

Role of Nonesterified Unsaturated Fatty Acids in the Pathophysiological Processes of Leptospirosis

Patricia Burth,¹ Mauricio Younes-Ibrahim,² Maria C. B. Santos,³ Hugo C. Castro-Faria Neto,⁴ and Mauro Velho de Castro Faria³

¹Department of Cellular and Molecular Biology, Biology Institute, Universidade Federal Fluminense, Niterói, ²Laboratório Integrado de Nefrologia, Faculdade de Ciências Médicas, and ³Department of Cell Biology and Genetics, Universidade do Estado do Rio de Janeiro, and ⁴Laboratory of Immunopharmacology, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

Organ malfunctions in patients with leptospirosis have been associated with the bacterial glycolipoprotein endotoxin and with its nonesterified unsaturated fatty acid (NEUFA) components. We examined the involvement of NEUFAs in the pathophysiological processes of leptospirosis. Patients showed a moderate increase in serum concentrations of oleic and linoleic acids but an important decrease in serum concentrations of albumin. A highly significant correlation between serum concentrations of creatinine or total bilirubin and the oleic-plus-linoleic acid:albumin ratio was revealed. We used the Na⁺,K⁺-ATPase inhibitory property of NEUFAs to test the capacity of serum to prevent the cytotoxic effects of NEUFAs in vitro. Albumin solutions and serum samples from healthy volunteers, but not serum samples from severely affected patients, were able to revert the Na⁺,K⁺-ATPase inhibition by oleic acid. On the basis of these data, we defined a “serum protection factor” that can be helpful in predicting NEUFA toxicity. Our data support the concept that the administration of human albumin to patients may be helpful in severe leptospirosis cases.

Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira* species. This disease is a public health problem mainly in tropical regions where it is endemic, with epidemic bursts occurring during the rainy-season floods [1].

After penetration into the host, pathogenic *Leptospira* species circulate in the blood before colonizing organs, mainly the kidneys and liver. This infection may progress asymptotically or with mild clinical manifestations. In its severe icterohemorrhagic form (i.e., Weil disease), acute renal failure is usually present and is

characterized by a typical paradoxical hypokalemia [2, 3], which probably is linked to the impairment of tubular Na⁺,K⁺-ATPase caused by the bacterial endotoxin. Leptospirosis jaundice also manifests in an unusual fashion: cholestasis with high bilirubin and low albumin concentrations in serum, together with normal or slightly increased transaminase levels and minimal histological damage, is frequently found, suggesting that there is predominant functional impairment of the liver [4–6]. The acute respiratory distress syndrome (ARDS) found in severe forms does not seem to be directly related to extensive lung bacterial colonization [7, 8].

The *Leptospira* lipopolysaccharide differs from those of most other gram-negative bacteria in that it has no biological effects. According to Vinh et al. [9], leptospiral glycolipoprotein (GLP) is the bacterial fraction displaying endotoxic properties. Although biochemical mechanisms involving the action of leptospiral endotoxins remain controversial [10], we have shown that GLP is a potent and specific Na⁺,K⁺-ATPase inhibitor [11, 12] and that some lipid GLP components (i.e., palmitoleic, palmitovaccenic, and oleic acids) are the active Na⁺,K⁺-ATPase inhibitors. However, this does not imply that GLP exerts the same effect as unbound

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Reprints or correspondence: Prof. Mauro Velho de Castro Faria, Dept. de Biologia Celular e Genética, Universidade do Estado do Rio de Janeiro, Instituto de Biologia Roberto Alcântara Gomes, Pavilhão Haroldo Lisboa da Cunha, Sala 210, Rua São Francisco Xavier, 524 Maracanã, Rio de Janeiro 20550-013, Brazil (castrofaria@nitnet.com.br).

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nonesterified unsaturated fatty acids (NEUFAs) [13]. Instead, it appears that NEUFAs may have deleterious effects in their own right.

Nonesterified fatty acids (NEFAs) are transported in the blood while bound to albumin, which ordinarily neutralizes their cytotoxic effects. Nevertheless, reports associating high blood NEFA levels (oleic acid in particular) with the pathophysiological processes of some diseases have been published [14, 15]. We investigated serum concentrations of NEFAs during acute leptospiral infection and, in particular, the correlation between some serum NEUFA levels (or the NEUFA:albumin molar concentration ratios) and indicative parameters of kidney and liver functions. Furthermore, we also developed an *in vitro* model comparing the protective properties of serum from healthy individuals and patients with Weil disease with those of human albumin solutions after the inhibition of a standardized Na^+, K^+ -ATPase preparation caused by oleic acid. A test measuring the protective capacity of a serum sample against NEUFA toxicity is proposed.

MATERIALS AND METHODS

Serum NEFA quantification. Serum levels of the predominant NEFAs—palmitic, oleic, linoleic, palmitoleic, and miristic acids—were determined by use of high-performance liquid chromatography (HPLC). The extraction of fatty acids from 100- μL serum samples and the formation of bromophenacyl derivatives were performed exactly as described by Puttman et al. [16]. The internal standard margaric acid (C_{17}) was added to serum samples before extraction. Fatty acid derivatives dissolved in acetonitrile were injected onto a reverse-phase, free fatty acid HP 3.9 \times 150-mm column (Waters Corporation). The column was previously equilibrated with 65% acetonitrile in water and eluted with this same solvent mixture for 30 min at 1.0 mL/min. UV detection was performed at 254 nm, and chromatograms were analyzed by use of the Pro-Star program (Varian). Standard solutions of miristic, palmitoleic, linoleic, palmitic, oleic, margaric, and stearic acid derivatives were used to calibrate the system. Whenever necessary, corrections for differences in the molar absorption coefficients, at 254 nm, of fatty acid bromophenacyl derivatives, were introduced in concentration calculations.

Serum albumin, creatinine, and bilirubin assays. Serum albumin was quantified by use of the colorimetric procedure described by Doumas et al. [17], using human serum albumin standards. Commercial kits purchased from Sigma were used for the determination of serum creatinine (Sigma catalog 555-A) and total and conjugated bilirubin (Sigma catalog 605-C).

Na^+, K^+ -ATPase preparation. A Na^+, K^+ -ATPase-enriched fraction (specific activity, 100 μmol inorganic phosphate [Pi] formed/h/mg of protein and <2% ouabain-insensitive activity) was prepared from guinea pig kidneys, according to Jørgensen's procedure for rabbit kidneys [18]. The final enzyme suspension

(adjusted to 0.35 mg of protein/mL) was prepared in a solution containing 30 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 1% sucrose. Aliquots of this suspension were poured into dark flasks, lyophilized, and stored sealed in a nitrogen atmosphere at -20°C . The lyophilized preparation could be stored for >6 months without losing its activity. Freeze-dried aliquots suspended in water (0.35 mg of protein/mL) and stored at 0°C could be used up to 24 h after resuspension.

Na^+, K^+ -ATPase assay. Enzyme activity was determined in microtiter plates in triplicate assays. The final incubation volume (120 μL) contained 110 mmol/L NaCl, 20 mmol/L KCl, 5 mmol/L MgCl_2 , 50 mmol/L Tris-HCl (pH 7.6), enzyme preparation, and 5 mmol/L ATP. For every assay, blanks containing 3.6 mmol/L ouabain were processed. The spectrophotometric enzyme assay, as well as the oleic acid inhibition profile, were performed as described by Burth et al. [13]. Oleic acid-Tris salt solutions were prepared by dissolving the fatty acid in water with vigorous stirring and adding Tris base until the pH reached 9.5; next, the pH of the clear solution was carefully corrected to 7.6 with diluted HCl. The amount of the enzyme preparation used in all experiments (3.5 μg of protein) corresponded to an activity of 0.38–0.40 μmol Pi formed/h in control assays (100% enzyme activity). Preparations were preincubated for 10 min before starting enzyme reactions with ATP. In some experiments, just before the preincubation, 2 μL of serum and/or 2 μL of albumin solution was introduced into assay mixtures already containing oleic acid. The fatty acid was omitted in the 100% activity controls. In other experiments, oleic acid was replaced by GLP.

Preparation of the GLP. Growth of *Leptospira interrogans* serovar *canicola* (strain RU10) and the leptospiral GLP preparation were performed as described elsewhere [13].

Total protein assay. Total protein in preparations was assayed as described by Peterson [19].

Drugs, reagents, and solvents. Human albumin solutions were prepared from a 20% commercial human albumin solution (Immuno). A portion of this albumin was also delipi-

Table 1. Concentrations of the main circulating non-esterified fatty acids in serum from patients with leptospirosis and in serum from control subjects.

Fatty acid	Control subjects (n = 17)	Patients with leptospirosis (n = 40)
Miristic	23.3 \pm 14.7	31.2 \pm 16.8
Palmitoleic	49.2 \pm 21.1	59.4 \pm 20.2
Linoleic	83.3 \pm 19.5	134.2 \pm 55.0 ^a
Palmitic	94.4 \pm 20.4	126.9 \pm 41.7
Oleic	87.5 \pm 13.4	142.8 \pm 48.6 ^a
Stearic	63.2 \pm 13.1	73.2 \pm 22.0

NOTE. Data are mean \pm SD micromoles per liter.

^a $P < .005$, vs. control samples (unpaired Student's *t* test).

Table 2. Disease categories based on the severity of hepatic and renal injuries.

Disease category ^a	No. of patients	Total serum bilirubin level, mean, mg/dL	Serum creatinine level, mean, mg/dL	Serum albumin level, mean, g/dL
A (mild or absent icterus and ARF)	10	1.2	1.1	4.3
B (moderate icterus and ARF)	16	13.7	2.7	2.9
C (severe icterus)	2	34	4.8	2.3
D (severe ARF)	6	16.4	6.7	2.7
E (severe icterus and severe ARF ^b)	6	41.7	6.6	2.1
No disease	17	0.98 ± 0.26 ^c	0.88 ± 0.15 ^c	5.2 ± 0.47 ^c

NOTE. ARF, acute renal failure.

^a Total serum bilirubin levels <10, 10–29, and ≥30 mg/dL characterized mild or absent, moderate, and severe icterus categories, respectively. Serum creatinine levels <1.7, 1.7–5.9, and ≥6 mg/dL characterized mild or absent, moderate, and severe ARF groups, respectively.

^b Severe pulmonary syndrome was present in 4 patients in this category.

^c Data are mean ± SD.

dated, as described by Chen [20]. Delipidated albumin was concentrated by centrifugation in Amicon ultrafilters (10 kDa cutoff; Centriprep 10). The summed concentration of the 6 NEFAs in this commercial albumin was determined by HPLC, as described above. The calculated NEFA:albumin molar ratio of the original human albumin was 0.57; it decreased to <0.1 after delipidation.

Fatty acid standards, bovine albumin, human IgG, and all other reagents were purchased from Sigma. Solvents (HPLC grade) were obtained from Merck.

Serum samples. Serum samples from patients with leptospirosis were obtained from the Pedro Ernesto University Hospital (Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil). All serum samples were tested for leptospirosis by use of a microagglutination technique. Samples were kept frozen at –70° C and were used within a week. Serum samples from 40 patients (34 men and 6 women; 19–60 years old) presenting with different degrees of disease severity were studied.

Control serum samples were prepared from blood collected from 17 healthy volunteers (10 men and 7 women; 20–55 years old). The research protocol of this work received previous approval of the Ethics Committee of the Hospital Universitário Pedro Ernesto, University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

Statistics. An unpaired Student's *t* test was used for determining the statistical significance of the increase in levels of circulating NEFAs in patients' serum samples, compared with levels in control serum samples (table 1). Pearson's correlation coefficients (*r*) and 2-tailed *P* values assessing the significance of the correlation between the parameters studied were calculated by use of the GraphPad Prism program (version 3.0; GraphPad).

RESULTS

Because the primary aim of the present study was to investigate NEUFA toxicity associated with altered hepatic and renal func-

tions during leptospiral infection, the 40 patients were grouped in 5 categories (A, B, C, D, and E), according to their serum levels of total bilirubin and creatine, as shown in table 2.

Compared with concentrations in control serum samples, augmented oleic and linoleic acid concentrations (+63% and +61%, respectively) were noted in serum samples from the 40 patients with leptospirosis (figure 1; table 1). The other NEFAs (miristic, palmitoleic, palmitic, and stearic acids) which, together with oleic and linoleic acid, constitute >90% of all circulating NEFAs [21], had smaller increases. The summed serum concentrations (mean ± SD) of these 6 NEFAs increased from

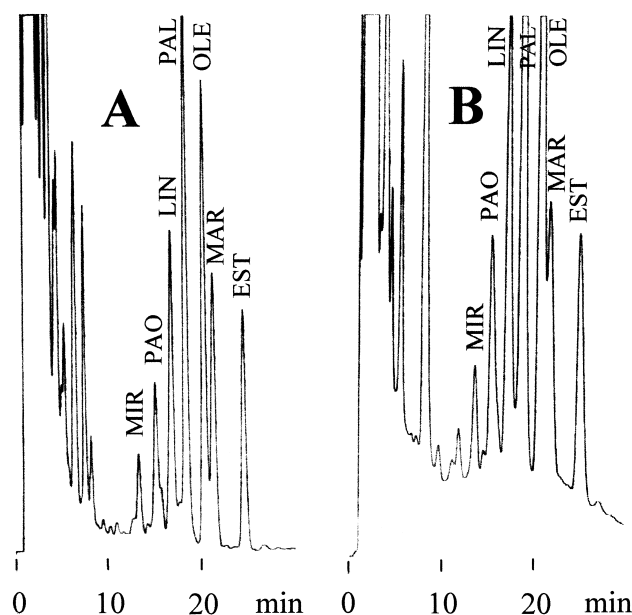


Figure 1. Representative chromatograms showing the distribution of nonesterified fatty acids in a serum from a healthy person (A) and from a patient with severe leptospirosis (B). The following fatty acids are identified in graphs: miristic (MIR), palmitoleic (PAO), linoleic (LIN), palmitic (PAL), oleic (OLE), margaric internal standard (MAR), and stearic (EST).

401.5 ± 82.3 μmol/L in the control group to 596 ± 168.5 μmol/L in patients. The highest concentration was 780 μmol/L, in a patient with Weil disease. These moderately increased fatty acid concentrations cannot be considered as solely accounting for the cytotoxic effects of NEUFAs. Because patients also had remarkably low serum albumin levels, we investigated the correlation between NEFA:albumin molar ratios and the severity of kidney and liver impairment. As shown in figure 2, analysis of serum from patients with mild-to-severe renal or hepatic injuries showed a significant correlation between creatinine or total bilirubin and oleic acid concentrations, especially the oleic acid:albumin and oleic-plus-linoleic acid:albumin molar ratios. Statistical significance was lower when just oleic acid concentrations were considered but increased substantially when correlation coefficients for oleic-plus-linoleic acid:albumin ratio versus creatinine ($r = 0.84$) or versus bilirubin ($r = 0.78$) were calculated. Mean (±SD) oleic acid:albumin and oleic-plus-linoleic acid:albumin molar ratios measured in 17 control serum samples were 0.13 ± 0.035 and 0.25 ± 0.068, respectively.

We searched for an in vitro test that would measure the capacity of serum albumin to prevent the toxic effects of NEUFAs and considered the potent Na⁺,K⁺-ATPase inhibitory property of oleic acid. We therefore used this fatty acid to inhibit a

standardized preparation of the ATPase as a tool to detect protective effects exerted by serum samples and human albumin solutions. Indeed, we found that patients' serum samples had a decreased protective capacity against the deleterious effects of NEUFAs. Although control serum samples completely suppressed enzyme inhibition by oleic acid, at concentrations as high as ~0.125 mmol/L, this protection was absent when serum samples from severely affected patients were tested (figure 3A). These patients, who were included in category E (table 2), had oleic-plus-linoleic acid:albumin molar ratios ≥1.0 and severe pulmonary distress. Human albumin at a concentration matching that found in control serum samples (866 μmol/L) also afforded significant protection. The efficacy of delipidated albumin was somewhat higher in this regard (figure 3B). Figure 3 also shows an unambiguous increase in the protective effect when the albumin concentration in incubation mixtures already containing serum samples from severely affected patients was corrected to the level expected from serum from healthy individuals (14 μmol/L).

These results led us to propose a simple test, essentially using the experimental protocol shown in figure 3A, to measure the protective capacity of serum against toxicity of NEUFAs by fixing the oleic acid concentration in the preincubation mixture

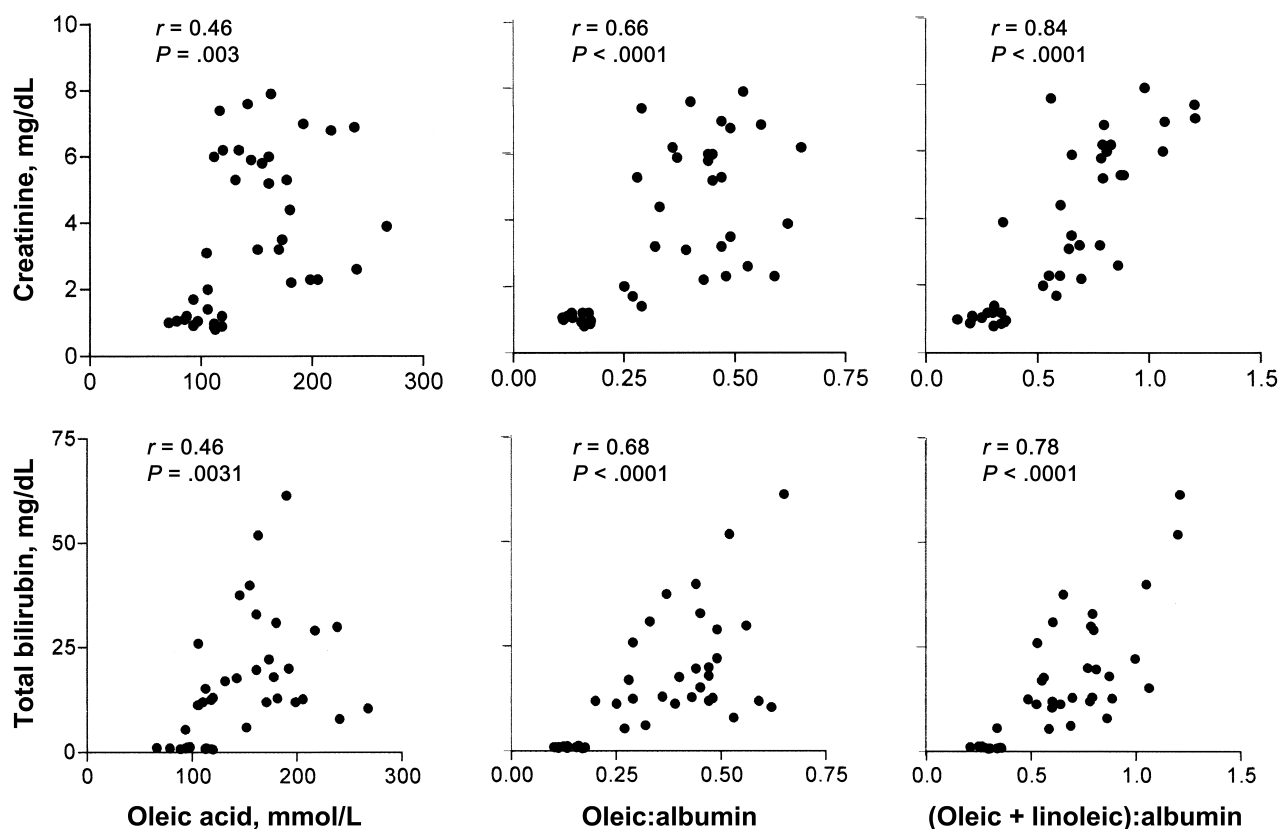


Figure 2. Correlation between creatinine or total bilirubin concentrations with oleic acid and oleic acid:albumin or oleic-plus-linoleic acid:albumin molar ratios in serum samples from 40 patients with leptospirosis. Pearson's correlation coefficients (r) and 2-tailed P values are shown.

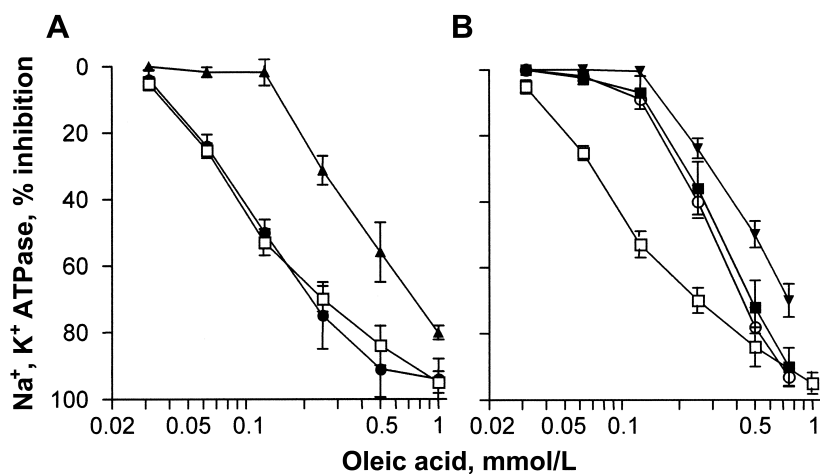


Figure 3. Protective capacities of serum from healthy individuals and patients with leptospirosis, as well as of human albumin solutions, against the inhibition of Na⁺,K⁺-ATPase by oleic acid *in vitro*. Experimental details are described in the text. The oleic acid inhibition curve (□) is shown in both graphs (mean ± SD of 10 different determinations). *A*, Additions to the preincubation mixture include 2 μL of healthy individuals' serum (▲) and 2 μL of serum from patients with severe pulmonary distress included into category E (●) (see table 2 for a description of the categories). Each point is the mean ± SD result of 4 different serum samples. *B*, Additions to the preincubation mixture include 2 μL of 866 μmol/L original human albumin solution (■) and 2 μL of 866 μmol/L delipidated albumin (▼). The albumin concentration in a preincubation mixture already containing 2 μL of a patient's serum (○) (the same samples in panel *A*) was corrected to the mean control serum value (14 μmol/L) by adding an adequate amount of original human albumin solution to the mixture. Each point is the mean ± SD of 4 different experiments.

at 0.1 mmol/L. Thus, the serum protection factor (SPF) could be calculated by the formula $SPF = A - B/A$, where *A* is the percentage of enzyme inhibition in the presence of 0.1 mmol/L oleic acid (in our conditions, *A* was equal to 53% ± 2.8%, the mean ± SD of 25 determinations), and *B* is the percentage of enzyme inhibition in presence of 0.1 mmol/L oleic acid plus 2 μL of serum to be tested.

Since control assays (100% activity controls) used to calculate *B* contained the corresponding serum samples, any eventual interference with the Na⁺,K⁺-ATPase activity that could be attributed to other serum components was minimized. Accordingly, the highest SPF was 1.0, characterizing the presence of control serum samples. In the 17 control serum samples studied, *B* was 0%. Otherwise, the lowest factor was 0, indicating the absence of serum protection against oleic acid inhibition. A statistically significant negative correlation was obtained when the SPF values of 12 patients with mild, intermediate, and severe infection were plotted against their respective serum bilirubin and creatinine concentrations (figure 4).

At this point, we also considered the effect of human albumin in protecting against the Na⁺,K⁺-ATPase inhibitory property of GLP. Vinh et al. [9] have shown that the bovine albumin present in fibroblast culture medium protects these cells against the cytotoxicity of GLP. As shown in figure 5, 53% inhibition of the Na⁺,K⁺-ATPase activity was obtained when an amount of GLP corresponding to 0.6 μg of GLP was added to the incubation mixture. This inhibition was completely reverted in the presence

of 24 μg (final concentration, 3.3 μmol/L) of human or bovine albumin. Another human serum protein, IgG, was ineffective.

DISCUSSION

High serum concentrations of NEFAs—oleic acid in particular—have been implicated in the development of ARDS and have been associated with pathological conditions, including trauma, sepsis, pancreatitis, and embolism. Accordingly, it is known that intravenously injected oleic acid induces ARDS in experimental animals [14–22]. Impairment of alveolar cell Na⁺,K⁺ transport may be involved in this disturbance, since Na⁺,K⁺-ATPase activity plays an important role in suppressing pulmonary edema [23, 24]. Although mechanisms responsible for the cytotoxicity of NEFAs are not completely understood [15], it is known that they can alter membrane fluidity and interfere with the activity of diverse transport systems [25, 26], including Na⁺,K⁺ active transport [27]. In addition, *in vivo* effects may also be mediated by NEFA metabolites, and epoxy derivatives of linoleic acid may be involved in linoleic acid-induced cellular toxicity [28], possibly through specific interaction with the Na⁺,K⁺ pump [29].

Under physiological circumstances, circulating NEFAs are not toxic, because they are transported bound to albumin. Nonetheless, deleterious effects may be present *in vivo* in situations of increased NEFA:albumin ratios, as a result of the saturation of albumin-binding sites. Molar ratios of ~2.0 are associated with the severity of some diseases [15], whereas ratios of ~2.5 are associated with systemic disorders in preeclampsia [30]. On

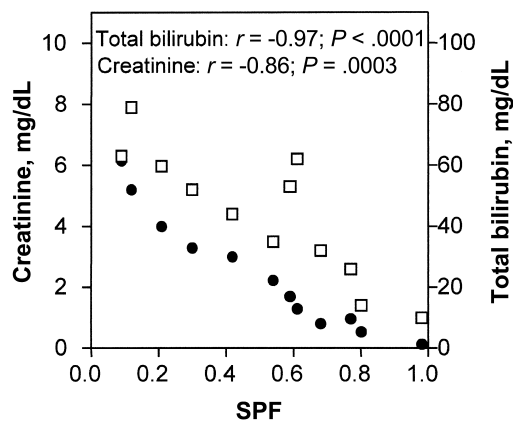


Figure 4. Correlation between the serum protection factor (SPF) and the respective serum total bilirubin (●) or creatinine (□) concentrations in 12 patients with leptospirosis divided into categories A (2 patients), B (4 patients), C (2 patients), D (1 patient), and E (3 patients); the categories are described in table 2. Pearson's correlation coefficients (*r*) and 2-tailed *P* values for bilirubin and creatinine are shown in the graph.

the basis of our data, over the course of moderate or severe leptospiral infections, we can expect total NEFA:albumin and oleic-plus-linoleic acid:albumin molar ratios to be >2.0 and >1.0, respectively.

As can be calculated from data shown in figure 3, in experiments containing control serum samples or albumin solutions, the *in vitro* Na⁺,K⁺-ATPase inhibitory response to oleic acid was detected only when the calculated oleic acid:albumin molar ratio was >9:1. This value seems to be substantially higher than the NEFA:albumin ratios expected to induce cytotoxicity *in vivo*. However, the degree of the enzyme inhibition by oleic acid *in vitro* would be changed by altering incubation conditions, such as the enzyme concentration in the incubation mixture, and there likely are differences between an extensively purified enzyme preparation and the same enzyme embedded in an intact cell membrane.

Figure 3 also shows that the NEUFA-binding capacity of serum samples from severely affected patients, as judged by the capability of reversing Na⁺,K⁺-ATPase inhibition by oleic acid, was absent. We believe this lack of oleic acid-binding capacity derives not only from an increased NEFA:albumin ratio but also from the large increase in other competing albumin ligands, such as bile pigments. Oleate and bilirubin compete for albumin binding [31], and, as can be calculated from our data, an ~4-fold increase in the serum oleic-plus-linoleic acid:albumin molar ratio found in some patients corresponded to an ~100-fold increment in the total bilirubin:albumin molar ratio. The striking negative correlation between serum bilirubin levels and SPF, as revealed in figure 4, also favors this view.

Leptospiral hepatic injury evolves mainly as a functional liver impairment. Histological lesions and increases in serum concentrations of transaminases are not prominent, as in other

forms of infectious hepatitis. The elevated serum concentrations of bilirubin (mainly in the conjugated form) in this disease may be related to altered liver membrane transport. Although mechanisms of organic anion transport through liver membranes remain controversial [32], compelling evidence points to an ATP-dependent and/or membrane-driven active transport of conjugated bilirubin through canalicular membranes [33] and transporter-mediated uptake of unconjugated bilirubin by the human liver [34]. Furthermore, increases in serum concentrations of NEFAs may also derive from membrane transport disturbances, since studies from several laboratories have demonstrated energy-dependent, Na⁺-linked NEFA uptake into hepatocytes [35].

All these findings strengthen and complement our previous proposal involving crucial pathophysiological events associated with leptospiral infection [11–13]. In brief, after colonization of organs, lysis of leptospira following the host immune response would release GLP. Because GLP is a potent and specific Na⁺,K⁺-ATPase inhibitor [11], the impairment of the Na⁺,K⁺ active transport certainly plays an important role in this initial process. Resulting metabolic disorders would cause increased NEUFA and bilirubin serum levels and decreased albumin concentrations, leading to albumin saturation. In this way, the enhanced hepatic and renal dysfunctions, as well as other systemic disturbances found in severe cases, may also be linked to NEUFA-related toxic effects. The proposed SPF test can be helpful in predicting possible consequences associated with NEFA-related cytotoxicity, not only during a leptospiral infection but also in all other diseases in which a high serum fatty acid:albumin ratio is present.

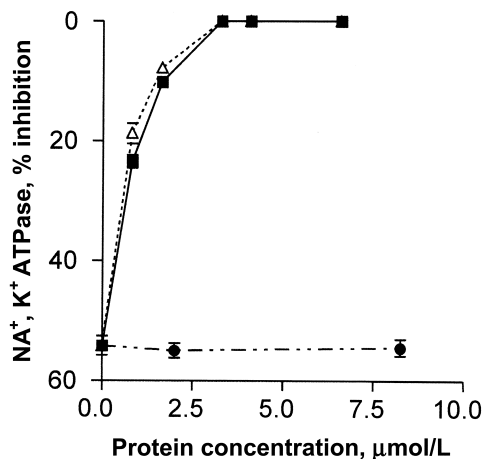


Figure 5. Protective effect of albumin against the inhibition of Na⁺,K⁺-ATPase by the leptospiral glycoprotein (GLP). All assays, except 100% activity controls, contained an amount of GLP corresponding to 0.6 μg of GLP protein. Additions to the preincubation mixture included human albumin (■), bovine albumin (△), and human IgG (●). Each point is the mean ± SD of 4 different experiments.

Finally, administration of human albumin to patients with Weil disease may be a useful therapeutic aid. As we have shown in the present work, the common commercial human albumin preparation, although it is stabilized by a short-chain fatty acid salt (caprilate), efficiently protects against both NEUFA- and GLP-related cytotoxic effects in vitro. In a single case study of severe leptospiral jaundice treated with exchange blood transfusion and albumin infusion in 1969, Murphy [36] reported that the addition of albumin improved the effectiveness of the treatment. This was attributed to an increase in the number of binding sites for bilirubin.

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