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Immunology

Regulation of cellular function by products of lysosomal enzyme activity: Elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes

(cytotoxic cells)

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ABSTRACT **L-Leucine methyl ester (Leu-OMe) is a lysosomotropic compound that irreversibly removes natural killer cell (NK) function from human peripheral blood mononuclear cells. This effect was dependent on the presence of mononuclear phagocytes (Mϕ) or polymorphonuclear leukocytes (PMN) and was prevented by lysosomal inhibitors such as chloroquine or NH_4Cl. When Mϕ or PMN were incubated with Leu-OMe, a product was formed that eliminated all NK function from mixed lymphocyte populations. This effect did not require the presence of Mϕ or PMN and was not prevented by lysosomal enzyme inhibitors. Thin-layer chromatography and mass spectral analysis revealed that this NK-toxic product was L-leucyl-L-leucine methyl ester (Leu-Leu-OMe). When human lymphocytes were exposed to >50 μM Leu-Leu-OMe for 15 min, all NK function was irreversibly eliminated. Other dipeptide methyl esters containing nonpolar L amino acids caused similar effects, but substitution with amino acids containing polar or charged side chains or with D stereoisomers produced compounds that had no toxic effect on NK. These findings indicate that Mϕ and PMN can regulate NK function by releasing the dipeptide condensation product Leu-Leu-OMe generated from Leu-OMe via a lysosomally mediated mechanism. The data show that specific products of lysosomal enzyme activity may have potent effects on the function of adjacent cells.**

Mononuclear phagocytes (Mϕ) and polymorphonuclear leukocytes (PMN) are specialized cell types that have the ability to ingest and degrade particulate material, including invading microorganisms and cellular debris. To accomplish these tasks, both cell types are endowed with an array of degradative enzymes. In addition to this scavenger function, both cell types also generate products that can modulate the function of lymphoid cells under certain circumstances.

We recently reported a unique example of such modulatory interactions between Mϕ, PMN, and lymphoid cells (1). It was found that either Mϕ or PMN could interact with the lysosomotropic compound, L-leucine methyl ester (Leu-OMe) and generate a new product with the capacity to ablate human natural killer cell (NK) function from a mixed cell population. When Mϕ-depleted human lymphocytes were exposed to Leu-OMe directly, no elimination of NK function was noted. Although Mϕ and PMN have previously been observed to suppress NK function by producing prostaglandin E_2 (2), reactive oxygen metabolites (3), or other heat-stable soluble factors (4), the Mϕ/PMN-mediated effect of Leu-Ome was distinct from that caused by these agents and appeared to be mediated by a newly synthesized cytotoxic product that removed all effector NK from human peripheral blood mononuclear cells (PBM) (1).

In the present studies, the mechanism of Mϕ- or PMN-mediated Leu-OMe-induced removal of NK from human PBM was examined. It was found that human Mϕ and PMN generate the dipeptide condensation product, L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) from Leu-OMe via a lysosomally mediated process. This compound was found to deplete irreversibly all NK function from Mϕ-depleted lymphocytes. Other dipeptide methyl esters containing nonpolar L amino acids were found to cause similar effects, whereas dipeptide methyl esters containing polar amino acids or glycine were much less active. These results further delineate a model system in which a dipeptide methyl ester with selective toxicity for NK cells can be generated by the action of Mϕ or PMN on Leu-OMe.

MATERIALS AND METHODS

Cell Preparations. PBM were separated from heparinized venous blood of healthy donors by centrifugation in sodium diatrizoate/Ficoll gradients (Isolymph, Gallard Schlesinger, Carle Place, NY). Monocyte-enriched populations (Mϕ) were prepared from glass-adherent cells, and Mϕ-depleted lymphocytes, from the nonadherent cells remaining after incubation in glass Petri dishes and passage through nylon wool columns (5). PMN were collected by resuspending peripheral blood cells that penetrated sodium diatrizoate/Ficoll gradients and removing erythrocytes by dextran sedimentation and hypotonic lysis (1).

Synthesis of Amino Acid and Peptide Methyl Esters. L-[U-^{14}C]Leucine (270–330 mCi/mM, 1 Ci = 37 GBq; ICN) or various dipeptides (purchased from Sigma; United States Biochemical, Cleveland, OH; or Serva, Heidelberg) were incubated with an excess of 3 M HCl in anhydrous methanol at 4°C for 72 hr as described (1) to synthesize [^{14}C]Leu-OMe and peptide methyl esters used for these studies. They were stored in methanol under nitrogen at −20°C. Immediately before use of the esters, methanol was removed by evaporation under a stream of nitrogen. Amino acid methyl esters and Asp-Phe-OMe* were purchased directly from Sigma. Aqueous isotonic stock solutions of these reagents were made as described (6).

All cell exposures to the amino acids, dipeptides, or their methyl esters were carried out by suspending cells in Dulbecco's phosphate-buffered saline (P_i/NaCl) and incubating them at room temperature with the reagent. After incuba-

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Abbreviations: Mϕ, mononuclear phagocyte(s); PMN, polymorphonuclear leukocyte(s); NK, natural killer cell(s); PBM, peripheral blood mononuclear cell(s); CI, chemical ionization.

*Unless otherwise noted, all optically active amino acids are of L configuration.

tion, the cells were washed twice with Hanks' balanced salt solution and resuspended in RPMI 1640 medium (Inland Laboratories, Fort Worth, TX) supplemented with 10% fetal bovine serum (Microbiological Associates) for assay of function.

Cytotoxicity Assay. NK activity against K562 target cells was assessed in a 3-hr ^{51}Cr-release assay and percent specific lysis was calculated as previously described (1). Percent of control cytotoxicity was calculated using the formula: (experimental % specific lysis/control % specific lysis) × 100.

Generation and Purification of Leu-OMe Metabolites. Mϕ or PMN (25×10^6 per ml) were suspended in P_i/NaCl and incubated with 25 mM Leu-OMe for 20 min at 22°C. Cell suspensions then were centrifuged at 1000 × *g* for 10 min, and the supernatants were lyophilized at −70°C and 100 millitorr (13.3 Pa) pressure. In some experiments, Leu-OMe-treated Mϕ or PMN were sonicated to increase the yield of the reaction product. Samples were then extracted with methanol for application to TLC plates (200 μm × 20 cm^2, Analtech, Newark, DE). Following development with chloroform/methanol/acetic acid (19:1:12.5 by volume), 1-cm bands were eluted with methanol, dried under nitrogen, and resuspended in 1 ml of P_i/NaCl.

Mass Spectral Analysis. Mass spectra were obtained with a Finnegan model 4021 automated EI/CI, GC/MS system coupled to an Incos data system. Methane was used as the reagent gas for chemical ionization (CI) mass spectral analysis.

RESULTS

Previously, the addition of Leu-OMe to human PBM was shown to cause rapid death of Mϕ and NK but not T or B lymphocytes (1, 6). Amino acid methyl esters are known to be lysosomotropic compounds (7–9), and in previous studies it was found that the lysosomal inhibitors chloroquine and NH_4Cl prevented Leu-OMe-induced Mϕ toxicity (6). To assess whether these agents similarly prevented formation of any NK-toxic products, we carried out the following experiments.

PBM were incubated with various potential NK-toxic agents for 30 min in the presence or absence of various lysosomal inhibitors, washed to remove the inhibitor, incubated for 18 hr to permit recovery from any transient inhibition caused by lysosomotropic agents, and then tested for NK activity. As previously demonstrated (1), neither chloroquine, nor NH_4Cl, nor Ile-OMe had any substantial permanent effect on NK function (Fig. 1). In contrast, 5 mM Leu-OMe eliminated all NK activity. This activity of Leu-OMe was largely prevented by chloroquine, NH_4Cl, or Ile-OMe. The product(s) generated by Mϕ or PMN after exposure to Leu-OMe also completely removed all NK activity from PBM. In contrast to the effect noted with Leu-OMe, the lysosomal inhibitors did not protect NK from the action of this product(s). Additional experiments indicated that extracts from sonicated Mϕ or PMN had no effect on NK function in

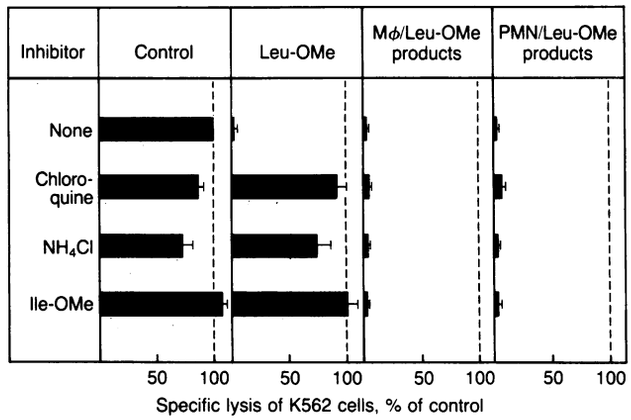

FIG. 1. Inhibitors of lysosomal enzyme function prevent generation of an NK-toxic product. PBM (5×10^6 per ml) were incubated either with medium or with medium containing 100 μM chloroquine, 30 mM NH_4Cl, or 5 mM Ile-OMe for 10 min at 22°C before addition of either medium (control), 5 mM Leu-OMe, or the supernatant (20% final concentration) from Mϕ (25×10^6 per ml) or PMN (25×10^6 per ml) that had been incubated with 25 mM Leu-OMe for 30 min. The PBM were incubated with these agents for another 30 min at 22°C, washed, and then cultured for 18 hr at 37°C before assay for ability to lyse K562 cells. Data are expressed as percentage of control cytotoxicity (mean + SEM, $n = 3$) observed with an effector/target cell ratio of 40:1 (results at other ratios were similar).

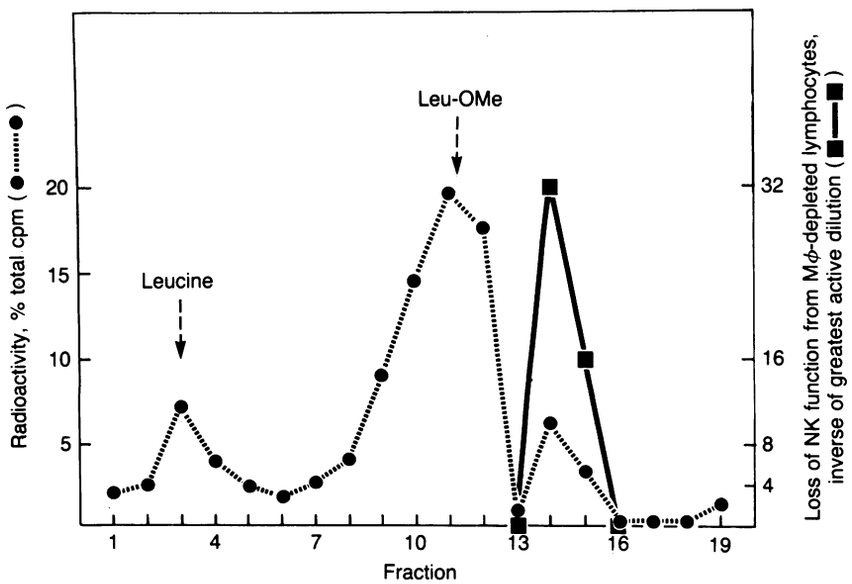

FIG. 2. Elimination of NK function is mediated by a metabolite of Leu-OMe. PMN (25×10^6 per ml) were incubated with 25 mM [^{14}C]Leu-OMe for 30 min, and the supernatant then was harvested for TLC analysis. Mϕ-depleted lymphocytes (2.5×10^6 per ml) were exposed to various dilutions of each TLC fraction for 30 min, washed, and cultured for 2 hr prior to cytotoxicity assay at an effector/target cell ratio of 20:1. Samples were considered to contain an NK-toxic product when percent specific lysis was <25% of control.

this system whereas the supernatants or sonicated-cell extracts of Leu-OMe-treated PMN or Mϕ also depleted NK from Mϕ-depleted lymphocytes. These results therefore suggested that interaction of Leu-OMe with the lysosomal compartment of Mϕ or PMN produced a product that was directly toxic to NK through a mechanism that was no longer dependent on lysosomal processing within either the NK or an additional cell type.

When the NK-toxic property of Mϕ/Leu-OMe or PMN/Leu-OMe incubation mixtures was further evaluated, it was found that this activity was stable in aqueous solutions for >48 hr at 4°C but labile at 100°C, retarded on Sephadex G-10 columns, dialyzable through 1000 molecular-weight-cutoff membranes, and extractable by chloroform/methanol (3:1, vol/vol). As shown in Fig. 2, when [^{14}C]Leu-OMe was incubated with PMN and the supernatant subsequently was fractionated by TLC, three major peaks of ^{14}C activity were found. Two of these corresponded to Leu-OMe itself and to leucine, whereas the third represented a new product. This third peak accounted for <10% of the total ^{14}C-labeled material. When Mϕ-depleted lymphocytes were exposed to each TLC fraction, the third peak was found to contain all NK toxic activity. This NK-toxic activity not only appeared to be ^{14}C-labeled but was also ninhydrin-positive, suggesting that it was a metabolite that retained an amino group as well as part of the carbon structure of Leu-OMe. An identical ^{14}C-labeled ninhydrin-positive product was detected by TLC of Mϕ/Leu-OMe incubation mixture supernatants or of methanol extracts of sonicated Mϕ/Leu-OMe. The production of this metabolite by PMN or Mϕ was inhibited by chloroquine, NH_4Cl, or Ile-OMe (data not shown).

The nature of this product was examined by mass spectroscopy. When the TLC-purified, NK-toxic fraction was subjected to CI mass spectral analysis with CH_4 as the reagent gas, peaks at m/z 259 (MH^+, parent molecular ion), 287 ($M+C_2H_5^+$), and 299 ($M+C_3H_5^+$) indicated the presence of a compound of M_r 258 (Fig. 3*A*). The presence of peaks at m/z 244 (MH^+-CH_3) and 272 ($M+C_2H_5^+-CH_3$) suggested that this compound contained a methyl ester group. Furthermore, the persistence of peaks corresponding to leucine (MH^+ 131, $M+C_2H_5^+$ 159) and Leu-OMe (MH^+ 146, $M+C_2H_5^+$ 174), in spite of careful TLC purification of the NK-toxic product from any free leucine or Leu-OMe present in the crude supernatants of the incubation mixtures, suggested that a condensation product of Leu-OMe such as Leu-Leu-OMe (M_r 258) was present in the NK-toxic fraction isolated after incubation of PMN or Mϕ with Leu-OMe.

When Leu-Leu-OMe was synthesized from reagent grade Leu-Leu, it was found to have TLC mobility identical to NK-toxic fractions of Mϕ/Leu-OMe and PMN/Leu-OMe incubation mixtures. Furthermore, its CI mass spectrum (Fig. 3*B*) was identical to that of the M_r 258 compound found in these fractions.

Experiments were therefore undertaken to confirm that Leu-Leu-OMe was the product generated by Mϕ or PMN from Leu-OMe that was responsible for the selective ablation of NK function from human lymphocytes. Leu-Leu-OMe was synthesized by incubation of Leu-Leu in methanolic HCl. TLC analysis revealed <2% contamination of this preparation with leucine, Leu-Leu, and Leu-OMe, and CI mass spectral analysis (Fig. 3*B*) revealed no contaminants of other molecular weights. In the representative experiments shown in Fig. 4, Mϕ-depleted lymphocytes were exposed to various concentrations of Leu-Leu-OMe for 15 min at room temperature and then were washed and assayed for ability to lyse K562 cells. No NK function could be detected in lymphocyte populations exposed to >50 μM Leu-Leu-OMe. As previously demonstrated (6), exposure of such Mϕ-depleted lymphocyte populations to 100-fold greater concentration of leucine or Leu-OMe had no irreversible effect on NK function. Leu-Leu or the D stereoisomer D-Leu-D-Leu-OMe also had no inhibitory effect. Although Leu-Leu-Leu-OMe caused dose-dependent loss of NK function, 5-fold greater concentrations of this tripeptide methyl ester were required to cause an effect equivalent to that of Leu-Leu-OMe. When lymphocyte populations exposed to various concentrations of Leu-Leu-OMe were analyzed further, it was found that exposure to >50 μM Leu-Leu-OMe resulted in the loss of K562 target binding as well as complete depletion of cells stained by Leu11b, an anti-NK monoclonal antibody (data not shown). Thus, the Mϕ- or PMN-generated product of Leu-OMe that is directly toxic for human NK is the dipeptide condensation product Leu-Leu-OMe.

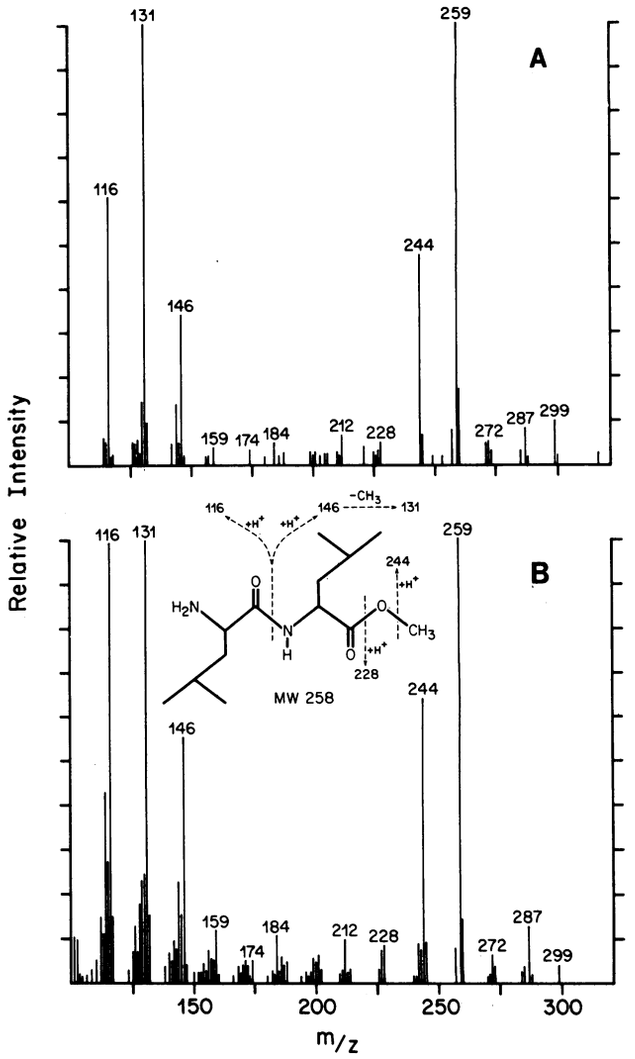

FIG. 3. CI mass spectra of TLC fractions (see Fig. 2) with NK-toxic activity (*A*) and of Leu-Leu-OMe synthesized from reagent grade Leu-Leu (*B*).

In previously reported studies, Leu-OMe was unique among a wide variety of amino acid methyl esters in its ability to cause Mϕ- or PMN-dependent elimination of NK function from human PBM (1). The identification of Leu-Leu-OMe as the Mϕ-generated metabolite responsible for this phenomenon suggested either that Mϕ or PMN did not generate the corresponding dipeptide methyl esters from other amino acids or that Leu-Leu-OMe was unique among dipeptide methyl esters in its toxicity for NK. Therefore, experiments were carried out to assess the effect of other dipeptide methyl esters on NK function. The methyl esters of a variety of dipeptides were synthesized and analyzed for the capacity to deplete NK function. Each dipeptide methyl ester was as-

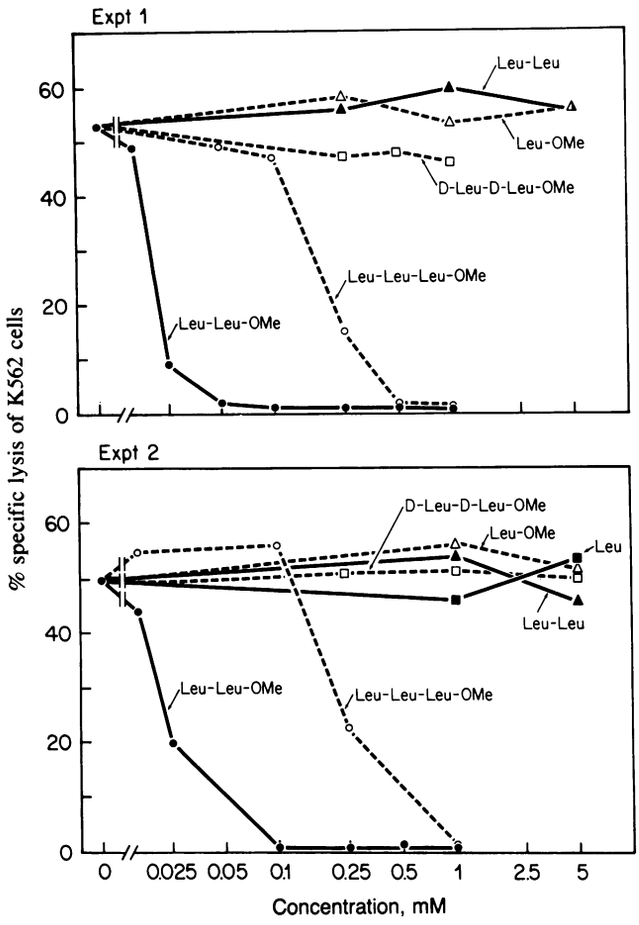

FIG. 4. Loss of NK function after exposure to Leu-Leu-OMe. Mϕ-depleted lymphocytes (2.5×10^6 per ml) were incubated for 15 min with the indicated concentrations of leucine-containing compounds. Cells were then washed, cultured at 37°C for 2 hr (Expt. 1) or 18 hr (Expt. 2), and then assayed for NK activity at an effector/target cell ratio of 20:1.

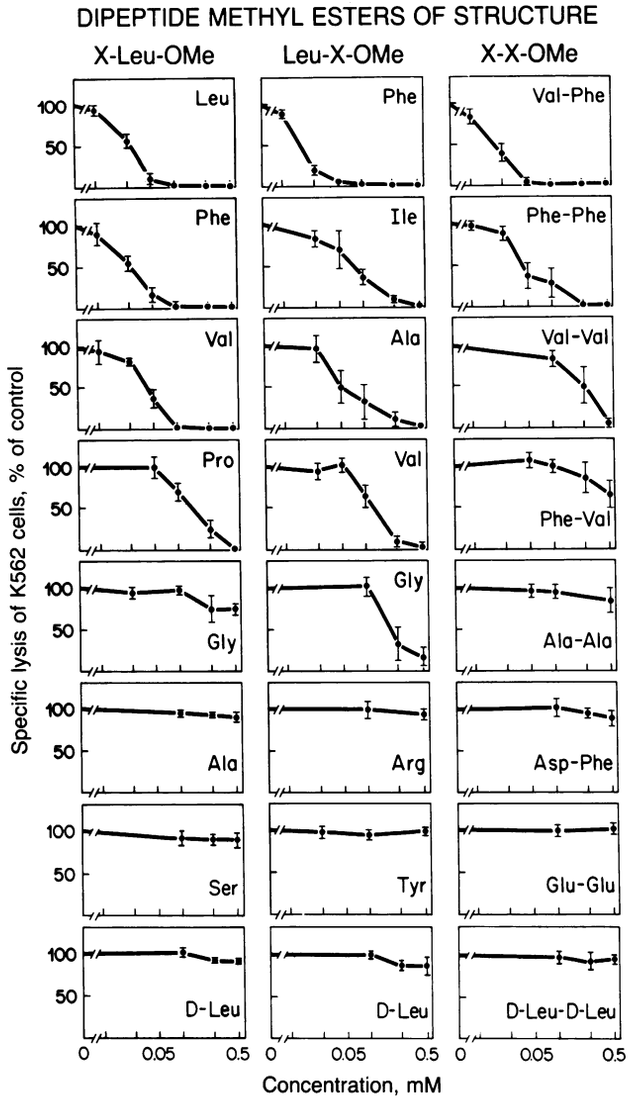

FIG. 5. NK toxicity of dipeptide methyl esters. Mϕ-depleted lymphocytes were treated with various concentrations of dipeptide methyl esters as outlined in the legend to Fig. 4. Results are given as mean ± SEM of at least three separate experiments with each compound. Amino acid residue(s) X for each compound tested is given within the corresponding panel.

sessed in a minimum of three experiments. As is shown by the results displayed in Fig. 5, Leu-Leu-OMe is not the only dipeptide methyl ester that exhibits NK toxicity. When amino acids with nonpolar R groups were substituted for leucine in either position, the resulting dipeptide methyl ester generally displayed at least some degree of NK toxicity. In particular, Leu-Phe-OMe, Phe-Leu-OMe, Val-Phe-OMe, and Val-Leu-OMe produced concentration-dependent depletion of NK function at concentrations comparable to those at which Leu-Leu-OMe was active. The sequence of active amino acids was important, however, as evidenced by the finding that Phe-Val-OMe was markedly less active than Val-Phe-OMe. Similarly, Leu-Ala-OMe was NK-toxic, whereas 10-fold greater concentrations of Ala-Leu-OMe had no NK-inhibitory effects. Furthermore, Phe-Phe-OMe was less NK-toxic than either Leu-Phe-OMe or Phe-Leu-OMe, and Val-Val-OMe was less active than either Leu-Val-OMe or Val-Leu-OMe, yet Val-Phe-OMe was among the most potent of the NK-toxic dipeptide methyl esters. Thus, conformation aspects of the dipeptide methyl esters also seem to be important in producing the observed NK toxicity.

When glycine or amino acids with polar or charged R groups replaced leucine, the resulting dipeptide methyl esters had either greatly reduced NK toxicity, as in the case of Gly-Leu-OMe or Leu-Gly-OMe, or no observed NK-inhibitory effects, as in the case of Leu-Arg-OMe, Leu-Tyr-OMe, Ser-Leu-OMe, or Asp-Phe-OMe. Furthermore, when the D stereoisomer was present in either position of a dipeptide methyl ester, no toxicity for NK was observed (Fig. 5). When unesterified dipeptides were assessed for their effect on NK function, as in the case of Leu-Leu (Fig. 4), up to 5 mM concentrations of Leu-Phe, Phe-Leu, Val-Leu, and Val-Phe had no effect on NK survival or lytic activity (data not shown).

Previous experiments had shown that compounds such as Val-OMe or Phe-OMe or combinations thereof did not delete NK function from human PBM (1), despite the current finding that dipeptide methyl esters containing these amino acids were potent NK toxins. To determine whether Mϕ or PMN could generate the relevant dipeptide methyl esters from these amino acid methyl esters, TLC analysis of the supernatants of Mϕ and PMN incubated with these compounds was carried out. It was found that Mϕ and PMN did generate detectable amounts of dipeptide methyl esters from these L amino acid methyl esters. However, when equal concentrations of Leu-OMe, Val-OMe, or Phe-OMe were added to Mϕ or PMN, the concentrations of Val-Val-OMe generated were 50–80% those of Leu-Leu-OMe, and Phe-Phe-OMe was detected at only 10–30% the levels of Leu-Leu-OMe. Dipeptide

methyl esters were not generated from D amino acid methyl esters.

DISCUSSION

These studies show that the elimination of NK function following incubation of human PBM with Leu-OMe is mediated by a Mϕ- or PMN-generated condensation product, Leu-Leu-OMe. This compound is one of several dipeptide methyl esters that display toxicity for NK. Unlike other modulators of NK activity previously shown to be generated by Mϕ or PMN (2–4), this class of compounds selectively kills all NK effector cells, as shown by the rapid depletion of all cells with K562 target binding activity and all cells that can be stained by Leu11b or OKM1, monoclonal antibodies that react with NK (10, 11), from human lymphoid populations exposed to Leu-OMe plus Mϕ (1) or Leu-Leu-OMe alone.

As is the case for the majority of weakly basic alkylamines, methyl esters of amino acids and dipeptides are lysosomotropic (6–9, 12). Furthermore, these compounds cause lysosomal disruption in isolated subcellular organelles (7, 8), intact cells (6), and whole organ preparations (9). Such an action explains the loss of lysosomal function within cells exposed to amino acid methyl esters (9). In particular, the lysis of tumor cell targets by NK is dependent on intact lysosomal function (13) and can be inhibited by a wide variety of amino acid methyl esters (1). This inhibition of NK function was observed over the same concentration range (10^{-3}–10^{-2} M) at which these compounds cause lysosomal disruption (6–9) and was found to be entirely reversible for amino acid methyl esters such as Ile-OMe and Val-OMe. In the absence of Mϕ or PMN, even Leu-OMe was found to have only a transient and fully reversible inhibitory effect on NK function (1). Thus, lysosomal disruption alone was not found to lead to death or permanent inactivation of NK.

The observation that, in the presence of freshly isolated Mϕ or PMN, Leu-OMe causes irreversible loss of NK function (1) suggested that an NK-toxic product might be generated from Leu-OMe by these cells. The current studies confirm this hypothesis. They show that PMN or Mϕ generate Leu-Leu-OMe when exposed to Leu-OMe. Submillimolar concentrations of Leu-Leu-OMe but not Leu-Leu, leucine, Leu-OMe, or the D stereoisomers of Leu-Leu-OMe were found to remove all NK function from Mϕ-depleted lymphocyte populations.

The finding that chloroquine and NH_4Cl inhibit Mϕ or PMN generation of this NK-toxic product suggests that it is formed within the acidic lysosomal compartments of these cells. Indeed, when human PMN were fractionated by sedimentation techniques, the ability to convert Leu-OMe to Leu-Leu-OMe was co-isolated with the granule fraction (data not shown). Although the trapping and concentration of Leu-OMe within lysosomes undoubtedly facilitates this process, it is likely that the condensation is enzymatically mediated as well, since no conversion of D-Leu-OMe to a corresponding dipeptide methyl ester was detected. These findings are in agreement with those of Goldman and Kaplan (7), who have observed that rat hepatic lysosomal enzyme preparations metabolize Leu-OMe but not D-Leu-OMe via a dipeptide intermediate.

Although a variety of dipeptide methyl esters were toxic to NK, Mϕ and PMN were most capable of generating such a metabolite from Leu-OMe. The lack of NK toxicity after addition of Phe-OMe or a wide variety of other L-amino acid methyl esters (1) to PBM appears to be caused by lack of production of adequate quantities of the dipeptide methyl ester. Thus, although Val-OMe is converted to Val-Val-OMe at a slightly lower rate than that for the leucine-containing compounds, the fact that NK toxicity requires 10-fold greater concentrations of Val-Val-OMe than of Leu-Leu-OMe is likely to account for the previous observations that addition of 1 mM Leu-OMe to human PBM eliminated NK function, whereas up to 25 mM Val-OMe had no adverse effect on NK survival or function (1). In contrast, although Phe-Phe-OMe is a relatively more potent NK toxin than Val-Val-OMe, it is generated at an even lower rate from Mϕ or PMN, thus accounting for the lack of NK toxicity when PBM are incubated with Phe-OMe.

The finding that Leu-Leu-OMe toxicity for NK is not prevented by inhibitors of lysosomal function suggests that further lysosomal processing of the dipeptide methyl ester is not a requisite step in its toxic effect on the NK cell itself. Additional results suggest, however, that toxicity of dipeptide methyl esters requires cellular metabolism or receptor recognition by the NK. Thus, dipeptide methyl esters containing D-amino acids were devoid of NK toxic potential. Since L-amino acids with nonpolar but not polar R groups could be substituted for leucine in a dipeptide methyl ester and still maintain NK toxicity, it is likely that membrane permeability or actual localization within hydrophobic regions of the cell is also of importance in the toxic action of these molecules. The exact mechanism whereby Leu-Leu-OMe and other dipeptide methyl esters damage NK and the basis for the apparent specificity of this effect remain to be elucidated.

The present studies delineate an experimental system for studying the modulation of NK function. Although a variety of pharmacologic agents have been shown to alter NK function, the dipeptide methyl esters described in this communication are unique in possessing the ability to kill NK rapidly under circumstances in which they are clearly not toxic to a variety of other cell types (1, 6, 9). Thus, further analysis of the actions of these compounds should provide additional insights into the unique functional characteristics of NK as well as provide methods for studying the modulation of the function of these cells.

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