The Action of Leucyl-Leucine Methyl Ester on Cytotoxic Lymphocytes Requires Uptake by a Novel Dipeptide-Specific Facilitated Transport System and Dipeptidyl Peptidase I–Mediated Conversion to Membranolytic Products

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Summary

The mechanism of toxicity for cytolytic lymphocytes of Leu-Leu-OMe and related dipeptide derivatives was examined. Selective inhibition of dipeptidyl peptidase I (DPPI), a lysosomal thiol protease highly enriched in cytotoxic lymphocytes, prevented all natural killer (NK) toxic effects of such agents. However, many DPPI substrates were found to possess no NK toxic properties. For some such agents, this lack of NK toxicity appeared to be related to the lack of uptake by lymphocytes. In this regard, Leu-Leu-OMe was found to be incorporated by lymphocytes and monocytes via a saturable facilitated transport mechanism with characteristics distinct from previously characterized mammalian dipeptide transport processes. This novel transport process was found to be specific for dipeptides composed of selective L-stereoisomer amino acids and enhanced by hydrophobic ester or amide additions to the COOH terminus of dipeptides. Maximal rates of Leu-Leu-OMe uptake by T8 and NK cell–enriched peripheral blood lymphocytes (PBL) were four- to sixfold higher than for T4-enriched PBL or PBL depleted of Leu-Leu-OMe-sensitive cytotoxic lymphocytes. All dipeptide amides or esters with NK toxic properties were found to act as competitive inhibitors of [^{3}H]Leu-Leu-OMe uptake by PBL. However, some NK nontoxic DPPI substrates were found to be comparable with Leu-Leu-OMe in avidity for this transport process. Such agents were noted to possess one or more hydrophilic amino acid side chains and were found not to mediate red blood cell lysis when subjected to the acyl transferase activity of DPPI. Thus, uptake by a dipeptide-specific facilitated transport mechanism and conversion by DPPI to hydrophobic polymerization products with membranolytic properties were found to be common features of NK toxic dipeptide derivatives. The presence of a previously unreported dipeptide transport mechanism within blood leukocytes and the selective enrichment of the granule enzyme, DPPI, within cytotoxic effector cells of lymphoid or myeloid lineage appear to afford a unique mechanism for the targeting of immunotherapeutic reagents composed of simple dipeptide esters or amides.

Cells with cytotoxic potential contain a unique array of granule-associated proteins. Thus, specialized cytoplasmic granules containing pore-forming molecules and other toxins, have been noted to be common features of cytotoxic effector cells of lymphoid or myeloid lineage (1–5). A number of serine esterases also appear to be selectively expressed within the lysosome-like granules of cytotoxic lymphocytes (6–10). In addition, we have recently found that the lysosomal thiol protease, dipeptidyl peptidase I (DPPI)[1] is present at far higher levels in cytotoxic lymphocytes than in cells without cytolytic potential or not of bone marrow origin (11).

The presence of high concentrations of DPPI within cytotoxic effector cells was noted in the course of studies designed to delineate the mechanisms of the toxic effects of L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) on these cells. The selective toxic effects of Leu-Leu-OMe on human or murine lymphoid or myeloid cells with cytolytic potential had previously been defined (12–15), and were found to relate to their unique capacity to metabolize this immunosuppressive agent. After exposure to Leu-Leu-OMe, cytotoxic lymphocytes generated membranolytic products of the structure $(Leu\text{-}Leu)_n\text{-}OMe$, where $n \geq 3$ (11) by the acyl transferase activity of the lyso-

[1] *Abbreviations used in this paper:* DPPI, dipeptidyl peptidase I; Mø, monocytes.

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somal thiol protease, DPPI, which is present at far higher levels in these cells than in cells without cytotoxic potential (11). Examination of a panel of dipeptide methyl esters for the capacity to cause similar toxicity toward cytolytic cells indicated that all active compounds could be substrates for DPPI (11, 12). However, additional dipeptide esters such as Leu-Tyr-OMe or Ser-Leu-OMe, which are putative substrates for DPPI, were found to cause no toxicity for cytolytic cells (12). This observation suggested that other properties of dipeptide esters or other aspects of the processing of these compounds must also be involved in the mechanism whereby Leu-Leu-OMe and other dipeptide ester substrates of DPPI selectively kill cytotoxic lymphocytes and myeloid cells. The present studies were designed to delineate the metabolism of dipeptide esters by cytotoxic cells in greater detail and, thereby, the mechanism of toxicity of Leu-Leu-OMe for lymphocytes and myeloid cells with cytolytic potential.

Materials and Methods

Medium. For cell culture, RPMI 1640 (Hazelton, Dutchland, Inc., Denver, PA) supplemented with 10% FCS, penicillin G (200 U/ml), gentamicin (10 μg/ml), and L-glutamine (0.3 mg/ml) was used. For incubations with various dipeptide derivatives, PBS supplemented with 1 g/liter glucose was used, except where noted.

Reagents. Leu-Leu-NH_2, Gly-Phe-β-naphthylamide, Gly-Arg-β-naphthylamide, N-α-benzyloxycarbonyl (Z) Arg-Arg-β-naphthylamide, and Asp-Phe-OMe were purchased from Sigma Chemical Co., St. Louis, MO. All other dipeptide methyl esters were synthesized from appropriate dipeptides (Sigma Chemical Co.) by esterification in 3 M methanolic HCl, as previously described (12). [^{14}C]Methanol (ICN Radiochemicals, Irvine, CA) was used to synthesize [^{14}C]methyl ester derivatives of selected dipeptides. Purity of [^{14}C]methyl ester derivatives was confirmed by thin layer chromatography (12). [4,5-^{3}H]Leu-Leu-OMe (60 mCi/mM) was custom synthesized by New England Nuclear, Boston, MA. Leu-Leu-OBenzyl and Leu-Leu-$NHCH_3$ were synthesized from amino acid precursors by C. DeWitt Blanton, University of Georgia College of Pharmacy, Athens, GA. The compounds were isolated as the hydrochloride salts, and the structures were confirmed by elemental analysis for carbon, hydrogen, nitrogen, and chlorine, with results found to be within ± 0.4% of theoretical values. The respective melting points were found to be 144–146°C (Leu-Leu-OBenzyl) and 223–225°C (Leu-Leu-$NHCH_3$). Gly-Phe-CHN_2 was purchased from Enzyme Systems Products, Livermore, CA. Purified bovine DPPI was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. mAb 66.1 (anti-CD4) was obtained from hybridoma ascites provided by Patrick Beatty, Seattle, WA, and OKT4 (anti-CD4), OKT8 (anti-CD8), and L243 (anti-HLA-DR) were obtained from culture supernatants of hybridoma cells obtained from the American Type Culture Collection, Rockville, MD. Leu-11b (anti-CD16) was purchased from Becton Dickinson Monoclonal Centers, Inc., Mountain View, CA.

Cell Preparations. PBMC were separated from heparinized venous blood of healthy donors by centrifugation on sodium diatrizoate/ficoll gradients (Isolymph; Gallard Schlesinger, Carle Place, NY). Monocyte (Mø)-enriched populations were prepared from glass-adherent cells, and Mø- and B cell–depleted PBL were prepared from PBMC by passage through nylon wool columns as described (16). $CD8^+$ T cell (T8 cell)–and NK-enriched lymphocytes were prepared by 66.1 and L243 + C′ treatment of PBL, and $CD4^+$ T cell (T4 cell)–enriched lymphocytes were prepared by incubation of PBL with OKT8 and L243, followed by panning on goat anti–mouse Ig–coated petri dishes and treatment with OKT8, Leu-11b, and C′, as previously described (13, 14).

Cytotoxicity Assays. NK activity against K562 target cells was assessed in 4-h ^{51}Cr release assays, and percent specific cytotoxicity was calculated as previously described (16). In other experiments, human RBC were ^{51}Cr labeled and added to microtiter wells (10^6 RBC/well) in HBSS (200 μl/well) containing 25 mM Hepes and 2 mM DTT and the indicated concentrations of purified bovine DPPI and dipeptide esters or amides. After incubation for 4 h at 37°C, plates were centrifuged at 50 *g* for 5 min, 100 μl of culture supernatant were harvested from each well, and ^{51}Cr release and percent specific lysis calculated as previously detailed (16).

Assessment of Cell-associated [^{3}H]Leu-Leu-OMe. Cell populations (2.5×10^6/ml) were incubated at the indicated temperature with 0.2–1 μCi/ml [^{3}H]-labeled Leu-Leu-OMe and the indicated concentrations of unlabeled dipeptide esters for fixed time intervals. The cells were then centrifuged through silicone oil (SF1250; General Electric Co., Waterford, NY) to separate them from external unbound Leu-Leu-OMe. Aliquots of the PBS supernatant and the cell pellet were then assessed for [^{3}H]Leu-Leu-OMe content by liquid scintillation spectroscopy. Control tubes to which no cells had been added were analyzed to quantify the amount of unbound [^{3}H]Leu-Leu-OMe that passed through the silicone oil. All assessments were performed in triplicate, and cell-associated [^{3}H]Leu-Leu-OMe was expressed as mean specific cell-associated [^{3}H]Leu-Leu-OMe (mean cpm below silicone in tubes with cells added − cpm below silicone oil in tubes without silicone oil), or as an absolute quantity of Leu-Leu-OMe incorporated as calculated from the formula: Leu-Leu-OMe incorporated = [(concentration of Leu-Leu-OMe) (Volume of total incubation mixture)] [cpm (cell pellet) − cpm (background)/cpm (cell pellet) + cpm (supernatant)]. In some experiments, Leu-Leu-OMe incorporation was assessed over a broad concentration range, and kinetic constants (Km and Vmax) were calculated from Lineweaver-Burk plots.

Enzyme and Protein Assays. DPPI activity was assayed fluorometrically at pH 5.0 in 0.05 M sodium acetate buffer, 10 mM cysteine, 30 mM NaCl, and 1 mM EDTA with Gly-Phe-β-naphthylamide as substrate, as previously described (17). In selected experiments, the acyl transferase activity of DPPI was measured by incubation of purified DPPI or cell sonicates with 50 mM dipeptide ester, 25 mM DTT, 0.4 M hydroxylamine.HCl at pH 6.8 for 20 min at 37°C, as previously detailed (18). The reaction was stopped by addition of equal volumes of 20% trichloroacetic acid. The supernatant was then mixed with 1/2-vol 5% $FeCl_3.6H_2O$ in 0.1 M HCl and 1 vol H_2O, and the absorbance at 510 nM was measured. Cathepsin B was assayed at pH 6.3 in 0.1 M sodium phosphate buffer, 1 mM EDTA, 10 mM cysteine with Z-Arg-Arg-β-naphthylamide as substrate, as previously described (19). Protein content was assessed by reaction with bicinchoninic acid, as previously described (20).

Results

DPPI has been shown previously to possess acyl transferase activity that converts dipeptide amide or dipeptide ester substrates to polymerization products of the general structure $(R_1 - R_2)_n - NH_2$ or $(R_1 - R_2)_n - OR_3$ when R_1 is not arginine, lysine, or proline, and R_2 is not proline (21–24). The acyl transferase activity of DPPI has been demonstrated to play an essential role in Leu-Leu-OMe-mediated toxicity

for human cytolytic cells (11), but its potential role in mediating toxicity of other related compounds has not been determined. The experiments detailed in Table 1 were therefore carried out to examine the role of DPPI in the sensitivity of cytolytic cells to other dipeptide esters or amides. Since the sensitivity of NK cells to these compounds parallels that of CTL, for convenience, NK cells were used in these experiments (11, 13).

As demonstrated in experiment 1 (Table 1), incubation of human PBL with 50 μM Leu-Leu-OMe, Val-Phe-OMe, or Leu-Phe-OMe for 15 min at room temperature ablated NK function. The role of DPPI in the action of each of these dipeptide methyl esters was demonstrated by the finding that the NK toxic effects of Leu-Leu-OMe, Val-Phe-OMe, or Leu-Phe-OMe were prevented by preincubation of PBL with the irreversible specific inhibitor of DPPI, Gly-Phe-CHN_2 (25). Exposure of human PBL to 10^{-5} M Gly-Phe-CHN_2 for 30 min at 37°C caused >95% inhibition of DPPI activity with

Table 1. *Dipeptide Methyl Ester-mediated NK Cell Toxicity Is Prevented by the DPPI Inhibitor Gly-Phe-CHN_2 and by Ser-Leu-OMe, but not by Leu-Leu-NH_2*

Exp.	Lymphocyte preincubation*		NK function (percent specific lysis, K562‡)
	First addition	Second addition	
1	–	–	38
		50 μM Leu-Leu-OMe	2
		50 μM Val-Phe-OMe	3
		50 μM Leu-Phe-OMe	2
	10^{-5} M Gly-Phe-CHN_2§	–	33
		50 μM Leu-Leu-OMe	33
		50 μM Val-Phe-OMe	35
		50 μM Leu-Phe-OMe	34
2	–	–	42
		50 μM Leu-Leu-OMe	2
		250 μM Ser-Leu-OMe	40
		250 μM Leu-Tyr-OMe	43
		5 μM Gly-Phe-β-naphthylamide	0
		500 μM Gly-Arg-β-naphthylamide	44
3	–	–	51
		25 μM Leu-Leu-OMe	16
		50 μM Leu-Leu-OMe	4
		100 μM Leu-Leu-OMe	2
	250 μM Ser-Leu-OMe	–	54
		25 μM Leu-Leu-OMe	58
		50 μM Leu-Leu-OMe	53
		100 μM Leu-Leu-OMe	26
4	–	–	37
		50 μM Leu-Leu-OMe	0
	1 mM Leu-Leu-NH_2	–	41
		50 μM Leu-Leu-OMe	1

* PBL were suspended at 2.5–10 × 10^6/ml in PBS and incubated at room temperature for 30 min (Exp. 1) or 15 min (Exps. 2–4) in the presence of the indicated concentration of the first compound. The second compound was then added to the incubation mixture, and the cells were incubated for an additional 15 min at room temperature before washing with a greater than fourfold excess of HBSS at 0–4°C.

‡ Treated cells were resuspended in medium and cultured at 37°C for 2 h before assessment of the capacity to lyse K562 cells at an E/T ratio of 10:1.

§ After 30 min of incubation with diluent control (0.5% DMSO in PBS) or 10^{-5} M Gly-Phe-CHN_2, DPPI activity of the cell lysates was 14.6 and 0.3 nM naphthylamide released per 10^6 cells/h, respectively, whereas cathepsin B activity was 7.4 and 7.7 nmol naphthylamide/10^6 cells/h, respectively.

no significant effect on the activity of another lysosomal thiol protease cathepsin B (Table 1, footnote). These results are consistent with the conclusion that the capacity of a dipeptide methyl ester to serve as a substrate for DPPI was necessary for it to be toxic to NK cells. However, as demonstrated in experiment 2 (Table 1), exposure of PBL to the putative DPPI substrates, Ser-Leu-OMe or Leu-Tyr-OMe (22, 24), did not cause detectable NK toxicity, even when these compounds were present at concentrations fivefold higher than concentrations of Leu-Leu-OMe that ablated all NK function. DPPI has been shown to polymerize dipeptide amides as well as dipeptide esters (23). Consistent with this, we have found that both Gly-Phe-β-naphthylamide and Gly-Arg-β-naphthylamide are efficiently degraded by PBL lysates or purified bovine DPPI, with the latter a better substrate than the former (data not shown). Despite this, as demonstrated in experiment 2 (Table 1), NK function was ablated by exposure to modest concentrations of Gly-Phe-β-naphthylamide, whereas Gly-Arg-β-naphthylamide was not NK toxic, even at a 100-fold higher concentration. In addition, Leu-Leu-NH_2, another substrate of DPPI, was not NK toxic at 1 mM, as demonstrated in experiment 4 (Table 1). These results indicate that substrates of DPPI differ in their capacity to cause NK cell toxicity. Additional complexity in this system was indicated by the finding that Ser-Leu-OMe was able to inhibit the toxic effects of Leu-Leu-OMe on NK cells, even though it was not directly toxic for NK cells. Thus, as shown in experiment 3 (Table 1), PBL were completely protected from the NK toxic effects of 25–50 μM Leu-Leu-OMe by 250 μM Ser-Leu-OMe, and partially protected from the effects of 100 μM Leu-Leu-OMe. By contrast, the presence of a 10-fold excess of Leu-Leu-NH_2 (experiment 4, Table 1) afforded no protection from the NK toxic effects of Leu-Leu-OMe. The inability of either Ser-Leu-OMe or Leu-Leu-NH_2 to exert toxic effects on NK cells, and the differences in their capacities to inhibit Leu-Leu-OMe-mediated NK toxicity, suggested that being a substrate for DPPI was not sufficient for a dipeptide methyl ester or amide to be toxic for NK cells. Other features of the molecule appeared to be necessary for this action.

Other possible steps involved in the toxicity of a dipeptide methyl ester for NK cells could be the uptake of the compound by the cell and/or the generation of a membranolytic molecule by the action of DPPI. The next experiments, therefore, examined the mechanism of the uptake of Leu-Leu-OMe by human PBL to determine whether a specific transport mechanism was involved. As detailed in Fig. 1, the quantity of cell-associated [^{3}H]Leu-Leu-OMe increased in a linear, time-dependent fashion over the first 30 min of incubation of human PBL with 250 μM Leu-Leu-OMe at room temperature. As demonstrated in Table 2, when incubations were carried out at temperatures ≤4°C, no accumulation of [^{3}H]Leu-Leu-OMe by PBL was detected. After incubation at 37°C, levels of cell-associated Leu-Leu-OMe were increased above those seen at 22°C (experiment 2, Table 2). These findings suggested that Leu-Leu-OMe was not simply binding to PBL by an energy- or temperature-independent process. Rather,

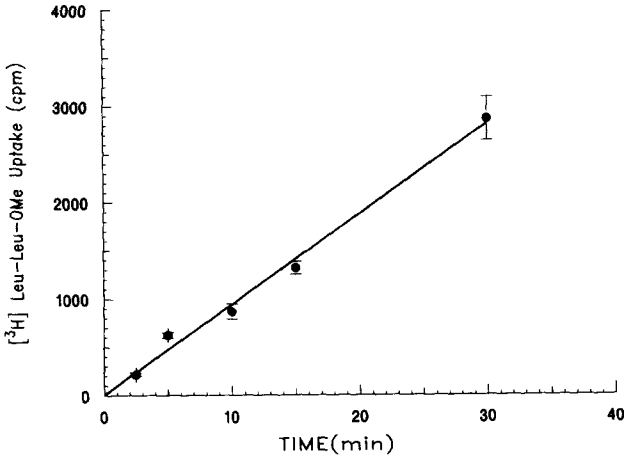

Figure 1. Time course of Leu-Leu-OMe incorporation by human PBL. Human PBL (2.5 × 10^6/ml) were suspended in PBS containing 0.75 μCi [^{3}H]Leu-Leu-OMe per milliliter and 250 μM unlabeled Leu-Leu-OMe. At the indicated time points, triplicate 1-ml aliquots of cell suspensions were centrifuged through silicone oil and cell-associated [^{3}H]Leu-Leu-OMe was assessed. Values are given for mean ± SE of triplicate determinations.

the time- and temperature-dependent increases in cell-associated Leu-Leu-OMe suggested that this compound was being internalized and retained by PBL.

When the concentration dependence of Leu-Leu-OMe uptake by various subpopulations of human PBMC was assessed (see Fig. 2), the quantity of Leu-Leu-OMe incorporated during a 15-min incubation at 22°C was noted to increase in direct proportion to the external concentrations until 100 μM was exceeded, at which point the saturable characteristics of this uptake process became apparent. Differences in the uptake of Leu-Leu-OMe by different subpopulations of PBMC were noted. Thus, T8 and NK cells incorporated three- to four-fold greater quantities of Leu-Leu-OMe than did T4 cells (see Fig. 2 and Table 3). Mø took up an intermediate amount of Leu-Leu-OMe compared with that taken up by T8- and NK-enriched cells and T4-enriched cells (Fig. 2 and Table 3).

Table 2. *Temperature Dependence of [^{3}H]Leu-Leu-OMe Uptake/Binding by Lymphocytes*

Exp.	Temperature	[^{3}H]Leu-Leu-OMe uptake/binding*
		nmol/10^6 cells
1	0°C	0.05
	22°C	0.77
2	4°C	0.02
	22°C	0.31
	37°C	0.52

* Cell-associated [^{3}H]Leu-Leu-OMe was assessed after 15-min incubations of PBL with 100 μM Leu-Leu-OMe at the indicated temperature.

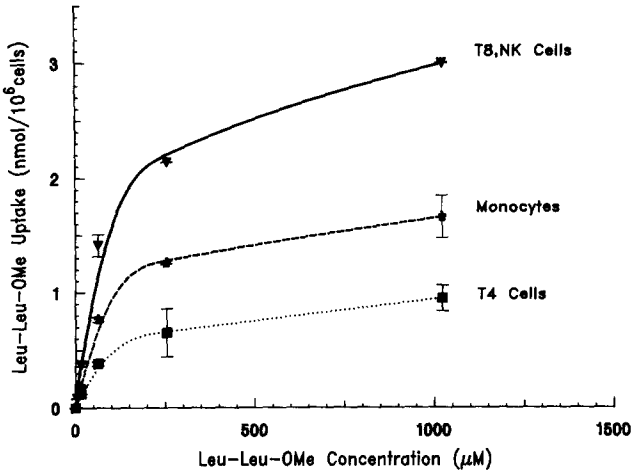

Figure 2. Concentration-dependent uptake of Leu-Leu-OMe by human Mø and lymphocytes. The indicated cell populations were incubated for 15 min at 22°C with varying concentrations of Leu-Leu-OMe and the quantity of Leu-Leu-OMe incorporated, as detailed in Fig. 1 and Table 2. Values are given for mean ± SE of triplicate determinations.

Despite the variability in maximal uptake of Leu-Leu-OMe by the various populations of PBMC, Leu-Leu-OMe incorporation in all cases was mediated by a saturable process with a Km of 100–200 μM (Table 3; additional experiments not shown). To determine whether cells with a greater capacity to take up Leu-Leu-OMe were the cells selectively killed by exposure to this agent, PBL were incubated in the presence or absence of 250 μM Leu-Leu-OMe for 15 min at 22°C and then washed and cultured for 18 h at 37°C before assessment of Leu-Leu-OMe uptake. These treatment conditions have been previously shown to result in the death of 20–50% of PBL (13). The majority of PBL, however, remain viable and functionally intact (13). As illustrated in Table 3, Leu-Leu-OMe-resistant PBL depleted of NK and CTL by Leu-Leu-OMe treatment (12, 13) were noted to incorporate Leu-Leu-OMe at approximately half the rate of untreated control PBL.

Table 3. *Rates of Leu-Leu-OMe Uptake and DPPI Content of Monocytes and Lymphocyte Subpopulations*

Cell population*	DPPI content	Kinetics of Leu-Leu-OMe uptake‡	
		Km	Vmax
	nmol naphthylamine released/10⁶ cells/h	*μM*	*nmol/ 10⁶ cells/h*
T8, NK cells	63.1	152	16.0
Mø	25.0	190	9.0
PBL	24.7	101	5.5
T4 cells	7.7	113	4.1
Leu-Leu-OMe-resistant PBL	5.6	100	2.7

* Data are provided for the cell populations isolated from a single peripheral blood sample.
‡ Kinetic constants calculated by least squares fit in Lineweaver Burk plots of Leu-Leu-OMe uptake assessed at concentrations of 4, 16, 64, 256 and 1,024 μM. All coefficients of correlation were >0.95.

As demonstrated by the data detailed in Table 3, cell populations with higher DPPI concentrations (T8, NK cells, or Mø) generally were found to incorporate [^{3}H]Leu-Leu-OMe at greater rates than was observed for relatively DPPI-deficient T4 cells or Leu-Leu-OMe-treated PBL. Thus, T8- and NK-enriched PBL were found to contain approximately eightfold more DPPI activity and to incorporate Leu-Leu-OMe at a fourfold greater rate than did T4-enriched PBL. In addition, Leu-Leu-OMe-resistant PBL had a four- to fivefold lower DPPI content and incorporated Leu-Leu-OMe at approximately half the rate of control PBL. Despite the apparent correlation between DPPI content and Leu-Leu-OMe incorporation, the Leu-Leu-OMe uptake measured in these studies was not simply a reflection of retention of Leu-Leu-OMe products of DPPI metabolism. Thus, in the experiment detailed in Fig. 3, unseparated PBL or T8- and NK-enriched populations were preincubated with 10^{-5} M Gly-Phe-CHN_2. Although incubation with Gly-Phe-CHN_2 routinely leads to >95% inhibition of intracellular DPPI activity (Table 1), Leu-Leu-OMe uptake by cells treated with this specific DPPI inhibitor was not significantly altered. Thus, Leu-Leu-OMe uptake appears to involve mechanisms distinct from the enzymatic activity of DPPI.

Peptide transport processes have been described in a variety of mammalian tissues (26). The experiments detailed in Table 4 were carried out to determine whether the uptake

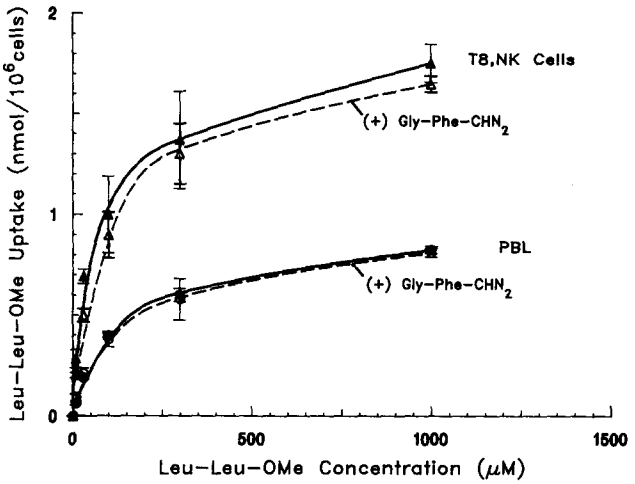

Figure 3. Concentration-dependent uptake of Leu-Leu-OMe is not affected by pretreatment with the DPPI inhibitor Gly-Phe-CHN_2. The indicated cell populations were incubated for 1 h in the presence or absence of 10^{-5} M Gly-Phe-CHN_2. The cells were then washed and assessed for concentration-dependent Leu-Leu-OMe uptake, as detailed in Fig. 2.

Table 4. *Metabolic Requirements of Leu-Leu-OMe Uptake by Human PBL*

Composition of incubation medium (150 mM)	Leu-Leu-OMe uptake*
	pmol/10⁶ cells/15 min
PB‡, pH 7.4	1,344 ± 237
PB, pH 7.0	717 ± 41
PB, pH 6.0	412 ± 43
PB, pH 7.4, 0.1% Sodium Azide	1,447 ± 76
PB, pH 7.4, 0.1% Glucose	1,307 ± 136
NaCl, 25 mM Hepes, pH 7.4	1,385 ± 116
CholineCl, 25 mM Hepes, pH 7.4	1,416 ± 132

* Leu-Leu-OMe uptake was assessed after incubation of PBL for 15 min at 22°C in the presence of 100 μM Leu-Leu-OMe. Values are given for mean ± SE of triplicate determinations.
‡ PB, phosphate buffer composed of Na_2PO_4 and $NaHPO_4$.

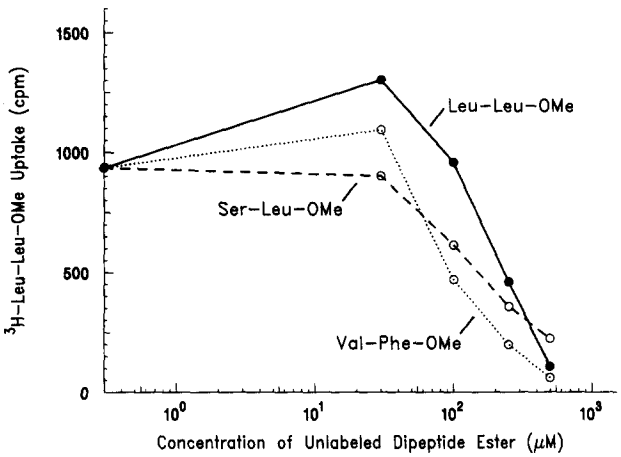

Figure 4. [^{3}H]Leu-Leu-OMe uptake is competitively inhibited by Leu-Leu-OMe, Ser-Leu-OMe, or Val-Phe-OMe. PBL (2.5×10^6/ml) were suspended in PBS containing 0.75 μCi/ml [^{3}H]Leu-Leu-OMe (sp act, 60 mCi/mM) and the indicated concentrations of unlabeled dipeptide esters. After a 15-min incubation at 22°C, cells were spun through silicone oil, and [^{3}H]Leu-Leu-OMe incorporation was assessed. Values displayed represent means of triplicate determinations. In all cases, SEs were <15% of mean values.

of Leu-Leu-OMe by human PBL is mechanistically similar to dipeptide transport processes previously characterized in the intestinal tract, renal tubule, or the brain (26–31). Intestinal and renal tubular dipeptide transport has been shown to be an active proton-coupled process that proceeds optimally at extracellular pH of 5.5–6.0 (27, 28, 31). However, as indicated by the results detailed in Table 4, Leu-Leu-OMe uptake by human PBL is decreased rather than increased when extracellular pH is decreased from 7.4 to 6.0. Furthermore, rates of Leu-Leu-OMe uptake were unaffected by addition of the metabolic inhibitor, sodium azide, or by the presence or absence of extracellular glucose or sodium, additionally demonstrating that its uptake by PBL proceeds by a mechanism that is different than the aforementioned dipeptide transporters (26–31).

Additional experiments were carried out to assess the specificity of this transport process. Various structural analogues of Leu-Leu-OMe were examined for the capacity to inhibit the uptake of [^{3}H]Leu-Leu-OMe. The results of experiments depicted in Fig. 4 demonstrate that uptake of [^{3}H]Leu-Leu-OMe is competitively inhibited not only by addition of excess unlabeled Leu-Leu-OMe, but also by excess unlabeled Val-Phe-OMe or Ser-Leu-OMe. The virtually identical concentration-dependent patterns of inhibition of [^{3}H]Leu-Leu-OMe uptake by these three unlabeled dipeptide methyl esters suggested that these compounds were competing for a common facilitated transport mechanism. Additional experiments were carried out to explore the structural characteristics of compounds that competitively inhibit [^{3}H]Leu-Leu-OMe uptake in greater detail. As demonstrated by the results in Table 5, [^{3}H]Leu-Leu-OMe uptake is competitively inhibited not only by excess quantities of unlabeled Leu-Leu-OMe, but also by high concentrations of the dipeptide Leu-Leu and by other esters of Leu-Leu such as Leu-Leu-OBenzyl. However, neither Leu-OMe nor Leu-Leu-Leu-OMe were observed to compete with lymphocyte incorporation of Leu-Leu-OMe. Furthermore, neither the D-stereoisomer of Leu-Leu-OMe nor the amide analogue Leu-Leu-NH_2 inhibited uptake of the dipeptide methyl ester. Thus, the saturable process whereby Leu-Leu-OMe is incorporated into PBL is restricted to dipeptides or dipeptide derivatives.

In addition, as demonstrated by the experiments in Table 6, not all dipeptide methyl esters composed of L-stereoisomers of naturally occurring amino acids can effectively compete with [^{3}H]Leu-Leu-OMe uptake. Thus, excess concentrations of unlabeled Pro-Leu-OMe or Asp-Leu-OMe had no discernible effect on the uptake of [^{3}H]Leu-Leu-OMe. Of note, nei-

Table 5. *Competitive Inhibition of [^{3}H]Leu-Leu-OMe Uptake by Other Derivatives of Leu-Leu*

Unlabeled compound	Percent inhibition of [^{3}H]Leu-Leu-OMe uptake*
Leu-OMe	−10
Leu-Leu	44
Leu-Leu-OMe	72
D-Leu-D-Leu-OMe	7
Leu-Leu-OBenzyl	87
Leu-Leu-NH_2	8
Leu-Leu-Leu-OMe	6

* Uptake of 10 μM [^{3}H]Leu-Leu-OMe was assessed in the presence of 250-μM concentrations of the indicated unlabeled compounds. Results are the means of two to four separate triplicate determinations. In all cases, SEM was ≤10%.

Table 6. *Competitive Inhibition of [³H]Leu-Leu-OMe Uptake by Other Dipeptide Esters*

Unlabeled compound	Percent inhibition of [³H]Leu-Leu-OMe uptake*	NK toxicity‡ (LD50)
		μM
Pro-Leu-OMe	−18	>250
Asp-Phe-OMe	8	>250
Leu-Leu-OMe	72	35
Leu-Phe-OMe	72	29
Val-Phe-OMe	75	24
Ser-Leu-OMe	84	>250
Leu-Tyr-OMe	86	>250

* Assessed as detailed in Table 3. In all cases, SEM was ⩽10%.
‡ Calculated from previously published data (12) LD50 determined from the concentration required to ablate 50% of cytotoxicity/activity of NAC assayed against K562 targets at a 20:1 E/T ratio.

ther Pro-Leu-OMe nor Asp-Phe-OMe caused significant NK toxicity, whereas compounds such as Val-Phe-OMe and Leu-Phe-OMe not only shared the NK toxicity of Leu-Leu-OMe, but also appear to share the same lymphocyte uptake pathway, as they are virtually identical to unlabeled Leu-Leu-OMe in the capacity to inhibit the uptake of [³H]Leu-Leu-OMe. Lack of affinity for this facilitated transport pathway, however, could not explain the lack of NK toxicity of Leu-Tyr-OMe or Ser-Leu-OMe, as these compounds were noted to inhibit [³H]Leu-Leu-OMe incorporation very effectively.

The results suggested that for some dipeptide esters such as Ser-Leu-OMe or Leu-Tyr-OMe, lack of toxicity for DPPI-rich cytolytic lymphocytes could not be explained by the inability to be taken up by lymphocytes or by the lack of known substrate affinity for DPPI. Experiments were therefore carried out to determine whether DPPI-generated metabolites of such dipeptide esters exhibited membranolytic potential. As previously reported and as detailed in Fig. 5 (*upper left*), when ^{51}Cr-labeled human RBC were incubated with varying concentrations of Leu-Leu-OMe alone or with DPPI alone, no damage to cell membranes was apparent. However, in the presence of 250 μM or more Leu-Leu-OMe and DPPI, lysis of RBC was noted. This membranolytic effect has been demonstrated previously (11) to be mediated by Leu-Leu-OMe polymerization products of the general structure $(Leu\text{-}Leu)_n$-OMe, when $n \geqslant 3$.

Leu-Leu-NH_2 proved to be an equally good substitute for production of membranolytic DPPI metabolites, whereas the benzyl ester derivative of Leu-Leu was found to be an even more effective substrate for this process. Substitution of D-stereoisomers of Leu into both positions of Leu-Leu-OMe or either the NH_2-terminal or penultimate position of this substrate (data not shown) led to no DPPI-catalyzed membranolytic effects. The lack of RBC lysis in the presence of DPPI and D-stereoisomers of Leu-Leu-OMe likely is related to the fact that the acyl transferase activity of this enzyme is seen only with dipeptide substrates composed entirely of L-stereoisomer-containing amino acids (22, 24). However, use of this assay system in additional experiments revealed that DPPI-catalyzed RBC lysis was observed when NK-toxic dipeptide esters such as Leu-Phe-OMe or Val-Phe-OMe were used, but not when the putative DPPI substrates Ser-Leu-OMe or Leu-Tyr-OMe were used. That compounds such as Ser-Leu-OMe indeed bind to the active site of DPPI is shown by the results of the experiment detailed in Fig. 6. In this experiment, excess quantities of Ser-Leu-OMe were noted to inhibit RBC lysis mediated by the combination of DPPI and 250–1000 μM Leu-Leu-OMe. In additional experiments, Leu-Tyr-OMe also was observed to inhibit RBC lysis mediated by DPPI + Leu-Leu-OMe (data not shown).

A number of additional dipeptide esters and amides were also screened in each of these assays to determine the nature of active compounds in greater detail. As summarized for representative compounds in Table 7, competitive inhibition of [³H]Leu-Leu-OMe uptake was seen with dipeptides or dipeptide methyl esters composed of uncharged L-stereoisomer amino acids. Addition of somewhat more hydrophobic benzyl ester or β-naphthylamide derivatives favored competition with the transporter responsible for Leu-Leu-OMe incorporation by PBL, whereas simple amide or methyl amide derivatives demonstrated no avidity for this transport system. Only dipeptide esters or amides composed entirely of nonpolar R group L-stereoisomer amino acids were converted to RBC lytic products by DPPI. Thus, all NK toxic dipeptide derivatives have been noted to act both as competitive inhibitors of [³H]Leu-Leu-OMe uptake by human PBL and to serve as substrates for DPPI-catalyzed lysis of human RBC. In contrast, all dipeptide ester or amides that at a concentration of 250 μM exert no effect on NK cell function have proven to be inactive in one or both of these assays. Finally, NK cells pretreated with the specific DPPI inhibitor Gly-Phe-CHN_2 are resistant to any discernible toxic effects of any of the dipeptide esters or amides screened in these studies.

The results summarized in Table 7 imply that Ser-Leu-OMe and Leu-Tyr-OMe lack NK toxic effects because DPPI polymerization products of these compounds lack membranolytic effects. To verify that this was the basis for the lack of NK toxicity of these compounds, the experiments detailed in Table 8 were performed. Uptake of these compounds by cytolytic cells was directly measured, as was their capacity to serve as substrates for DPPI-mediated transamidation. The results of these studies indicate that rates of incorporation of Ser-Leu-O[^{14}C]Me and Leu-Tyr-O[^{14}C]Me by T8- and NK-enriched PBL are comparable with the rate of uptake of Leu-Leu-O[^{14}C]Me. Furthermore, both of these compounds served as substrates for transamidation by DPPI or by lysates of cytotoxic cells. The rates of DPPI-mediated acyl transferase activity observed with Ser-Leu-OMe or Leu-Tyr-OMe as substrates were at least as great as the rates observed with Leu-Leu-OMe as a substrate. Thus, the results indicate that Ser-Leu-OMe and Leu-Tyr-OMe are taken up by T8- and NK-

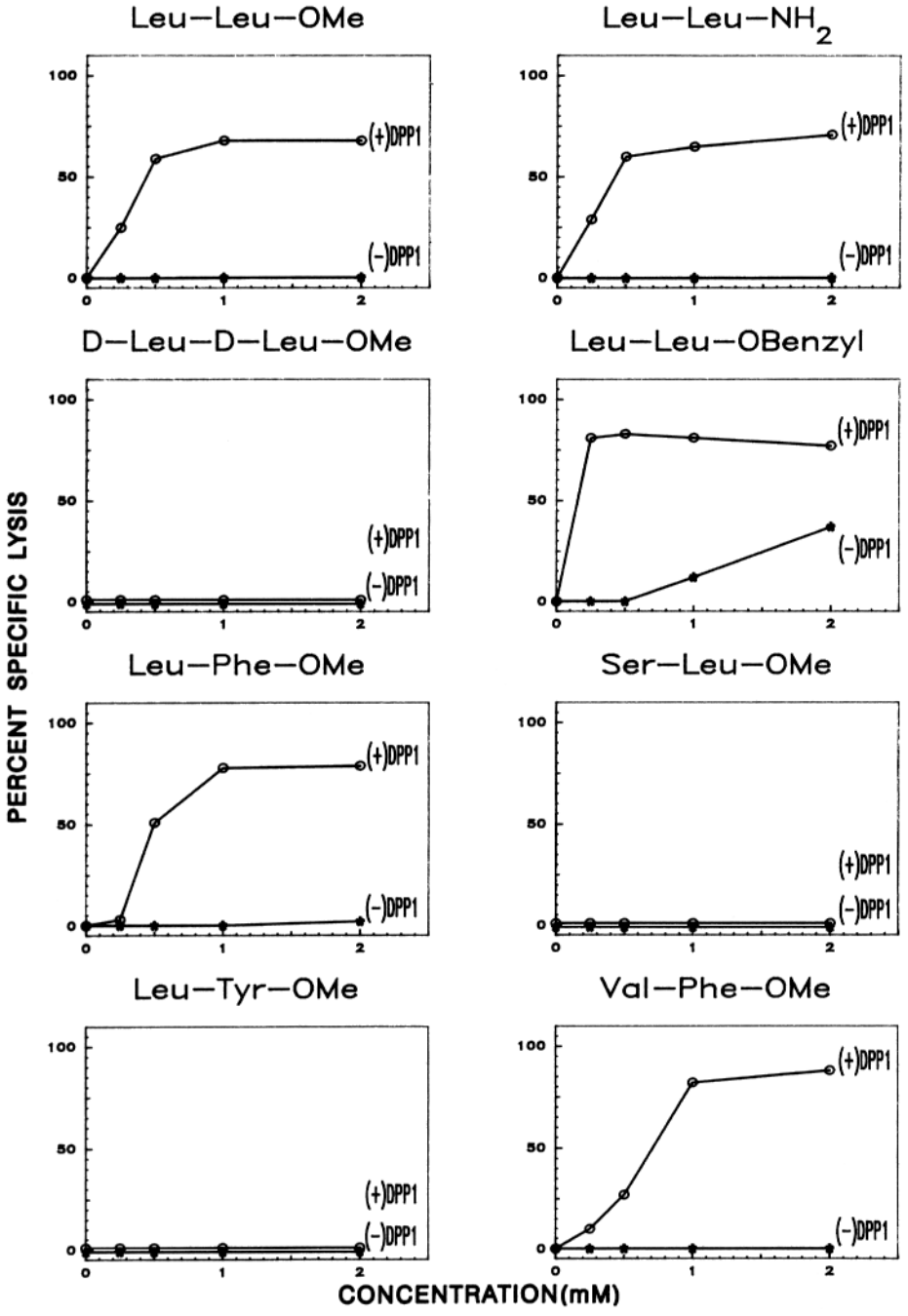

Figure 5. RBC lysis is mediated by DPPI metabolites of some but not all ester or amide derivatives of Leu-Leu. ^{51}Cr-labeled human RBC were incubated in the presence (O) or absence (*) of 8×10^{-4} U/ml of DPPI, and the indicated concentrations of dipeptide esters or amides for 4 h at 37°C and were then assessed for percent specific lysis.

enriched PBL and are suitable substrates for transpeptidation by the DPPI activity present within cytotoxic lymphocytes. The inability of the compounds to exert toxicity for cytolytic cells, therefore, appears to result from the lack of membranolytic properties of the transpeptidation products.

Discussion

The present studies elucidate several common characteristics of NK toxic dipeptide esters and amides. The results not only document the metabolic steps involved in the toxicity to cytotoxic cells, but have also delineated a new transport system for dipeptides. The observations indicate that uptake via a facilitated transport mechanism and conversion to hydrophobic polymerization products by the granule protease, DPPI, are essential steps in the mechanism, whereby dipeptide esters mediate toxic effects toward cytolytic lymphocytes. These findings not only provide a basis for design and synthesis of dipeptide analogues that may mimic the unique immunosuppressive effects of Leu-Leu-OMe, but also provide

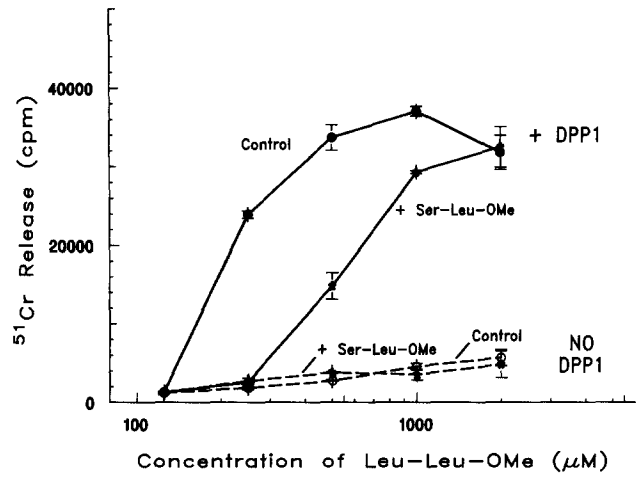

Figure 6. Ser-Leu-OMe competitively inhibits DPPI-catalyzed production of membranolytic metabolites from Leu-Leu-OMe. RBC lysis was assessed as detailed in Fig. 6 in the presence (——) or absence (----) of DPPI with no competing Ser-Leu-OMe (O) or with 2 mM Ser-Leu-OMe (*) present in the assay. Values are given for mean ± SE of triplicate determinations.

new insights into the manner in which lymphocytes process these small peptide derivatives.

It has been demonstrated previously that active transport of di- and tripeptides occurs in mammalian small intestine, as well as in tissues or cells isolated from a variety of other organs (26). A number of pharmaceutical agents of analogous structure, including β-lactam antibiotics and the angiotensin-converting enzyme inhibitor captopril, are also transported through these peptide carrier systems (32). Peptide transport in intact small intestine or renal tubules is mediated by an active, low affinity process with a Km in the 5–10 mM range (32–34). When dipeptide transport has been assessed in intestinal or renal brush border vesicles, the Km of this process has been found to be ~1 mM (35). In contrast, the present studies indicate that human PBL incorporate Leu-Leu-OMe via a relatively high affinity transport mechanism with a Km of 100–200 μM. Furthermore, no change in the rate of Leu-Leu-OMe uptake by PBL was noted in the presence of azide and/or in the absence of extracellular glucose or Na^+. Thus, we have found no evidence to suggest that dipeptide or dipeptide ester uptake by human PBL is an energy-requiring process. Rather, the transport process appears to facilitate movement of molecules down a concentration gradient. Thus, when lymphocyte volume estimated by light scatter was used to determine the overall intracellular concentration of Leu-Leu-OMe achieved after a 15-min incubation, it was found to approximate no more than 5% of the extracellular concentration. Therefore, the present data regarding lymphocyte uptake of Leu-Leu-OMe are most consistent with a facilitated diffusion process mediated by a high affinity carrier. Furthermore, the absence of a requirement for extracellular Na^+ and the lack of enhancement of PBL dipeptide transport in the presence of decreased extracellular pH suggests that unlike amino acid or dipeptide transport in other cell types, Leu-Leu-OMe uptake by PBL was not coupled to Na^+ or H^+ gradients (27–31). A high affinity peptide transport process with Km and Vmax values similar to those detected in the present studies has been observed in astroglial-rich brain cell cultures (29). However, dipeptide uptake by astroglial cells has been demonstrated to be an energy-dependent process that is inhibited by extracellular Na^+ depletion, by inhibition of ATP production, or by the addition of a variety of tri- or oligo-peptides (29). Therefore, this astroglial cell peptide transport system, as well as dipeptide transport mechanisms previously characterized in the intestine or renal tubule, appear to be distinct from the dipeptide-specific facilitated transport process elucidated in the present studies.

Wheras simple dipeptides such as Leu-Leu appear to utilize the same PBL transport process as Leu-Leu-OMe, the present studies indicate that binding to this transporter is facilitated by nonpolar ester or amide additions to the COOH terminus of dipeptides. The lack of competitive inhibition of Leu-Leu-OMe uptake by excess concentrations of Leu-OMe or Leu-Leu-OMe suggests that this transport process is strictly specific for dipeptide derivatives. As not all dipeptide esters competitively inhibit Leu-Leu-OMe uptake, the membrane carrier responsible for this process appears to be selective with regard to dipeptides that can be transported. In the current studies, this transport system was assessed for the capacity to internalize Leu-Leu-OMe and other dipeptide ester or amide substrates of DPPI. It is also possible, however, that under physiologic conditions, the same peptide carrier may be responsible for facilitating egress of dipeptide products of protease-mediated degradation of oligopeptides or proteins.

In addition to characterizing the mechanism for internalization of NK toxic dipeptide derivatives, the present studies also demonstrate that NK toxicity mediated not only by Leu-Leu-OMe, but also by a variety of other dipeptide esters or amides, is prevented by preincubation of these cells with a specific inhibitor of the lysosomal thiol protease, DPPI. In previous studies, it has been demonstrated that this enzyme is responsible for conversion of Leu-Leu-OMe to oligomers of the general structure $(Leu\text{-}Leu)_n$-OMe (11). The present studies confirm that serving as a substrate for the transpeptidase activity of DPPI is an essential characteristic of NK toxic dipeptide esters or amides. However, this attribute is not sufficient to induce NK toxic effects. Thus, DPPI substrates such as Ser-Leu-OMe and Leu-Tyr-OMe mediated no NK toxic effects when present at concentrations well in excess of concentrations of Leu-Leu-OMe that cause ablation of all NK activity. These agents are equal to Leu-Leu-OMe in the capacity to inhibit [^{3}H]Leu-Leu-OMe uptake by PBL, and therefore appear to utilize the same transport mechanism. Furthermore, direct measurements of Ser-Leu-OMe and Leu-Tyr-OMe uptake by PBL reveal that lymphocyte incorporation rates of these dipeptide esters are equal to or superior to that of Leu-Leu-OMe. Thus, the lack of NK toxicity is not related to any inability of NK cells to internalize these agents. However, unlike Leu-Leu-OMe and other dipeptide esters or amides composed of amino acids with relatively hy-

Table 7. *Comparison of the Avidity for Dipeptide Transport, Conversion by the Acyl Transferase Activity of DPPI to Membranolytic Products, and NK Toxicity of Various Dipeptide Derivatives*

Compound	Competitive inhibition of [^{3}H]Leu-Leu-OMe uptake*	Dipeptidyl peptidase I catalyzed lysis of human RBC‡	Toxicity for human NK cells§	Toxicity for Gly-Phe-CHN_2 treated NK cells‖
Leu-Leu-OMe	+ +	+ +	+ +	−
Leu-Leu	+	−	−	−
Leu-Leu-NH_2	−	+ +	−	−
Leu-Leu-$NHCH_3$	−	−	−	−
Leu-Leu-OBenzyl	+ +	+ +	+ +	−
D-Leu-L-Leu-OMe	−	−	−	−
L-Leu-D-Leu-OMe	−	−	−	−
Leu-Phe-OMe	+ +	+ +	+ +	−
Leu-Tyr-OMe	+ +	−	−	−
Val-Phe-OMe	+ +	+ +	+ +	−
Pro-Leu-OMe	−	−	−	−
Asp-Phe-OMe	−	−	−	−
Ser-Leu-OMe	+ +	−	−	−
Gly-Phe-OMe	+	+	+	−
Gly-Phe-βnaphthylamide	+ +	+ +	+ +	−
Gly-Arg-βnaphthylamide	−	−	−	−

* Assayed as detailed in Tables 5 and 6; >66% inhibition, + +; >33% inhibition, +; <33% inhibition, −.
‡ Assayed as detailed in Fig. 6; >33% RBC lysis at 250 μM, + +; <33% RBC lysis at 250, >33% lysis at 1 mM, +; <33% lysis at 1 mM, −.
§ NK toxicity assessed as detailed in Table 1. >50% loss of NK function, E/T 10:1; dipeptide concentration 50 μM, + +; <50% loss of NK function at 50 μM but >50% at 250 μM, +; <50% loss NK function at 500 μM, −.
‖ Toxicity assessed as above for cells preincubated for 30 min at 37°C with 10^{-5} M Gly-Phe-CHN_2 before exposure to 50–500-μM concentrations of the indicated compound.

drophobic R groups, DPPI substrates with polar R group amino acids in either position do not serve as adequate substrates for DPPI-catalyzed lysis of RBC. These polar R group–containing dipeptide esters, however, are capable of competitively inhibiting RBC lysis mediated by the combination of DPPI and Leu-Leu-OMe, and can serve as substrates for the acyl transferase activity of DPPI, as has been previously indicated (22, 24). These results, therefore, suggest that (Ser-Leu)$_n$-OMe or similar polymers form within the granule compartment of cytotoxic lymphocytes but have no mem-

Table 8. *Ser-Leu-OMe, Leu-Tyr-OMe, and Leu-Leu-OMe Are Taken up by T8- and NK-enriched PBL and Converted to DPPI-mediated Transamidation Products at Comparable Rates*

Dipeptide ester	Rate of uptake by T8, NK cells*	Rate of transamidation by purified bovine DPPI‡	Rate of transamidation by T8, NK Cell sonicates§
	pmol/10^6 cells/5 min	*nmol hydroxamate/20 min*	
Ser-Leu-OMe	280	3,660	490
Leu-Tyr-OMe	340	2,450	380
Leu-Leu-OMe	220	1,850	240

* Uptake of 250 μM Ser-Leu-O[^{14}C]Me (sp act 1.29 mCi/mM), Leu-Leu-O[^{14}C]Me (sp act 1.74 mCi/mM), and Leu-Leu-O[^{14}C]Me (sp act 1.34 mCi/mM) by T8- and NK-enriched PBL was assessed during a 5-min incubation at 22°C.
‡ Purified bovine DPPI was present at a concentration of 10^{-2} U/ml.
§ T8- and NK-enriched cells (10^7/ml in saline) were sonicated and mixed with equal volumes of substrate buffer solutions containing 100 mM dipeptide ester, 50 mM DTT, and 0.8 mM hydroxylamine·HCl. Reactions mixtures containing equal concentrations of T8 and NK cell sonicates treated with 10^{-4} M Gly-Phe-CHN_2 were used as blanks so that only DPPI-mediated transmidation could be assessed.

branolytic properties, and hence, no toxic effects. Thus, the generation of membranolytic products from dipeptide esters or amides only occurs with DPPI substrates uniformly composed of hydrophobic amino acids.

Of note, in the ^{51}Cr-labeled RBC assay in which this membranolytic effect has been directly observed, DPPI-catalyzed RBC lysis is only observed in the presence of >250 μM concentrations of dipeptide ester or amide substrates. Despite this, the calculated intracellular concentration of Leu-Leu-OMe achieved after exposure to NK toxic concentrations of Leu-Leu-OMe may be as low as 1–5 μM. Several factors may account for this apparent discrepancy. The lysosomotropic characteristics of amino acid and peptide esters have been previously well characterized (36, 37). Therefore, although average intracellular concentrations of Leu-Leu-OMe achieved after exposure of T8- and NK-enriched PBL to 50–100 μM Leu-Leu-OMe may be only 1–5 μM, the concentration of this agent within the DPPI-containing granule compartment of these cells may be much higher. In addition, introduction of membranolytic extensively hydrophobic $(Leu\text{-}Leu)_n$-OMe metabolites into appropriate cell membranes may be far more efficient when these DPPI products are produced within a cell rather than in the external aqueous medium used in the ^{51}Cr-labeled RBC lysis assays. These results, therefore, are still consistent with a primary role for the membranolytic effects of $(Leu\text{-}Leu)_n$-OMe products in the generation of Leu-Leu-OMe-mediated killing of cytolytic lymphocytes. It remains to be determined whether the membranolytic $(Leu\text{-}Leu)_n$-OMe, n >3 products of this enzyme trigger other unique enzymatic events in cells with endogenous cytolytic capacity that also contribute to the restricted spectrum of toxicity of Leu-Leu-OMe and related compounds.

The toxic effects of Leu-Leu-OMe on human and murine cells have been noted to be highly selective with only cytotoxic lymphocytes and cells of myeloid origin killed after brief exposures to 50–500 μM Leu-Leu-OMe (13, 15). In contrast, Th cells, B cells, and a variety of cells of non–bone marrow origin remain functionally intact after such exposures (13, 15). The present studies demonstrate that facilitated transport of the dipeptide ester is an essential step in the toxicity caused by these agents. Both Leu-Leu-OMe-sensitive and Leu-Leu-OMe-resistant cell types transport dipeptides with a similar avidity (Km) but with two- to sixfold disparate capacity (Vmax). Thus, differential rates of Leu-Leu-OMe uptake are likely to contribute to the selective nature of this toxicity. 8–10-fold differences in DPPI content of Leu-Leu-OMe-sensitive and Leu-Leu-OMe-resistant cells have also been noted (11). Therefore, it is likely that the relative enrichment of this granule enzyme within cytotoxic lymphocytes and myeloid cells also plays a significant role in determining susceptibility to Leu-Leu-OMe and related compounds. Whereas DPPI-like activity has been demonstrated in a host of mammalian organs and cell types, the detection of marked enrichment of this enzyme within cytotoxic lymphocytes and myeloid cells, as well as the existence of a facilitated transport system to deliver substrates of this enzyme, provide a unique means of targeting agents with the capacity to selectively delete cytotoxic cells.

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