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Enfuvirtide effects on human erythrocytes and lymphocytes functional properties‡

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Abstract: Enfuvirtide (T-20) is the first inhibitor of human Immunodeficiency Virus type-1 (HIV-1) entrance on a target cell approved for clinical use. Recent studies indicated that its action mechanism involves the interaction with the membrane surface, increasing the concentration in the site of action. In the present study, the *in vitro* interaction between enfuvirtide and blood cells of healthy human donors, namely erythrocytes and lymphocytes, and the peptide effect on plasma and lymphocyte suspensions supernatant ions were evaluated, in order to better characterize the action of this peptide. Enfuvirtide causes a decrease in the concentration of hemoglobin and in the percentages of methemoglobin and carboxyhemoglobin, together with increased values of P_{50}, pCO_2, and [HCO_3^-], and significant decreases of pO_2 and pH, in blood plasma. The supernatants of lymphocyte suspensions derived from blood incubated with enfuvirtide presented a decrease in pH and [HCO_3^-]. Fluorescence anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), used to assess erythrocyte and lymphocyte membrane fluidity, did not yield enfuvirtide-induced changes (an effect could be expected due to peptide partition to lipid bilayers). Erythrocytes incubated with high enfuvirtide concentrations showed a significant decrease in osmotic fragility. As for erythrocyte deformability, enfuvirtide leads to increased elongation indexes for low shear stress values, whereas for high shear stress values it has the opposite effect. Despite the observed statistically significant variations in several parameters, these enfuvirtide-induced changes are not expected to lead to any detectable biomedical outcome for enfuvirtide-treated patients. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: enfuvirtide; T-20; HIV; erythrocyte; lymphocyte

INTRODUCTION

Human Immunodeficiency Virus type-1 (HIV-1) enters the host cell through a multistep mechanism mediated by the viral envelope glycoproteins complex formed by gp120 and gp41 [1]. The glycoprotein complex gp120 binds to CD4 and to a coreceptor, usually CCR5 [2] or CXCR4 [3]. This interaction causes a conformational change in gp41 that ultimately leads to the fusion of the viral and cellular membranes. The glycoprotein complex gp41 has two α-helical regions with heptad repeat sequences (HR1 and HR2) that form a six-helix bundle, critical for the fusion process [4,5].

Enfuvirtide (formerly known as T-20), a synthetic peptide derived from the HR2 region of gp41 (residues 638–673), has been shown to inhibit virus–cell and cell–cell fusion *in vitro* (for a recent review on enfuvirtide molecular mode of action and clinical trials, see Ref. 6). Following successful phase I and II clinical trials, the TORO 1 [7] and 2 [8] phase III clinical trials set the conditions for the fast approval of enfuvirtide for clinical use. These studies demonstrated the significance of the antiretroviral and immunologic benefits of the addition of enfuvirtide to optimized antiretroviral regimens in patients with HIV-1 infections resistant to previously established therapies, and in patients who had previously received multiple antiretroviral drugs. The most common adverse events associated with enfuvirtide administration reported are injection-site reactions, including pain, discomfort, erythema, induration, nodules, cysts, pruritus, and ecchymosis [7,8].

The most widely accepted model of action for enfuvirtide assumes that it binds to the complementary residues in HR1, preventing the formation of the six-helix bundle and blocking the entry process [9,10]. However, enfuvirtide shows a more potent activity against HIV-1 than other C-peptides that contain the coiled-coil cavity binding region, supposed to be essential for inhibition [10,11]. Recent studies demonstrated enfuvirtide's high and selective partition to lipid membranes and indicated that membranes play a role in enfuvirtide action, namely, by increasing concentration in its site of action [12,13]. These and other findings conducted to alternative or additional models for enfuvirtide action at the molecular level [12].

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To further assess the influence of the peptide inside the organism and to evaluate the outcome of its insertion in biological membranes, we studied the *in vitro* interaction between enfuvirtide and blood cells of healthy human donors, particularly erythrocytes and lymphocytes, and the peptide effect on plasma and lymphocyte suspension supernatant ions. These measurements allowed us to better characterize the peptide and its action inside the organism, an important task considering the significance of enfuvirtide as a breakthrough in the antiretroviral therapy against HIV.

MATERIALS AND METHODS

Reagents

Enfuvirtide was a kind gift from Roche (Palo Alto, CA, USA). The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), and fura-2 acetoxymethyl ester were from Molecular Probes (Eugene, OR, USA). ATP, luciferin-luciferase preparation, NADH, and pyruvic acid were obtained from Sigma (St. Louis, MO, USA). All other reagents were from Merck (Darmstadt, Germany).

Blood Samples

Venous blood was collected with anticoagulant (10 UI heparin/ml blood) from 30 healthy male human donors (in fasting condition), with their previous informed consent.

Evaluation of Enfuvirtide Effects on Plasma and Erythrocyte Parameters

Blood samples ($N = 15$) were divided into aliquots and incubated at 37 °C for 30 min with different enfuvirtide concentrations (0, 1, 10, 100, and 1000 ng/ml). This peptide has reported IC_{50} values ranging from 2 to 200 ng/ml for indicator cell lines and primary cells. It is clinically administrated in a dose of 90 mg twice daily. Considering the progressive decrease in its concentration in the blood stream after the local higher concentration on the injection site, all the enfuvirtide concentrations tested in the present work are within the therapeutically relevant range of concentrations.

Hemoglobin, carboxyhemoglobin, and methemoglobin concentrations, P_{50} (oxygen tension at 50% hemoglobin saturation), pO_2, pCO_2, plasma pH, and plasma concentrations of K^+, Na^+, Ca^{2+}, and HCO_3^- were determined using an ABL 505 electrode system connected to an OSM 3 hemoximeter (Radiometer, Copenhagen, Denmark). Hematocrit was calculated using a micro-hematocrit centrifuge (ALC, model 4223, Milan, Italy). Plasma osmolalities were determined using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Blood smears were stained with Wright's solution and imaged on an optical microscope.

In order to evaluate the erythrocyte osmotic fragility (erythrocyte susceptibility to hemolysis in hypotonic solutions), aliquots of each sample were added to different NaCl solutions. After incubation at 37 °C for 45 min and centrifugation for 10 min at 1500 g, the absorbance of the supernatants was measured at 540 nm in a Spectronic 10UV spectrophotometer (Genesys, Rochester, NY, USA). The absorbance of the samples incubated with 0.90 and 0.00% NaCl were indicative of the occurrence of 0 and 100% of hemolysis, respectively, and were used to convert the absorbencies obtained for the other NaCl concentrations to percentage of hemolysis, leading to a sigmoidal hemolysis (%) *versus* [NaCl] plot. The median corpuscular fragility (MCF) is defined as the NaCl concentration for which there is a 50% hemolysis.

As previously described [14], erythrocyte aggregation indexes were determined at 5 and 10 s after dispersion of the blood sample, in triplicate, in a MA1 aggregometer (Myrenne, Roetgen, Germany) and in order to evaluate erythrocyte deformability, erythrocyte elongation indexes were measured in a Rheodyn SSD defractometer (Myrenne, Roetgen, Germany) at eight different shear stress values.

Membrane fluidity was assessed by fluorescence anisotropy measurements, as previously described [14,15], using the fluorescent membrane probes DPH and TMA-DPH. Intracellular calcium concentrations ($[Ca^{2+}]_{int}$) were measured by a spectrofluorimetric method using the acetoxymethyl ester of the fluorescent probe fura-2, as recently described [15]. All the fluorescence spectroscopy measurements were conducted in a Hitachi F-3000 fluorescence spectrophotometer (Tokyo, Japan). All the fluorescence intensity data points are the averages of two identical aliquots, corrected by blank subtraction.

Acetylcholinesterase (AChE) enzyme activity was measured by Ellman's method [16], previously adapted to lymphocyte [17] and erythrocyte [18] AChE.

The hemolysis rate assay was adapted from the work described by Saldanha *et al.* [19]. Erythrocytes were resuspended in a 20% hematocrit in 0.90% NaCl. The suspension was added to NaCl solutions of different hypotonicities. The variation of transmittance at 690 nm with time was used to calculate the pseudo-first order rate constant (k_{obs}), calculated by fitting the transmittance variation between two consecutive readings ($\Delta T = T_{t+\Delta t} - T_t$) *versus* time ($t$) plot to an exponential decay equation:

$$\Delta T = A e^{-k_{obs} t} + b \quad (1)$$

where A and b are also fitting parameters. The second order rate constant (k) corresponds to k_{obs}/[NaCl].

Evaluation of Enfuvirtide Effects on Lymphocyte Parameters

Accordingly with the previous set of studies, blood samples ($N = 12$) were divided into aliquots and incubated at 37 °C for 30 min with the different enfuvirtide concentrations. Lymphocytes were isolated in a Ficoll-Paque gradient and counted on a Cell-Dyn 1600 hematology analyzer (Abbott, Abbott Park, IL, USA).

The supernatant of suspensions of $\approx 10^6$ lymphocytes/ml in 0.90% NaCl was used to determine osmolality, pH, pO_2, pCO_2 and K^+, Na^+, Ca^{2+} and HCO_3^- concentrations. A suspension of $\approx 10^{12}$ lymphocytes/ml in 0.90% NaCl was prepared for the determination of membrane fluidity (DPH and TMA-DPH fluorescence anisotropy), $[Ca^{2+}]_{int}$, and AChE activity. A suspension of 1.2×10^6 lymphocytes/ml in 0.2 M Tris-HCl buffer pH 8.0 was used to determine lactate dehydrogenase (LDH) activity, following an adaptation of Beutler's method [20]. Each suspension was incubated with 0.2 μM NADH

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at 37 °C for 10 min. A second incubation (3 min at 37 °C) was carried out in the presence of 1 μM sodium pyruvate. The enzyme activity was calculated from the slope of the variation of the absorbance at 340 nm with time. A suspension of 1.5×10^6 lymphocytes/ml in 0.90% NaCl deproteinized with 0.8% trichloroacetic acid was used for intracellular ATP quantification by luciferase-luciferin bioluminescence assay, using a 1250 Luminometer from LKB Wallac (Turku, Finland).

Statistical Analysis

Paired sample *t*-tests were used to evaluate significance ($p \leq 0.005$) of the experimental variations.

RESULTS

Enfuvirtide Effects on Plasma and Erythrocyte Parameters

Data obtained for plasma and erythrocyte parameters are shown in Table 1. The microscopic observation of blood smears did not show any differences between the aliquots analyzed, nor did the rate of hemolysis assay (Figure 1). There were also no significant differences ($p > 0.05$) in the values of hematocrit, osmolality, AChE enzyme activity, plasma concentration of Na^+, intracellular concentration of Ca^{2+}, aggregation index, and fluorescence anisotropy (both for DPH and TMA-DPH). In the presence of enfuvirtide, there were significant ($p < 0.05$) decreases in hemoglobin concentration, methemoglobin, pO_2, and plasma concentration of K^+, when compared to control. Carboxyhemoglobin decrease was only significant for enfuvirtide concentrations higher than 1 ng/ml. Each increase in enfuvirtide concentration resulted in a significant pH decrease. There are also significant increases in Ca^{2+} and HCO_3^- plasma concentrations between all samples, except for enfuvirtide concentrations higher than 100 ng/ml. Each increase in enfuvirtide concentration resulted in significant increases of pCO_2 and P_{50}.

The osmotic fragility sigmoid results are shown in Figure 2. There were no differences in hemolysis percentage between the aliquots incubated with NaCl concentrations higher than 0.32%, reflected in similar MCF values for all samples (data not shown). However, aliquots incubated with 1000 ng/ml of enfuvirtide showed a significant decrease in hemolysis percentage at NaCl concentrations below 0.32%, when compared with the remaining ones. Also the deformability assay revealed the existence of significant differences between the aliquots analyzed (Figure 3). For shear stress values below 1.20 Pa, the erythrocyte elongation index (EEI) is significantly higher when the aliquots are incubated with enfuvirtide concentrations above 1 ng/ml (except for 0.30 Pa), whereas the EEI of the samples incubated with enfuvirtide are significantly lower than the control for the highest shear stress value (60.00 Pa).

Table 1 Mean values ± standard deviation ($N = 15$) of the plasma and erythrocyte parameters under evaluation

Parameter	[Enfuvirtide] (ng/ml)				
	0	1	10	100	1000
[Hb] (g/dl)	14.43 ± 0.69	14.21 ± 0.68	14.29 ± 0.61	14.23 ± 0.72	14.13 ± 0.74
Hematocrit (%)	46.9 ± 2.6	46.4 ± 2.3	46.7 ± 1.9	46.7 ± 2.4	46.6 ± 2.1
MetHb (%)	1.65 ± 0.10	1.58 ± 0.11	1.59 ± 0.13	1.56 ± 0.10	1.56 ± 0.10
COHb (%)	1.77 ± 1.02	1.76 ± 1.00	1.72 ± 1.01	1.72 ± 1.01	1.73 ± 1.00
Osmol. (mOsmol/kg)	293 ± 5	293 ± 3	294 ± 2	293 ± 3	294 ± 4
P_{50} (mm Hg)	21.85 ± 1.51	23.01 ± 1.21	23.65 ± 1.10	24.20 ± 0.74	24.11 ± 0.94
pO_2 (mm Hg)	37.8 ± 15.8	36.7 ± 16.3	35.2 ± 12.6	34.9 ± 11.4	34.8 ± 11.9
pCO_2 (mm Hg)	34.6 ± 4.5	37.8 ± 4.6	39.6 ± 4.4	41.3 ± 4.0	42.1 ± 4.0
pH	7.451 ± 0.037	7.427 ± 0.037	7.415 ± 0.033	7.405 ± 0.029	7.397 ± 0.028
AChE (U/min/mg Hb)	308 ± 18	303 ± 25	314 ± 22	298 ± 36	307 ± 24
$[K^+]_{ext}$ (mM)	4.07 ± 0.32	3.96 ± 0.31	3.99 ± 0.34	3.95 ± 0.32	3.95 ± 0.32
$[Na^+]_{ext}$ (mM)	139.4 ± 1.4	139.1 ± 1.2	139.3 ± 1.3	139.6 ± 1.2	139.5 ± 1.2
$[Ca^{2+}]_{ext}$ (mM)	1.159 ± 0.038	1.172 ± 0.037	1.179 ± 0.035	1.186 ± 0.033	1.187 ± 0.034
$[Ca^{2+}]_{int}$ (nM)	81 ± 41	85 ± 38	81 ± 41	86 ± 42	85 ± 47
$[HCO_3^-]_{ext}$ (mM)	23.6 ± 1.6	24.4 ± 1.5	24.8 ± 1.5	25.3 ± 1.3	25.3 ± 1.3
Aggregation index (%)	—	—	—	—	—
5 s	6.71 ± 1.67	6.44 ± 1.49	6.48 ± 1.46	6.56 ± 1.72	6.52 ± 1.77
10 s	11.77 ± 2.88	10.99 ± 2.74	11.29 ± 2.48	11.16 ± 2.23	11.42 ± 3.02
Fluorescence anisotropy	—	—	—	—	—
DPH	0.335 ± 0.026	0.316 ± 0.027	0.348 ± 0.022	0.313 ± 0.046	0.320 ± 0.022
TMA-DPH	0.325 ± 0.016	0.335 ± 0.017	0.320 ± 0.012	0.341 ± 0.007	0.328 ± 0.017

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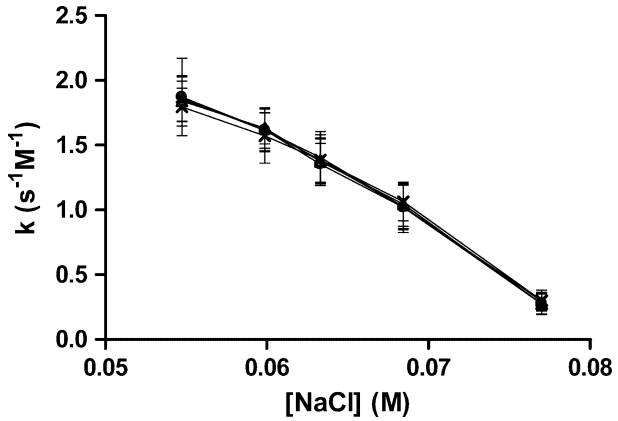

Figure 1 Hemolysis rates at different NaCl concentrations, obtained for different enfuvirtide concentrations: ■, 0 ng/ml; ▲, 1 ng/ml; Δ, 10 ng/ml; ×, 100 ng/ml; ○, 1000 ng/ml. The values plotted are means ± standard deviations ($N = 15$).

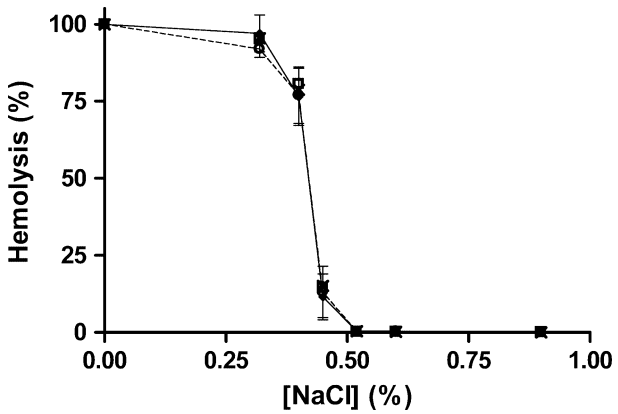

Figure 2 Hemolysis percentages at different NaCl concentrations, obtained for different enfuvirtide concentrations: ♦ (solid line), 0 ng/ml; □, 1 ng/ml; ▲, 10 ng/ml; ×, 100 ng/ml; ○ (dashed line), 1000 ng/ml. The values plotted are means ($N = 15$), except for 0 and 1000 ng/ml of enfuvirtide, which are means ± standard deviations ($N = 15$). For 0.32% NaCl, the hemolysis rate of the aliquots incubated with 1000 ng/ml enfuvirtide is significantly ($p < 0.05$) lower than all the other aliquots.

Enfuvirtide Effects on Lymphocyte Parameters

Table 2 presents the data obtained for the lymphocyte parameters. There were no significant differences in the pO_2, pCO_2, osmolality, AChE enzyme activity, LDH activity, and fluorescence anisotropy values (both for DPH and TMA-DPH). K^+, Na^+, and Ca^{2+} supernatant concentrations, and Ca^{2+} and ATP intracellular concentrations, were also not significantly different between the samples tested. There were significant decreases in pH and Ca^{2+} supernatant concentration when the aliquots were incubated with enfuvirtide (independently of the concentration). The HCO_3^- supernatant concentration was also significantly lower in the sample incubated with 1000 ng/ml, when compared to the remaining ones.

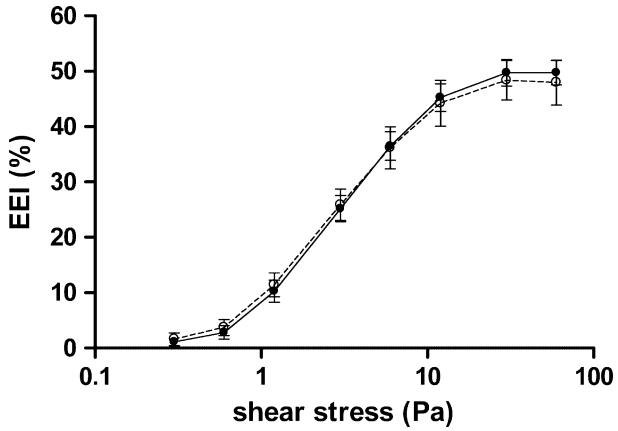

Figure 3 Erythrocyte elongation indexes (EEI, a parameter used to quantify erythrocyte deformability) at different shear stresses, obtained at the absence (●, solid line) and presence (○, dotted line) of 1000 ng/ml of enfuvirtide. The values plotted are means ± standard deviations ($N = 15$). Note the shift in erythrocyte elongation indexes between lower and higher shear stress values.

DISCUSSION

The results presented here show a significant effect of enfuvirtide on diverse blood parameters. These effects may be related to some of the adverse events reported in clinical trials with patients undergoing enfuvirtide treatment [7,8].

According to our results, enfuvirtide causes a decrease in the concentration of hemoglobin and in the percentages of methemoglobin and carboxyhemoglobin, without any significant change in the hematocrit. Other effects reported in this work are the significant increases of P_{50} and pCO_2, and the significant decreases of pO_2 and pH in blood plasma (Table 1). All these parameters are related to each other and also to the extracellular concentration of HCO_3^- [21–23], which shows a significant progressive increase in whole blood incubated with enfuvirtide. The pH values stay well above the ones considered to be indicative of acidosis (pH < 7.35) [21,24,25]. Despite the increase in HCO_3^- concentration, the levels are also within the normal range (22–26 mM) [22,23]. The decreased pH and the increased HCO_3^- concentration are in agreement with the increase in pCO_2. As for the partial pressure of oxygen, we observed that increasing levels of enfuvirtide decrease pO_2. This can be related with the lower affinity of hemoglobin for oxygen, indicated by the increase in P_{50} values, according to the oxyhemoglobin dissociation curve [24].

When testing the supernatant of a lymphocyte suspension derived from blood incubated with enfuvirtide, we found no changes in these parameters, except for a decrease in pH and HCO_3^- concentration. However, the only value of HCO_3^- concentration significantly lower than the control sample is

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Table 2 Mean value ± standard deviation ($N = 12$) of the lymphocyte parameters under evaluation

Parameter	[Enfuvirtide] (ng/ml)				
	0	1	10	100	1000
pO_2 (mm Hg)	185.9 ± 3.2	185.9 ± 4.1	184.9 ± 4.3	185.8 ± 4.1	186.1 ± 3.4
pCO_2 (mm Hg)	6.08 ± 0.36	6.05 ± 0.48	6.01 ± 0.51	5.95 ± 0.38	6.06 ± 0.41
pH	6.327 ± 0.134	6.257 ± 0.154	6.252 ± 0.142	6.286 ± 0.165	6.254 ± 0.152
Osmol. (mOsmol/kg)	273 ± 13	272 ± 13	274 ± 14	274 ± 13	274 ± 14
$[K^+]_{ext}$ (mM)	0.16 ± 0.05	0.18 ± 0.04	0.18 ± 0.04	0.18 ± 0.04	0.17 ± 0.05
$[Na^+]_{ext}$ (mM)	148.6 ± 2.5	148.3 ± 2.4	148.3 ± 2.1	148.4 ± 1.9	148.4 ± 1.9
$[Ca^{2+}]_{ext}$ (mM)	0.131 ± 0.033	0.124 ± 0.032	0.121 ± 0.032	0.124 ± 0.038	0.119 ± 0.033
$[Ca^{2+}]_{int}$ (nM)	81 ± 52	78 ± 60	77 ± 55	87 ± 63	88 ± 54
$[HCO_3^-]_{ext}$ (mM)	0.30 ± 0.11	0.27 ± 0.08	0.27 ± 0.08	0.27 ± 0.10	0.26 ± 0.10
AChE (U/min/mg Hb)	300 ± 140	303 ± 116	352 ± 60	415 ± 80	310 ± 42
Fluorescence anisotropy	—	—	—	—	—
DPH	0.333 ± 0.016	0.344 ± 0.020	0.348 ± 0.017	0.330 ± 0.014	0.334 ± 0.022
TMA-DPH	0.331 ± 0.030	0.334 ± 0.035	0.341 ± 0.025	0.335 ± 0.025	0.340 ± 0.025
LDH activity (/min)	0.0103 ± 0.0036	0.0096 ± 0.0029	0.0093 ± 0.0035	0.0100 ± 0.0032	0.0097 ± 0.0042
$[ATP]_{int}$ (μM)	2.42 ± 1.02	2.59 ± 0.96	2.36 ± 1.10	2.32 ± 1.27	2.47 ± 1.28

that for 1000 ng/ml enfuvirtide, which does not suggest a high impact of enfuvirtide on this parameter. Changes in this environment, as opposed to blood plasma, can be due to a direct effect of the peptide on the lymphocytes, which means that the cells can be releasing acid compounds to the extracellular milieu.

For the correct functioning of the body, it is important to maintain a stable concentration of electrolytes both in intra- and extracellular fluids [21]. Their global concentration can be assessed by the osmolality measurements, which in our study are not affected by the incubation with enfuvirtide, neither in blood plasma nor in the supernatant of a lymphocyte suspension. Beside the hydrogencarbonate anion mentioned before, other important electrolytes were evaluated: sodium (the most abundant in blood plasma), potassium, and calcium ions. Our results show that sodium concentration is not altered by enfuvirtide, as expected, since it is the major contributor for osmolality [21], but blood incubation with enfuvirtide does affect both Ca^{2+} and K^+ extracellular concentrations in blood plasma, increasing the former and decreasing the latter. Since both ions play important roles in cell and body functions, alterations in their concentrations can have severe consequences for the organism. Extracellular potassium is involved in heart and muscular functions, and a fall of potassium plasma levels (hypokalemia) can cause muscular weakness and cardiac irregularity and failure [21,23,24]. However, normal levels of extracellular K^+ are between 3.5 and 5.0 mM, which mean that the observed decrease of this electrolyte, although significant for samples incubated with enfuvirtide, is not enough to induce hypokalemia. The levels of extracellular calcium, an electrolyte important for normal nerve and muscle function [21,23], also stay within the normal range despite the significant increase observed for the incubation with enfuvirtide. Hypercalcemia (high levels of plasma calcium) is associated with the formation of kidney stones, the occurrence of nausea, hypertension, cardiovascular abnormalities, and mental changes [21,23,24]. Nevertheless, enfuvirtide administration is not likely to induce calcium levels high enough for these conditions. The level of intracellular calcium was also measured, due to its importance as a key signaling molecule, but no significant changes were reported. Regarding the lymphocyte suspension, we found that the supernatant concentration of calcium is significantly decreased in the samples incubated with enfuvirtide, but the cytoplasm concentration of calcium in these cells is not altered. We may assume the occurrence of calcium mobilization to the lymphocyte endoplasmic reticulum or mitochondria.

Factors pertaining to the hemorheological properties of blood were also assessed. The measurements of erythrocyte and lymphocyte membrane fluidity did not yield any enfuvirtide-induced changes, although an effect would be possible from the reported peptide propensity to insert in lipid bilayers [12]. The rate of hemolysis, which is influenced by erythrocyte aggregation and membrane fluidity [26], had no change.

The samples incubated with the highest amount of enfuvirtide showed a significant decrease in osmotic fragility for highly hypotonic solutions (without significant MCF variations), indicating that enfuvirtide might exert some protective effect on the erythrocytes, which prevents them from undergoing hemolysis. The osmotic fragility of erythrocytes mainly depends on the erythrocyte surface area to volume ratio. The spherocyte

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erythrocyte morphology, acquired as a result of the erythrocyte swelling, bursts at higher saline concentrations (its ability to take in water before stretching the surface membrane is more limited than normal), and it is, therefore, particularly susceptible to osmotic lysis. On the contrary, reticulocytes are more resistant to hemolysis due to their higher surface area. The observed decrease in hemoglobin concentration (without hemolysis) in the presence of enfuvirtide can be related to a decrease in the number of erythrocytes. Such a variation, associated with the absence of hematocrit variations observed, could eventually result from a higher erythrocyte surface area to volume ratio, due to a larger proportion of reticulocytes, undetectable when blood smears are observed on the microscope. This possible slight morphological change may lead to the decreased osmotic fragility in the presence of a high concentration of enfuvirtide (higher resistance to hemolysis in hypotonic solutions).

Erythrocyte deformability describes the ability of erythrocytes to alter their shape in response to applied shear stress, whether it is imposed by a stationary barrier (e.g. entrance in a capillary blood vessel) or by the momentum of viscous fluid flow [27]. This parameter is primarily regulated by the erythrocyte internal viscosity, its surface area to volume ratio, and viscoelastic properties of the erythrocyte membrane–cytoskeleton complex. A dual effect on erythrocyte deformability was observed in the present work: enfuvirtide increases the EEI for low shear stress values, whereas for high shear stress values it has the opposite effect. Regarding erythrocyte factors influent on deformability, we may hypothesize changes in membrane properties as well as in surface area to volume ratio to explain the increased elongation index and changes in erythrocyte internal viscosity, leading to the decrease in deformability [27]. This has consequences on blood flow, since these measurements mimic the pressures in arteries, veins, and capillaries [28]. Low shear stress values correspond to vessels with a larger diameter, and in these conditions it is mainly the aggregation of erythrocytes, which we have already determined is not affected by enfuvirtide, that influences most of the blood flow. On the other hand, for high shear stress values, which represent bed capillaries, deformability plays an important role, and the fact that enfuvirtide decreases this parameter may compromise microcirculation [28].

As indicators for cell viability and activation, we measured the activity of the AChE enzyme responsible for the hydrolysis of acetylcholine [29], the activity of LDH, and the intracellular concentration of ATP, with negative results for the three parameters. AChE is found to be associated to the membranes of many cell types, including erythrocytes and lymphocytes, and its activity is a good indicator of cell viability [29]. LDH activity is also an indicator of cell integrity and functionality [30]. The internal concentration of ATP was measured in the lymphocyte population in an attempt to determine if the incubation with enfuvirtide has a lymphocyte activation effect.

Overall, our results lead us to the conclusion that enfuvirtide does not affect blood-, erythrocyte-, and lymphocyte-related parameters in a severe way. Despite the observed statistically significant variations in several parameters, these changes are not expected to cause any detectable biomedical outcome. The values obtained for the samples incubated with enfuvirtide are still within the normal range for the parameters evaluated. Therefore, we did not find any effect that could result in a serious condition for enfuvirtide-treated patients, which is in agreement with the finding in TORO 1 and 2 clinical trials that enfuvirtide is well tolerated by patients [7,8]. Although an extensive analysis of adverse events in patients receiving enfuvirtide was made in these clinical trials, it would be interesting to perform the same type of analysis shown here using peripheral blood from those patients, as a way to confirm our results *in vivo*.

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