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A Thymic Metallo-Peptide Influences Lymphocyte, Monocyte and Thymocyte Responses

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Abstract

Non-covalent metal-peptide interactions are critical in peptide assembly, folding, stability, and function. Zinc has chemical, structural and regulatory functions in biological systems. The present study investigated the changes caused by the addition of Zn^{2+} on the biological activity of a thymic peptide on immune cells. For this purpose, we exposed different cells to 10^{-10} M peptide and different concentrations of Zn^{2+}, Mg^{2+} and Cu^{2+} for 24 and 48 h, and monitored the proliferative and phagocytic activities of the treated cells. We also performed NMR and chromatography analysis of the peptide in the presence of Zn^{2+} and other ions. Peptide activity increased in the presence of Zn^{2+}, Mg^{2+} or Cu^{2+}, and this increase was over 100-fold in the presence of Zn^{2+}. NMR studies indicated that the peptide exhibited field displacements: Glutamic acid (D) to low-field NMR (Δδ+0.027 ppm), and both aspartic acid (E) and the leucine with a terminal carboxylic acid (L^8) to high-field NMR (Δδ-0.016 and -0.051 ppm, respectively). In addition, the retention time in HPLC decreased in the presence of ions. Our findings show that the peptide loses its biological activity in the presence of a zinc-chelating agent. That is, the presence of zinc and other metals to a lesser extent is essential for the activity of the peptide. This unexpected dependence on zinc appears to be due to the active form of the peptide-zinc complex, for which we propose the name of "immuno-modulator metallo-peptide" (IMMP).

Keywords: Thymic humoral factor; Divalent cations; Immuno-modulator metallo-peptide; Zinc; Metallopeptide

Abbreviations: Con A: Concanavalin A; DTPA: Diethylenetriaminepentaacetic acid; DMSO: Dimethyl sulfoxide; EC_{50}: half maximal effective concentration; EDTA: Ethylenediaminetetraacetic acid; FCS: Fetal Calf Serum; HPLC: High-performance liquid chromatography; IMMP: Immune Modulator Metallo Peptide; LPS: Lipopolysaccharide; MTS: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR: Nuclear magnetic resonance; PBMC: Peripheral blood mononuclear cells; PHA: Phytohemagglutinin; PI: Propidium iodide; RP-HPLC: Reversed-phase-HPLC; TFA: Trifluoroacetic acid; Zn^{2+}-complex: L^1EDGPKFL^8 peptide plus zinc

Introduction

Metal-ligand interactions are critical components of the metallopeptide assembly, folding, stability, electrochemistry, and function. Zinc serves chemical, structural, and regulatory roles in biological systems. Experimental zinc deprivation leads to generalized lymphoid hypoplasia, rapid thymic involution (particularly of the cortex), and decreased production of thymic hormones, impaired lymphocyte proliferative capacity after phytomitogen stimulation, and reduced antibody and cell-mediated responses [1-4]. Divalent cations, polypeptides and basic proteins have all been proposed to be natural mitotic stimulants, both *in vivo* and *in vitro*, serving to initiate cell division [5-8]. The thymic octapeptide Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu (L^1EDGPKFL^8) has been implicated in augmenting T-cell functions, such as the response to T-cell lectins and mixed lymphocyte reactions; it also increases interleukin-2 production by T-cells [9]. However, the reported concentration range in which it operates *in vitro* is very broad. We, investigated whether any metal could modify the activity of the peptide or not.

It is important to examine the contribution of trace metal cations to peptide-induced biological activity. We carried out biological and biophysical studies to determine if there is a possible metal-peptide physical interaction and a synergistic effect between the peptide and those metallic ions that improve peptide function. The aim of this study was to investigate whether the function of immune cells was modified by the presence of the peptide-Zn^{2+} complex *in vitro*.

Material and Methods

Reagents

RPMI-1640, nonessential amino acids, fetal calf serum (FCS), L-glutamine, antibiotics and 0.25% trypsin were obtained from Gibco BRL Inc., (Grand Island, N.Y., U.S.A.). Ultra pure LPS from Escherichia coli O111:B4 was from Alexis Biochemicals (San Diego, CA, U.S.A.). Phytohemagglutinin (PHA), Concanavalin A (ConA), Diethylenetriaminepentaacetic acid (DTPA), Dimethyl sulfoxide (DMSO), as well as the Ficoll-Hypaque and Ficoll-Paque Plus gradients were from Sigma-Aldrich Co. (St. Louis, Missouri, U.S.A.). Trifluoroacetic acid (TFA), $ZnCl_2$, $MgCl_2$, $CuCl_2$, and $MnCl_2$ were of analytical grade and obtained from Merck (Whitehouse Station, NJ. U.S.A.) as sulfates. Peptide Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu was synthesized by New England Peptide (Gardner, MA, U.S.A.).

Cell cultures and treatment

Two cell lines were used for this study: THP-1 cells (TIB-202 ATCC) derived from acute monocytic leukemia and Jurkat cells

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(TIB-152 ATCC) from acute T cell leukemia. In addition, we also used human lymphocytes, newborn rat thymic cells (thymocytes), and human peripheral blood mononuclear cells (PBMC). The PBMC and lymphocytes were obtained from healthy donors using Ficoll-Hypaque and from Ficoll-Paque Plus gradients, respectively; the cells were washed and suspended in appropriate culture medium. The cells were cultured in RPMI-1640 that was supplemented with nonessential amino acids, 10% FCS, L-glutamine (2 mol/L), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Five hundred thousand viable cells were plated in 25 cm^2 culture bottles and maintained at 37°C under an atmosphere of 5% CO_2 and 95% air. Fresh medium was added every 2 d, and the cells were harvested and diluted 5-fold every 7 d.

Newborn rat thymic cells (thymocytes) were obtained by passing thymic tissue through a 100 micron mesh. The cells were then washed and suspended in RPMI-1640 medium that was supplemented with nonessential amino acids, 10% FCS, L-glutamine (2 mol/L), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). The cells were seeded in a tissue culture flask at 37°C with 5% CO_2 in humidified air.

THP-1 cells were co-stimulated with LPS (0.5 μg/ml), the human lymphocytes were co-stimulated with PHA (1 μg/ml), and the rat newborn thymocytes co-stimulated with Con A (2 μg/ml). Next, the cells were treated with peptide (10^{-10}M) or peptide (10^{-10}M) plus Zn^{2+} (10 nM) for 24 h. In another experiment, previously co-stimulated cells were treated with peptide (10^{-10}M) alone or peptide (10^{-10}M) plus (10^{-8}M) Zn^{2+}, Mg^{2+}, Cu^{2+} or Mn^{2+}.

Cell proliferation assay

Cell proliferation was measured using an MTS assay [10]. Briefly, the cells were cultured in a flat-bottomed 96-well plate (Corning, 2×10^5 cells/well), co-stimulated and treated with peptide in the absence or presence of divalent ions, and maintained at 37°C under an atmosphere of 5% CO_2 in humidified air. After 24 h, Cell Titer 96® AQueous One Solution Reagent (Promega, PR China) was added to each well according to the manufacturer's instructions. After 4 h in culture, the cell viability was determined by measuring absorbance at 490 nm using a VERSA max Tunable microplate reader.

Phagocytic function

PBMC phagocytic functions were determined as described earlier [10]. Briefly, human PBMC were pretreated with peptide (10-^{10}M) plus Zn^{2+} (10^{-8}M) in the presence or absence of DTPA (5 μM) for 24 and 48 h. Two 0.25 ml aliquots of cell suspension were placed in small incubation chambers, prepared on microscopic slides and incubated for 30 min at 37°C and 5% CO_2. The chambers were then rinsed three times with 0.5 ml MEM that had been pre-warmed at 37°C. Next, they were incubated with living, non-opsonized yeast cells that had also been prepared in MEM (PBMC: yeast cells ratio 1:40), as targets. Lastly, the boiled yeast cells were added in a solution of 5 μg/ml propidium iodide (PI). Phagocytosis was quantified using flow cytometry. At least 10,000 PBMC were observed. As the intensity of PI is proportional to the amount of yeast phagocytized, we identified the peak channel fluorescence in 540 nm (FL2). The results are expressed as the peak channel in FL2.

HPLC analysis

Lyophilized peptide was dissolved in distilled water (final concentration 15 mM) and analyzed using a C18 (3.5 μ×4.6×75 mm) RP-HPLC column. Samples of 5 μg in 50 μl of each preparation were eluted. The elution was performed in A (TFA 0.2% in water) and B (Acetonitrile), changing the mobile phase composition from 5% B to 50% over a period of 65 min at a flow rate of 1 ml/min. All of the steps were carried out at room temperature. The fractions were collected and monitored by UV at 210 nm.

NMR spectroscopy

Studies using Nuclear Magnetic Resonance (NMR) were carried out on Varian Unity Inova equipment with proton frequencies of 400 MHz and 699,815 MHz at a temperature of 25°C. The spectra were obtained in D_2O and, in certain cases, D_2O-H_2O mixtures using DSS and $CDCl_3$ as an external reference for ^1H and ^{13}C, respectively.

Peptide $L^1EDGPKFL^8$, as a white solid (13.7 mg, 1.5×10^{-5} mol), was dissolved in deuterated water (1 ml) to give a homogeneous solution of pH 1.8. The pH was adjusted to a value of 7.2 (corresponding to pD6.8) with small additions of Na_2CO_3(s). A volume of 700 μl of this solution (*ca.* 15 mM) was transferred to a 5 mm NMR tube for the acquisition of the ^1H, ^{13}C, COSY, TOCSY and gHSQC spectra.

One molar equivalent of $ZnCl_2$ (53 μl aliquot of 0.2 M solution in D_2O) was added to 700 μl of 15 mM $L^1EDGPKFL^8$ peptide in D_2O. The pH of the solution did not change significantly after the addition. Both ^1H and ^{13}C NMR spectra were acquired.

Statistical analysis

Each experiment was performed in duplicate and repeated at least three times. The data are presented as the mean ± SEM. Statistical significance was determined using Student's paired t-test for comparison between two groups or ANOVA (analysis of variance) following the Student–Newman–Keuls test for comparisons among three or more groups with SPSS software version 11.5 (SPSS Inc., Chicago, IL). A value of $p<0.01$ was considered to be statistically significant.

Results

Conformational changes determined using NMR

We used NMR analysis and circular dichroism (CD) to examine the conformational characteristics of the peptide $L^1EDGPKFL^8$ in the presence of Zn^{2+}. Due to the length of the peptide it was not expected to present secondary structure; indeed CD experiments showed a typical spectrum of a random coil chain that is not affected by the presence of Zn^{2+} (Figure 1). These studies strongly suggest that the free peptide in aqueous solution is flexible and can rapidly attain equilibrium between multiple conformations.

The analysis by NMR was performed on the metal-free peptide (in DMSO solution and in aqueous medium) using one or two dimensional (^1H, ^{13}C) spectra. It is worth noting that only the amino acids that contain a free carboxylic acid exhibit changes in chemical shifts in the presence of zinc ions (Figure 2). Glutamic acid (D) shifts in a lower field ($\Delta\delta = +0.027$ ppm), while aspartic acid (E) and the leucine with a terminal carboxylic acid (L^8) are displaced in higher fields ($\Delta\delta = -0.016$ and -0.051 ppm, respectively). These changes suggest that Zn^{2+} and the peptide residues interact through non-covalent bonds.

Further titrations experiments were performed to determine the association constant of the polypeptide $L^1EDGPKFL^8$ with Zn^{2+}, monitoring the possible changes in the ^1H spectrum of the Hα and amide signals (see procedure iv). Although some changes were observed in the chemical shift displacements of the Hα residues

belonging to the carboxylic acids, they could not be used to calculate the equilibrium constant. To observe the amide region (NH-CH), a second titration experiment with Zn^{2+} $L^1EDGPKFL^8$ peptide was performed in a D_2O:H_2O (90:10) mixture. Again, there were small changes in the region of the NH groups containing a carboxylic acid residue; for example, the group of signals that appeared approximately 8.2 ppm was modified in the presence of Zn^{2+}; however, the overall shift could not be used for the specific identification of the amino acid residue and its displacement to calculate the constant.

Physicochemical parameters for the complex

Reverse phase high performance liquid chromatography (RP-HPLC) was used to examine certain physicochemical parameters of the zinc-peptide complex. NMR analysis identified specific interactions between the carboxylic groups of the polypeptide and the zinc ion. Hypothesizing that peptide-Zn^{2+} interaction could modify the carboxylic group charges and therefore lessen the overall peptide polarity, RP-HPLC was tested to know if the addition of Zn^{2+} could modify the retention time of the peptide. In this experiment, the retention time was higher for peptide alone, while peptide plus Zn^{2+}, Mg^{2+} or Cu^{2+} eluted more rapidly. This suggests that the peptide in the presence of Zn is more polar, and then eluted more rapidly (Table 1).

The effect of the complex on cell proliferation

To examine the effect of zinc on cell proliferation, co-stimulated immune cells were incubated in the presence of the $L^1EDGPKFL^8$ peptide (10^{-12} to 10^{-6} M), Zn^{2+} (10^{-8} M), or a combined treatment of the $L^1EDGPKFL8$ peptide plus zinc (Zn^{2+}-complex). After 24 h, proliferative responses of cells were measured using an MTS assay. Figure 3 shows the effect of different treatments on cell proliferation in cells co-stimulated with LPS (0.5 µg/ml) for THP-1 cells, PHA (1 µg/ml) for Jurkat cells, and Con A (2 µg/ml) for neonatal rat thymocytes. THP-1 cells treated with different concentrations of peptide showed an increase in cell proliferation (70%) beginning at a concentration of 10^{-8} M (EC_{50}=2.77e^{-8} M) ($p<0.05$). When cells were treated with the Zn^{2+}-peptide complex (10^{-12} to 10^{-6} M) (Zn^{2+} (10^{-8} M)), they exhibited an increase in cell proliferation (80%) that began at 10^{-10} M (EC_{50}=6.37e^{-11} M) ($p<0.05$). The peptide also increased cell proliferation (≈ 50%) in PHA co-stimulated human Jurkat cells at a concentration of 10^{-7} M (EC_{50}=7.80e^{-8} M) ($p<0.05$). The Zn^{2+}-peptide complex increased cell proliferation in PHA co-stimulated Jurkat cells beginning at 10^{-11} M and reached its maximum effect (59%) at 10^{-10} M (EC_{50} of 7.47e^{-}

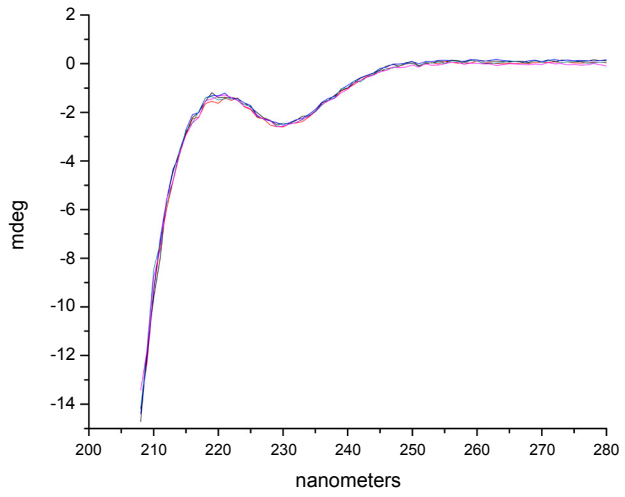

Figure 1: Overlay of CD spectra for the metal-free peptide $L^1EDGPKFL^8$ (75 µM) and in the presence of $ZnCl_2$ (25 to 125 µM).

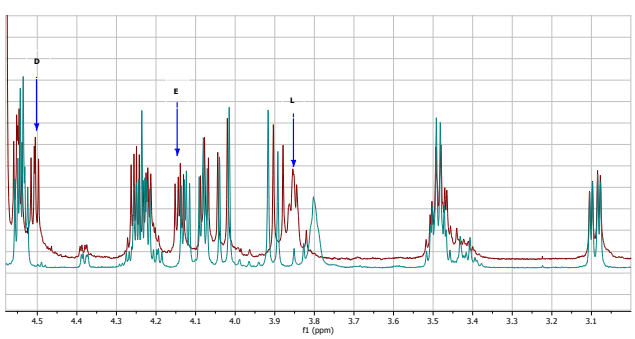

Figure 2: NMR ^1H spectrum (700 MHz) for peptide $L_1EDGPKFL_8$ in D_2O. Red line: peptide $L_1EDGPKFL_8$, 15 mM; blue line: peptide $L_1EDGPKFL_8$, 15 mM and $ZnCl_2$, 15 mM. Expansions on H's regions are marked, showing which amino acids had chemical shift displacement in the presence of a molar equivalent of $ZnCl_2$.

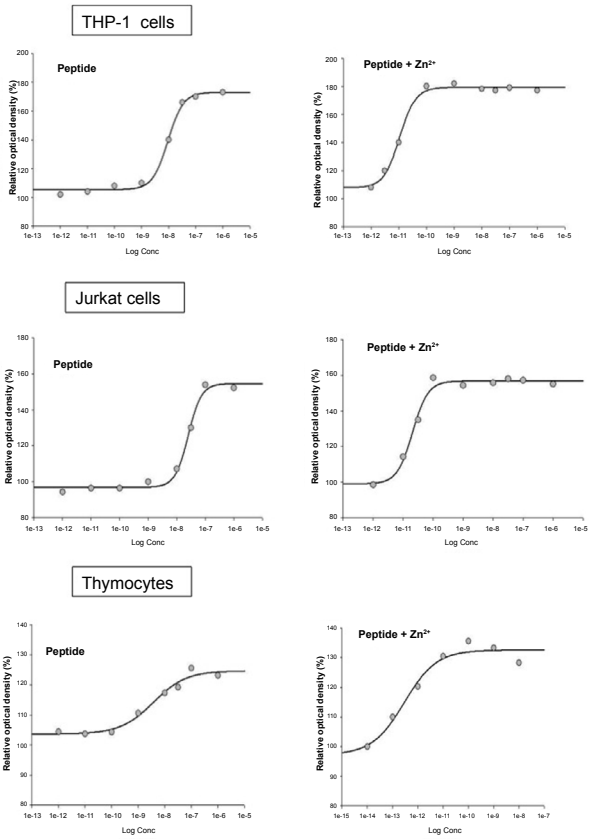

Figure 3: Dose-response curve for peptide $L^1EDGPKFL^8$ and the Zn^{2+}-peptide complex. The cells were stimulated in the presence of different co-stimulatory ligands: LPS (0.5 µg/mL) for THP-1 cells, PHA (1 µg/mL) for Jurkat cells, and Con A (2 µg/mL) for newborn rat thymocytes. The EC_{50} values (half stimulatory concentrations) are indicated. Each curve is representative of 3 independent experiments.

^{11} M) ($p<0.05$). In neonatal rat Con-A stimulated thymocytes, the peptide increased cell proliferation at 10^{-7} M ($EC_{50}=1.93e^{-8}$ M), while the combined Zn^{2+}-peptide complex treatment (10^{-12} to 10^{-6} M) (Zn^{2+} (10^{-8} M)) increased cell proliferation at 10^{-10} M (35%) ($EC_{50}=2.34e^{-11}$ M) ($p<0.05$). According to the present results, the presence of Zn^{2+} in conditioned media has a similar effect on cell proliferation but a 1000-fold lower peptide concentration. Zn^{2+} alone did not modify co-stimulated cell proliferation for any of the studied cells (Table 2); probably peptide activity previously detected contamination due to zinc or other cations in the culture medium. The Zn^{2+}-peptide complex acted as a modulator of the response or a co-stimulant of other primary stimulus; in other words, the Zn^{2+}-peptide complex only had activity on activated cells.

The effect of zinc deprivation

To confirm the role of zinc on the Zn^{2+}-peptide complex activity, we co-stimulated THP-1 cells, Jurkat cells and human lymphocytes in the presence or absence of DTPA, an extracellular zinc chelant. As primary stimuli we used LPS (0.5 μg/ml) for THP-1 cells and PHA (1μg/ml) for Jurkat cells and human lymphocytes. The cells were then treated with the peptide (10^{-10} M) plus different concentrations of Zn^{2+} (10^{-12} to 10^{-8} M) in the presence or absence of DTPA (5 μM). As expected, the combined treatment with the Zn^{2+}-peptide complex (peptide plus zinc) increased cell proliferation in a dose-response manner in all of the studied cells. The effect began at the zinc:peptide ratio of 0.1:1 and peaked at 1:1; this effect was sustained at higher ratios. Unsurprisingly, extracellular Zn^{2+} chelation by DTPA inhibited the peptide co-stimulatory activity on the proliferation of these cells (Table 2). Jurkat cells and THP-1 cells responded similarly to human lymphocytes. The EC_{50} for Zn^{2+} for the three cell types was 1.9 e^{-11} M.

The effect of complex on phagocytic function

To further explore the effect of Zn^{2+} on peptide activity with respect to non-proliferative activity, we examined the complex's effect on PBMC phagocytic function. We added the zinc-peptide complex to human PBMC that had previously received or had not received DTPA (5 μM) and that had been cultured for 24 and 48 h. At the final time point, we added boiled yeast in a solution of PI and examined the cells using flow cytometry to quantify yeast phagocytosis (Table 3). As expected, co-stimulation of PBMC with the Zn^{2+}-peptide complex (10^{-10} M) in the presence of Zn^{2+} (10^{-8} M) for 48 h significantly increased the phagocytosis of yeast (4.3-fold) ($p<0.05$). Moreover, as expected, the increase in phagocytosis induced by the complex was abolished by the presence of DTPA (Table 3).

The effect of Mg^{2+}, Cu^{2+}, or Mn^{2+} peptide

To determine if the zinc may be replaced by another divalent cation in the metallo-peptide complex, we incubated THP-1 cells co-stimulated with LPS in the presence of the peptide (10^{-10} M) in combination with different concentrations of the divalent cations (10^{-10} to 10^{-7} M). Unsurprisingly, peptide activity was higher in the presence of Zn^{2+} (10^{-10} M) and as the concentration of the ion increased ($EC_{50}=4.08e^{-11}$ M) ($p<0.05$) (Figure 4). When THP-1 cells co-stimulated with LPS were treated with peptide plus Mg^{2+} or Cu^{2+}, they displayed a similar cell proliferation pattern as peptide plus Zn^{2+}, but the efficacy was ten times lower ($EC_{50}=2.25e^{-10}$ M for Mg^{2+} and $EC_{50}=2.82e^{-10}$ M for Cu^{2+}) ($p<0.05$). Mn^{2+} did not modify the peptide's activity. The presence of peptide plus Zn^{2+} in conditioned THP-1 cells co-stimulated with LPS induced cell proliferation more effectively, and a lower dose was required.

Discussion

Zn^{2+} is a divalent cation found inside cells and in the extracellular medium. Zinc is a structural constituent, tightly bound to numerous proteins, including zinc enzymes, growth factors, cytokines receptors, transcription factors, and zinc storage proteins, and is essential for the biological activity of such proteins [11-13]. Emphasizing the physiological relevance of Zn to life, a human genome bioinformatics study revealed that approximately 10% of all proteins may bind to Zn [14-17]. Here, we carried out biological and biophysical studies using several co-stimulated immune cell types to assess a possible physical interaction and synergistic effect between the Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu peptide and metallic ions, particularly Zn^{2+}.

Ours results strongly suggest that the free peptide in aqueous solution is flexible and can rapidly attain equilibrium between multiple conformations. It is worth noting that only the amino acids that contain a free carboxylic acid suffer changes in chemical shifts in the presence of zinc ions. Our results showed that zinc changed certain physico-chemical properties of the peptide, such as overall peptide polarity. These results assume that zinc and the peptide interact through non-covalent bounds, resulting in a molecular complex.

	Peptide alone	Peptide+Zn^{2+}	Peptide+Mg^{2+}	Peptide+Mn^{2+}	Peptide+Cu^{2+}
Time (min)	17.92 ± 0.01	17.69 ± 0.06*	17.88 ± 0.02*	17.85 ± 0.06	17.75 ± 0.12*

Peptide (L1EDGPKFL^{8}) (100 μg/ml) alone or with ions in a ratio 1:10 was analyzed in a C18 column. The value represents median ± SD of retention time (min) of the peptide in 20 independent runs. *$p<0.01$ compared with the control group

Table 1: The effect of different ions on the retention time of peptide in RP-HPLC.

	THP-1 cells (% of control)			Jurkat cells (% of control)			Human lymphocytes (% of control)		
Zn^{2+} [M]	Zn^{2+}	Peptide + Zn^{2+}	Peptide + Zn^{+2} + DTPA	Zn^{2+}	Peptide + Zn^{2+}	Peptide + Zn^{+2} + DTPA	Zn^{2+}	Peptide + Zn^{2+}	Peptide + Zn^{+2} + DTPA
Control	100 ± 4.6	100 ± 2.5	99 ± 6.0	100 ± 3.3	100 ± 3.4	95.0 ± 1.4	100 ± 3.2	100 ± 5.1	100 ± 4.3
10^{12}	100 ± 3.2	102 ± 5.2	101 ± 4.6	100 ± 1.9	105 ± 1.3	97.0 ± 1.2#	99 ± 2.7	98.5 ± 2.2	98.5 ± 2.7
10^{11}	101 ± 1.6	135 ± 10*	104 ± 5.1#	99 ± 3.1	120 ± 3.3*	96.5 ± 1.8#	100 ± 5.1	114.2 ± 3.6*	101.4 ± 2.1#
10^{10}	99 ± 2.7	190 ± 10*	104 ± 4.8#	100 ± 2.8	143 ± 3.4*	96.8 ± 1.4#	100 ± 3.2	158.5 ± 5.6*	100.0 ± 3.7#
10^{9}	102 ± 4.7	192 ± 12*	105 ± 3.1#	101 ± 2.6	140 ± 1.6*	94.3 ± 1.2#	100 ± 5.1	154.2 ± 2.9*	99.2 ± 4.5#
10^{8}	101 ± 3.8	195 ± 10*	103 ± 4.2#	100 ± 3.6	145 ± 1.4*	95.6 ± 1.4#	101 ± 5.6	155.7 ± 2.1*	101.4 ± 5.8#

The cells were treated with Zn^{2+}, peptide (10^{-10} M) + Zn^{2+} (10^{-12} to 10^{-8} M) or peptide + Zn^{2+} (10^{-12} to 10^{-8} M) + DTPA (5 μM) for 24 h. The control group was grown without any of the above. The results are presented as the mean ± SD of 3 independent experiments. *$p<0.01$ in comparison with the Zn^{2+} group; # $p<0.01$ with peptide + Zn^{2+} group

Table 2: The effect of DTPA (zinc chelation) on the Zn^{2+}-peptide complex-induced stimulation of cell proliferation.

Time (h)	Control	Peptide + Zn^{2+}	Peptide + Zn^{2+} + DTPA
24 h	40 ± 3	56 ± 2*	38 ± 4#
48 h	43 ± 3	185 ± 18*	44 ± 3#

The cells were treated with peptide (10^{-10} M), Zn^{2+} (10^{-8} M) or DTPA (5 μM) for 24 and 48 h. The value represents the mean ± SD of the mean channel fluorescence (540 nm) of 3 independent experiments. *p<0.01 compared with the control group; #p<0.01 with peptide + Zn^{2+}

Table 3: The effect of the peptide - Zn^{2+} complex on PBMC phagocytosis in the presence or absence of DTPA.

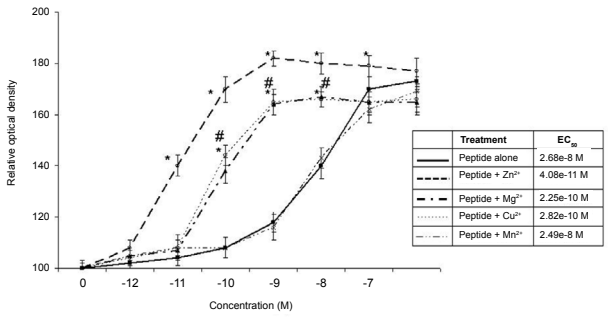

Figure 4: Comparative effect of different concentration of Zn^{2+}, Mg^{2+}, Cu^{2+}, and Mn^{2+} on cell proliferation of THP-1 cells co-stimulated with peptide (10^{-10} M) and LPS (0.5 μg/ml) for 24 h. The results are presented as the mean ± SD of 3 independent experiments. *P <0.01 in comparison with the control group; #P<0.01 with the peptide alone group.

We found that the activity of the peptide depends of the presence of divalent cations, especially zinc. We studied two activities of the metal-peptide complex: (i) cell proliferation in rat thymocytes, human lymphocytes, Jurkat T cells and monocytic cells; and (ii) phagocytosis in monocytic cells (THP-1 cells). In all cases, we demonstrated that Zn^{2+} increased the efficacy and the potency of the peptide in a dose-dependent manner. The zinc-peptide ratio for obtaining higher efficacy ranged from 1:1 to 1:100; the maximum response occurred at a concentration of 10^{-10} M. Our assays showed that the peptide alone had little or no biological activity and that any activity that was observed disappeared when DTPA was added to the culture media. These results indicate that the activity of the peptide alone, probably due to contamination with zinc or other cations in the growth medium or serum used to supplement it, allowing the formation of metal-peptide complexes at low concentration, which are responsible for the activity.

Our results showed that in the absolute absence of Zn^{2+}, the peptide lost its biological activity; in contrast, in the presence of Zn^{2+}, peptide activity is restored. It has been reported that rapidly replicating lymphocytes in the thymus could be particularly sensitive to the functional anomaly of the zinc-rich metallo-enzymes required for cell proliferation, indicating that Zn^{2+} plays an important role for maintaining the activity of the immune system [18,19]. It has been previously reported that DTPA, a chelator that is similar in structure to EDTA but with a 100-fold stronger affinity to certain divalent cations, including zinc and copper [20], does not affect intracellular Zn^{2+} because it induces Zn^{2+} chelation only in the extracellular medium [21]. To complement this approach, we performed yeast phagocytosis assays, which have not been described as being dependent on Zn^{2+}. For this reason, we expected that changes in phagocytosis would not be related to Zn^{2+} deficiency or the lack of this ion. The human PBMC pool contains cells that are able to phagocytize yeast and bacteria. Our studies found that PBMC cells treated with peptide plus Zn^{2+} increased their phagocytic function after 48 h and that this effect was completely nullified in the presence of DTPA. This result indicates that is not only cell proliferation that depends on the presence of Zn^{2+} but also other immune functions, such as phagocytosis [22,23].

The present data demonstrate the importance of metals in the biological activity of the peptide Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu *in vitro*. It was observed that this peptide lost its biological activity after incubation with a chelating agent but maintained its activity in the presence of various divalent ions, particularly Zn^{2+}, Mg^{2+}, and Cu^{2+}. It is now widely accepted that trace elements, such as Zn^{2+}, Mg^{2+} and Cu^{2+}, exert a powerful and apparently specific influence on the thymus, T lymphocytes and cellular immunity, resulting in strong or weak immunomodulation on cell-mediated responses in both human and animal systems [24-30]. Zn^{2+} deficiency has multiple consequences on the immune system; these effects are similar to those observed after thymectomy, either in neonatal, young or adult rats, or when the deficiency is induced during adulthood. One of the most important functions of Zn is related to its antioxidant role and its participation in the antioxidant defense system [31,32]. Experimental magnesium deficiency in rats induces a clinical inflammatory syndrome characterized by leukocyte and macrophage activation as well as the release of inflammatory cytokines and acute phase proteins [33,34]. Additionally, it is known that the immune system requires Cu^{2+} to perform several functions, although little is known regarding the direct mechanism of action of this ion [35]. Diets deficient in copper have been associated with a significant decrease in the proliferation of peripheral blood mononuclear cells and an increase in the percentage of circulating B cells ($CD\ 19^{+}$) [36].

The physical interaction between Zn^{2+} and the examined peptide was directly shown in NMR studies. We found that the addition of Zn^{2+} to the peptide led to a glutamic acid (D) shift in a low-field, as well as the shifting of aspartic acid (E) and a leucine with a terminal carboxylic acid (L_8) in a high field. Thus, our results showed that Zn^{2+} and the peptide interact through a non-covalent bond that produces a change in certain physicochemical parameters of peptide, for example, its polarity and the retention time in RP-HPLC. The addition of inorganic salts causes a linear increase in surface tension, as the entropy of the compound-solvent interface is precisely controlled by the surface tension [37,38]. It is therefore possible that the addition of inorganic salts containing not only Zn^{2+} but also Mg^{2+} and Cu^{2+} could decrease retention time for the peptide. The polarity of the peptide alone is higher than when it is combination with Zn^{2+}, Mg^{2+} or Cu^{2+}. This change in the polarity could be responsible for the shift of the three carboxylic groups in the peptide, which enhances its co-stimulatory potency. We observed that the highest biological activity of the Zn^{2+}-peptide complex was obtained when the peptide and Zn^{2+} were mixed at a molar ratio of 1:1, suggesting a stoichiometric interaction between them.

Our experiments do not fully document the type of binding between the peptide and the metal ions. However, we observed that only three metals (Zn^{2+}, Mg^{2+}, and Cu^{2+}) were able to induce the full reactivation of peptide. These metals are known to induce tetra-coordinated complexes with organic molecules [39-41] and could therefore stabilize a precise conformation of the peptide to make it fully biologically active. A better understanding of the coordination geometry of metal ions and peptide will most likely be derived from physicochemical methods, such as X-ray crystallography or Raman spectroscopy [42].

The present studies indicate that peptide Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu, in the absence of divalent cations, is essentially biologically inactive. This peptide requires Zn^{2+} to be active, and the

addition of Zn^{2+} enhances peptide activity in a dose-response manner. From a physicochemical perspective, this addition produces certain conformational changes in the peptide and decreases molecular polarity. Our findings have identified an unexpected peptide-Zn^{2+} dependency; and this molecular complex is required for immune function.

These results indicate the existence of two peptide forms: the first is deprived of Zn^{2+} and is biologically inactive, while the second contains Zn^{2+} and is biologically active. We propose the name "immuno-modulator metallo-peptide (IMMP)" for the latter. The importance of the presence of Zn^{2+} with respect to the biological activity of the peptide should be taken into consideration when the latter is used for preclinical and clinical evaluation.

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