2 assays used here.

Ubenimex was a strong inhibitor of leucine aminopeptidase and aminopeptidase B of cell plasma membrane[32,33]. The effect of ubenimex in enhancing IL-1 and IL-2 release from immuno-competent cells may be exerted through the interaction of ubenimex with leucine aminopeptidase and/or aminopeptidase B on the cell surface. Further studies to clarify the mechanism of action of ubenimex in this direction are underway.

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