Medullasin Enhances Human Natural Killer Cell Activity

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ABSTRACT Medullasin, a new serine protease found in bone marrow cells, increased markedly human natural killer cell activity. Whereas the natural killer cell activity measured immediately after the treatment with medullasin remained almost on the same level as the control, an incubation at 37°C for several hours increased markedly the natural killer cell activity of the lymphocytes treated with medullasin. Enhancement of the natural cytotoxicity was caused by the treatment with physiologic concentrations of the protease (5–20 μg/ml). Inhibitors of medullasin such as phenylmethylsulfonyl fluoride and elastatinal prevented the activation of natural cytotoxicity. Depletion of lymphocytes bearing Fc receptors for IgG abolished the enhancement of natural killer cell activity by medullasin. Interferon activity was not detected in the supernatant of lymphocyte cultures stimulated with medullasin. The medullasin enhanced further the natural killer cell activity of lymphocytes stimulated with interferon. Medullasin activity was detected neither in unstimulated nor stimulated (by concanavalin A or phytohemagglutinin) human lymphocytes. The protease was released easily from human mature granulocytes into culture medium. It is considered from these results that the level of human natural killer cell activity is regulated by medullasin released by mature granulocytes.

INTRODUCTION

A new serine protease was found in bone marrow cells including both erythroblasts and granulocytes (1). It was crystallized from human bone marrow cells, and therefore named medullasin (from Latin medulla ossium, bone marrow). The molecular weight of the protease is 31,800, and optimum pH 8.5. Histidine, serine, and one carboxyl group were shown to be essential to the protease activity. It resembles elastase, without being identical with it. Equimolar amounts of α_1-antitrypsin inhibit the protease. From subcellular fractionation studies using rat erythroblasts as the material, medullasin is considered to be located on the inner mitochondrial membrane. Medullasin in erythroblasts was shown to participate in the development of a certain kind of anemia (2, 3). In the course of investigating the biologic significance of medullasin in granulocytes we found that levels of medullasin in granulocytes were elevated in certain pathological conditions such as chronic inflammations and cancer (submitted for publication). Furthermore, medullasin was found to enhance markedly human natural killer cell activity.

Natural killer cells, which show cytotoxicity against target cells without prior sensitization, are considered as one of the primary defense mechanism of animals against cancer and viral infections (4–7). In human peripheral blood they are considered to be the lymphocytes which bear the receptor for Fc portion of IgG but lack immunoglobulins on the cell surface (8–10). It is postulated that natural killer cells play equal or more important roles than T and B lymphocytes in the natural conditions of defense mechanisms against carcinogenesis and viral or bacterial infections (11, 12). The natural killer cell activity is reported to be modified by a variety of factors. Interferon (13, 14) and interferon inducers, such as viral infections, tumor cells, Bacille Calmette-Guérin(BCG), *Corynebacterium parvum*, and certain chemicals (15–20) enhance natural killer cell activity. Also tuftsin and interleukin-2 were reported to stimulate natural cytotoxicity of mice (21, 22). On the other hand, cyclophosphamide, hydrocortisone, and cholera toxin inhibit natural killer cell activity (23, 24). Also protease inhibitors and protease substrates were reported to inhibit cytotoxic T lymphocyte activity (25), antibody-dependent cell-mediated cytotoxicity (26), and natural killer cell activity (27). Furthermore, macromolecular protease inhibitors, such as α_1-antitrypsin and α_1-antichymotrypsin, inhibit human natural cytotoxicity (28). It is considered from these results that proteases may be involved in lymphocyte-mediated cytolysis.

In this paper we have examined the effect of medullasin on human natural killer cell activity, and discussed the biologic significance of this effect.

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METHODS

Preparation and assay of medullasin. Medullasin was purified and further crystallized from bone marrow cells obtained from resected human ribs according to the method described (1). After removal of polyethylene glycol by CM-cellulose column chromatography the protease was dissolved in 0.05 M acetate buffer (pH 5.0) and stored at 4°C. Since medullasin inactivates specifically the apo-form of certain pyridoxal enzymes, medullasin activity in mature granulocytes was measured by the use of apo-ornithine transaminase as substrate (1, 2). Mature granulocytes (5×10^6–1×10^7) suspended in 3 ml of phosphate-buffered saline (pH 7.5) were sonicated at 50 W for 15 s in a 50-ml centrifuge tube (model W 185, Branson Sonic Power Co., Danbury, CT). From the precipitate obtained by centrifuging the sonicated solution at 20,000 *g* for 15 min the protease was extracted with 1 ml of 0.5 M potassium phosphate buffer (pH 7.0) at 37°C for 30 min. One unit was defined as the amount of protease inactivating 50% of apo-ornithine transaminase under the conditions described (1). Tubes containing < 6 U of medullasin activity were used for the determination of activity, and the reaction was stopped at 10 min to determine the remaining ornithine transaminase activity.

Preparation of lymphocytes and mature granulocytes. Heparinized peripheral blood (30–50 ml) obtained from normal donors was diluted with equal volume of saline. It was layered on top of an equal volume of Lymphoprep (Nyegaad & Co. AS., Oslo, Norway), and centrifuged at 400 *g* for 30 min at room temperature. The mononuclear cell fraction formed between the sample and Lymphoprep was washed in saline by centrifugation, and suspended in 6–10 ml of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (Flow Laboratories, Rockville, MD). It was then applied on Sephadex G-10 column (2.5 × 9 cm) equilibrated with RPMI 1640 containing 10% fetal calf serum. The cellular fraction containing lymphocytes (97–99%) with a minimum contamination of monocytes (1–3%) was eluted from the column with the same medium. Lymphocytes thus obtained were washed two times in saline by centrifugation, and suspended in RPMI 1640 containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Mature granulocytes were prepared from the precipitate obtained by the Lymphoprep centrifugation described above. The precipitate, which contained both erythrocytes and mature granulocytes, was suspended in equal volume of plasma, and layered on top of an equal volume of Lymphoprep. It was left at room temperature for 30–50 min, during which time a layer was formed between the sample and Lymphoprep. The layer was collected and washed two times in saline by centrifugation to obtain mature granulocytes (94–97% mature granulocytes and 3–6% lymphocytes with a minimum contamination of erythrocytes)

Treatment of lymphocytes with medullasin. Lymphocytes (2×10^6/ml) suspended in 1 ml of RPMI 1640 supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) were treated with various amounts of medullasin (3–70 μg/ml) at 37°C for 30–180 min in a humidified 5% CO_2 incubator. The reaction was terminated by the addition of 10% fetal calf serum containing inhibitors to this protease. The lymphocyte suspension was further incubated for various intervals at 37°C in a humidified 5% CO_2 atmosphere before the assay for cytotoxicity against target cells. Tubes not containing protease served as control. Treatment with trypsin (10–50 μg/ml) was performed in the same way as described above.

Inhibitor studies. Phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and elastatinal (Institute of Microbiology, Tokyo) were used to inhibit medullasin activity in lymphocyte suspensions. Phenylmethylsulfonyl fluoride (0.1 mM) or elastatinal (20 μg/ml) was added to each tube containing medullasin (10 μg/ml) and lymphocytes (2×10^6) in a final volume of 0.3 ml. It was incubated at 37°C in a humidified 5% CO_2 atmosphere. After 60 min 10 ml of phosphate-buffered saline (pH 7.5) was added to each tube, and was centrifuged at 250 *g* for 10 min. After removing the supernatant 1 ml of RPMI 1640 containing both 10% fetal calf serum and antibiotics was added to the pellet, and the tubes were further incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to measure cytotoxicity against target cells. Tubes without medullasin served as control.

Enrichment and depletion of lymphocytes bearing Fc receptors. Rabbit IgG fraction containing the antibody against ox erythrocytes was diluted to a concentration just below that causing an agglutination of erythrocytes. To the pellet of ox erythrocytes (2×10^9), washed two times in phosphate-buffered saline by centrifugation, 1 ml of the IgG fraction was added and incubated for 30 min at 37°C. After incubation the erythrocytes were washed twice in phosphate-buffered saline by centrifugation and suspended in RPMI 1640 (2×10^9/ml). To 1 ml of the lymphocyte suspension in RPMI 1640 ($4–5 \times 10^7$/ml) 1 ml of antibody coated ox erythrocytes was added, and incubated at 37°C for 40 min after centrifugation at 140 *g* for 5 min. Tubes containing ox erythrocytes without antibody instead of those coated with antibody served as control. After incubation the cell suspensions were layered on top of an equal volume of Lymphoprep, and centrifuged at 400 *g* for 30 min. The cell layer formed between the sample and Lymphoprep was collected and washed two times in phosphate-buffered saline by centrifugation to obtain lymphocytes without Fc receptors (>95% of lymphocytes beared no Fc receptors). 2 ml of water was added to the pellet obtained by the Lymphoprep centrifugation followed immediately by the addition of 2 ml of two times concentrated RPMI 1640 medium. It was washed two times in phosphate-buffered saline by centrifugation to obtain the fraction enriched in lymphocytes bearing Fc receptors (50–60% of lymphocytes beared Fc receptors).

Determination of natural killer cell activity of lymphocytes cultured with mature granulocytes. Tubes containing both lymphocytes (1.5×10^6/ml) and mature granulocytes (1.3×10^6/ml) in 2 ml of RPMI 1640 supplemented with 20% autologous serum and antibiotics were incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Tubes without mature granulocytes served as control. After incubation the cell suspensions (10 ml) were applied on Sephadex G-10 column (2.5 × 9 cm) equilibrated with RPMI 1640 containing 10% autologous serum. The eluate from the column contained negligible amount of granulocytes (<2%). Lymphocytes obtained from the eluate of the column were washed two times in RPMI 1640, suspended in RPMI 1640 (2×10^6/ml) supplemented with 10% fetal calf serum and antibiotics, and further incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to be assayed for natural killer cell activity.

Measurement of the release of medullasin from granulocytes. Zymosan particles (Sigma Chemical Co.) preopsonized by incubating with fresh serum at 37°C for 30 min were washed twice in Hanks' solution. Mature granulocytes (3×10^7) obtained from normal donors were incubated at 37°C for 30 min in the presence or absence of zymosan (2 mg/ml) in 1 ml of RPMI 1640 (pH 7.5). After centrifugation medullasin activity was measured in the supernatant.

Interferon. Human interferon-β produced in human diploid foreskin fibroblasts was supplied from Toray Ind. Inc., Tokyo. Human interferon-α prepared by Dr. K. Cantell (Helsinki) was kindly supplied by Dr. S. Kobayashi (Toray Basic Research Laboratories, Kamakura, Japan). Interferon titers in the supernatant of lymphocyte cultures were kindly measured by Dr. T. Nagashima (Toray Basic Research Laboratories, Kamakura, Japan) by inhibition of the cytopathic effect of sindbis virus on a FL cell monolayer according to the method of Havell and Vilček (29). Antiviral units were expressed as the reciprocal of the titer inhibiting 50% of the cytopathic effect using National Institutes of Health standard of human interferon-α.

Cytotoxicity assay. K-562 cells (myeloid leukemia) and Molt-4 cells (T-cell leukemia) were used as the target cells for ^{51}Cr-release assay. Target cells ($1–5 \times 10^6$) suspended in 0.2 ml of RPMI 1640 containing both 10% fetal calf serum and antibiotics were incubated with 75 μCi ^{51}Cr ($Na_2{}^{51}CrO_4$, Japan Atomic Energy Research Institute, Ibaragi, Japan) at 37°C for 2 h. The labeled target cells were then washed three times in phosphate-buffered saline (pH 7.5) and resuspended in RPMI 1640 medium containing both 10% fetal calf serum and antibiotics at 2.5×10^5 cells/ml. Various number of effector cells (0.16 ml) and 1×10^4 target cells were added to 13×100-mm tubes in triplicate in a final volume of 0.2 ml, and were incubated at 37°C for 5 h in a humidified 5% CO_2 atmosphere. Maximum ^{51}Cr release was determined by the addition of 0.16 ml of 10% Triton X-100, and spontaneous ^{51}Cr release by the addition of 0.16 ml of medium alone to the tube in triplicate instead of the lymphocyte suspensions. The values of cytotoxicity were calculated from the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}.$$

Spontaneous release ranged between 10 and 20% of the maximum. The cytotoxic activity of lymphocytes was also expressed as lytic units per 10^6 lymphocytes. One lytic unit is the number of cells required to cause 30% specific ^{51}Cr release.

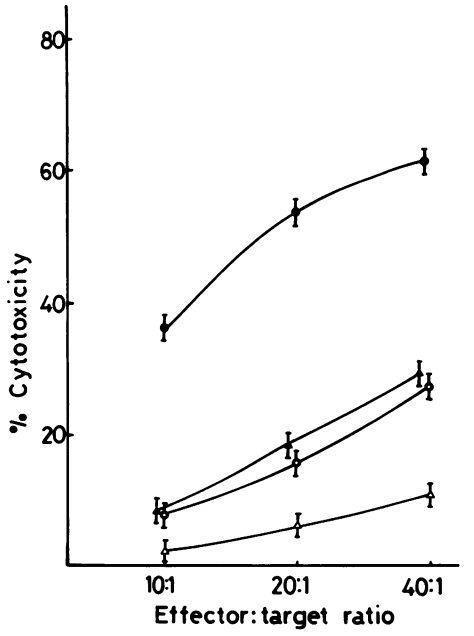

FIGURE 1 Effect of treatment with medullasin on natural killer cell activity. Lymphocytes (2×10^6/ml) suspended in RPMI 1640 were treated with medullasin (20 μg/ml) at 37°C for 60 min in a humidified 5% CO_2 incubator. After stopping the reaction by adding 10% fetal calf serum lymphocyte suspensions were further incubated at 37°C for 24 h before the assay for the cytotoxicity (Methods). Each natural killer cell activity was assayed using the following cells: ○, lymphocytes treated in the absence of medullasin (target cells: K-562); ●, lymphocytes treated with medullasin (target cells: K-562); △, lymphocytes treated in the absence of medullasin (target cells: Molt-4); and ▲, lymphocytes treated with medullasin (target cells: Molt-4). Bars, standard deviation of triplicate determinations.

RESULTS

Enhancement of natural killer cell activity by the treatment with medullasin. Natural killer cell activity of lymphocytes obtained from normal donors was markedly enhanced by the treatment with medullasin when the cytotoxicity was measured 24 h after the treatment. A typical result is shown in Fig. 1. The stimulatory effect of medullasin was equally observed whether K-562 cells or Molt-4 cells were used as target cells.

Dependence of the stimulatory effect of medullasin on the incubation period after the treatment. Fig. 2 shows that the enhancement of natural cytotoxicity by medullasin depends upon incubation periods after the treatment. Stimulatory effect of medullasin on the natural cytotoxicity was not significant when examined immediately after the treatment with medullasin. Enhancement of the natural killer cell activity by medullasin became evident when lymphocytes treated with medullasin were incubated at 37°C for 6 h in a humidified 5% CO_2 atmosphere. The effect of medullasin treatment was maximum at 24-h incubation, thereafter the stimulatory effect gradually decreased. When the mononuclear cell fraction (84–92% lymphocytes and 8–16% of monocytes) obtained by centrifuging the peripheral blood on Lymphoprep was used for the treatment with medullasin, rather inhibitory effect was observed when the cytotoxicity was measured immediately after the treatment. However, the stimulatory effect of medullasin was almost the same as that obtained by using pure lymphocytes when the natural killer cell activity was measured 24 h after the treatment.

Dose dependence. The relationship between the amounts of medullasin used and enhancement of natural killer cell activity was examined (Fig. 3). Maximum stimulation was observed when lymphocytes were treated with 20 μg/ml of medullasin for 1 h.

Dependence on incubation time. Natural killer cell activity of lymphocytes treated with 20 μg/ml of medullasin for various hours is shown in Fig. 4. Maximum stimulation was obtained when lymphocytes were treated for 60 min with medullasin.

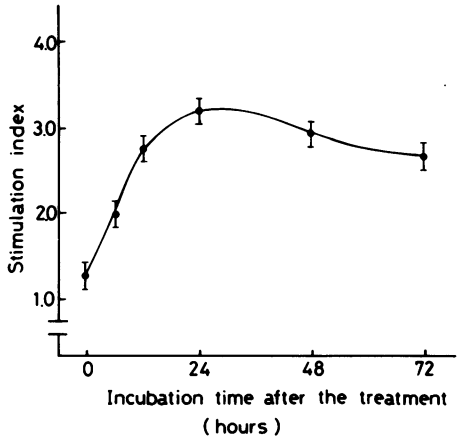

FIGURE 2 Dependence of the stimulatory effect of medullasin on the incubation time after the treatment. Lymphocytes (2×10^6/ml) suspended in RPMI 1640 were treated with medullasin (20 μg/ml) at 37°C for 60 min. After stopping the reaction by adding 10% fetal calf serum lymphocyte suspensions were further incubated at 37°C for various hours in a humidified 5% CO_2 atmosphere. At each incubation period lymphocytes were removed to be assayed for the cytotoxicity against K-562 cells. Stimulation index shows the ratio of the natural killer cell activity expressed as lytic units measured using the lymphocytes treated with medullasin to that measured using the lymphocytes treated in the absence of medullasin. Bars, standard deviation of triplicate determinations.

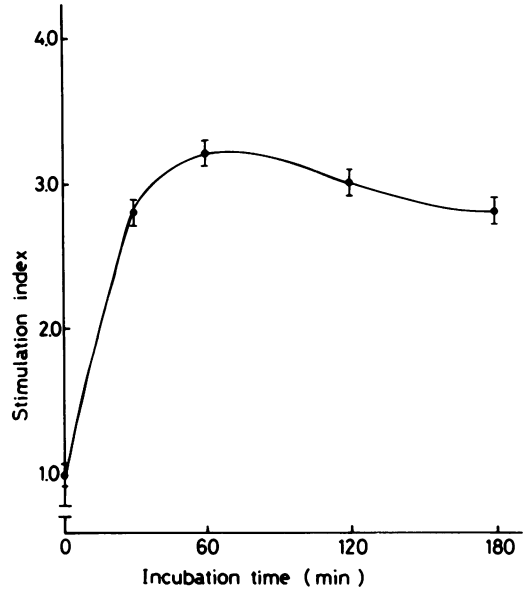

FIGURE 4 Stimulation of the natural killer cell activity as a function of incubation time. Lymphocytes (2×10^6/ml) suspended in RPMI 1640 were incubated at 37°C for various hours in a humidified 5% CO_2 atmosphere in the presence of medullasin (20 μg/ml). The reaction was terminated and assayed for the natural killer cell activity as described in Fig. 3. Stimulation index shows the same as that in Fig. 2. Bars, standard deviation of triplicate determinations.

Effect of medullasin inhibitors. The effect of medullasin inhibitors was examined to realize whether the stimulatory effect of medullasin on natural killer cell activity depends on its protease activity. Whereas an incubation of lymphocytes with phenylmethylsulfonyl fluoride (0.1 mM) at 37°C for 1 h did not affect the natural killer cell activity, treatment of lymphocytes with medullasin (10 μg/ml) for 1 h at 37°C in the presence of phenylmethylsulfonyl fluoride (0.1 mM) remarkably inhibited the enhancement of natural cytotoxicity (Fig. 5). (Medullasin activity was inhibited by 85% in the presence of 0.1 mM phenylmethylsulfonyl fluoride.) Furthermore, although an incubation of lymphocytes with elastatinal (20 μg/ml) at 37°C for 60 min had no effect on the natural killer cell activity, treatment of lymphocytes with medullasin in the presence of elastatinal (20 μg/ml), the inhibitor of this protease, markedly diminished the stimulatory effect of medullasin (medullasin activity was decreased by 75% in the presence of 20 μg/ml of elastatinal). Also both phenylmethylsulfonyl fluoride and elastatinal were not cytotoxic at these concentrations to lymphocytes as determined by trypan blue dye exclusion test (>98% of lymphocytes were viable).

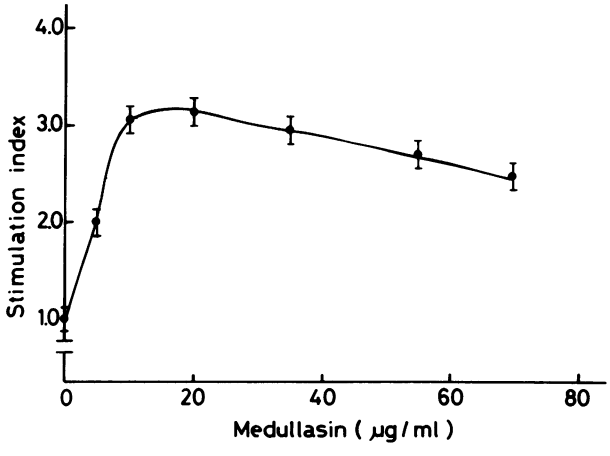

FIGURE 3 Stimulation of the natural killer cell activity as a function of medullasin concentrations. Lymphocytes (2×10^6/ml) suspended in RPMI 1640 were incubated at 37°C for 60 min in the presence of various amounts of medullasin. The reaction was terminated by adding 10% fetal calf serum, and lymphocyte suspensions were further incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to be assayed for cytotoxicity against K-562 cells. Stimulation index shows the same as that in Fig. 2. Bars, standard deviation of triplicate determinations.

Effect of trypsin treatment. Lymphocyte obtained from normal donors were treated with various amounts of trypsin (10–50 μg/ml) at 37°C for 60 min (Methods). The natural killer cell activity measured immediately after the treatment was decreased as compared with that of controls. Furthermore, the natural cytotoxicity measured 24 h after the treatment remained also below control levels.

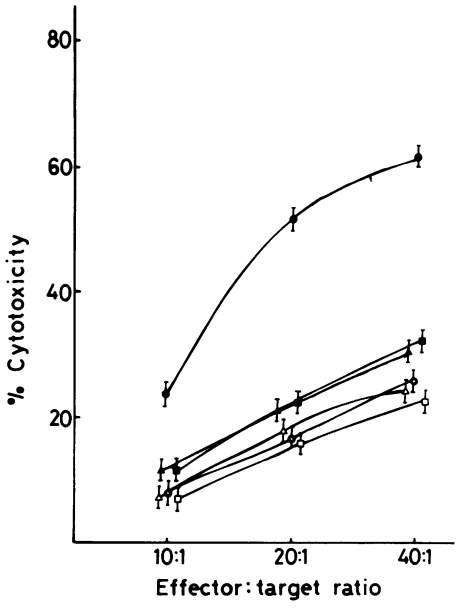

FIGURE 5 Effect of protease inhibitors on the stimulatory effect of medullasin. Lymphocytes (2×10^6) suspended in 0.3 ml of RPMI 1640 containing medullasin (10 μg/ml) were incubated in the presence of phenylmethylsulfonyl fluoride (0.1 mM) or elastatinal (20 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. After 60 min 10 ml of phosphate-buffered saline (pH 7.5) was added to each tube, followed by centrifugation at 250 *g* for 10 min. To the pellet 1 ml of RPMI 1640 containing both 10% fetal calf serum and antibiotics was added. Each tube was further incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to be assayed for the cytotoxicity against K-562 cells. Each tube contained the following lymphocytes: ○, lymphocytes treated in the absence of both medullasin and inhibitors; ●, lymphocytes treated with medullasin in the absence of inhibitors; △, lymphocytes treated with phenylmethylsulfonyl fluoride in the absence of medullasin; ▲, lymphocytes treated with medullasin in the presence of phenylmethylsulfonyl fluoride; □, lymphocytes treated with elastatinal in the absence of medullasin; and ■, lymphocytes treated with medullasin in the presence of elastatinal. Bars, standard deviation of triplicate determinations.

Effect of medullasin on the natural killer cell activity of lymphocyte subpopulations. Lymphocytes eluted from Sephadex G-10 column were fractionated as described in Methods into two groups; that containing lymphocytes devoid of Fc receptors and that enriched in lymphocytes bearing Fc receptors. Effect of medullasin treatment on the natural killer cell activity of each subpopulation is summarized in Table I. The natural killer cell activity of both lymphocytes eluted from Sephadex G-10 column and those contained in the fraction enriched in lymphocytes bearing Fc receptors was significantly stimulated by the treatment with medullasin. On the contrary, the natural killer cell activity of lymphocytes devoid of Fc receptors was negligible and the activity was not stimulated significantly by the medullasin treatment.

TABLE I
Enhancement of the Natural Killer Cell Activity of Lymphocyte Subpopulations by Medullasin

Lymphocytes	Lytic units* Medullasin treatment‡ ⊖	⊕	Stimulation index§
Lymphocytes eluted from Sephadex G-10 column	1.8±0.1	5.8±0.3	3.2
Lymphocytes bearing Fc receptors	3.6±0.2	11.7±0.6	3.3
Lymphocytes without Fc receptors	<0.02	<0.02	

* Lytic units per 10^6 lymphocytes. One lytic unit was defined as the number of cells required to cause 30% of specific ^{51}Cr release under the conditions described in Methods.
‡ Treatment of lymphocytes with medullasin was performed as described in Fig. 1.
§ Stimulation index shows the same as that in Fig. 2. Each value of lytic units was expressed as mean±SD of triplicate determinations.

Medullasin activity in lymphocytes. Medullasin activity in lymphocytes was measured using lymphocytes eluted from Sephadex G-10 column as the enzyme source. No medullasin activity was detected. Also the protease activity was not detected even when the mononuclear cell fraction containing 8–16% of monocytes besides lymphocytes was used for the enzyme source. Furthermore, the mononuclear cell fraction (2×10^6/ml) obtained from Lymphoprep centrifugation was incubated in RPMI 1640 medium containing 10% autologous serum at 37°C for 48–72 h in a humidified 5% CO_2 atmosphere in the presence of concanavalin A (Sigma Chemical Co., 10 μg/ml) or phytohemagglutinin (Gibco Laboratories, 15 μg/ml). No medullasin activity was detected in these cultured lymphocytes. Also no medullasin activity was detected in the culture medium when the mononuclear cell fraction was incubated in RPMI 1640 medium without serum in the presence of concanavalin A or phytohemagglutinin at 37°C for 48–72 h in a humidified 5% CO_2 atmosphere.

Release of medullasin into culture medium from mature granulocytes. The release of medullasin from granulocytes was examined by incubating mature granulocytes in RPMI 1640 medium in the presence or absence of zymozan (2 mg/ml). As shown in Fig. 6, medullasin release from mature granulocytes was prominent (25% of total) when incubated at 37°C for 30 min in the presence of zymosan. Considerable amounts of medullasin (3.5%) were also released even when mature granulocytes were incubated in the absence of zymosan.

Natural killer cell activity of lymphocytes cultured with granulocytes. As shown in Fig. 7, lymphocytes cultured with mature granulocytes for 24 h revealed elevated natural killer cell activity as compared with those

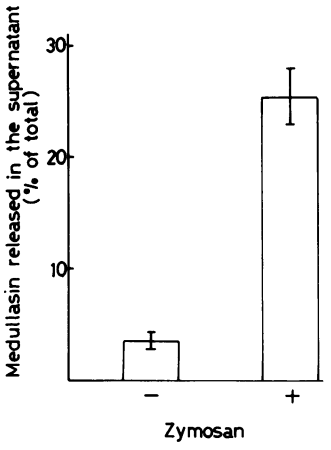

FIGURE 6 Release of medullasin from mature granulocytes into culture medium. Mature granulocytes (3×10^7) suspended in 1 ml of RPMI 1640 (pH 7.5) were incubated at 37°C for 30 min in the presence or absence of preopsonized zymosan particles (2 mg/ml). After centrifugation the medullasin activity in the supernatant was measured, and compared with total activity. Bars, standard deviation of triplicate determinations.

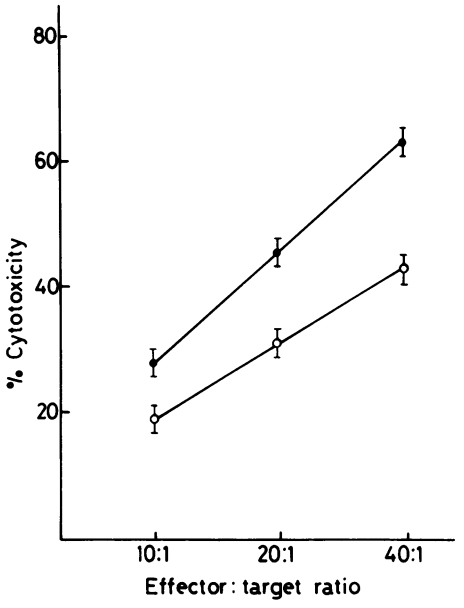

FIGURE 7 Enhancement of the natural killer cell activity by the incubation with granulocytes. Lymphocytes (1.5×10^6/ml) were incubated in the presence or absence of mature granulocytes (1.3×10^6/ml) in 2 ml of RPMI 1640 supplemented with 20% autologous serum and antibiotics at 37°C for 24 h in a humidified 5% CO_2 atmosphere. After incubation granulocytes were removed from lymphocyte suspensions by Sephadex G-10 column chromatography (Methods). Lymphocytes were then suspended in RPMI 1640 (2×10^6/ml) supplemented with both 10% fetal calf serum and antibiotics, and further incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to be assayed for the natural cytotoxicity. Each tube contained the following lymphocytes: O, lymphocytes incubated without granulocytes; ●, lymphocytes incubated with granulocytes. Bars, standard deviation of triplicate determinations.

cultured without granulocytes when measured after 24-h incubation at 37°C in a humidified 5% CO_2 atmosphere.

Interferon titers in the supernatant of lymphocyte cultures. The titer of interferon in the supernatant of lymphocyte cultures stimulated by the medullasin treatment was negligible (<5 U/ml), although natural killer cell activity of the lymphocytes was markedly enhanced as compared with that of lymphocytes not treated with medullasin.

Effect of medullasin on the natural killer cell activity of the lymphocytes stimulated with interferon. Various amounts of interferon-α were added to the lymphocyte suspensions treated with medullasin (20 μg/ml) for 60 min at 37°C, followed by an incubation for 24 h at 37°C in a humidified 5% CO_2 atmosphere to be assayed for natural killer cell activity. The same amounts of interferon-α were also added to the lymphocyte suspensions not treated with medullasin. Results are shown in Fig. 8. The medullasin enhanced further the natural killer cell activity of lymphocytes stimulated with interferon in all ranges of interferon concentrations. The same results were obtained when interferon-β was used instead of interferon-α.

DISCUSSION

There have been many reports suggesting that proteases are involved in cytolysis mediated by lymphocytes. The activity of cytotoxic T lymphocytes was reduced by inhibitors of serine protease (25, 30). Also both antibody-dependent cell-mediated cytotoxicity and natural killer cell activity were inhibited by serine protease inhibitors and substrates (26–28). A cytotoxic serine protease was isolated from unstimulated human lymphocytes (31). It is considered from these reports that certain serine proteases in lymphocytes may participate in lymphocyte-mediated cytolysis. Medullasin, however, is different from other proteases, which are assumed to be present in lymphocytes, because its activity was detected neither in pure lymphocytes nor in the mononuclear cell fraction containing 8–16% of monocytes. Furthermore, since stimulation of lymphocytes with concanavalin A or phytohemagglutinin failed to induce medullasin activity, it is considered that the protease is present neither in lymphocytes nor monocytes.

Results of experiments fractionating lymphocytes into subpopulations revealed that medullasin enhances natural killer cell activity of lymphocytes bearing Fc receptors. Therefore, medullasin is considered to stimulate the activity of classical natural killer cells (8–10). The amount of medullasin required for the enhancement of natural killer cell activity is physiologic, since medullasin concentration in peripheral blood of nor-

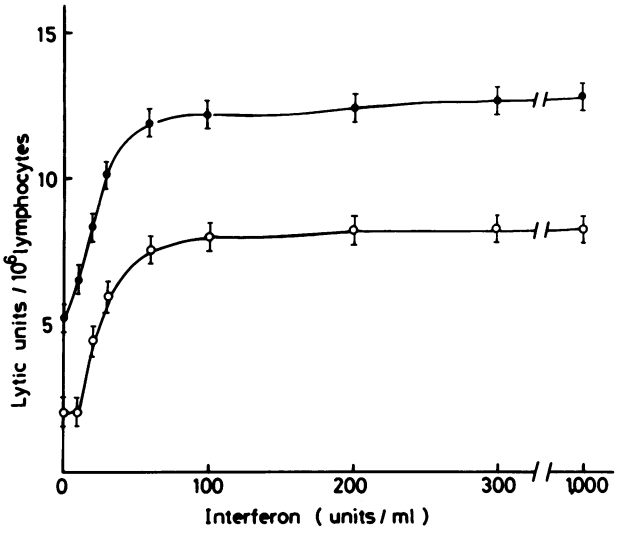

FIGURE 8 Effect of medullasin on the natural killer cell activity of lymphocytes stimulated with interferon. Tubes containing lymphocytes (2×10^6/ml) in RPMI 1640 were treated with 20 μg/ml medullasin at 37°C for 60 min, followed by the addition of 10% fetal calf serum, antibiotics, and various amounts of interferon-α. Control tubes were those treated in the absence of medullasin. Tubes were then incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere to be assayed for the natural cytotoxicity. Each tube contained the following lymphocytes: O, lymphocytes stimulated with interferon; and ●, lymphocytes treated with medullasin and further stimulated with interferon. Stimulation index shows the same as that in Fig. 2. Bars, standard deviation of triplicate determinations.

mal donors is 5–10 μg/ml. A large amount of medullasin was released from mature granulocytes into culture medium when incubated in the presence of zymosan. Considerable amount of the protease was released also into culture medium even when granulocytes were incubated at 37°C for 30 min in the absence of zymosan. Although medullasin activity is inhibited completely in plasma owing to the presence of plasma protease inhibitors (1), it is quite possible that medullasin in mature granulocytes acts directly on the surface of lymphocytes because of the close relationship between granulocytes and lymphocytes in hemopoietic organs, peripheral blood, and various other tissues. In fact, lymphocytes cultured with granulocytes in the presence of serum showed an elevated natural killer cell activity when measured after 24 h incubation at 37°C. It is considered from these results that the natural killer cell activity is regulated in vivo by the medullasin of granulocytes. Medullasin activity in mature granulocytes is the highest in man as compared with that in other animals (1). Therefore, it seems probable that the natural killer cell activity in man is more intimately regulated by the medullasin than that in other animals.

The natural killer cell activity was reported to be modified by a variety of agents as described in the Introduction. Effects of proteases on the natural killer cell activity were also reported. Although natural cytotoxicity of lymphocytes obtained from lymph nodes was resistant to trypsin treatment (32), that of lymphocytes obtained from peripheral blood decreased by the treatment with trypsin, pronase, and papain (8, 33). Incubation of lymphocytes treated with trypsin at 37°C for 3 h in autologous serum restored the natural killer cell activity to original levels, but did not increase it above control levels (33). Therefore, the effect of medullasin on the natural killer cell activity is considered to be quite different from that of these proteases described above.

An enhanced cytotoxicity brought about by medullasin treatment is not attributable to a direct effect on target cells, because medullasin activity was completely inhibited by the addition of fetal calf serum before the contact with target cells, and also because an increment of the cytotoxicity was not brought about immediately after the treatment with the protease. It also seems improbable that medullasin stimulated directly natural killer cells in releasing their lysosomal enzymes to lyse target cells, since the protease did not enhance the natural cytotoxicity immediately after the treatment. Since interferon enhanced natural killer cell activity immediately after the treatment (13), stimulatory effect of medullasin on natural killer cells seems different from that of interferon. In fact, negligible activity of interferon was demonstrated in the supernatant of lymphocyte cultures that were stimulated by medullasin treatment. Furthermore, as shown in Results, medullasin further enhanced the natural killer cell activity of lymphocytes stimulated with interferon. These results indicate that the enhancement of natural killer cell activity by medullasin treatment is not mediated through interferon production. Since granulocytes obtained from rats injected with BCG as well as those from rats during carcinogenesis showed elevated medullasin activity (manuscript in preparation), it is also possible that several natural killer cell-activating agents which have been attributed to an enhanced production of interferon may act through activation of this protease of granulocytes. Medullasin also activated RNA and DNA synthesis of human lymphocytes (will be published elsewhere) and therefore, this protease may stimulate natural killer cell activity directly or through enhanced production of lymphokines (22). Medullasin, like interferon (34), may also stimulate prenatural killer cells into becoming mature natural killer cells. The mechanism of enhancement of the natural killer cell activity by medullasin is presently under investigation.

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