Trauma Decreases Leucine Enkephalin Hydrolysis in Human Plasma

RETO BABST, LUCILLA BONGIORNO, MARIO MARINI, L. GIORGIO RODA, GIULIO SPAGNOLI and ALESSANDRA URBANI

Research Division, Department of Surgery, University of Basel, Basel, Switzerland (R.B., G.S.); Dipartimento di Medicina Sperimentale, Università degli Studi “Tor Vergata,” Rome, Italy (L.B.); and Dipartimento di Neuroscienze, Università degli Studi “Tor Vergata,” Rome, Italy (M.M., L.G.R., A.U.)

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ABSTRACT

Plasma hydrolysis of leucine enkephalin was evaluated, together with several cellular immune parameters, in a homogeneous group of human subjects who had undergone severe trauma (proximal femur fracture); data obtained were compared with those obtained in an age-matched control group. In the experimental group, immediately after hospitalization, substrate hydrolysis was reduced with respect both to the control subjects and the same patients 4 weeks after the trauma. Chromatographic separation of the enzymes active on leucine enkephalin showed that the reduction of substrate hydrolysis is mainly attributable to the decrease in the activity of enkephalin-degrading enzymes, principally of aminopeptidases, per se, whereas the role of the low-molecular-weight plasma inhibitors is only minor. In the same subjects, several of the immunological parameters measured underwent modifications that may be considered stress related. However, the absence of a quantitative relationship between reduction in hydrolysis and modifications of immune parameters does not support the hypothesis of a direct relationship between these two sets of data.

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ABBREVIATIONS: ANOVA, analysis of variance; CV, coefficient of variation; df, degree of freedom; GH, growth hormone.
different receptors: in immunocompetent cells, \( \beta \)-endorphin binds both opioid and nonopioid receptors, inducing stimulatory effects (van den Bergh et al., 1993) and inhibitory effects (van den Bergh et al., 1993; Shaker et al., 1994), respectively. In addition, immune effects of the opioid peptides are both peripherally and centrally mediated. The existence of differences between in vivo and in vitro effects, such as those described by Bayer et al. (1992), has indeed been explained by the existence of both direct and indirect interactions: in the first case, the immunocompetent cell receptors are involved; in the second case, mechanisms such as the stimulation of the hypothalamo-pituitary axis (Bazzetti et al., 1989b), leading to the release of steroid hormones (Cupps and Fauci, 1982). Central neurons innervating immune-relevant tissues (Weber and Perth, 1989) can also be involved in these interactions.

Because modulation of blood immunocompetent cell activity is carried out by the opioid peptides present in this tissue, these peptides are exposed to hydrolysis by three different groups of enzymes: plasma soluble peptidases, peptidases present in the membrane of immunocompetent cells (Amoscato et al., 1989), and peptidases released by immunocompetent cells (Marini et al., 1992). The presence of these enzymes causes rapid hydrolysis of the opioid peptides, whose half-life in human plasma is approximately 5 min for enkephalins and 40 min for \( \beta \)-endorphin.

The activity of plasma proteolytic enzymes is contrasted by inhibitors present in the tissue itself. These have mainly been studied regarding the enzymes that process plasma proteins, yet the low-molecular-weight inhibitors appear to contrast with the activity of the plasma enzymes active on opioid peptides (Rossetti et al., 1988), as well as the activity of the enzymes released by immunocompetent cells (Marini et al., 1992). The role of plasma proteolysis inhibitors in controlling peptide-mediated signaling has been studied at the whole-organism level: Chipkin and Coffin (1991) and Saksida et al. (1993) attributed the antinociceptive effect of proteolysis inhibitors to the decrease induced by these substances in the degradation of plasma opioid peptides. Katoh et al. (1992) found that nonhydrolyzable enkephalin analogs, or a combination of proteolysis inhibitors and opioid peptides, can modify the behavior of mice under stressful conditions. Moreover, the hydrolysis pattern of peripheral opioid peptides is altered by environmental conditions: Young et al. (1989) described increased degradation of \( \beta \)-endorphin after chronic stress, whereas Schulteis and Martinez (1993) reported that external stimuli increase leu-enkephalin hydrolysis and modify the ratio between the hydrolysis by-products that are formed.

Taken together, the above data support the hypothesis that there exists a relationship between stressful events, the status of the immune system, and the degradation of plasma immunoreactive peptides. In other words, it seems possible to hypothesize that the stress-induced differences in the status of the immune system may modify the enzyme degradation of neuropeptides. The data that follow attempt to verify this hypothesis, analyzing the possible relationship among stress, hydrolysis of plasma peptides, and the status of the immune system. The substrate used was leu-enkephalin, and the model was a homogeneous group of volunteers who had undergone a single traumatic event. The results obtained were compared with those obtained with age-matched control subjects. In the same subjects, several immune parameters were measured, and the possible relationship between modification of hydrolysis and modification of immune-related indexes was examined by analyzing the correlation between these two sets of data.

Materials and Methods

Sample Population

The sample population that was studied consisted of 11 women (mean age, 80.0 years; S.D., 7.3 years) who had been hospitalized for proximal femur fracture. Before trauma, all patients were in apparently good health and did not present with any major syndrome. All were classified with regard to physical independence (Schoening and Iversen, 1968) and cerebral function (Folstein et al., 1975); in both cases, values were judged as normal for the age group. Analgesic treatment consisted of methadone (100 \( \mu \)g/kg) administered s.c. All patients underwent surgery under local anesthesia within 24 h of the accident. Postoperative treatment included thromboprophylaxis with heparin (3000 IU/day), antibiotics (2 g of cefamandol twice; Eli Lilly, Indianapolis, IN), early physiotherapy, and enteral nutrition. Paracetamol (500 mg) or diclofenac (25 mg) was given as required. In the context of a parallel clinical study, five patients were treated with i.v. growth hormone (GH; 0.2 IU/kg/day; Pharmacia, Erlangen, Germany). Analyses performed to verify whether GH treatment affected substrate hydrolysis and the alteration of immune parameter did not indicate the existence of a relationship between these data: curves were identical within experimental error, and analyses of variance (ANOVA) did not indicate a statistically significant relationship between these parameters (\( F = 1.293 \) to 0.011 and \( P = 271 \) to .917, respectively). Therefore, GH-treated and naive subjects were treated as a homogeneous sample. The control population consisted of six women (mean age, 80.3 years; S.D., 13.4 years). Also, in this case, physical independence and cerebral function scores were normal for the age group. The administration of pharmacologically active substances, with the exception of cardioactive ones, was excluded for 5 days before the samples were taken.

Blood Collection

Blood referred to as \( t_0 \) was obtained immediately after hospitalization, simultaneous with analgesic treatment. Because hospitalization was limited to an average of 16 days, samples referred to as \( t_0 \) were obtained on an outpatient basis, 28 days after trauma. Once collected, blood was transferred to ice, and centrifuged (100g for 10 min). Plasma was stored at \(-70^\circ\text{C}\) until used.

Enzyme Assay

Enkephalin-degrading enzymes were quantified on the basis of the hydrolysis by-products formed. When necessary, samples were dialyzed at 4°C against 10 mM HEPES, 100 mM NaCl, 2.5 \( \times \) \( 10^{-5} \) M \( \text{ZnCl}_2, \) \( \text{pH} \) 7.2. Tritiated leu-enkephalin (5 \( \mu \)l corresponding to 3.5 \( \times \) \( 10^{-12} \) moles) was added to 50 \( \mu \)l of each sample. Reactions were carried out at 37°C for 30 min and stopped with 5 \( \mu \)l of acetic acid (Marini et al., 1997). Intact leu-enkephalin and its labeled hydrolysis by-products were quantified by thin-layer chromatography as described below.

Chromatographic Procedures

Thin-Layer Chromatography. Thin-layer chromatography was performed on aluminum-backed Kieselgel 60 sheets (E. Merck, Darmstadt, Germany). The samples (4 \( \mu \)l), together with 2 \( \times \) \( 10^{-9} \) mol each of leu-enkephalin, Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Gly-Gly-Phe used as internal standards, were applied to each spot. Chromatograms were developed at 50°C with 70:30:10:1.7 2-butyl alcohol/acetic acid/water/acetonitril and stained with 0.1% ninhydrin in acetone (Marini et al., 1997). Sheets were cut according to the standards, transferred to scintillation vials with the addition of 3 ml of...
applied to the column, and eluted at 2.2 ml in a 5-

fractionated, as well as separated from the low-molecular-weight tiphase; Wallac Oy, Turku, Finland), they were counted for tritium.

Temperature. After the addition of 3 ml of scintillation cocktail (Optiphase; Wallac Oy, Turku, Finland), they were counted for tritium activity. In agreement with known data, the results obtained indicate the presence of all three classes of enkephalin-hydrolyzing enzymes: aminopeptidases, dipeptidylaminopeptidases, and dipeptidylcarboxypeptidases (e.g., Hambrook et al., 1976).

Immunophenotyping

Peripheral blood mononuclear cells were obtained by centrifuging whole venous blood drawn from the patients under investigation with use of a density gradient (Lymphoprep; Nyegard, Oslo, Norway). Peripheral blood mononuclear cells were resuspended at 10^6 ml^-1 in RPMI medium to which 2 x 10^{-3} M glutamine, 100 mg ml^-1 kanamycin, and 10% human AB serum had been added. Cells were then cultured for 72 h in 96-flat-well plates (Costar, Cambridge, MA) in the presence of 1 μg/ml^-1 purified anti-CD3 mitogenic monoclonal antibodies (CB3G; courtesy of Professor F. Malavasi, Turin, Italy). Titrated thymidine (0.5μCi/well) was then added to the cells, which were further incubated for 6 h, harvested, and counted for tritium to measure de novo DNA synthesis. The stimulation index was calculated as the ratio between the mean number of counts per minute measured in anti-CD3-stimulated cultures and that measured in unstimulated controls. Expression of leukocyte surface markers was evaluated with monoclonal antibodies on whole blood samples. Heparinized blood (50 μl) was incubated for 15 min at room temperature in the presence of optimal dilutions of fluorochrome-labeled monoclonal antibodies (Becton-Dickinson, San Jose, CA) directed against CD14 (Leu-M3), CD45 (all isotypes), CD3, CD4, CD8, CD16, CD19, and HLA-DR (L243, specific for a monomorphic epitope). A red cell lysis solution was added to the blood, and incubation was prolonged for an additional 10 min. Cells were then washed twice and resuspended in paraformaldehyde 0.2% in 137 mM NaCl and 8 mM Na phosphate, pH 7.0 (phosphate-buffered saline). Specific fluorescence was measured with a fluorochrome activated cell scan analysis (FACSscan) fluorocytometer equipped with Lysis II software (Becton Dickinson). Granulocytes, monocytes, and lymphocytes were identified by their physical characteristics (FSC and SSC cytometric parameters), coupled with differential binding of CD14 and CD45 monoclonal antibodies (Spagnoli et al., 1995). This characterization allowed the analysis of individual "gated" leukocyte populations. Absolute counts of specific subsets were obtained by combining percent data measured by FACS analysis with the absolute leukocyte number obtained by automatic counting. Mean fluorescence intensity quantitatively estimates the binding of fluorochrome-labeled monoclonal antibodies to their molecular targets. This was evaluated on specific cell subsets from different samples after staining and fluorocytometer acquisition under identical conditions.

Data Analysis

Regression analyses were performed by a linear polynomial procedure of variable degree; related equation systems were solved using a modified Gram-Schmidt algorithm. Parametric statistics were calculated according to standard procedures. ANOVA was calculated by means of a commercial software package (Cohort Software, Minneapolis, MN), using one-way or two-way randomized complete blocks or completely randomized models. A homogeneity of variance test (Bartlett’s test) was routinely performed before ANOVA.

Materials

Leu-enkephalin, Tyr-Gly, and Tyr-Gly-Gly were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland); Tyr was obtained from Serva Feinbiochemica GmbH (Heidelberg, Germany). Tritiated leu-enkephalin was obtained by isotope exchange reaction followed by steric exclusion chromatography. All other materials were obtained through local suppliers and used without further purification.

Results

Hydrolysis in Whole Plasma

Leu-Enkephalin Hydrolysis. The possible variations of leu-enkephalin hydrolysis associated with the sequence of events common to all patients (henceforth referred to as “trauma”) were measured by incubating the tritiated pentapeptide in the presence of whole plasma obtained from normal control subjects and from traumatized patients; in the latter case, blood was drawn twice, immediately after hospitalization (t0) and 28 days after the trauma (t28). The relative amount of the intact peptide, and of its relevant hydrolysis by-products (Tyr, Tyr-Gly, and Tyr-Gly-Gly), was quantified as described in Enzyme Assay. The amount of substrate hydrolyzed was different under the three conditions tested (Fig. 1). Higher hydrolysis was measured in the control subjects: on average, 9.96 x 10^{-13} mol ml^{-1} min^{-1} (S.D. = 4.19 x 10^{-14}, CV = 4.2%), a value consistent with other data relative to this age group (Babst et al., 1998). The hydrolysis measured in plasma obtained from trauma-
tized subjects was lower than that measured in the controls: 6.86 \times 10^{-13} \text{ mol/ml} \cdot \text{min}^{-1} (\text{S.D.} = 1.73 \times 10^{-13}, \text{CV} = 25.2\%) \text{ at } t_0 \text{ and } 8.05 \times 10^{-13} \text{ mol/ml} \cdot \text{min}^{-1} (\text{S.D.} = 1.73 \times 10^{-13}, \text{CV} = 21.5\%) \text{ at } t_{28}. \text{ Therefore, the substrate hydrolyzed immediately after trauma (} t_0 \text{) was 68.7\% of that hydrolyzed in the control subjects, whereas that hydrolyzed after clinical recovery (} t_{28} \text{) was 80.6\% of the substrate hydrolyzed in control plasma and 117.3\% of that hydrolyzed at } t_0 \text{ (Fig. 1, inset).}

The above data indicate a lower hydrolysis of leu-enkephalin in traumatized compared with control subjects, as well as immediately after trauma compared with after clinical recovery. To verify the statistical significance of the variations observed, these data were analyzed by ANOVA as described in Data Analysis. ANOVA results indicate a very low probability of the null hypothesis (} F = 64.676, P = .0005, df = 5 \text{ for controls and } t_0; F = 17.076, P = .0000, df = 5 \text{ for } t_{28} \text{ and } t_0); \text{ conversely, the association between hydrolysis reduction and the grouping of the subjects is statistically highly significant. As indicated by the dispersion indexes reported above and the error bars in the main panel of Fig. 1, data dispersion is slightly higher in the traumatized subjects than in the control subjects. Because the two groups are age and sex homogeneous and the control group is notably smaller, this phenomenon could be related to individual differences in the response to trauma. Regardless of the reason, data dispersion can only reduce, not increase, the significance of the ANOVA results (i.e., leading to a } P \text{ value higher than that reported above).}

**Formation of Hydrolysis By-Products.** The decrease in substrate concentration shown above is paralleled by the appearance of the three N-terminal hydrolysis by-products: Tyr, Tyr-Gly, and Tyr-Gly-Gly (Fig. 1). The formation of these peptides indicates the involvement of all three classes of enzymes known to hydrolyze leu-enkephalin: aminopeptidases, dipeptidylaminopeptidases, and dipeptidylcarboxypeptidases (Hambrock et al., 1976). The data shown in Fig. 1, inset, indicate that in } t_0 \text{ plasma, the activity of aminopeptidases is 71\% of the value measured in the controls, whereas in } t_{28} \text{ plasma, it is 86\% of that value (121\% of that measured in } t_0 \text{ plasma). For dipeptidylaminopeptidases, these values are 73\%, 79\%, and 108\%; for dipeptidylcarboxypeptidases, they are 40\%, 60\%, and 150\%, respectively. Therefore, the above-reported decrease in substrate hydrolysis is supported by a decrease in the activity of all three classes of enkephalin-degrading enzymes, which, on a percentage basis, is particularly evident for dipeptidylcarboxypeptidases. As a result of these variations, the relative contribution to substrate hydrolysis of aminopeptidases, dipeptidylaminopeptidases, and dipeptidylcarboxypeptidases (65\%, 25\%, and 10\% in the control subjects) is 67\%, 27\%, and 6\% in } t_0 \text{ plasma and 68\%, 25\%, and 7\% in } t_{28} \text{ plasma. Therefore, although the trauma-induced reduction of dipeptidylcarboxypeptidase activity is the highest on a percentage basis, the low activity of these enzymes makes its impact on substrate hydrolysis moderate, and the variations in the relative activity of the three enzyme classes are likewise only moderate.}

**Hydrolysis in Fractionated Plasma**

**Leu-Enkephalin Hydrolysis.** The modifications induced by trauma on the distribution of plasma enkephalin-degrading enzymes were analyzed in five donors, randomly chosen from the traumatized group, and in the whole control group. Plasma was fractionated as reported in Steric Exclusion Chromatography, and the relative activity of the three classes of enkephalin-degrading enzymes was analyzed as described in Enzyme Assay. Again, the data obtained indicate an increase in the amount of leu-enkephalin intact at reaction end (Fig. 2a) in traumatized compared with control individuals: in } t_0 \text{ plasma, the average amount of substrate hydrolyzed by the fractionated enzymes is 64\% of that measured in control plasma; in } t_{28} \text{ plasma, it is 84\% of that measured in control plasma (151\% of that measured in } t_0 \text{ plasma). In } t_0 \text{ plasma, the amount of substrate hydrolyzed decreases unevenly through the whole elution volume of the enzymatically active species: although the hydrolysis envelope is nearly symmetrical with respect to } K' = 0.58 \text{ (approximately 75\% of the reduction is measurable at } K' \text{ lower, and only 25\% at } K' \text{ higher, than this value) (Fig. 2a, inset), causing the asymmetrical narrowing of the enkephalin envelope evident in the main panel of Fig. 2a. On the contrary, after recovery (i.e., in } t_{28} \text{ plasma), the amount of substrate hydrolyzed decreases at } K' \text{ lower than 0.54, whereas it increases at } K' \text{ higher than that value. Because the decrease and increase are nearly identical (Fig. 2a, inset), the activity of the enzymes eluted at lower } K' \text{ (i.e., of higher apparent molecular weight) is lower, and recovers more slowly, than the activity of the enzymes eluted at lower apparent molecular weight.}

**Hydrolysis By-Products.** In column-fractionated } t_0 \text{ plasma, the activity of aminopeptidases (Fig. 2b), dipeptidylaminopeptidases (Fig. 2c), and dipeptidylcarboxypeptidases (Fig. 2d) is 64\%, 62\%, and 67\% of the values measured in control plasma, respectively. In } t_{28} \text{ plasma, the activity of these enzyme classes is 84\%, 93\%, and 63\% of the value measured in control plasma (131\%, 150\%, and 94\% of that measured in } t_0 \text{ plasma). These data indicate a quantitatively uniform reduction in the activity of the three enzyme classes immediately after trauma, followed by a less homogeneous recovery. Specifically, at } t_{28}, \text{ the recovery of dipeptidylaminopeptidase activity appears to be nearly complete, whereas the activity of dipeptidylcarboxypeptidase is even lower than that measured at } t_0. \text{ Enzyme Distribution. The insets in Fig. 2, a and b, indicate that the reduction in the activity of aminopeptidases is almost inverse to the increase of intact leu-enkephalin. Specifically, the narrowing of the leu-enkephalin hydrolysis envelope observable in Fig. 2a is mainly determined by the absence in both } t_0 \text{ and } t_{28} \text{ samples of the two shoulders of aminopeptidase activity evident in the controls at } K' = 0.25 \text{ and } K' = 0.75 \text{ (Fig. 2b); in addition, in both } t_0 \text{ and } t_{28} \text{ plasma, the aminopeptidases eluted at lower } K' \text{ undergo a reduction that is more noticeable than that of the aminopeptidases eluted at higher } K'. \text{ Finally, the differences in the activity of aminopeptidases noticeable between } t_0 \text{ and } t_{28} \text{ (Fig. 2b, inset) indicate a preferential recovery of the enzymes eluted at high } K'. \text{ A trauma-associated shift toward higher } K' \text{ (i.e., lower apparent molecular weights) is evident in the case of dipeptidylaminopeptidases as well (Fig. 2c). These enzymes are represented by two groups that are eluted at } K' = 0.40 \text{ and 0.54 in control plasma and at } K' = 0.42 \text{ and 0.67 in traumatized plasma, respectively. Between } t_0 \text{ and } t_{28} \text{ (i.e., during...}
recovery), the relative amount of the enzymes eluted at $K' = 0.67$ increases, whereas that of the enzymes eluted at $K' = 0.42$ is unchanged. As a result, recovery induces a shift toward lower apparent molecular weight also in the case of these enzymes.

Dipeptidylcarboxypeptidases are represented in the controls by two rather broad groups, eluted at $K' = 0.36$ and $K' = 0.67$, and in traumatized plasma by a single main group eluted at $K' = 0.42$. The variations in the apparent molecular weight of these enzymes are nearly symmetrically distributed both before and after $K' = 0.49$ for $t_0$ and 0.59 for $t_{28}$. These variations are conducive to a narrowing of the activity envelope, more noticeable in the case of $t_{28}$ plasma, which once again indicates a recovery of enzyme activity that appears uneven from the standpoint of apparent molecular weight. However, the limited overall importance of dipeptidylcarboxypeptidases in substrate hydrolysis implies a correspondingly limited contribution to the variations of the hydrolysis envelope described above.

In conclusion, the changes in the amount of substrate hydrolyzed, and in the hydrolysis by-products formed, appear to be associated with detectable variations in the relative amount and in the apparent molecular weight distribution of all three classes of enzymes capable of hydrolyzing the substrate.

**Fig. 2.** Hydrolysis of leu-enkephalin and formation of its hydrolysis by-products as a function of the elution volume of steric exclusion chromatography fractionated plasma. a, leu-enkephalin. b, Tyr. c, Tyr-Gly. d, Tyr-Gly-Gly. In all panels, gray lines represent controls, dotted black lines represent $t_{28}$, solid black lines represent $t_0$, and crosses indicate experimental points. Insets, difference between control and $t_{28}$ plasma (thin lines) and between control and $t_0$ plasma (thick lines). Chromatographic and assay conditions are as described in Materials and Methods.
**Plasma Protein.** Differences between control and traumatized plasma are also noticeable in terms of total protein (Fig. 3). These are to be found almost exclusively at low $K'$ (less than approximately 0.47) because the elution volume of the substances centered at $K' = 1.63$ is not compatible with proteinaceous material. Measured as the integral of the absorbance at 280 nm across the whole elution volume of the column, protein is 71% of the control in $t_0$ plasma, whereas it is 86% of the control, or 122% of $t_0$, in $t_{28}$ plasma. These differences are more evident if the analysis is restricted to the material eluted between $K' = 0.00$ and $K' = 0.47$, that is, within the elution volume of the bulk of the enkephalin-degrading enzymes. Measured in this region, protein in $t_0$ plasma is 57% (S.D. = 46, CV = 114) of the control. In $t_{28}$ plasma, it is 86% (S.D. = 121, CV = 138) of the control, or 149% of the value measured at $t_0$. These data indicate a reduction in the protein (or, more precisely, of the absorbance at 280 nm) in the region between $K' = 0.00$ and $K' = 0.47$ in traumatized compared with control plasma, a reduction that recedes perceptibly with recovery. The statistical significance of the relationship between absorbance at low $K'$ measured at $t_{28}$ and that measured at $t_0$ was analyzed by ANOVA. The results obtained ($F = 64.915$, $P = .0125$, df = 5) indicate that the variations observed between the two sets of data are statistically significant. Finally, the hypothesis that the reduction in absorbance at low $K'$ is related to the reduction in the activity of enkephalin-degrading enzymes was verified by calculating polynomial regressions of variable degree between these two sets of data. The results obtained ($r = 0.828, 0.863, 0.907, and 0.999$ for first to fourth degree) indicate a strong relationship between the two variables, together with deviations from linearity that may indicate the involvement of multiple factors.

**Low-Molecular-Weight Inhibitors**

Because steric exclusion chromatography fractionation separates the enzymes from the low-molecular-weight proteolysis inhibitors, a comparison of the data obtained in the presence of fractionated and whole plasma may allow the evaluation of the possible role of these substances. Specifically, in fractionated plasma (i.e., in the absence of inhibitors), the ratio between the substrate hydrolyzed by the three enzyme classes is slightly different from that seen in whole plasma (Table 1): under all three conditions tested, the activity of aminopeptidase is higher, that of dipeptidylaminopeptidase is lower, and that of dipeptidylcarboxypeptidase is higher in fractionated (Fig. 4) than in whole plasma (Fig. 1). These data can be interpreted as indicating inhibition of aminopeptidases (more evident in the controls) and of dipeptidylcarboxypeptidases (more evident at $t_0$), paired to null (or extremely low) inhibition of dipeptidylaminopeptidases. Because the differences measured are small, these data suggest a minor role of inhibitors in determining the trauma-associated reduction of hydrolysis. Consequently, this phenomenon appears to be principally caused by the per se reduction in enzyme activities described above.

**TABLE 1**

Hydrolysis by-products formed in the presence of whole and column-fractionated plasma

<table>
<thead>
<tr>
<th></th>
<th>$Y_{t_0}$</th>
<th>$Y_{t_{28}}$</th>
<th>$Y_{G_{t_0}}$</th>
<th>$Y_{G_{t_{28}}}$</th>
<th>$Y_{GG_{t_0}}$</th>
<th>$Y_{GG_{t_{28}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>67.9</td>
<td>66.8</td>
<td>24.5</td>
<td>27.2</td>
<td>7.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Fractionated</td>
<td>70.6</td>
<td>70.8</td>
<td>18.6</td>
<td>21.3</td>
<td>10.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Difference</td>
<td>-2.7</td>
<td>-4.0</td>
<td>+5.9</td>
<td>+5.9</td>
<td>-3.3</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

Whole, whole plasma; Fractionated, column-fractionated plasma; Difference, difference between whole- and column-fractionated plasma; $t_0$ and $t_{28}$, days from trauma. Peptides identified by one-letter code.
Effect on Immunocompetent Cells

The possible effect of trauma on peripheral blood immunocompetent cells was measured as described in *Immunophenotyping*. Results obtained were analyzed by ANOVA, as described in *Data Analysis*. In the case of 6 of the 18 parameters measured, the probability of the null hypothesis was significantly low (P between 0.0003 and 0.0033; Table 2). These were increase in absolute numbers of leukocytes, granulocytes, and monocytes; decrease in lymphocytes over total leukocytes; increase in lymphocytes B over total lymphocytes; and decrease in the HLA-DR surface expression in monocytes. The amount of NK lymphocytes over total lymphocytes was reduced by 23%, whereas the absolute number of activated T lymphocytes was reduced by 34%; in both cases, P was around .30. Finally, the variation of all other parameters measured was ±15% or less, associated with P ≥ .44.

The possible relationship among the six statistically significant immune parameters listed above and the reduction in hydrolysis was analyzed by calculating polynomial regressions of degree ranging from 1 to 4. Regression coefficients ranged from a minimum of 8.1 × 10⁻⁴ to a maximum of 0.682, indicating the absence of any significant relationship between the indexes measured and the reduction of substrate hydrolysis. A similar analysis was performed on the above-listed immune parameters, taken in pairs. Except in the single case of leukocyte and granulocyte absolute counts (r = 0.826), calculated correlation coefficients ranged between 0.106 and 0.596. These values indicate an extremely limited correlation between each pair of indexes; thus, their quantitative variations can be taken to be practically independent of each other.

Discussion

The data reported in *Results* indicate that the whole sequence of events indicated as trauma modifies the enzyme hydrolysis of plasma leu-enkephalin. Specifically, it reduces the amount of the peptide degraded and modifies the pattern of its hydrolysis by-products. The reduction in leu-enkephalin hydrolysis is mainly caused by a decrease in the activity of the enzymes involved, especially aminopeptidases, whereas the role of the low-molecular-weight inhibitors active on these enzymes is limited. The modified pattern of leu-enkephalin hydrolysis by-products can be related to the variations occurring between the rates of activity of the different enzymes capable of hydrolyzing the substrate. These variations, in turn, appear to be caused by trauma-associated shifts in the relative activity of the three classes of enkephalin-degrading enzymes, as well as by changes in the distribution of the active species within each class. Because of the technique used for enzyme separation (steric exclusion chromatography), this latter phenomenon is manifested as a reduction in the activity of the enzymes eluted at higher apparent molecular weight, which is more evident than that of the enzymes eluted at lower apparent molecular weight.

In addition to reducing the hydrolysis of leu-enkephalin and altering the ratio between its hydrolysis by-products, trauma modifies several immune parameters that can be related to stress. The low probability of the null hypothesis indicated by ANOVA of the trauma-associated alterations of substrate hydrolysis and of several of the immune indexes analyzed suggests that the variations induced in these two sets of data may be related to a common cause. Specifically, it is tempting to postulate that the stress associated with the sequence of events occurring before taking the blood samples may induce both sets of effects. This interpretation is, however, contrasted by the poor correlation found between decrease in hydrolysis and variations of immune parameters. The values of these correlation indexes indicate that the quantitative variation of one set of variables is not matched by a proportional variation of the other set. This datum cannot be assumed, however, as an indication of discordant variations of the two groups of variables; that is, the variations subsequent to trauma of both hydrolysis reduction and immune indexes are homogeneously positive or negative, and the probability that both sets are (independently) connected with the trauma is statistically significant. As mentioned above, the variations in the two sets of data may be connected by a direct causal relationship or may be linked indirectly. Finally, because the statistical probability indicates just a probability, the two sets of data may be totally unrelated. It seems, however, unreasonable to assume that modifications of metabolic parameters as complex as those examined here may depend on a single factor. In addition, the metabolic processes that relate the above-mentioned variations of enzyme activity to trauma are essentially unknown. Furthermore, in the case of the immune system, the existence of multiple regulatory factors is self-evident. Finally, the regression coefficients measured between the variations of the different immune parameters are just as low as those calculated between hydrolysis variation and immune indexes. At least to some degree, these results may be rationalized in terms of the nonhomogeneous status of the immune system in different donors, and the diphasic effect described for many neuropeptides in relationship to their role as immunomodulators, as detailed in the introduction.

In showing environment-induced modifications of peripheral opioid peptides, the data reported are in agreement with the few studies existing on this topic (Martinez and Weinberger, 1988; Young et al., 1989; Schulteis and Martinez, 1993). The differences between the data reported here and those referred to above, which were obtained with rat plasma, may be accounted for at least in

| TABLE 2 |
| Trauma-associated variations of immune parameters |
| Lets AC | Grnlc AC | Lymps % Lets | HLA-DR | Lymps B % Lymps | Mncts AC |
| Δ%   | +59.3 | +130.2 | −55.8 | −42.1 | +72 | +47.9 |
| S.D. | 65.9 | 98.6 | 41.9 | 21.7 | 43.0 | 74.2 |
| CV  | +66.2 | +63.6 | −92.8 | −55.4 | +82.5 | +132.9 |
| P   | 0.0003 | 0.0005 | 0.011 | 0.0046 | 0.0153 | 0.0330 |

Lcts AC, leukocytes absolute counts; Grnlc AC, granulocytes absolute counts; Lymps % Lets, lymphocytes as percentage of leukocytes; Lymps B % Lymps, lymphocytes B as percentage of total lymphocytes; Mncts AC, monocytes absolute counts.
part by the intraindividual differences in the degradation pattern of peripheral enkephalins (Bolacchi et al., 1996). In addition, differences in the stressful stimuli (e.g., chronic versus acute, single versus repeated) are known to influence some of their effects (e.g., Laudenslager et al., 1983; Sacerdote et al., 1994); therefore, the different experimental layouts may be responsible for the opposite direction of the effects reported. Another factor to be accounted for is the composition of the sample. The present study was conducted on a set of subjects who were particularly homogeneous regarding sex, age, and clinical history. This is both a drawback and an advantage: as reported just above, the effect of trauma varies in both intensity and direction with variations of the stressful stimuli. Therefore, homogeneity of both trauma and subsequent events should reduce stress-associated variability. On the minus side, the details of the specific mechanisms described, especially the reduced role of inhibitors, can be partially specific to the age (Babst et al., 1998), if not the sex (Marini et al., 1997), of the subjects under study.

In conclusion, it seems unlikely that communication between two major systems such as the nervous and immune systems would be anything but complex. Therefore, the lack of a clear relationship between different, although possibly related, effects such as those demonstrated above is to be expected. However, this implies that determination of the mutual dependence of these factors may require the accumulation of an impractically large body of data, at least until the mechanisms linking stress and the immune system and linking stress and enzyme activity are known in better detail. Finally, it seems worth noting that the lack of a definite tridimensional structure of relatively small peptides in polar solvent makes their hydrolysis relatively unspecific and that the enzymes that degrade enkephalins are also active on other low-molecular-weight peptides (Roscetti et al., 1988). Therefore, at least in principle, the data reported here, which were obtained using leu-enkephalin, may be extended to other blood peptides of suitable molecular mass. Likewise, it was obtained using leu-enkephalin, may be extended to other low-molecular-weight peptides (Roscetti et al., 1988).

References


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Send reprint requests to: L. Giorgio Roda, Dipartimento di Neuroscienze, Università degli Studi “Tor Vergata,” Via di Tor Vergata 135, 00133 Rome, Italy. E-mail: lbroda@linet.it